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# Handbook of Process Chromatography

Development, Manufacturing,  
Validation and Economics



Lars Hagel, Günter Jagschies and  
Gail Sofer



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Validation and Economics

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Validation and Economics

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# Preface

Biotherapeutics have become the main drivers for the pipeline of the pharmaceutical industry, and carry hope for millions of patients for a better life with, or even cure from, diseases for which there are no existing effective treatments. This group of medicines includes, but is not limited to, protein and nucleic acid-based therapeutics as well as different classes of vaccines and gene therapy treatments. Today, there are a few hundred licensed biotherapeutics produced from genetically engineered cells and over a thousand are in pre-clinical and clinical studies.

Process chromatography provides companies developing or manufacturing biological pharmaceuticals tools to fulfil high requirements on safety and quality of active ingredients. This book discusses process chromatography tools and their capabilities; and it does so with all the main interrelations between different phases of manufacturing kept in mind.

Since the first edition of this book, the field has matured significantly and has seen a number of important new aspects in business strategies, manufacturing framework and use of new technology. Emphasis has shifted away from the focus on individual steps and their technical performance to a more comprehensive process operational view. Statistical tools are now used to establish robust operating parameters, and analytical methods have been significantly improved.

In this book, we take a holistic approach to describe purification processes by considering the biopharmaceutical industry and its needs, the types of products and the sources from which they are produced, other technologies that are used prior to purification and some other technologies, such as filtration that complement chromatography, which is still the workhorse of downstream purification.

In Chapter 1, we address the state of the biopharmaceutical industry today. This sets the stage for the subsequent chapters. Much has changed. For example, the whole concept of follow-on products, generic approaches and platform technologies were not even topics of interest when the first edition of this book was published.

Chapter 2 describes process capability from the perspective of several functional departments, the market needs for biopharmaceuticals and some production setups for manufacturing at different scales during the various stages of development and production. The ability of mammalian cell and microbial substrates to meet the future market demands is explored, and the use of multi-product facilities described.

Chapter 3 presents process design concepts that enable development of a process suitable for manufacturing biotherapeutics. We discuss, among other topics, the importance of risk assessments, the design of a logical purification strategy and characterization studies. Expression systems used to produce biopharmaceuticals, with an emphasis on the most commonly used hosts, *E. coli* and CHO, are discussed in terms of productivity and types of products made today.



Separation technologies that are discussed in Chapter 4 include both chromatography and filtration. An overview of currently used recovery steps is followed by a discussion on basic chromatography techniques and their optimization and scale-up.

In Chapter 5, in-process and final product analytical methods are presented. Specific analytical tools applied to monoclonal antibodies and nucleic acid products, such as DNA plasmids, are described. Process analytical technologies (PAT), method validation, setting specifications and the use of standards are also addressed.

Chapter 6 addresses the always-important issues of cleaning and sanitization of chromatography resins, reusable filters and equipment. This chapter is followed by validation (Chapter 7), which includes cleaning validation for chromatography columns.

An appendix to the Chapter 7 provides a summary of activities from pre-clinical to post-licensure for biopharmaceutical production from genetically engineered mammalian cells. Current validation trends, which are discussed, may influence future validation costs.

Economy of production has become more and more important since our previous edition. The economics is addressed in Chapter 8.

Chapter 9 on basic properties of biological molecules has been updated to include viral and DNA-based therapeutics and highlights properties of some important type of biopharmaceuticals.

Chapter 10 discusses optimization of separation processes based on well-established chromatography theory. Influence of experimental parameters may be simulated with the software tutorials supplied on a CD-ROM.

Our final two chapters address chromatographic equipment and column packing. New equipment designs, improved automation and pack-in-place columns are discussed.

Appendices with detailed references for nomenclature of liquid chromatography, reduced numbers used in process engineering, validation activities during development and the simulation tutorial complete the content of the second edition of *Handbook of Process Chromatography*.

We hope you will find the content of this book helpful for your daily work and as your reference in the coming decade.

Uppsala, Sweden and Warren, NJ, USA  
Lars Hagel, Günter Jagschies and Gail Sofer

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# Biopharmaceuticals Today

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## 1.1 INDUSTRIAL CONTEXT

In our introduction to the 1st edition of this book in 1997 we wrote: ‘In the rush to get a product to market, process optimization and validation are often sacrificed’, which indicated a certain lack of maturity in the biopharmaceutical field at that time. Those days are gone. A significant portion of biopharmaceutical drug development is now performed by experienced big pharma and a small number of established biopharma companies. In addition, there are hundreds of small companies and start-up institutions without sufficient competence to bring novel biopharmaceuticals all the way to market; but today, even these organizations have full access to the required expertise from contract manufacturing and clinical research organizations.

Consequently, the routines followed by this industry have matured. Where there was once mere science and many remaining unknown issues for manufacturing protein-based drugs, there are now sets of tools, the challenges are well understood and relevant information is in the public domain. Process chromatography, the main topic of this handbook, is one of the most important tools due to its close relation to end-product quality and safety. Well-managed platforms with overall workflow and specific methods are being established for every aspect of process development, analytical tasks and even regulatory procedures. These platforms are upgraded in a carefully controlled fashion following progress in science and applicable technology. Knowledge gaps are closing rapidly.

In addition to biochemical and biological science, process engineering is beginning to dominate the field as protein production loses most of the ‘mysteries of biology’ and moves towards predictable and controllable production operations. This is not, yet, the situation with emerging gene and cell-based candidate therapeutic agents, where the uncertainties and inexperience reflect the situation that existed for protein drugs just two decades ago. The design of work strategies and methods for these novel medicines still constitutes a major challenge for their developers, as well as for regulators evaluating the associated risks.

The following sections in this chapter provide a brief history and describe the current biopharmaceutical business. Our description is qualitative, i.e. where there are business-related numbers, they are merely intended to create a snapshot that may help to illustrate or exemplify our points. We are perfectly aware that this industry is moving too fast to

expect such numbers and many technical details to become much more than a faint trace of history. However, the principles laid out in this book will remain valid and have not, in fact, changed that much since the last edition.

## 1.2 OVERVIEW OF BIOPHARMACEUTICAL HISTORY

The history of commercial biopharmaceuticals reaches back no more than 80 years, and has seen real progress only in the last 20 years or so. However, biological medicines have already changed the landscape of healthcare more than any other drug development, and they will continue to do so. We only describe vaccines and protein drugs here, but current and future developments arising from the hundreds of ongoing studies of gene- and cell-based therapies are likely to provide breakthroughs in the next 15–20 years.

### 1.2.1 Protein drugs

The very first modern protein drug was insulin, which was isolated from animal pancreas in 1921 and resulted in Nobel Prizes for Banting and McLeod in Toronto and the Danish professor August Krogh. When faced with the problem of mass producing this breakthrough drug, the already established US pharma company Eli Lilly and the three co-founders (one of whom was August Krogh) of the company known today as Novo Nordisk, simultaneously but independently recognized the opportunity to develop and expand their businesses through access to a life-saving treatment of diabetes [1].

The trigger to the availability of the next important group of protein-based pharmaceuticals was the need to treat wounded soldiers during the Second World War and the Korean War, which led to the development of industrial scale, extraction-based fractionation methods by Cohn [2]. Subsequently mass production for human serum albumin, immunoglobulin fractions and, later, coagulation factors VIII and IX to treat haemophilia A and B became a reality.

Beginning in the early 1970s, chromatography was first proposed and then gradually implemented for production of these plasma derivatives [3]. At the same time, a novel business field emerged, i.e. the supply of purification technology, including commercial chromatography media, for biologics. Companies providing this technology included Pharmacia Fine Chemicals, Bio-Rad and Whatman.

The breakthrough for protein therapeutics came in the mid-1970s with the development of genetically engineered cells as production sources. These new production sources have gradually replaced natural protein sources that have limited availability and inherent infection risks. The development of methods to engineer DNA opened the door to previously unheard of possibilities for recombinant protein production [4].

Recombinant human growth hormone (hGH) and insulin, both well-studied therapeutics with approved biologic counterparts from natural sources, were targeted early because of the ease of their production and large markets for these products. Herbert Boyer transformed *Escherichia coli* cells with a recombinant plasmid in 1973 and later founded Genetic Engineering Technology (Genentech, S. San Francisco, CA, USA).

His team, with the help of their licensing partner Eli Lilly (Indianapolis, IN, USA), obtained a license to market the first human recombinant protein, recombinant human insulin (Humulin), in 1982.

In the following years, among many other proteins, six versions of interferon- $\alpha$ , - $\beta$  and - $\gamma$  that entered clinical trials in the 1980s ultimately gained approval in the United States for chronic hepatitis C infection, hairy cell leukaemia, chronic granulomatous disease and multiple sclerosis.

Human tissue plasminogen activator (tPA) was the first complex, glycosylated therapeutic protein to be produced in mammalian cells (Activase®, Genentech, 1987). Another breakthrough in the use of mammalian cells occurred in 1983 when the gene coding for erythropoietin (EPO) was identified by a team headed by Fu-Kuen Lin at Amgen. Recombinant DNA technology was used to express EPO in Chinese hamster ovary (CHO) cells utilizing large numbers of roller bottles. Recombinant EPO was launched by Amgen as Epogen® in 1989 for treatment of anaemia that results from chronic renal failure. This early success story was dependent on decades of academic research studies on EPO from natural sources [5, 6].

Most biopharmaceuticals approved during the 1980s and early 1990s were unaltered murine monoclonal antibodies or simple replacement proteins of unaltered amino acid sequence, such as insulin, blood factors, interferons and EPOs. The 1990s witnessed the approval of some engineered products. This trend has accelerated over the past 5 years and, within this period, 29 of the 65 approvals (44%) were for products engineered in some way to modify the pharmaceutical features of the drugs. Prominent among recently approved engineered products displaying altered amino acid sequence are various chimeric and humanized antibodies, and engineered insulins. Interferons represent a notable category of products that are altered by post-translational modifications to prolong the half-life in the patient and reduce the dose as a direct consequence. Specifically, this is accomplished by covalently attaching polyethylene glycol (PEG) to the protein backbone [7].

Monoclonal antibodies (Mab) have developed into the predominant class of proteins investigated for new therapies (Figure 1.2). The first therapeutic Mab to enter the market was Ortho Biotech's Orthoclone OKT3®, a mouse hybridoma antibody approved in 1986. The breakthrough for Mabs coincided with the invention of technology to partially or fully humanize the antibodies and express them as recombinant proteins, mainly from mammalian CHO cells and from NS0, a myeloma cell line derived from murine B lymphocytes. Mammalian cell culture of Mabs is now performed in fermentors up to 12,000 L or greater with batch sizes between 5 and 50 kg. Annual production for selected Mab therapeutics is expected to reach a ton scale. Mabs come close to insulin in production scale. However, the reasons for producing these therapeutics in large quantities are quite different. They are related to dose per patient for Mabs and to the size of the patient population for insulins. Table 1.1 lists therapeutic antibodies and some of their important characteristics.

During the 20-year period described here, the design of downstream processes and the installations used for production purposes have matured significantly. One achievement is a reduction in the average number of chromatography steps, typically from four to three. And today, some companies present processes with only two steps. Complementary filtration and conditioning steps have been eliminated where possible. Virus clearance steps have been integrated into downstream processes.

**Table 1.1**

Monoclonal antibodies, humanized and fully human antibodies mark the final breakthrough for antibody therapies (information derived from drug monographs)

	Mouse 100% murine	Chimeric 30% murine	Humanized 5% murine	Fully human 0% murine
Examples	Orthoclone, 1986 Panorex, 1995 Zevalin, 2002	Remicade, 1998 Rituxan, 1997 Erbitux, 2004	Synagis, 1998 Herceptin, 1998 Avastin, 2004	Humira, 2002
Effectiveness	Low	High	High	High

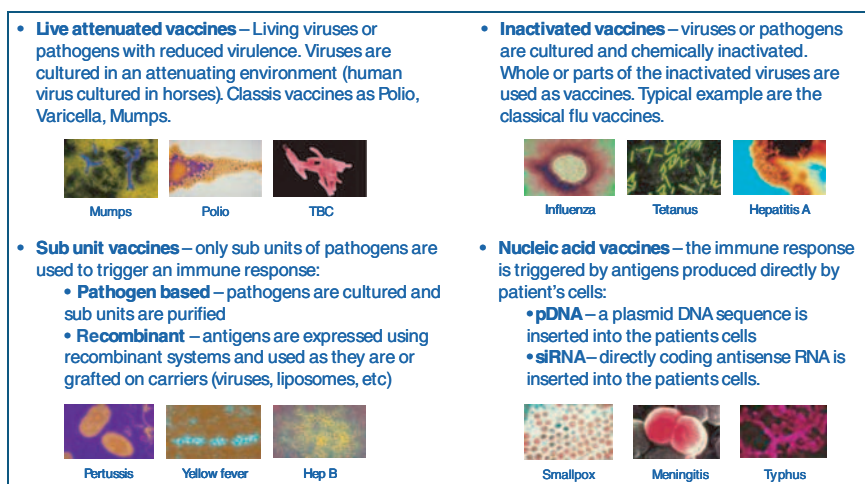
While a lot of hope had been placed on the power of affinity chromatography, its true potential is only realized in Mab processes for which a proteinaceous ligand, Protein A, was produced for the initial capture step. A few processes use immunoglobulin affinity ligands or immobilized metal affinity chromatography (IMAC). However, apart from Mab processes, ion exchangers are the work horses of industrial purification schemes. Hydrophobic interaction chromatography (HIC) plays a niche role in final stages of certain purification schemes. Size exclusion chromatography (SEC) is used in a few vaccines purification processes, and it is also used for some therapeutic proteins to achieve the removal of impurities of significantly different molecular size, e.g. aggregates. Reversed phase chromatography (RPC) is used with insulin and a few other relatively small proteins and has an unrivalled selectivity for removal of trace amounts of homologous impurities.

Current, commercially available Protein A resins, IMAC resins, ion exchangers and RPC resins are capable of handling batch sizes of 50 kg or more, often 10–20 times more than just a decade ago. This capability makes chromatography well suited in technical terms for production of proteins in multi-ton scale (see Chapter 2).

### 1.2.2 Vaccines

In classic terms, a vaccine is a substance that contains antigenic components that may be attenuated or inactivated whole organisms or components of those organisms (see Figure 1.1). Vaccines may also be synthetic antigens. A vaccine stimulates production of an active immune response against that antigen.

The pioneer of modern vaccination was the English physician Edward Jenner. The concept of vaccinations to prevent disease dates back to 1796. In that year, Jenner noted that dairymaids who had caught cowpox (a minor disease), could not catch smallpox (a fatal disease). Jenner then took diseased matter from the hand of Sarah Nemes, a local dairy-maid who had become infected with cowpox, and inserted this matter into the cut arm of James Phipps, a healthy 8-year-old boy. The boy then caught cowpox. Forty-eight days later Jenner injected smallpox matter into the boy. It had no effect. This was the first recorded vaccination. The widespread use of vaccination eventually led to the eradication of smallpox as a threat to human health. The closing years of the nineteenth century and the early years of the twentieth century were marked by the achievements of scientists like



**Figure 1.1** Map of vaccine categories. Classic live attenuated and inactivated vaccines, recombinant subunit vaccines and nucleic acid vaccines are in a category of medicines that carry strong hopes for disease prevention.

Louis Pasteur, who showed that attenuated forms of bacteria, produced by serial culture, could be used for immunization.

Most first-generation vaccines were based on the pathogenic organism that they are intended to combat. The first step in making a vaccine is to separate the immune-stimulant effects of the organism from its capacity to cause disease. This usually means isolating or creating an organism, or part of one that is incapable of causing disease but still retains the antigens responsible for inducing the patient's immune response. Vaccine preparation typically involved growing organisms in cultures of mammalian host cells or in eggs, harvesting the organism by centrifugation and filtration, inactivation, e.g. with chemical additives and conditioning with tangential flow filtration (TFF). Purification steps, such as those used for proteins were rarely included and were limited to centrifugation, filtration or density gradient ultracentrifugation (e.g. influenza vaccine). The first generation of vaccines caused repeated occurrence of side effects, considered by many to be too severe. The introduction of recombinant DNA technology widened the possibilities for vaccine production. Today, antigens can be produced separately, e.g. as recombinant proteins, and later recombined into a subunit vaccine (see Figure 1.1) for proper presentation to the immune system (e.g. Merck & Company, Recombivax HB®, 1986 and GSK, LYMERix™, 1998). In recombinant form, toxic pathogens are designed to become safe (e.g. GSK, Pediarix™, 2002). The introduction of these approaches led to the current generation of vaccines with increased safety and more reliable manufacturing. Modern recombinant vaccines are produced using advanced purification processes that include chromatography.

Mass production of vaccines has recently been given a great deal of attention, namely for influenza. There is concern that current technology would not enable production of sufficient vaccine doses if a large epidemic or a pandemic occurred. This fear is driving



billions of USD of investment into the development of new manufacturing processes and will lead to a paradigm shift in vaccine manufacturing. The current basis of influenza vaccine manufacturing is growing massive quantities of virus in fertilized hens' eggs, a commercial process that has been in place for decades. The surge capacity that will be needed for a pandemic response cannot be met by egg-based vaccine production alone, as it is impractical to develop a system that depends on hundreds of millions of 11-day-old specialized eggs on a standby basis.

In contrast, cell-culture manufacturing technology can be applied to influenza vaccines. Cell culture is used today to produce most other viral vaccines (e.g. polio vaccine, measles–mumps–rubella vaccine and chickenpox vaccine). Using cell culture, viruses can be grown in bioreactors containing large numbers of cells in growth media. The surge capacity afforded by cell-based technology is independent on availability of natural source material and can be adjusted to vaccine demand.

Among the companies developing cell-culture processes are Baxter, Aventis Pasteur, Medimmune and Chiron, all of whom are expected to launch vaccines based on novel production methods within the coming years.

The field of vaccines is undergoing a revolution, and vaccination has the potential to become the most promising future route for preventing and treating both infectious and non-infectious diseases.

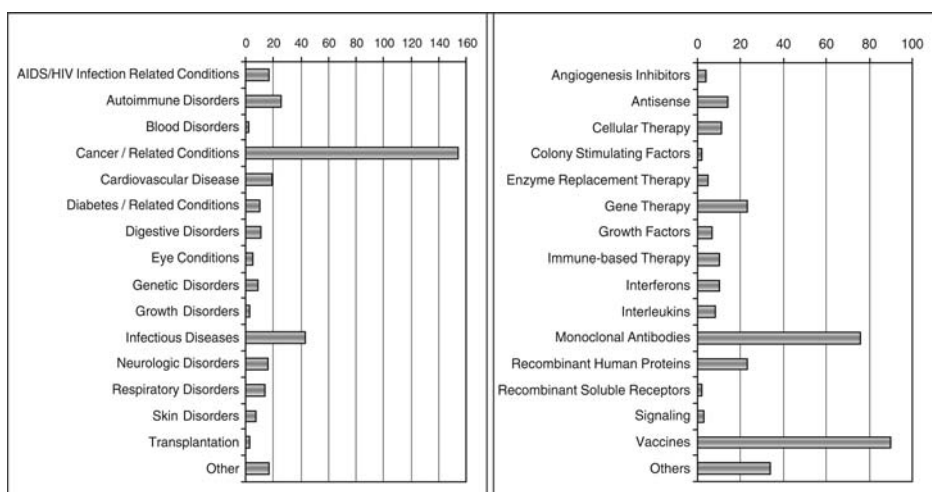
The term 'vaccine' today covers many different active ingredients, such as whole cells, virus, virus-like particles, antigens or fragments of antigens, plasmid DNA, Mabs and other proteins and conjugated molecules (see Figure 1.1). They all have one thing in common; they interact with the immune system. Modern vaccines under investigation are directed not only against infectious diseases, but also against cancer, cardiovascular disease and diabetes. Most of these vaccines are manufactured using purification processes to ensure quality and safety. Consequently, this enables implementation of robust downstream manufacturing processes for this new type of biopharmaceuticals.

### 1.3 BIOPHARMA BUSINESS ENVIRONMENT

During the second half of the past century, we have seen a breath-taking increase in the understanding of biological and biochemical foundations of health and disease. Almost all established pharmaceutical companies, and a countless number of entrepreneurs, have embarked on projects to develop novel medicines on the basis of this growing knowledge. In this section, we provide a snapshot of the situation, evaluate both the successes and the challenges and extract a number of practical conclusions for the development of future manufacturing processes.

#### 1.3.1 Biopharma pipeline: the promise

With 324 biotechnology medicines in clinical development [8] by member companies of the Pharmaceutical Research and Manufacturing Association (PhRMA), well over 500 global clinical development projects, and another 700–800 projects in pre-clinical stages [9], the pipeline seems to be rich in both new drugs and new therapeutic concepts (Figure 1.2).



**Figure 1.2** The 2004 biotechnology medicine pipeline as published by PhRMA [8].

Cancer and its associated conditions have by far the greatest industry focus, followed by infectious diseases including AIDS/HIV. Vaccines and Mabs form the largest categories of products that are in development. The PhRMA survey lists 44 vaccines directed towards cancer, 26 projects addressing autoimmune disorders including arthritis and 16 studies directed at neurologic disorders such as Alzheimer's, Parkinson's and multiple sclerosis. The four industry leaders in vaccines are estimated to spend more than 750 MUSD a year on vaccine R&D. For some of these companies, this is as much as a fivefold increase over vaccine R&D in 1992.

Today, approximately one fourth of new drugs coming in the market are biopharmaceuticals, and the ratio is expected to increase rapidly during the coming decades. These drugs have benefited some 300–400 million people worldwide. The number of publicly traded companies in the US involved in biopharmaceutical development and/or manufacturing has now surpassed 300 with almost 200,000 employees.

### 1.3.2 Launched biopharmaceuticals: delivering on promises

In the United States alone, between 1994 and 2010, revenues from biopharmaceutical drugs are predicted to increase from 11 to 52 billion USD, a very strong growth prognosis [4]. Table 1.2 lists the global top selling biopharma drugs and product categories for 2006, i.e. those that sell at more than one billion USD per annum. It is predicted that global annual sales of biological medicines will soon reach 100 billion USD [9].

The initial successful biopharma protein products (recombinant insulin, EPO, interferon and clotting factors) are still very important income generators. Table 1.2 also lists three Mabs for cancer therapy, two Mabs and one fusion protein for rheumatoid arthritis, one Mab directed against multiple sclerosis, the first Mab used against infectious disease and

**Table 1.2**

Top selling biopharmaceuticals 2006

Branded product (product category)	Companies	2006 US \$M	Medical indication
EPOs	Amgen, Roche, J&J	11,651	Anaemia
Insulins	Eli Lilly, Novo Nordisk, Sanofi-Aventis	9186	Diabetes
Interferons	Schering-Plough, Roche, Biogen Idec Bayer-Schering, Merck-Serono	6788	Multiple sclerosis
Enbrel	Amgen, Wyeth	4379	Rheumatoid arthritis
Remicade	J&J, Schering-Plough	4253	Rheumatoid arthritis
Rituxan (MabThera)	Genentech	3996	Cancer
Neulasta/Neupogen	Amgen	3923	Neutropenia
Clotting factors	Novo Nordisk, Wyeth, Bayer, Baxter	3726	Haemophilia, bleeding
Lovenox	Sanofi-Aventis	3299	Coagulation inhibition
Herceptin	Genentech	3243	Cancer
Avastin	Genentech	2446	Cancer
Growth hormones	Pfizer, Novo Nordisk, Eli Lilly, Serono	2021	Growth disorders
Humira	Abbott Laboratories	>2000	Rheumatoid arthritis
Prevnar/Prevenar	Wyeth	1961	Vaccine
Synagis	MedImmune	>1100	Anti-RSV
Cerezyme	Genzyme	1007	Gaucher's disease

also the very first vaccine product to ever reach the biopharma list of block busters. Proteins such as long-lasting insulin, PEGylated interferon and EPO and TNF fused with the Fc fragment of an immunoglobulin are modifications of today's top selling biopharmaceuticals and represent the next generation. Many more Mabs are also expected to rapidly populate the top selling lists.

Prevnar, a vaccine to prevent invasive pneumococcal disease in small children (Wyeth, Feb 2000), is currently the only blockbuster vaccine. Table 1.3 lists some leading companies in the vaccine field and the size of their businesses. The sum of the top vaccine businesses is smaller, however, than that of the top-selling protein drug product category (all EPOs).

Of the 15 vaccines approved since 1996, analysts considered only four products (rotavirus, Lyme disease, pneumococcal and meningococcal vaccines) to be innovative, and two of these are no longer on the market. The remaining 11 vaccines represent incremental improvements in previously existing products, such as combination products that reduce the number of injections given to children or intranasal delivery for influenza vaccine. These improvements increase the choice of vaccine products available for prevention of a specific infection and even diseases like cancer.

**Table 1.3**

Vaccine manufacturers, sales of vaccines in 2005

Company	\$M in 2005
Sanofi Pasteur	2062
Wyeth (Prevnar/Prevenar only)	1510
GSK	1390
Merck	1103
Chiron	604
Solvay	136
Berna	102

Recently, the quadrivalent human papillomavirus (HPV) vaccine Gardasil® against cervical cancer has been approved (Merck, 2006).

Several major issues discourage development of innovative vaccines. These include product benefit-to-risk profile, liability and return on investment. However, with the current trends in vaccine development described above, we are likely to see the vaccine business accelerate significantly.

Although not visible from the blockbuster statistics, a key part of the biopharma success story has been the treatment of rare ‘orphan’ diseases, i.e. those defined in the United States as conditions affecting fewer than 200,000 individuals. In the United States, 56% of biotechnology products launched between 1982 and 2000 were first approved for orphan drug indications, compared with just 14% of all new chemical entities (NCEs),<sup>1</sup> i.e. drugs of classic chemical type. Patients with orphan diseases such as cystic fibrosis, Crohn’s disease, non-Hodgkin’s lymphoma and haemophilia are among those who have benefited from advances in biopharmaceuticals. Biopharmaceuticals are well suited for treating enzyme deficiencies and metabolic conditions in small patient populations. Cancers and inherited genetic disorders, accounting for more than a third of orphan drug designations, continue to be important focus areas of biotechnology research.

### 1.3.3 Chromatography products used for making biopharmaceuticals

At the same time that biopharmaceuticals were turning into a major opportunity for both the industry and patients, the large-scale chromatography products’ business was also developing.

Based on our background in this industry, we estimate that the size of this particular supply business segment is approximately 500 MUSD (2005), which would represent

<sup>1</sup> A NCE is a chemical molecule developed by the innovator company in the early drug discovery stage, which after undergoing clinical trials could translate into a drug that could be a cure for some disease. Synthesis of NCE is the first step in the process of development of a drug.

**Table 1.4**

Supply companies for large-scale chromatography in biopharma manufacturing and some of their products used for manufacturing

Company	Chromatography products in biopharma manufacturing
Bio-Rad Laboratories	Ceramic hydroxyapatite, ion exchangers
EKA Chemicals	Reversed phase chromatography (RPC) resins
GE Healthcare <sup>a</sup>	Resins for all chromatography techniques, columns, skids, UF membranes and skids
Merck KGaA	Ion exchangers
Millipore	Protein A resins, columns, skids
Pall	Membrane adsorbers, columns, skids
Sartorius	Membrane adsorbers
Tosoh Bioscience	Ion exchangers, hydrophobic interaction chromatography resins

<sup>a</sup>Formerly Pharmacia Biotech then Amersham Biosciences.

around 1% of the turnover of products produced with this technology. Table 1.4 lists chromatography suppliers with significant business in large-scale manufacturing operations and their main categories of products.

## 1.4 KEY BUSINESS ISSUES

The exciting scientific and technical advancements and the rich pipeline easily distract from the fact that progress occurs in a tough business environment and does, indeed, cost a lot of money. At a conference in May 2006, G. Gromo, Head of Discovery Research at Roche (CH) stated: ‘In 2005 the FDA approved only 20 new drugs, down from 36 in 2004. The decline in drug development came as spending on research by the Pharma industry reached a new high, passing 30 billion USD’. It also distracts from the fact that the vast majority of clinical projects fail, because as Dr. Gromo continued: ‘The increased knowledge about disease processes has created as many questions as answers’. In fact this observation has troubled the industry for some time.

There were only a few ‘easy wins’ and the number of diseases with a direct link between one gene or one protein and the cause of the disease is very limited. The complexity of discovery is increasing again. Medical indications with block-buster business potential are limited and competition in these high-gain areas is rapidly mounting. Medical indications for a small patient population do not always deliver profits with sufficient potential to pay back the very significant development costs.

### 1.4.1 Prime challenge, time and cost of new drug development

DiMasi *et al.* have reported the cost of development of a novel drug to be as high as 800 MUSD [10]. However, in the case of biopharmaceuticals, we expect this number to be lower due to smaller clinical studies and higher success rates.

The industry can no longer assume that the biopharmaceuticals of the future will continue to have shorter development times and higher success rates than chemically derived pharmaceuticals [11]. As noted earlier, many of the early biopharmaceuticals were protein-replacement therapies and/or recombinant versions of natural proteins for which therapeutic benefits had already been established. The transition from the selection of these early drug targets of established therapies to those with new therapeutic profile is contributing to prolonged development times for biopharmaceuticals.

Total development times for drug and biological products have been converging since the mid-1980s and are now averaging at 7–8 years [12], which reflects a significant increase for modern biopharmaceuticals. At the same time, the clinical success rate for biopharmaceuticals has declined and is now averaging around 30%, with Mab success rates being lower at 20–25%. These figures can be compared with typical success rates of 15–20% for NCEs [13].

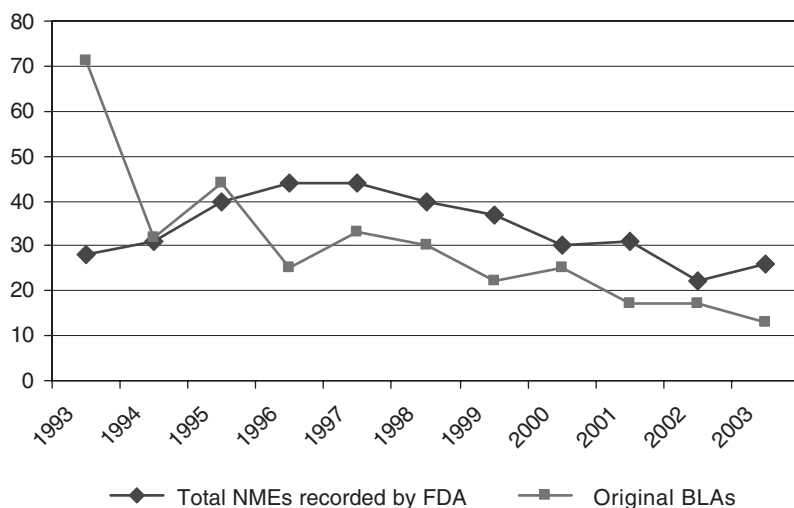
There is an ongoing, world-wide debate about the cost of healthcare systems and potential solutions to the threat of collapse of those systems in highly industrialized countries. S. Burrell has summarized the problem in a way that relates only to a specific issue in large US corporations, but is still very illustrative of the overall importance: ‘General Motors can’t compete as healthcare costs per car are \$1700 higher than at Toyota’ [14]. The biopharmaceutical industry is part of the health care system, and is feeling significant pressure to manage its costs and contribute to an overall reduction of healthcare costs. Growing pressures to contain costs come from managed care organizations in the United States and pricing and reimbursement authorities abroad. These pressures, in turn, also increase pressure on firms to get new drugs to market sooner and reduce development costs, while still providing clear advantages in safety, efficacy or economic value.

Considering the few truly agreed upon facts in this debate and the scope of this book, one may state that the industry is challenged to increase the efficiency and reduce the costs of its manufacturing processes. Another conclusion is that there is a great need to get things right the first time and be as efficient as possible even in development. One week of delay for a 1 billion USD biopharmaceutical represents around 20 MUSD of lost revenue in its first year on the market. This has a number of indirect effects on the discussions in this book and our recommendations for the development of a purification process, the workflow and the priorities of optimization.

### 1.4.2 Other significant business challenges

Despite a great future potential and the ethics of supporting a better life for patients, today’s biopharmaceutical business has left behind the early days of ‘financial innocence’ and is a business just as any other with owners who have high expectations on profit and growth from their investment, further emphasizing the pressure on efficiency and costs in the industry. The challenges this industry is facing are becoming at least as clear as the promises.

Business analysts believe that the rich pipeline will not enable companies to drive growth in line with expectations, and they assume that companies will have to focus on the introduction of product variants to improve their financial performance. Biopharmaceutical



**Figure 1.3** During the last several years, the number of new molecular entities (NMEs)<sup>2</sup> and biologics license applications (BLAs)<sup>3</sup> submitted to FDA has declined significantly [15].

product variants include those such as inhalable insulins for more convenient medication and PEGylated proteins to prolong drug activity post-injection.

In the past decade, the numbers of New Molecular Entities (NMEs) and new biologicals submitted to the regulatory authorities such as the United States Food and Drug Administration (US FDA) has seen a continuous downwards trend (Figure 1.3). This is happening at the same time that R&D investments are rapidly increasing.

In agreement with the business analysts, the US FDA stated in a 2004 report [15] that ‘developing products targeted for important public health needs, prevention indications or individualized therapy is becoming increasingly challenging’ and adds: ‘if the costs and difficulties of medical product development continue to grow, innovation will continue to stagnate or decline, and the biomedical revolution may not deliver on its promise of better health. In fact, with rising health care costs, there is now concern about how the nation can continue to pay even for existing therapies. Similar trends have been observed by regulatory agencies worldwide’.

As drug development becomes more complex and expensive, developers must concentrate available resources on fewer projects. Fewer development projects, in turn, lead to fewer new drug approvals [16]. Early termination of unpromising R&D projects remains a key challenge for drug companies and an imperative if they want to remain competitive [12].

<sup>2</sup> NME is used synonymously with NCE, new chemical entity.

<sup>3</sup> BLA: A request for permission to introduce, or deliver for introduction, a biologic product into interstate commerce (FDA).

The competitive pressure for companies developing novel biopharmaceuticals has increased dramatically. Effective market exclusivity for first-in-class drugs declined fivefold since the 1970s—from an average of 8.2 years in the 1970s to 1.8 years in 1995–1998. Second-generation drugs often provide a therapeutic advantage over first-in-class drugs and can be expected to decrease the advantage of a first-to-market business position [16]. Second-generation drugs are based on novel innovations and developments.

Biosimilars, also called follow-on proteins, biogenerics, etc., are reaching the market after the end of patent protection for the innovator's product. Biosimilars are products that are essentially copies of the original protein drug. The regulatory pathway for approval for these products has been established in the EU, but, at this time, is not clear in many other regions of the world. Insulin, interferon and EPO, all among the top biopharmaceutical economic performers, are the first products being developed as biosimilars.

The extent of, and even the need for, clinical trials for biosimilars will be dictated, in part, by the complexity of the product and the patient indication. Reduction of clinical studies is likely to be the key to lower development costs for biosimilars. Production facilities and processes will, however, not be significantly different in technical or cost terms from the original manufacturing processes, since purity and safety of the product must be assured. Where differences in facility costs exist, for example as a result of lower engineering fees in a country like India, the original inventors may decide to produce in low-cost areas as well or use the economical advantage of a more efficient second-generation manufacturing process.

Competition brings another burden to this business, namely intellectual property protection. Patent protection is sought in biotechnology wherever possible, i.e. anything from gene sequences to methods used for purification. The alternative is to maintain knowledge as trade secrets. While absolutely necessary to manage the risk of operating in a competitive and highly challenging scientific environment, this aspect also adds a significant cost and time loss, not only to run the IP machinery itself, but also through invention and re-invention of a countless number of aspects of research and development at every individual company in the field. In other words, IP management presents a barrier to improving the global efficiency of drug development.

Manufacturing capacity investments are considered a major business issue for companies in this field. In addition to facility and equipment aspects, there are human resource issues. In a survey performed by BioPlan Associates among 100 biopharmaceutical manufacturers around the globe [17], respondents stated that the cost of building, validating and operating a manufacturing facility was considered the single most important barrier to establishing production. Technical reasons were negligible in this respect. On the other hand, there was strong emphasis on improving both cell culture and downstream processing to make the facility more efficient once an investment is made. This clearly points to facility utilization as the most important factor for cost optimization and de-bottlenecking in an existing production facility. Related to this, organization and managerial bottlenecks were described as availability of trained production staff and the need for a stronger focus on production efficiency including product yield. M.E. Kamarck provides a review of alternative approaches applied to overcome the production capacity challenge and proposes



flexibility from outsourcing to CMOs as well capacity sharing between industry leaders as a key future strategy [18].

On a final note related to challenges, the US government has recently published a Proposer Information Pamphlet looking for proposals to develop a system that would be suitable for what is called ‘advanced manufacturing of pharmaceuticals’ [19]. Such a system has been defined by a number of highly challenging specifications and should be capable of producing three million doses of an antibody therapeutic or vaccine against a previously unknown agent within 12 weeks after the agent has been provided to the manufacturer. For the vaccine, the cost of goods should be  $<1$  USD/dose, for the Mab the costs should be  $<10$  USD/dose. There should be no quality compromises when the products are compared to currently marketed vaccines or Mabs. Knowing that under optimal conditions, current time lines are at least 18–24 months from cell line creation to first pilot scale production at a cost of goods for Mabs ranging from 100 to 300 USD/g (one dose may well be equivalent to 1 g), one may be tempted to call this science fiction. However, it is perfect to describe the dream and the nightmare, the ultimate scientific and technical challenges for this industry related to manufacturing capabilities.

### 1.4.3 Biosafety and general drug safety issues

As noted earlier, among the very first modern biopharmaceuticals were proteins derived from human plasma, namely human serum albumin, immunoglobulin fractions and coagulation factors. In the mid-1980s, the world experienced incidents of both HIV and Hepatitis infections from coagulation factors. Those infections were linked to infected plasma donors. Ever since, the biopharmaceutical industry has been challenged with close to zero-risk requirements paralleled only by the aircraft industry. This risk reduction requirement is related, in particular, to virus safety and verification of the removal of potential viral safety risks. Typically, every manufacturing process should include two mechanistically independent virus clearance steps, which may be needed in addition to the purification steps. The process should be demonstrated to clear all known virus classes (see Chapters 3 and 7). It is unlikely that these requirements will disappear or even be slightly reduced. Biosafety cannot be debated.

Radical changes in drug development and testing in humans are being discussed [20]. It is proposed that the current process with large-scale confirmatory clinical trials may have reached the end of its useful life, as it is not always effective in protecting patients, e.g. from non-efficacious drugs and significant side effects. An approach is described where, in the future, disease models, target validation and biomarker knowledge allow shorter clinical studies and more rapid introduction of a drug with greater patient surveillance (Figure 1.4).

Legal systems are hindering such radical improvements in clinical studies as they tend to create a climate of non-forgiving liability. In addition, the modern press literally jump at all bad news. Thus, the will for risk-taking in the biopharmaceutical industry is severely limited. And the same applies to regulatory bodies and their political leadership who would have to clear the way for such changes.

	Current Model	Future Model Targets
Time from Target Identification to Launch	10-12 years	~3-5 years
Pre-launch Cost of Development	~\$800 million	<\$200 million
Success Rate from Entry into Man	5-10%	50% or better

**Figure 1.4** A disease-led approach to drug development will massively reduce the time and resources that are required [20].

#### 1.4.4 Regulatory issues

The US FDA and the European Union's European Medicines Agency (EMA) are the highest profile regulatory bodies. Regulations include a wide range of directives and guidance documents that address basic procedural and behavioural aspects summarized as current Good Manufacturing Practice (cGMP). They also provide non-binding guidance on certain classes of drugs such as Mabs and issues such as virus safety.

In essence, regulations and regulatory bodies are supposed to function to evaluate the quality of medicines and to protect the public from avoidable health risks that could be inherent in these medicines. In addition to providing regulations, authorities attempt to assure drug quality by inspecting manufacturing sites and reviewing relevant documentation and operating procedures.

Many key regulatory issues have been globally harmonized by the International Conference on Harmonization (ICH) guidelines. However, many companies experience approval and inspection procedures that are largely uncoordinated between different parts of the world, which in turn creates major additional costs on the path to market approval without adding a true value.

Guidance documents rarely provide sufficiently detailed instructions about 'what to do' and 'how to do it'. In addition, inspections are unlikely to prevent all potential quality problems. In order to maintain high quality, everyone in the industry needs to stay updated on regulatory matters and the related science.

More recently, the FDA has introduced its Process Analytical Technology (PAT) initiative, which is expected to have significant future influence on the generation of process knowledge during development and on process-control strategies during manufacturing [21]. PAT is intended to facilitate innovation and efficiency in pharmaceutical development, manufacturing and quality assurance. The PAT framework is founded on process understanding and risk-based regulatory decisions by industry and the Agency. A main

effect of this initiative is a potential simplification of regulatory submissions for process improvements. There is also the possibility that, in the future, there will be a reduction of validation requirements, which would be replaced with continuous real time quality assurance.

## **1.5 PROCESS CHROMATOGRAPHY WITHIN AN INDUSTRIAL CONTEXT**

Process chromatography is used to develop and manufacture biopharmaceuticals, biologicals for diagnostics purposes and, more recently, certain food products. It is used to provide a profitable supply of medicinal and other high-value products with strictly regulated quality and safety requirements.

As opposed to just using technical performance criteria, decisions related to the use of process chromatography are driven by sets of rules, which largely originate from manufacturing experience, risk management, environmental aspects, general financial and in particular, cost of goods sold (COGS) considerations. The relevant regulations also influence the use of chromatography.

Although chromatography technology has its roots in biochemical science and can cope with vastly different conditions and an enormous variety of complex and sensitive biological mixtures, its application in biomanufacturing brings important constraints to process design.

### **1.5.1 High-level managerial strategies**

Managers should identify and implement the best way for their companies to achieve success and deliver on expectations. In biopharma research, development and manufacturing, some of the following strategies are frequently used.

Contract Research Organizations (CROs) and Contract Manufacturing Organizations (CMOs) offer flexible resources. The risk of investing in a failure is lowered considerably and the high-attrition rate of biopharma development projects appears less formidable when outsourcing is used. In fact, renting manufacturing capacity is a trend that is not limited to the use of CMO service; several major biopharmaceutical firms have entered into agreements to manufacture other companies' products [22].

In the US, top companies are considering carrying out up to two-thirds of their FDA-regulated clinical trials at lower cost CROs abroad [16]. In a similar fashion, investments into manufacturing capabilities can be postponed or avoided by utilizing CMOs with ready-to-use installed capacity, experienced staff and advanced cell lines and production methods. With these resources, biopharma companies can reduce inefficient and costly re-invention.

Puerto Rico, Ireland and Singapore have developed into hubs for biopharma manufacturing. Strong, biotech-focused support to investors in those countries has created infrastructure, competence and fiscal advantages that have attracted many of the major biopharmaceutical firms as well as a large number of small and mid sized companies.

High-level technical strategies or specific solutions that can significantly increase project throughput in development and the output from manufacturing facilities are being used in most established biopharma companies. More drug candidates need to be put through development and unsuccessful ones need to be rapidly terminated to improve the quality of the pipeline and reduce expensive late stage failures to a minimum.

Risks from uncertainties in planning for production capacity need to be mitigated and related capital expenditures optimized. According to calculations presented by H. Levine [23], utilizing only 50% of a facility can cost 2–3 MUSD per month. Inadequate capacity that results in a loss of sales can lead to operating profit losses as high as 40–45 MUSD per month. This leads to the conclusion that as long as product is saleable, profit losses can have a 10- to –20-fold higher priority for management decisions over costs from production.

### 1.5.2 Development project throughput

Portfolio management dictates an increased throughput of development candidates, preferably at constant or lower cost and with no increase in human resources. Compared to 5 years ago, a twofold to threefold increase in projects per year is not uncommon. This increase has an influence on the workflow used to cope with the challenge and will, in the foreseeable future, create a paradigm shift in development labs in the industry.

Microtiter plates and robots are being used to run large series of experiments in a short time. In process development for chromatography, this approach is used to select chromatography media for specific steps [24] and it is also used to develop and optimize purification parameters. In a 1-day screen, approximately 400 conditions for two steps can be tested, and this consumes less than 1 g of target monoclonal antibody. Conditions found using the robotic system can be confirmed with laboratory scale columns [25].

However, evaluating large amounts of experimental data from a highly automated robotic system in a meaningful fashion remains a challenge. New analytical and data evaluation bottlenecks are created. Further developments of high-throughput systems and modelling of process steps will thus be required to aid in the interpretation of results from massive parallel experiments. Addressing these issues successfully will also lead to more significant progress for the PAT initiative, in which process understanding is key to manufacturing improvements.

### 1.5.3 Manufacturing strategies

Manufacturing for clinical trials requires high quality but the related investments are not financially compensated and must, therefore, be carefully controlled. One manufacturing scenario that offers a good compromise in terms of fixed costs is the use of disposable hardware and flexible facility designs [26]. Tanks can be replaced with bags, piping can be replaced with tubing, disposable pump heads and detection cells can be used, and both cleaning and certain validation efforts can be minimized in a pilot facility. Installation lead times can be minimized and the hardware can easily be moved around based on a

day-to-day need. Disposable solutions offer indirect cost benefits due to flexibility, and they also offer a direct running cost advantage. However, the direct running cost advantage is dependent on scale. For example, it has been calculated that at 500 L cell culture scale, the cost advantage is in the range of 8–14% [27].

Genentech has presented their approach of segregating R&D-related pilot scale manufacturing from cGMP pilot scale manufacturing [28] to allow production of material for research and preliminary validation studies with shorter lead time, lower costs per run and less conflicts with the rigid scheduling of cGMP runs. The efficiency of an intensive operation with more than 400 runs per year and with more than 10 development projects can be boosted with such segregation.

Process intensification strategies are being implemented to maximize the utilization of existing facilities, reduce processing time per batch and eliminate any non-productive time in the facility. Process intensification refers to the elimination of unproductive activities in order to improve facility utilization. Process intensification is widely applied today, especially in very large-scale Mab or insulin manufacturing. In-line buffer preparation and use of buffers suitable for this approach have helped to obtain a 30–40% reduction in total buffer preparation and hold time requirements for equilibration buffers alone. Use of high-titre cell culture in combination with modern chromatography resins operated closer to the limits of capacity makes chromatographic capture of 20,000 L at 5 g/L feasible in Mab manufacturing at a contract manufacturing organization (CMO) [29]. All conditioning steps that do no more than preparing the intermediate product for the next manufacturing step are gradually disappearing from production schemes.

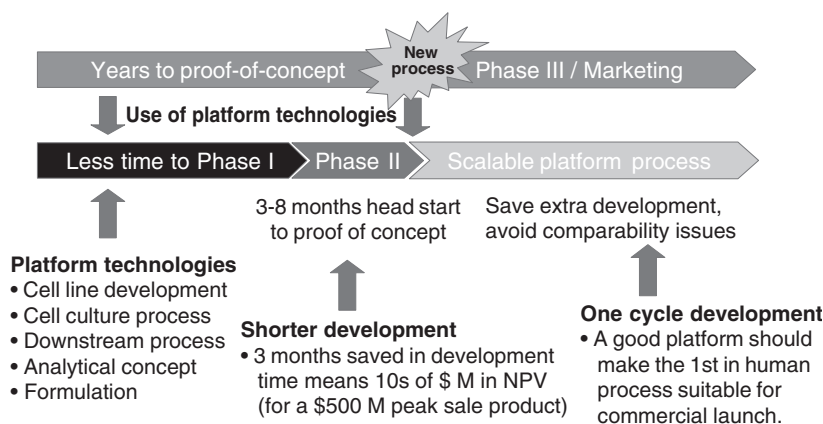
#### 1.5.4 Technology platforms

Technology platforms may be defined as a ‘standard’ set of conditions and methods applied to all molecules of a given class [30]. All raw materials and methods used in a platform should be regularly reviewed as science develops to make sure the technology remains current.

P. Bezy has reported time savings of 3–5 months using platforms for a fast-track development approach. Platforms were applied in all key aspects of development, including cell line development, cell culture, downstream processing, analysis and even filling. A head start of only three months into clinical trials can mean tens of MUSD of increased net present value (NPV) [28].

If the platforms used for clinical manufacturing can be used at commercial scale with little or no modification, then similar or even higher value can be gained once again and it will be less of a challenge to demonstrate comparability after scale changes are made (Figure 1.5). Although some modification may be required when using platform technologies for different molecules, e.g. different Mabs, major process changes should no longer be necessary upon scale-up of the pilot process.

At this time, platform strategies are mainly used for Mab development, since new candidate monoclonal antibodies are usually sufficiently similar in key characteristics. Typical Mab production platforms include the use of mammalian production cells such as CHO cells or murine myeloma NS0 cells, centrifugation for harvesting, Protein A affinity



**Figure 1.5** The value of technology platforms. Applying platform strategies to development and manufacturing promises the highest gains among the options to successfully manage the complexity of the R&D pipeline.

chromatography as first purification step, virus clearance using acid pH inactivation and virus filtration and usually two polishing steps using two orthogonal mechanisms of chromatography. The last step is typically TFF to formulate the purified bulk material. Analytical methods are usually similar for all Mab processes.

Since the chromatography resins and other materials are fixed in any given platform, the effort for basic development and optimization of each method is significantly lower than in a random approach to process design. Vendor certification and other regulatory aspects of development are addressed only once, and a great deal of experience with the process reduces human error to a minimum.

In the foreseeable future, platform strategies will most likely be used even for drug categories such as plasmid DNA-based vaccines, virus-based vaccines and fragments of monoclonal antibodies. At this time, recombinant proteins do not have a technology platform for purification that is unilaterally applicable. Instead, each process needs to be designed on a case by case basis as there is no efficient general-use capture step available.

### 1.5.5 Constraints on technology and product choices

As discussed in the previous section, technology platforms may dictate the choice of technology and specific chromatography media for a particular category of protein drug. However, there are a number of general aspects that are important to consider in selecting technology, and these are valid even if there is no established platform.

Tools such as process chromatography must generate robust processes that can tolerate starting material and chromatography resin variability. Robustness is verified in process validation. However, without proper selection criteria, there is a risk that a process will end up neither robust nor validatable.

**Table 1.5**

Key selection criteria for chromatography media in a production environment

Selection criteria	Explanations
Availability of production scale quantities	Estimated column filling in final scale and lead time to delivery
Batch-to-batch variability of chromatography media	Testing of several independent batches with final material acceptance criteria
Packing in production column	Instructions, services and proofs provided by vendor, experience of own team
Resistance to preferred CIP routines	Sodium hydroxide is the industry standard and offers best cost–performance ratio
Separation performance	Selectivity, binding capacity, product recovery, buffer consumption, process time
Cost of use	Life time in re-use regime, cost of buffers, purchasing price
Vendor	Successful vendor certification

In the case of chromatography resins, some of the main selection criteria are listed and explained in Table 1.5.

Technology choices are also limited by the use of an existing and equipped facility for a new product. The size of columns, the number of buffer tanks and environmental constraints at the facility may limit the choices, e.g. the number of buffers and buffer types, use of certain salts or use of technology requiring safety standards such explosion-proof installations. If space is limited, capacity needs may dictate the selection of chromatography media that can be scaled up with longer beds rather than the typically used wider columns.

## 1.6 SUMMARY

The degree of maturation in this industry suggests that everyone involved with the development of processes or with their application in manufacturing should understand the basic drivers that guide or even dictate technology choices and the regulated environment in which the technology is applied and business decisions are made.

In the many following chapters of this book, we will discuss process design and validation of processes in great detail and will attempt to provide advice for achieving robustness. We also present more details on the various opportunities and challenges described in this chapter. Readers are encouraged to use the information as a reference for decision making and understanding the regulated manufacturing environment in which their work is being performed. While many of the numbers presented here will change, it is unlikely that the mechanisms under which the industry operates and which we have tried to describe will change very much in the near future.



Process chromatography tools have improved in capacity and flow velocity that lead to higher productivity, but the strategy to achieve a robust process has remained very similar over the last decades. We anticipate that the tools will undergo further development and achieve even higher productivity. But with the introduction of multiproduct facilities and very large scale, intensively operated, dedicated facilities, it will also be very important to ensure that the individual process steps can be embedded into the facility and its routines. For most production facilities, this requires flexibility to make many products, significant reduction of non-productive activities and reduction of the cost of use. These factors will become equally important criteria for the development of novel chromatography media with further enhanced capacity and volume throughput. Robustness of the processes and quality of the produced protein drug, however, cannot be compromised, not today, and not in the future.

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## – 2 –

# Process Capability and Production Scenarios

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This chapter presents an overview of typical scenarios for product-development phases, market needs for biopharmaceuticals and some features of available technologies relevant to process capabilities. However, detailed process-design strategies including selection criteria for production organisms and their impact on the process scenarios and capabilities are discussed in Chapter 3.

## 2.1 PROCESS CAPABILITY

Any production scenario depends on the capability of the process to deliver product of the required quality in the quantity needed for the purpose at each moment in the life cycle of the product. Within most companies, however, a different perspective of process capability exists across the various functions, e.g. development and manufacturing.

### 2.1.1 The process scientist's perspective

A process-development scientist may state: each downstream process needs to be capable of 'handling' the product, which is either produced in mammalian cell culture or microbial fermentation or is provided from natural sources such as human blood plasma, eggs or transgenic materials. The term 'ability to handle' describes a technical capability to isolate the product from biomass, which may contain debris or other material causing a significant burden on the ability of the process to perform consistently, and also to remove any impurities and biosafety hazards from the isolated product in a manner that reproducibly yields the desired quantity of product with all of its critical quality attributes.

### 2.1.2 The production manager's perspective

A typical statement of a production manager might be: 'Process capability refers to the ability to produce the required quantity of the product. The product must be produced in

**Table 2.1**

Example of batch time for mammalian cell culture compared to downstream processing (DSP)

	Batch time	Batches/year <sup>a</sup>	Productivity factor
Mammalian cell culture	12.3 days	28	1
Downstream process	2 days	172	~6

*Note:* Dependent on the degree of process intensification, downstream processing has up to six times higher relative productivity assuming similar product quantities handled per batch (calculated as factor between batches/year for the two parts of the process).

<sup>a</sup>Single fermentor, 3 weeks facility downtime included.

due time and in the facilities available for the manufacturing operation. The overall production costs must not exceed what the product can bear when reaching the market place, while still delivering the expected profitability.<sup>7</sup> This aspect may initially be isolated from technical considerations such as product sources and methods used in manufacturing. It becomes possible to utilize straightforward modelling tools to predict capability and compare alternative production scenarios.

However, there are several interdependencies and the selection of technical options can have significant impact on the general process capability. As an example, Table 2.1 illustrates the challenge of linking mammalian cell culture with downstream processing, due to very different batch times of the two parts of the process. A small number of biopharmaceuticals (currently one marketed recombinant Factor VIII and one monoclonal antibody: Remicade<sup>®</sup> by Centocor, Johnson & Johnson) is being produced in continuous cell-culture processes with frequent but small-volume harvesting and subsequent proportionally adequate small-scale downstream processing. This option allows smooth linkage between cell-culture and downstream processing, but has not found widespread acceptance, probably due to the relative simplicity of fed batch processes and the progress made with their productivity.

The choice of production organism may have an impact on this issue, if the organism is not already dictated by critical product attributes such as glycosylation. Under certain circumstances, microbial systems may enable higher productivity at lower costs than mammalian systems, which are currently most widely used in manufacturing of complex proteins.

The example shown in Table 2.1 demands six fermentors to match the production capability of one downstream line in terms of batch frequency. For simplification, we assume that the product quantity from cell culture can be processed by the downstream line within the batch time (more details see later in this chapter). This example represents a high degree of process intensification as found in some dedicated facilities with full year operation at close to 100% utilization and may not be representative for the average currently operating manufacturing facility. However, it is representative in terms of what is possible today and is important to be aware of, in particular whenever downstream process capability is being debated versus the advances in upstream processes.

In order to achieve this high batch frequency, significant automation and a staffing level with 6–8 operators is required on each the upstream and the downstream processing line. Preparing culture media, maintaining the seed and inoculation trains for six fermentors,

preparing a variety of downstream buffers, sampling for analytics from the fermentors running in parallel and from up to ten different steps in the overall downstream sequence (see Chapter 3) and not the least documenting the operation requires significant staffing levels. Automation can partly replace staff, but not entirely.

The production manager may therefore face a different reality with a wide range of variation on the challenge: an existing facility design may not have foreseen the development of product titers from cell culture and the downstream line may not be dimensioned to handle the batch size from the fermentor. Instead of 2 days, the batch time for the overall downstream process may be as long as 5 days. Publicly reported cell-culture batch times vary between 12 and 20 days. Most processes reported to have titers above 3 g/L seem to run longer than 15 days [1]. Processes designed back in the eighties and early nineties may have batch times as low as 6 days at comparably very low product titer [2]. Processes as different as this may have to fit into the same facility. Automation as well as available staff may be limited, thus limiting the options to intensify the operation and run more batches in the same facility.

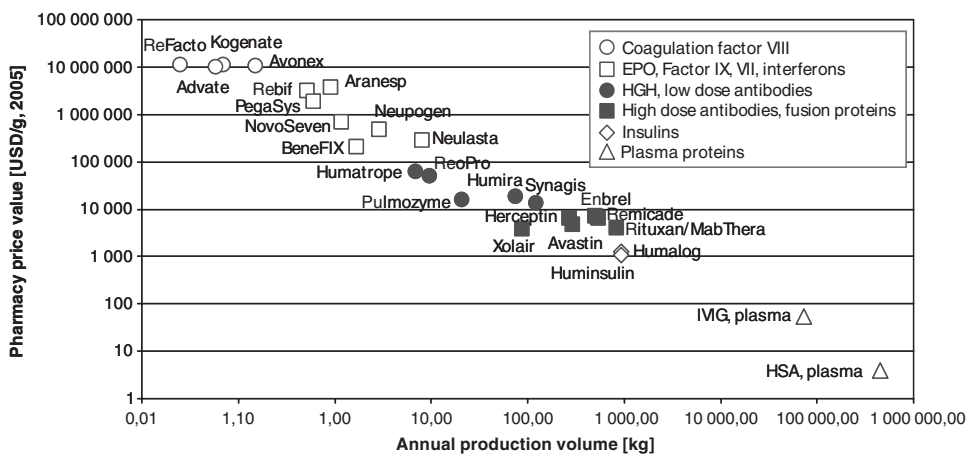
However, with product titers from cell culture increasing, a fermentor of a given volume can produce more product per batch and the downstream process needs to be scaled proportionally to accommodate this increase. Alternatively, downstream steps can be cycled several times at a smaller scale than needed for the total product quantity for each batch. The short batch-processing time enables this alternative and makes it attractive not the least when scale-up is considered expensive.

The production manager must ensure that an industrial process with sufficient process capability meets the necessary technical and regulatory requirements. Capabilities such as producing purity and safety cannot be compromised and are thus not really variables in biopharmaceutical manufacturing.

Management focuses on market and economy aspects. It is at this level where the benefits of planning for development and manufacturing and utilizing a well-designed process make the difference for the company's financial success. Preparing for process capability creates the match between technical ability and market needs as well as financial optimization. The decision for outsourcing versus in-house manufacturing, as well as the choice to build capacity dedicated to one product or instead more flexibly for multiple products are also part of the management challenge in this context.

### 2.1.3 How much product needs to be made?

This is the first question to be addressed in a hierarchy of considerations leading to an understanding of production scenarios. The answer to this question will vary between phases of development and among types of biopharmaceutical products produced. Manufacturing scale may range between a few hundred grams and metric tons annually. Figure 2.1 plots the estimated annual production volume for different biopharmaceuticals on the market today. The graph has been calculated from 2005 global sales information available from company annual reports and from selected pharmacy list prices in the US and Europe. Lowest prices between countries for large packs of the medicine have been chosen to simulate the official discounting of prices.



**Figure 2.1** Calculation of minimum production quantity of biopharmaceutical medicines. Product sales information from annual reports, pharmacy prices and package information are used in this calculation.

Six different categories of biopharmaceuticals can be differentiated. The first category contains recombinant anti-coagulation factors with a production need of  $\sim 100$  g/year and a sales value of  $\sim 10$  MUSD<sup>1</sup> per gram. The second category contains a number of recombinant proteins such as EPO<sup>2</sup> derivatives, interferons and coagulation factors VII and IX with a production quantity up to 10 kg/year and a sales value between 100 KUSD and 5 MUSD per gram. In a third category we find human growth hormone and some relatively low-dose monoclonal antibodies with annual sales of up to 100 kg and a sales price of 10–100 KUSD per gram. The fourth category contains the more typical high-dose monoclonal antibodies with production volumes between 100 kg and 1–2 metric tons. The sales value of such products lies between 4 and 10 KUSD per gram. Recombinant human insulins and their derivatives are placed in a fifth category. It seems that different insulins are produced at  $\sim 1$  ton per year for each of the market leading brands with a sales value of  $\sim 1000$  USD per gram of product. At a dose between 1.4 and 2.1 mg, the total annual demand for insulins in the industrialized world was estimated to be 4600 kg in the year 2000, i.e. in decent fit with this calculation [3]. Finally, certain human plasma proteins are produced at multiple metric ton scale: intravenous IgG annual production rate is 50–100 tons at a price of 50 USD per gram in the US market and human serum albumin sells at 5 USD per gram and the worldwide with a production scale is almost half a million metric tons annually.

Annual need is, however, not only a function of patient populations and dosage regimes. At the price levels found in Figure 2.1 annual need becomes a function of affordability as well (see discussion in Chapter 8.1.1).

<sup>1</sup> USD = US dollar, MUSD = million US dollar, KUSD = thousand US dollar.

<sup>2</sup> EPO = erythropoietin, therapeutic recombinant protein.

Table 2.2

Categories of biopharmaceuticals, estimated actual production quantities and sales values based on the calculations in Figure 2.1 adjusted with estimates on net revenue per gram produced product

Category number	Quantity per product and year	Sales per gram	Examples
1	Up to 0.5 kg	5–10 MUSD	Factor VIII
2	Up to 15 kg	50 K–5 MUSD	EPO, interferon, factor IX, VII
3	Up to 200 kg	5–50 KUSD	hGH, low-dose Mabs
4	Up to 3 tons	2–10 KUSD	High-dose Mabs
5	Up to 2 tons	0.5–1 KUSD	Insulins
6	50–500 tons	5–50 USD	iv IgG, HSA

Note: See discussion in text.

Due to the method of calculation chosen for Figure 2.1 the estimated production quantities reflect the amount of product that can be sold by the manufacturer. However, manufacturers may not be able to invoice at pharmacy price levels as they may work through a longer distribution channel. This would mean that their reported sales have to be calculated from more units produced at a lower sales value per unit.

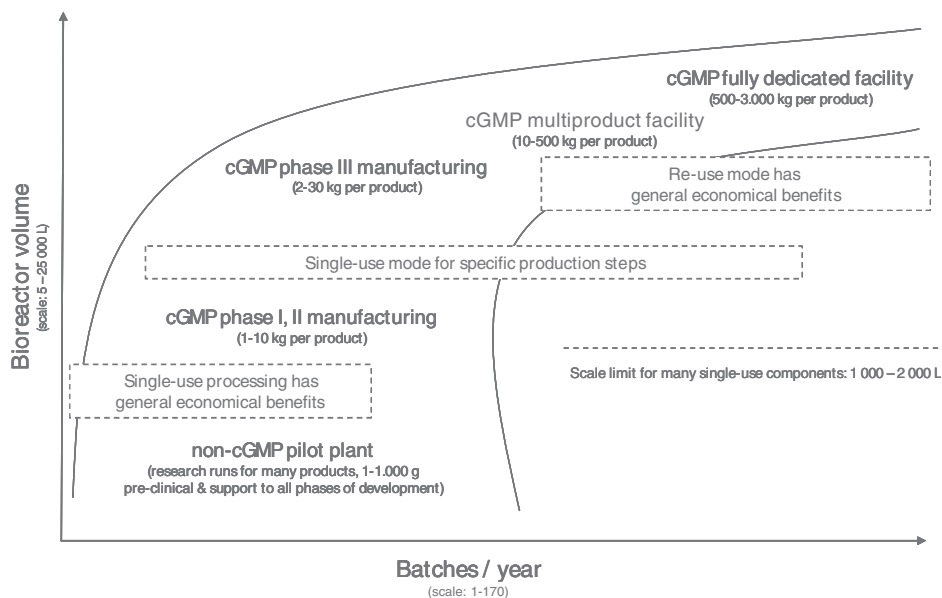
In addition, we assume that significant additional quantities need to be manufactured as a consequence of the following: for category one medicines, the amounts required for analysis and sampling may be multiples of the quantities going to patients. Proteins formulated at high concentration are particularly prone to losses to sampling and analysis as these are usually volume based. Dependent on the efficiency of the distribution and sales channels, protein drug shelf life may limit the portion of product than can be sold. In all categories (except human plasma products where actual plasma volumes processed are reported) this may lead to an increase of production needs versus what is plotted in Figure 2.1 of the order of 1.5- to 3-fold (see Table 2.2).

As a result of these considerations, it can be assumed that the current biopharmaceuticals sold in the greatest quantities are being manufactured at low single-digit metric ton scale, i.e. 1–3 tons per year. The smallest products probably do not require more than 500 g of annual production capacity.

Finally, the overall manufacturing process has a limited product yield in the range of 50–80%. Therefore, cell culture and fermentation need to deliver up to twofold more of the raw product than what can be filled into vials at the end of the production train.

2.2 PRODUCTION SETUPS

Figure 2.2 maps different situations for manufacturing at a range of scale and batch frequency, mostly under cGMP requirements. At the low end, preparative non-cGMP runs yielding between just 10 g and up to 1 kg are required to produce for pre-clinical studies and to support, e.g. characterization studies accompanying clinical trials and process



**Figure 2.2** Production scenarios: non-cGMP preparation for pre-clinical and characterization studies, phase I/II and phase III manufacturing under cGMP, regular manufacturing in multiproduct or in dedicated facilities.

development with appropriate quantities of drug substance. Each time, a few runs are set up on request. Over a year's time though, hundreds of individual runs need to be performed. It may thus be advantageous to segregate cGMP and non-cGMP operations once a company reaches a certain critical mass of development activities. This expedites development when it is not necessary to manufacture under the stringent environmental and scheduling requirements needed for cGMP compliance [4].

Clinical trials during phase I and II may require 1–5 kg of drug product, in rare cases up to 10 kg. This would include samples to be retained and assumes a somewhat lower process yield at an early stage of process optimization. The production of these quantities can conveniently be performed in one short campaign with a few batches. For monoclonal antibodies this may involve use of a 2000 L fermentor and 2–5 batches at a product titer of 1–2 g/L.

Clinical trials during phase III will require larger drug product quantities, perhaps as high as 10–30 kg, depending mainly on the size of the patient population to be tested and on the dosage regimes. Current regulatory concepts suggest that this manufacturing should take place at a production scale similar to the intended final scale. At this time, both upstream and downstream processes need to be fully optimized to mimic the process that will be submitted for approval once clinical studies are completed. Dependent on the product titer, manufacturing could be achieved with as few as five batches, e.g. at 5 g/L product titer in a 10,000 L fermentor. Alternatively, it may be decided to operate at smaller scale, but with higher batch frequency and better facility utilization.

Today, dedicated facilities are manufacturing products at any scale between several hundred kilograms and multiple tons. Regular manufacturing as such varies in even wider

ranges, from as little as a few hundred grams to multi-ton quantities in any given year. With the relatively low product titers that were achievable until recently, several large fermentors needed to be operated continuously in order to manufacture even smaller or mid scale products. An example for this category may be Enbrel, developed in the second half of the nineties. M. Kamarck [5] describes this case: 'From day one, demand for Enbrel exceeded expectations. Together, Amgen and Wyeth have brought online three large facilities for Enbrel manufacturing in capacity collaboration'. Boehringer Ingelheim, a CMO was contracted to provide even more manufacturing capacity. Figure 2.1 shows, however, that Enbrel is not produced in extraordinary quantities. Instead the issue really is that it was among the first substances in the large-volume class and was developed when product titers still were below 1 g/L. At titers currently achievable, keeping in mind that most monoclonal antibody products require annual production of no more than several 100 kg, manufacturing of most high-volume products could be performed in one facility—especially with some of today's largest installations with 4–6 times 15,000–20,000 L fermentor volumes.

The trend towards high product titers in cell culture can be utilized to reduce the batch numbers required to make a given quantity. In this case, facility utilization would be achieved through operating the plant with many different production processes and turning it into a multiproduct facility (see Section 2.3).

Figure 2.2 also puts labels related to single-use or re-use production scenarios next to the scale-based categories. Alternative to building very large bioreactors, one may decide to produce the medium quantity products (up to several hundred kg per year) in dedicated plants with no more than, e.g. 1000 to 2000 L fermentor scale and operate full-time for better facility utilization. At this bioreactor scale, the use of disposable fermentors, tanks, piping and devices for filtration and purification becomes a viable option. Use of plastic components for disposable operation is limited for mechanical stability and economical reasons beyond that scale. However, certain devices are used in disposable mode operation even at very large scale, for example virus filters and sometimes also membrane adsorbers. We will discuss economical aspects of this in Chapter 8. In general, re-use mode operation of process steps has economical advantages in large-scale scenarios with longer production campaigns. Single-use operation offers economical benefits when few batches are run and multiple re-use of raw materials is not possible.

At very large scale and in most dedicated facilities, fixed installations with stainless-steel equipment are currently preferred over disposables and plastics, but the trend towards higher titers facilitating production with fewer batches per product may change this landscape quite significantly in the future and favour multiproduct facilities with a high content of disposable equipment. Cost of change-over is another key driver favouring this development.

Finally, there is another constraint for this discussion about production scenarios, which is triggered by the development of product titers, i.e. whether one should run a few large batches, alternatively smaller but many batches. Analytical demands may currently be prohibitive for going towards more batches to produce a given quantity of product. Efforts for analysis are directly proportional to the number of batches and represent already up to a third of manufacturing costs. A further increase may eat up the advantage created by smart production setups. From the analytical department's perspective big batches are beautiful. A paradigm shift is needed to change this: it seems that a very significant reduction of analytical tests for the batch would be required. Extensive use of



process understanding leading to a good correlation between few assays and critical quality attributes of the product may be one route to achieve this.

### **2.2.1 Production setup for several hundred grams up to ten-kilogram scale**

In this category, we have grouped a large variety of different protein drugs with a similar variation of production sources and process designs (see Chapter 3). The choice of production source, if there is a choice, will largely determine the production scenario in general and also the specific process design with its harvesting and purification technology. However, at a general level and from a process capability perspective, it can be stated that there are no major questions and almost certainly no serious technical limitations or even stumbling blocks to be expected.

At the scale between grams and a few kg, annual quantities can be produced either in very few batches using large fermentors or in smaller production schemes operating permanently or in long campaigns. The Amgen process for EPO is run with roller bottles and can satisfy the demand, e.g. of 1 kg/year for the United States [6] as well as the need for the global market.

### **2.2.2 Production setup for several tens of kilograms up to multi-ton scale**

At this scale, there are questions that, when answered, lead to an understanding of optional production scenarios. How can the larger product quantities be manufactured? What are the principle options? How can cell culture and downstream processing be aligned to accommodate the production needs? Are there fundamental limits of implementation and, if so, where are they?

For the very high level of production, B. Kelley has described a model study for a 10 ton monoclonal antibody production scenario from mammalian cells [7]. At a titer of 5 g/L, six parallel 15,000 L fermentors and one downstream line were fully capable of such annual production. Making 20 tons would require no more than adding another downstream line with a space requirement as low as 400 m<sup>2</sup>. In this presentation, it was concluded that drug products at multiples of 10–100 ton scale would need unique specific production scenarios and process designs. The author questioned, however, that such a production scale would be needed for any of the biopharmaceuticals currently known to be in development.

With this reference as guidance, we will discuss monoclonal antibody manufacturing and mammalian cell culture as a model and analyse the capabilities of both the cell culture and the downstream process at this large-scale scenario.

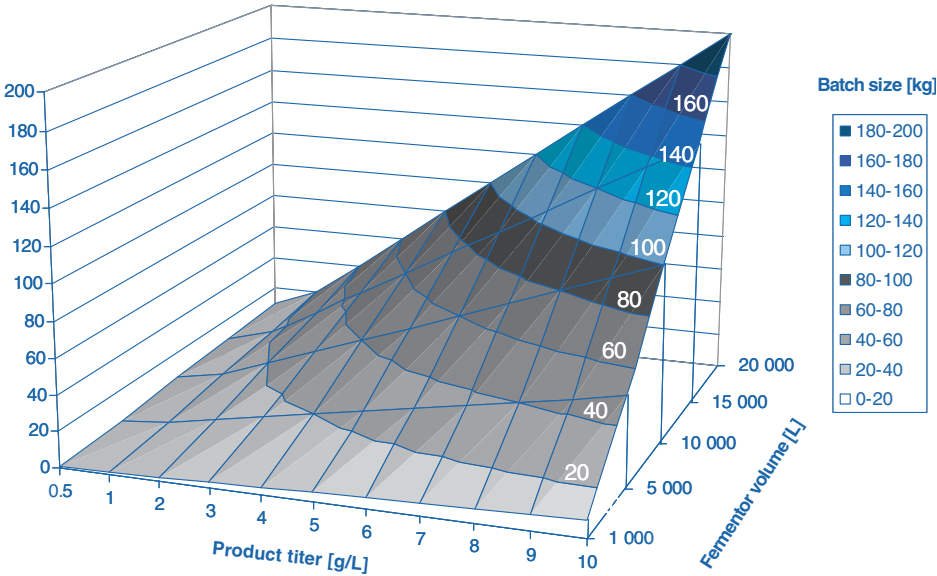
#### *Upstream process*

While production processes developed in the early-mid nineties yielded product titers below 1 g/L of cell-culture volume and while some more recent cell-culture processes have reached 2–3 g/L of product titer, it seems that products currently in clinical development

may well achieve production levels of 5 g/L or higher in highly optimized mammalian cell culture. Titrers of >10 g/L for mammalian cell culture are said to be in reach within the next decade. The development in cell-culture productivity has two main drivers: cell density in fed batch cultures increased from  $2 \times 10^6$  cells/ml (1986) to  $10 \times 10^6$  cells/ml (2004). In the same period specific productivity increased from below 10 pg/cell/day to around 90 pg/cell/day [2]. An efficient process has high cell density with high specific productivity.

B. Turner presented an overview of Mab cell-culture processes from different companies with regard to their product titers. Interestingly, all processes with titers higher than 3 g/L had batch times between 14 and 20 days [1]. Obviously, this would reduce the possible number of batches from a fermentor per year quite significantly compared to what we assume, i.e. 28 batches at 12 days (Table 2.1). With an extension of the duration of cell-culture processes in this way, purification processes could also run longer, e.g. as a result of increased batch cycling with smaller columns. However, product stability in process pools may become a constraint. It is very important to realize that titer is meaningful only as long as the desired biological activity is produced. If a significant portion of the product produced at high titer is biologically inactive, it may be better to chose conditions with lower stress for the producer cells.

Figure 2.3 illustrates the batch capacity of mammalian cell culture at different product titers and fermentor volumes. Assuming the near term future of product titer levels around 5 g/L one 2000 L fermentor could produce the annual market need for many rProteins in a single batch. One 15,000 L fermentor would deliver batch sizes of 75 kg and an annual capacity of almost 1700 kg (28 batches at 80% process yield). Six such fermentors could satisfy a—currently hypothetical—10 ton product need.



**Figure 2.3** Production fermentors and their batch capacity at different product titers.

**Table 2.3**

1000 and 10,000 kg annual production need for monoclonal antibodies, required and allowed batch frequency dependent on fermentor installations

Product to patient	1000 kg	Batches per fermentor				
Process yield	80%					
Titre	5 g/L					
	Fermentor volume					
Data	1000	2000	5000	10,000	15,000	20,000
One fermentor	250	125	50	25	17	13
Two fermentors	125	63	25	13	8	6
Four fermentors	63	31	13	6	4	3
Six fermentors	42	21	8	4	3	2
Eight fermentors	31	16	6	3	2	2
Product to patient	10,000 kg	Batches per fermentor				
Process yield	80%					
Titre	5 g/L					
	Fermentor volume					
Data	1000	2000	5000	10,000	15,000	20,000
One fermentor	2500	1250	500	250	167	125
Two fermentors	1250	625	250	125	83	63
Four fermentors	625	313	125	63	42	31
Six fermentors	417	208	83	42	28	21
Eight fermentors	313	156	63	31	21	16

*Note:* The white background marks allowed options; the dark background marks options beyond the current capability of mammalian cell culture. Values are calculated for a range of installation options from one to eight fermentors per downstream line. 12 days is the assumed batch time.

□ Required number of batches < allowed.

■ Required number of batches > allowed.

In Table 2.3 we illustrate two model scenarios with annual production needs of 1000 and 10,000 kg. For the lesser quantity, a dedicated facility with six 2000 L fermentors can produce sufficient quantities for the market. The 10 ton product requires essentially the same setup, but with six 15,000 L reactors. The 1000 kg scenario might even be run with disposable 1000 L fermentors. The higher number of batches required in this case can be achieved with ten fermentors operating at any given time. This may or may not mean that two downstream processing lines are needed. That need is dependent on the achievable degree of process intensification (removal of unproductive activity from the process) in this part of the process.

This discussion confirms that any currently anticipated product quantity can be manufactured with typical cell-culture bioreactors, assuming cell-culture productivity that already has been achieved in pilot scale setups [1].

Obviously, microbial fermentation systems could be at least as efficient due to their shorter batch times and somewhat higher achievable product titers, provided the product in question lends itself to production in bacteria or yeast.

Downstream process

How does the upstream scenario presented here match with the downstream purification process? The required surface of membranes as well as the volume of chromatography resins are proportional to the product quantity to be processed per batch and may reach hardware design limits with ever increasing batch sizes from the upstream process. Table 2.4 looks at chromatographic resins and calculates the column volume required to process a range of protein quantities per batch. At the same time, we illustrate the use of cycling to reduce the required column volume as compared to processing the whole batch in one run.

Table 2.4

Resin requirement for processing of one batch at different binding capacities

Capacity utilization 80%									
Batch (kg)	Low	Affinity resins	High						
			Low	Ion-exchange resins				High	
Resin capacity (g/L)									
5	20	30	40	50	60	70	80	90	100
10	313	208	156	125	104	89	78	69	63
20	625	417	313	250	208	179	156	139	125
30	1250	833	625	500	417	357	313	278	250
40	1875	1250	938	750	625	536	469	417	375
50	2500	1667	1250	1000	833	714	625	556	500
60	3125	2083	1563	1250	1042	893	781	694	625
70	3750	2500	1875	1500	1250	1071	938	833	750
80	4375	2917	2188	1750	1458	1250	1094	972	875
90	5000	3333	2500	2000	1667	1429	1250	1111	1000
100	5625	3750	2813	2250	1875	1607	1406	1250	1125
125	6250	4167	3125	2500	2083	1786	1563	1389	1250
150	7813	5208	3906	3125	2604	2232	1953	1736	1563
175	9375	6250	4688	3750	3125	2679	2344	2083	1875
200	10,938	7292	5469	4375	3646	3125	2734	2431	2188
	12,500	8333	6250	5000	4167	3571	3125	2778	2500

Many processes in operation today have batch sizes of 5–10 kg and can be processed in one cycle with the 800 L column used as an example for Table 2.4. In fact, at all resin-binding capacities from 20 to 100 g/L the columns can be much smaller for such batch sizes. Currently, the largest batch sizes are in the range of 50 kg and here an 800 L column packed with a resin of 30 g/L would require running at least three cycles per batch, e.g. in an affinity capture step. A modern ion exchanger with 100 g/L binding capacity and 800 L column volume could process 50 kg in one cycle. A first-generation industrial ion exchange resin still in operation in many plants would probably have around 50 g/L binding capacity and would reach a limit for processing in one cycle at batch sizes around 40 kg (column size may be larger than assumed in Table 2.4).

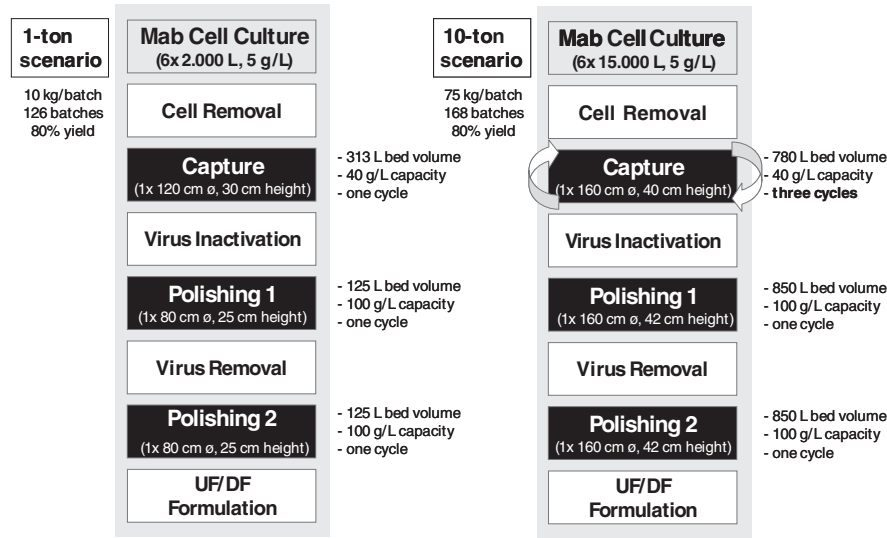
Table 2.4 allows looking into future scenarios, i.e. when more companies have reached titers of 5 g/L or even higher. It becomes obvious that product titers around 7.5 g/L used in currently installed 10,000–15,000 L reactors would lead to a milestone in batch size and process scale terms, since batch cycling would become a requirement even when using resins with very high binding capacity at 100 g/L or higher. Consequently, production scenarios with very high annual drug quantities would probably necessitate the use of more than just one downstream processing line in parallel to cope with the batch sizes within the allowed time frame of approximately 2 days.

In the 1000 kg annual product need scenario discussed above, an operation with six 2000 L fermentors was assumed feasible. At a titer of 5 g/L the batch size would be 10 kg, which could lead to a downstream processing setup as illustrated in Figure 2.4 (left), if the batch is to be processed in one run. Alternatively, one would use a ~800 L affinity column with 40 g/L capacity to handle the 75 kg monoclonal antibody from a 15,000 L fermentor with 5 g/L product titer in three cycles for the capture step. The subsequent polishing steps using modern ion exchangers at 100 g/L capacity could still manage this quantity in one cycle. This would support the 10 ton product scenario discussed earlier (Table 2.3).

Column dimensions in the two scenarios in Figure 2.4 are 120 cm in diameter with 30 cm bed height and 160 cm diameter with 40 cm bed height, respectively for the capture steps. Even in the 1 ton scenario, the capture column can be cycled and its volume reduced. The polishing columns have 80 cm diameter at 25 cm bed height and 160 cm diameter at 42 cm bed height, respectively. The largest feasible column would have a diameter of 200 cm and allow a bed volume of >1200 L. The scenarios in Figure 2.4 do not require any extraordinarily large equipment or extensive batch cycling.

Using 4–6 cycles per batch is current practice with affinity steps for Mab capture. Cycling is, in practice, often limited to the affinity purification of monoclonal antibodies with Protein A resins. Facing a future with larger batch sizes, one can conclude that higher capacity on the Protein A step is desirable to limit the need for extensive cycling regimes at this point in the downstream process. One limit for cycling may be in product stability while still in crude feedstream conditions and in the total time spent with the capture operation as a result of multiple cycle processing (Figure 2.4).

For a discussion of the process design used in the scenarios, please refer to Chapter 3. Notable capacity increase and operation with smaller columns can be achieved when changing operational mode from bind-elute (useful capacity limit at ~100 g/L) to flow-through mode where only impurities are adsorbed and more than 200 g of product per L



**Figure 2.4** Production setups confirming feasibility of chromatographic steps at 1 ton as well as 10 ton scale of annual production.

**Table 2.5**

Processing time and key characteristics for purification steps

	Capture	Polishing 1/2	Comments
Resin type	Affinity	Ion exchange	Typical choices
Capacity (g/L)	30–40 g/L	70–100 g/L	Latest-generation resins
Fluid velocity (cm/h)	300–500 cm/h	500–1000 cm/h	Latest-generation resins
Residence time (min)	5–8	2–5	Bed heights 30–40 cm
Processing time (h)	3–5	2–4	Time per cycle

*Note:* Without much optimization the longest time a chromatography step will take is 5 h for a complete cycle. Even with three cycles per batch, each column would become available to load the next batch after less than 24 h. In an automated plant prepared for intensive operation, this leaves a sufficient time buffer to operate the complete downstream process within 2 days and confirms the model calculations in this chapter as feasible.

of resin can be processed. The required column volume would then be reduced by a factor of 2–5 dependent on the amount and character of impurities to be cleared.

As a final feasibility check for the scenarios discussed in this chapter, we need to examine the processing time. The assumption in the discussion throughout this section is that fermentors can be harvested every second day and the downstream process takes 2 days to complete before the next batch is downloaded from the next fermentor.

Table 2.5 summarizes processing time information for the capture and polishing steps used in the scenarios above and confirms the feasibility of these model process setups.

The model of a three-step process for monoclonal antibodies used for this discussion of process capability can be run within 24 h as long as no batch cycling is needed. With 3–5 cycles for the capture step, the downstream process can still be operated within 48 h or 2 days. It is important, however, that all associated operations between chromatography steps are minimized. An ultrafiltration/diafiltration step to condition the load for the next column is an additional unit operation. Even pH and conductivity adjustments take time. Availability of labour for at least two shifts is also important for smooth implementation of these scenarios.

2.2.3 Multiproduct facilities

Instead of the huge 10 ton or even higher annual production scale for one single biopharmaceutical product, it seems more likely that many current and most future facilities regularly have to produce more than one product. In other words, the large batch sizes offered by high product titers in the upstream process will be exploited to make the needed quantities in fewer batches and gain production capacity for additional products. Multiproduct facilities are fully accepted from a regulatory perspective at this time, but they do require a number of precautions and certain related process-development efforts to ensure consistent quality and safe operation.

Well planned and documented change-over is a key activity in a multiproduct facility. Change-over limits the total time available for making product in the facility. Each product changeover typically requires about 2 weeks of downtime unless different production suites are used for different products. Where a company operates with a well-developed technology platform and focuses on the corresponding category of drug substance, e.g. Mabs, one may aim for 1 week change-over or even less. It helps if different processes do not have unusual unit operations giving rise to need for new equipment as part of the change-over and leading to increased technical and operational risk. However, change-over time can vary a lot with experience and with homogeneity of the portfolio to be manufactured: 2–6 weeks time may be the time range to consider in reality.

Assuming otherwise similar conditions as in the discussion in previous sections of this chapter, the calculation in Table 2.6 demonstrates that multiproduct facilities could cope

Table 2.6

Overview of multiproduct scenarios with change-over time of 2 weeks

Number of products	Number of change-overs	Number of batches/product	Annual production in 6 × 10,000 L facility at 5 g/L
2	2	79	3160 kg
4	4	36	1440 kg
8	8	15	580 kg

*Note:* All products in this simplified model calculation have equal production scale and product titer in cell culture is always 5 g/L. One extra annual downtime of 3 weeks is included. Process yield is 80%. Cell-culture batch time is 12 days.

with a variety of products, e.g. eight products with an average annual need of 500 kg, or higher quantities at a variety of different scales.

One particular aspect of a multiproduct setup is related to the need to maintain separate separation devices for each product, e.g. membrane cartridges and chromatography columns. This is currently unavoidable due to the strict regulatory views on cleanability and its verification on such large surfaces as membranes or beads. The risk for cross-contamination between products is seen as too high. Separation and purification devices that are re-usable for different drug products would be of significant advantage to the manufacturing industry provided the regulatory burden would not (over)compensate the technical benefit.

The use of disposable devices may be favoured in a multiproduct facility when the combinations of batch frequency per product and operation scale are suitable for the use of disposables in economical and handling terms. Disposable hardware definitely helps to reduce change-over efforts, since the need to clean and sanitize (or sterilize) equipment is eliminated. The time saving in this respect includes a large quantity of analytical samples taken to prove cleanliness of multi-use equipment. On the downside of disposable operation are the costs, especially when a somewhat larger number of batches are processed (see Chapter 8). In order to combine the advantages of single-use with re-use, a concept of ready-to-process equipment may be the best route. In such scenario, columns and membranes for the next campaign come pre-packed and sanitized or sterile and the previous campaign equipment is cleared out just as with disposables. The difference is that, e.g. the columns do not have to be exchanged for every new batch.

Finally, another alternative may be to use two separate downstream lines and several fermentors, which can be combined flexibly to make different products in parallel. This may not allow utilizing the installed capacity to the maximum in all situations. However, if costs are not driving towards highest possible efficiency, this alternative may indeed be the one with greatest convenience and lowest risk for quality in a multiproduct scenario.

## 2.3 PROCESS CAPABILITY CONCLUSIONS

In summary, the discussion illustrates that product quantities needed for today's biopharmaceuticals market or anticipated for the future (multiple 100 g to multiple tons per annum) can be manufactured using mammalian cell culture in combination with conventional downstream processing technology. The requirements we have built into the model are twofold: first, the use of current membrane and chromatography products as compared to first-generation products with significantly lower capability in terms of batch time and capacity is important. Second, it is assumed that the process steps are integrated with each other, i.e. that conditioning between the chromatography steps and other time consuming non-productive activities are minimized (see discussion of process integration in Chapter 3). Other steps, which we have not discussed so far such as virus filtration, may become the bottleneck once the purification steps have been well optimized and integrated with each other. Key features of the setups discussed here (for dedicated facilities)



include the installation of up to six fermentors per downstream line in order to maximize the utilization of the downstream process equipment. With large batch sizes, it may become advantageous to cycle the batch in two to six runs in order to avoid extreme dimensions of the capture column. The larger the annual production quantities being handled in a given facility, the more important it becomes to intensify the processing and to remove anything that is not productive in terms of adding to product quality. When moving to annual production of more than 10 tons, two parallel downstream processing lines may have to be used. However, such scenarios are currently not in sight [7].

The need for flexibility in non-cGMP operation and cGMP facilities for clinical manufacturing is driven by economic aspects, but is further emphasized by the ever increasing number of projects passing through development laboratories, pilot facilities and even through early clinical manufacturing in order to optimize the positive output from the R&D pipeline.

It is therefore very likely that highly flexible plant designs with intensive use of disposable solutions will prevail in these situations. There is not much time for designing the production process in scenarios like this. Process developers must aim for designs that cover preferably all but at least most of the molecules of the same category that enter development (platform processes). Even for non-platform processes, it is advisable purely on the basis of the analysis in this chapter that a very limited number of cell lines, membranes and chromatography resins are being used and that the number of different buffers to be prepared is kept to a minimum (see Chapter 3).

Another consideration leading towards flexible facility setups follows from the fact of increasingly mixed product life-cycle scenarios with more and more 'old' and 'new' products in the same facility. Process from different periods of technology development and process-design experience will meet in one facility and require either standardization or flexibility with convenient change-over. Process change for more standardization may not always be an option.

In more routine production with repeatedly large numbers of batches per product and long campaigns, additional development time can be afforded. Some of the advantages of flexibility and disposable operation disappear and are being replaced by—in those situations—lower cost fixed installations and re-use concepts. Process development needs to select methods that are feasible for these scenarios and should avoid forcing a change of concept between development phases, e.g. the use of a technology or product only available in disposable format while still in early-stage development. Cleaning and sanitization methodology needs to be developed. Companies may eventually decide to develop an entirely new process specifically for a very large manufacturing process, e.g. when the early-stage design is not considered economically acceptable. Since companies, also driven by regulatory demands, are often using the phase III process even for post-approval production, they may plan for post-licensure changes of the process to accommodate changes in demand or technology progress appropriately.

Biotherapeutic manufacturing processes with high annual production requirements generally require thorough optimization of all aspects that impact process economy. Monoclonal antibody and insulin production are the main examples for in today's biopharmaceuticals market. Details will be described in Chapter 8.

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## Process-Design Concepts

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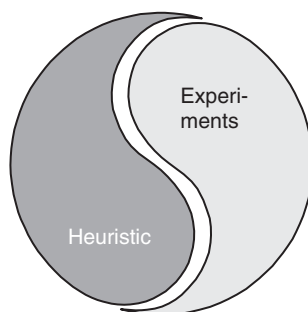
The objective of designing a manufacturing process for a biopharmaceutical product is to find the best tools and procedures to consistently and economically synthesize sufficient quantity of the target product, isolate it from the production system and then purify the product to the level of active pharmaceutical ingredient (API), which is also called bulk drug substance. That API must have well-understood characteristics, it must meet predetermined quality attributes and the manufacturing process must be robust.

Process design always requires two key components. First, experience from key disciplines involved in turning a drug candidate with research-level understanding into an approvable drug with fully understood characteristics. Second, a lot of experimental work is required. The former involves process development, manufacturing, analytical and validation staff. A ‘heuristic’ approach to process design helps to quickly cut through the huge amount of technical options and parameter variability, provided the process of applying the experience is coordinated. In the past, experimental work was addressed in a random fashion. Today, the approach is to apply systematic management, structured experimental planning and execution, high-throughput experimental and analytical tools and often modelling to produce more and better information and even to predict results. Together, these two components make process design more efficient and result in more reliable processes (Figure 3.1).

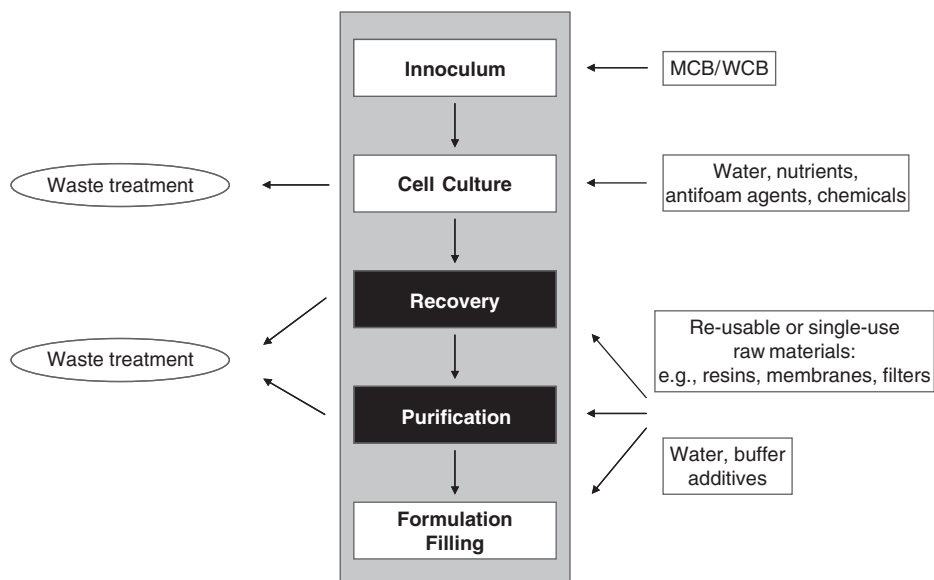
This chapter will guide you through the important aspects of process design. The scope of the book is limited to process chromatography. However, successful process designers always take into account the various interdependencies between upstream and downstream processes, as well as those between individual steps in the sequence. You will find tools to manage these interdependencies along with relevant technical guidance.

### 3.1 TYPICAL PROCESS DESIGN FOR BIOPHARMACEUTICALS

Manufacturing of modern biopharmaceuticals typically uses genetically engineered cells as the production source for the API. The most-common exception is human plasma. At this time, there are also a few projects with transgenic animals or plants as the production source. However, for the scope of this book the sources are similar, since they all use similar methods for recovery and purification, the so-called downstream process (Figure 3.2).



**Figure 3.1** Two components of process design, multi-disciplinary experience and efficient experimental work.



**Figure 3.2** Outline of a typical cell-culture process using genetically engineered cells to produce the active pharmaceutical ingredient (API), the combination of recovery and purification unit operations is usually referred to as ‘Downstream Processing’. Inoculation and cell culture form the upstream process.

Typically, the cell culture is started from a working cell bank (WCB), which has been prepared from a master cell bank (MCB). A seed train with several stages of increasing culture volume and mass leads to the final-scale fermentor or bioreactor production phase. Fermentation (microbial cells) and cell culture (mammalian and insect cells) require carefully controlled growth conditions that are supported by the culture medium, which contains nutrients and chemicals in dilute aqueous solution.

Whether mammalian or microbial cells are used, recovery of the product involves product isolation from the cell mass. Cell debris or whole cell removal is achieved with techniques such as centrifugation or filtration. Purification most often is achieved with chromatography and membrane-based techniques in an orthogonal combination of separation mechanisms.

A similar basic design is also applied to transgenic and insect cell production systems. The purification process provides the API,<sup>1</sup> which is then sterile filtered, formulated and filled into the final product containers.

## 3.2 MANAGEMENT FRAMEWORK FOR PROCESS DESIGN

Complementary to the Validation Master Plan (VMP, see Chapter 7), company management may decide to maintain master plans describing the company facilities, capabilities and key routines, e.g. for the development of new biopharma drugs. While the format of such plans is flexible, we suggest that one considers the following as important contents to be covered in a development plan.

### 3.2.1 Defining structure and workflow for process design

Designing one process that will take a company all the way from toxicology studies to a licensed product is an ideal. In reality, changes will need to be made during development. The final process evolves in several stages: first for producing material for pre-clinical studies, next for clinical manufacturing and finally for full scale, validated manufacturing of the product approved for marketing. During these different phases, process design requires careful coordination of the target goals with the level of knowledge and control over the process. Furthermore, one or more well-managed transfers between the development lab and internal or external manufacturing units will be required. Costly changes of the process and concomitant critical attributes of the product will result in a risk for delays or even failures of the project. Those types of changes need to be minimized during the progression from toxicology studies to licensed product. There is another stage, which we touch upon under process economy: post-approval changes.

Process design is usually performed first on individual manufacturing steps, which are then linked to yield a sequential flow of unit operations, each of which is optimized to process the product intermediate from the previous step. Development of the design for the synthesis process (upstream) and the recovery and purification process (downstream) is most often carried out by different groups, sometimes without coordinated timing of the optimization. Design of process steps may occur without sufficient awareness of the existing operation and the capability of large-scale equipment in manufacturing. To ensure smooth and economical operation, individual steps of the process need to be integrated during development. An early awareness of the final scale of manufacturing and its potential constraints is essential for successful development.

For these reasons and to keep the time and cost of process development under control, it is advisable to establish a management framework for process design that encourages the interaction between different groups, minimizes the risk from lack of coordination and defines procedures to be followed (workflow platforms) as well as preferred methods to be applied (technology platforms).

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<sup>1</sup> API, active pharmaceutical ingredient.

**Table 3.1**

Three core elements of process design for biopharmaceuticals

Element	Example	Comment
Selection of industrial tools	Cell lines, scalable resins	Documented evidence, audits, manufacturing experience
Selection of methods	Cell separation methods, purification methods, removal of proteases, viral clearance	Based on understanding of target solute and impurities, risk analysis, heuristic designs, experimental performance evaluation
Integration	Use of the same buffers in many steps, automated buffer preparation and column packing, use of disposables	Reduction of time-consuming associated activities, eliminate wasted efforts (LEAN <sup>a</sup> ) and reduce cost

<sup>a</sup>LEAN, a concept applied to create value. There are five steps: identify value from the end customers perspective, identify the stream of value creating steps and remove waste, create a flow of value creating steps, make the product the customer wants, improve continuously!

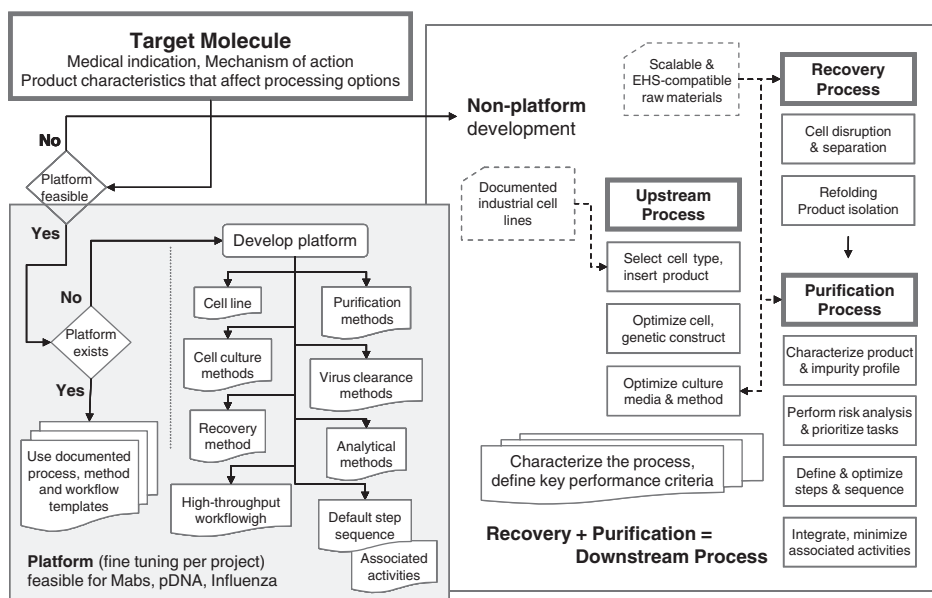
We propose a structured approach to process design to guide the process-development scientist and to make sure that the experience of process-development staff is institutionalized over time, i.e. turned from individually to corporately owned knowledge and into well-understood process solutions, ideally into technology and workflow platforms.

In principle, the design concept we propose contains three core elements (see Table 3.1). First, selection of industrial tools. This may include cell lines with full traceability of their origin and history of development. Processing and raw materials must also be selected, for example chromatography resins suitable for the intended scale of operation. Second, selection of the best methods for the intended purpose and optimization of those methods. Finally, integration of all steps, including minimization of all associated activities, such as buffer preparation or column packing.

Figure 3.3 provides a more detailed overview of the proposed workflow. The category of target molecule is determined mainly by the medical indication and the molecule's mechanism of action. The latter may also trigger requirements on features such as glycosylation patterns that will, in turn, influence the choice of production cell. Certain characteristics, notably stability, of the target molecule also influence the selection of processing methods.

For certain categories of target molecules such as monoclonal antibodies, plasmid DNA vaccines or influenza vaccines, platform approaches may be feasible. The process developer should be able to find instructions and guidance in the use of platform concepts referenced in the company's development plan. Platform technologies enable rapid development and reduce the risk for errors or omissions in the design and characterization of the process. However, even these processes, which utilize the same host (e.g. CHO cells), same recovery steps and purification modules, will require some fine tuning. Monoclonal antibodies, for example, may differ in their tendency to form aggregates and in initial levels of host cell impurities, such as DNA and host cell proteins (HCP).

Where a platform approach would be feasible, but does not exist, we recommend establishing a project for its development. The cell line, purification methods, analytical methods



**Figure 3.3** Structured process design: platform approaches can often be used for Mabs, plasmids and influenza vaccine. Other target molecules require more individual process designs.

and development workflow are some of the elements of a process that may become part of the overall technology platform. This can be a very successful development strategy, provided the category of target molecule allows re-use of these tools and methods every time a new candidate molecule from the same category is developed.

For target molecules, which do not lend themselves to a platform approach, such as many recombinant proteins other than monoclonal antibodies, it is still possible to follow a generic process-design guide with a powerful set of rules that reduce process failure, risk for product quality problems and time to reach an acceptable process.

Each process needs to be thoroughly characterized and the relations between process parameters and critical quality attributes (CQAs) of the product need to be well understood.

For the design of a non-platform process, the best place to start is with careful characterization of the target product and its impurity profile, followed by a risk assessment that leads to prioritization of the purification process tasks. Defining the different steps in the most appropriate sequence and optimizing them is the next activity. The steps of this initial process are then integrated. Process integration facilitates the transfer of intermediate product by optimizing the links between steps. At the same time, integration should reduce the amount of non-productive, associated activities required for each production step, such as preparation of different buffers, hold times caused by the need for re-buffering between steps or extensive column packing.

Many companies are organized such that development of the downstream process and the upstream process are carried out by separate development groups or teams. Product recovery is sometimes developed by the upstream team, up to the point where the intermediate



**Consult production and engineering whenever you:**

- ☐ use acidic buffers, esp. with chloride ions.
- ☐ use organic solvents.
- ☐ use "additives", e.g., detergents.
- ☐ require high pressure drop over the column.
- ☐ need narrow tolerances for process parameters.
- ☐ run at very wide flow rate range.
- ☐ use sensitive and expensive chromatography resins.
- ☐ have a complex fractionation pattern.
- ☐ use sophisticated level of automation.
- ☐ interpret chemical resistance information.

**Figure 3.4** Check list: production management checklist for the process-development team to prevent potential constraints after technology transfer.

product is free of cells and cellular debris. All the teams need to have the same understanding of the objectives of process design, namely, the generation of a robust manufacturing process with a defined API quality and a certain production capacity. Management may formulate a general, coordinative design dogma for everyone: the development of all process components must be performed with production purpose and scale in mind! The design must meet defined robustness objectives and lead to a validatable process.

Ideally, development teams as well as those groups to whom they transfer a process know about each other's activities through active interdisciplinary involvement, regular reporting and a number of practical check lists. Figure 3.4 provides an example of a check list from manufacturing to purification process development to avoid potential constraints in a specific production facility or in large-scale equipment in general.

Communicating with manufacturing can eliminate problematic scale-up issues. Manufacturing in-process measurement tools may not have the sensitivity of those used in development, and wetted materials and column distribution systems are often different from those used in development. What may be practical in the laboratory can lead to equipment design needs that are very tricky and costly to realize on the manufacturing floor, e.g. a complex product peak fractionation scheme easily realized with a laboratory fraction collector would need to be translated into a cascade of valves with a dead volume large enough to eliminate some of the resolution achieved by the purification step.

Cell-culture improvements made upstream can have a major impact on downstream processes. Changes in target product quality attributes, total protein load and impurity profile changes, such as the quantity of HCP, aggregates and new processing agents, are among the most likely changes that require adjustments to the downstream process. Adjustments may even include changing the scale and/or order of purification steps. Frequently, product titer is increased during the progression from early clinical manufacturing to full-scale manufacturing. This may result in unacceptable product variants and changes in product stability.

It is usually impractical to complete cell-culture development before downstream process development. Therefore, an incremental evolution of the upstream process in a limited number of stages agreed upon between upstream and downstream development teams may be the best approach.

Product and impurity characterization come early in the workflow suggested in Figure 3.3. In particular, product stability under a range of typical process conditions needs to be well

understood at a very early stage to avoid process-related issues that impact the product's biological activity. The analytical methods that are suitable for assessing stability and biological activity need to be available early in process design. The product development team needs to understand the capability of each assay to avoid making false conclusions, e.g. about satisfactory levels of impurity clearance. The analytical methods development team needs information about acceptable levels of removal of each key impurity category so that assays with sufficient sensitivity and specificity can be developed. (For further information on analytical methods, see Chapter 5.) A development timeframe should be developed by the analytics and validation teams. Those teams are also best suited to select assays that can support both ongoing development and process validation.

Finally, a word about reporting in a process-design project. It is often stated that something that is not documented does not exist! This is the dogma of the regulated industry as a whole. In a similar fashion, something that one team has not told the other is not a known fact. Every relevant question that has never been asked, answered and documented will leave one more open issue that may delay the project. Reports need to be written with the reader as the customer. They are not meant to make the author look good, but to inform the readers. Therefore, good reporting practice includes both writing what has been done and documenting what has been left out. The development records make the selection of methods and all major decisions on options clearly understandable and all open issues easily retrievable. Modern electronic document storage and retrieval systems are recommended to enable appropriate documentation management with the lowest risk for error and most efficient use of time.

### **3.3 PRODUCTION CELLS AND TYPICAL PRODUCT CHARACTERISTICS**

In this section, we briefly discuss the upstream process design. Guidance for selection of production cells and the typical impurity profiles are provided here. Since the product is created in the upstream process, this is also the best place to discuss the most-important characteristics that may lead to limits for subsequent recovery and purification steps.

#### **3.3.1 Production cells and their selection**

Production cells cannot be selected at random, e.g. in an attempt to improve economy. Often one is locked into mammalian cells because of the demands the protein drug places on the capability of the cellular protein synthesis and post-translational modifications. This is true for most monoclonal antibodies and for many viral vaccines. Significant investments would have to be spent in research to overcome these natural barriers and the chances of finding an industrially feasible solution are low.

Even when there is a choice, few companies seem to exercise it, possibly because they have an established cell line in their manufacturing operations and it is easier and faster to develop other products that can also be produced by those cells. One may conclude that is because of far-sighted planning of the company's therapeutic portfolio and a strategic selection of the cell line. It is more likely, however, that such decisions evolved in an evolutionary mode. Following 25 years of development and manufacturing experience with

**Table 3.2**

Overview of producer cells and systems established or tested in the biopharmaceutical industry

Production system	Examples of drugs	Productivity
Chinese hamster ovary cells (CHO)	Mabs, tissue plasminogen activator, erythropoietin (EPO)	3–5 g/L; >1000 kg/yr
BHK, NS0	Mabs	3–5 g/L; >1000 kg/yr
<i>Escherichia coli</i> ( <i>E. coli</i> )	Insulins, growth hormones, interferons	>1000 kg/yr
<i>Saccharomyces cerevisiae</i>	Insulin, Hepatitis B surface antigen vaccine	>1000 kg/yr
<i>Pichia pastoris</i>	Recombinant human serum albumin (rhSA)	>1000 kg/yr
Filamentous fungi	Fab antibody fragment	1.2 g/L
Insect cells	Vaccines, Mabs	
Transgenic plants		
Transgenic animals	Anti-thrombin alpha	300 kg/yr

genetically engineered cell lines, certain standard choices have been established. Table 3.2 lists the most frequently used cell lines, type of drug substance produced and scope of manufacturing.

The most common expression systems for production of recombinant DNA protein and peptide products are *Escherichia coli*, a Gram-negative bacterium, and Chinese Hamster Ovary (CHO) cells, a mammalian (rodent) cell line. Alternative systems include yeasts and other fungi, insect cells and transgenic animals and plants. Microbial cells are used mainly for proteins, which do not require post-translational modifications typically found in mammalian proteins.

It seems that many companies are moving to mammalian systems because a large part of the previous disadvantages related to costs and productivity have been overcome and also because companies increasingly include antibodies in their development programmes (see Chapter 1). In most cases, to make a safe and efficient Mab you need to make it in a mammalian system. The mechanism of action of the Mab dictates what molecular features it needs to have and, consequently, what production system capability is required. Mammalian cells are rapidly reaching capabilities sufficient for most biopharmaceuticals with cost levels approaching those for microbial cells. Costs ranging from 30 to 300 USD/g of product have been reported both for CHO (mammalian) and *E. coli* (bacteria) [1], i.e. for well-optimized processes. Other alternatives such as transgenic systems seem to be losing attractiveness in this respect. For example, while six 12,500 L fermentors could produce 4000 kg per year, a goat herd of 60 animals could only reach a productivity of 40 kg, i.e. a huge herd of 6000 animals would be needed to match the mammalian cell-culture facility [2].

Finally, in each cell line selection project, the history of the cell line and its genetic stability are issues that must be addressed to ensure production of consistent product and satisfy regulatory requirements. Cells must be of known origin and demonstrated to be sufficiently

stable to produce consistent product throughout the culture. Genetic stability determines the limit for *in vitro* cell age for production and is based on data derived from production cells expanded under pilot or full-scale conditions [3].

#### *Mammalian cells*

CHO cells are the most commonly used expression system for proteins that require post-translational modifications. CHO cells are used to produce several licensed monoclonal antibodies, tissue plasminogen activator (tPA) and erythropoietin (EPO), among others.

Glycosylation in rodent cells is not identical to that of humans, but CHO cell lines can produce human-like glycosylation. In addition to being able to make post-translational modifications, rodent cells can correctly fold and assemble human protein products that are secreted to the culture medium, which simplifies recovery steps. BHK (baby hamster kidney) cells, murine myeloma cell lines (NS0 and SP2/0) and a human cell line (human embryonic kidney—HEK) are also used for licensed products. Of the 12 biopharmaceuticals approved in 2004, four were produced in CHO, two in murine myeloma lines and one in a hybridoma line [4].

Expression levels from CHO or NS0 cell cultures in fed batch mode have reached 3–5 g/L in many development projects [5] and are projected to even exceed 10 g/L in the foreseeable future. The improvements have mainly been achieved through increased cell number (~10 fold) and specific productivity (~10 fold) [6].

While mammalian cells still require batch times of 10–20 days in batch culture due to their relatively slow growth rates, other disadvantages in comparison to microbial systems have been largely overcome in the recent decade, namely productivity and cost issues. Cell-culture media used for most mammalian cell cultures are now serum- and protein-free, synthetic media, thus further simplifying the purification process and reducing the costs significantly.

The human cell line PER.C6 (a human embryonic retinoblast cell) is being used in development of new protein products and has also been used to produce adenoviral vectors for gene therapy and a clinical HIV vaccine [7]. PER.C6 cultures have a very high productivity and they do not produce immunogenic glycan structures. Productivity in a continuous perfusion culture peaks up to 3 g/L per day [8].

#### *Escherichia coli*

*E. coli* is the most commonly used host for production of recombinant DNA proteins and peptides that do not require glycosylation or other post-translational modifications. *E. coli* is also used for production of gene-therapy plasmids and DNA-based vaccines. It has been used to produce several approved biopharmaceuticals, including insulin, growth hormone and various interferons. Fermentation media are relatively inexpensive, and expression levels are quite high, producing titers of 3–15 g/L. Rapid growth (hours to days) is achieved due to the high frequency of population doublings.

In bacteria, most products become insoluble and are found in aggregates, called inclusion bodies, which have some similarities with amyloid aggregates [9]. Protein products located in inclusion bodies require refolding after isolation of the inclusion bodies and resolubilization of the proteins. Refolding can be problematic and costly and is considered by

many to constitute a significant disadvantage compared to mammalian systems. Refolding requires large volumes of water and buffers that need very large tank space. Simultaneous renaturation and purification has been described for a recombinant human granulocyte colony stimulating factor expressed in *E. coli* [10]. Separation of correctly and incorrectly folded product is necessary.

In efforts to avoid refolding, *E. coli* has been engineered to secrete soluble protein products to the periplasmic space, but recovery operations often result in a high endotoxin level. Increasing the solubility of proteins expressed in *E. coli* can improve yield. A review describes protein quality in bacterial inclusion bodies and the approaches that are being used to obtain properly folded, soluble species [9]. Soluble products lack the terminal methionine typically found in proteins produced in *E. coli*. There is an effort underway to engineer *E. coli* to secrete products to the culture fluid [11].

### *Yeast*

There are currently two yeast expression systems that are being used to produce biotherapeutics, namely *Saccharomyces cerevisiae* and *Pichia pastoris*. *Saccharomyces* is used to produce a recombinant hepatitis B surface antigen vaccine and insulin. *Pichia* growth is regulated by methanol, and it has become the more popular of the two yeast expression systems. *Pichia* is being used to produce 12.5 tons of recombinant human serum albumin per year, due to its high expression levels and an efficient downstream process [12]. One estimate is that *Pichia* can produce 15 g/L [13].

Yeast expression levels are high when compared to those of mammalian cells, and growth typically takes days to a week. In some cases, refolding may be necessary. The growth medium is relatively inexpensive. Glycosylation is carried out by yeast, but the glycosylation pattern is different from that of humans in that there is no sialic acid and the sugars are different from those found in humans. However, a recent publication describes the production of human antibodies with specific human N-glycan structures in glycoengineered lines of *Pichia* [14].

### *Filamentous fungi*

Filamentous fungal systems are used to produce industrial enzymes. The potential for the use of filamentous fungi as host for production of biotherapeutics is addressed in a review article [15]. Production yields for a Fab antibody fragment were 1200 mg/ml using *Aspergillus niger*.

### *Insect cells*

The insect cell line *Spodoptera frugiperda* (Sf9) with a baculovirus vector has been employed to make vaccines that are still in clinical studies but nearing licensure. The system has also been used to make monoclonal antibodies [16]. In Japan, the silkworm is being used instead of Sf9 cells, but products are not yet used in humans [12].

Insect cell growth is about the same as that for yeast. Expression levels are relatively low, but proteins are secreted to the culture medium. Like yeast, insect cells do not have sialic acid and have sugars different from those of humans.

*Transgenic plants*

Sugars in plants and humans are significantly different, which causes concerns related to immunogenicity for plant-produced biotherapeutics. However, human-like glycosylation has been achieved in genetically engineered tobacco [17]. The problems, solutions and opportunities for using plants to produce biopharmaceuticals, including the issue of immunogenicity, have been reviewed [18]. Another publication describes global trends in plant transgenic science and technology [19]. Regulatory issues are rather complicated for plant-derived therapeutics. For example, in the U.S., regulatory agencies that are concerned with plant-made biopharmaceuticals include FDA, USDA and EPA [20].

*Transgenic animals*

In June, 2006, the first biotherapeutic produced in transgenic animals was given a positive opinion by the European regulatory authorities, who recommended it be given a marketing license. The product is an anti-thrombin alpha, which inhibits blood coagulation. Protein products are expressed in milk. Removal of lipids is a significant issue, but technology has been developed for their removal. It has been estimated that at a production scale of 300 kg/year, the cost of goods for transgenic manufacturing is 6 USD/g, compared to 48 USD/g for cell culture [21]. Production in transgenic animals takes more time than cell culture due to the time required for establishment of the founder animal and offspring, but transgenic animals can provide very high productivity. Regulatory issues are complex due to potential immunogenicity of non-human glycosylation patterns and risks associated with animal adventitious agents. Companies working with transgenic systems have addressed these concerns.

**3.3.2 Typical impurity profiles**

In general, one distinguishes between product-related and process-related impurities (see Table 3.3). They differ both in origin and strategy for prevention or removal.

*Product-related impurities*

Product-related impurities may be present as a result of the cellular synthesis process or be created during the process as a consequence of enzymatic activity or certain process conditions such as pH of the product solution. For example, aggregated forms of a monoclonal antibody could be produced during cell culture or formed under extreme process conditions, such as rapid pH change during elution from a Protein A capture column or simply as a consequence of very high protein concentration.

Potential modifications to proteins include aggregation, misfolding and/or random disulphide bridge formation, deamidation of certain amino acids, oxidation of methionine and post-translational modifications such as glycosylation, phosphorylation and acylation. Any of these modifications may lead to micro-heterogeneous forms of the product, which may or may not be acceptable. Purity demands will be dependent on the efficacy, potency and safety profiles of the various forms. Removal of product-related impurities is among the most challenging tasks for purification technology as it usually requires resolution of substances with very similar behaviour in any separation method.

Table 3.3

Process and product-related impurities	
Process-related impurities	Product-related impurities
Cell-culture nutrients, chemicals	Dimers, multimers, aggregates
Host cell proteins	Misfolded product and/or product with random disulphide bridge forms
Proteolytic enzymes, other enzymatic activity	Deamidated product variants
Endotoxins	Product with oxidation of methionine
Cellular DNA, other nucleic acids	Product with heterogeneity of post-translational modifications such as glycosylation, phosphorylation and acylation
Virus	Enzymatic degradation products
Cell debris, lipids	
Antifoams, antibiotics	
Leakage, e.g. from affinity columns	
Extractables, e.g. from plastic surfaces	
Water, buffers	

*Note:* Analytical methods to detect and quantify these impurities are discussed in Chapter 5.

Table 3.4

Certain process-related impurities are dependent on the selected product source						
	Tissue	Plasma	Transgenic milk	Mammalian cells	Micro-organism cytoplasm insoluble	Micro-organism secreted
Cell debris	+				+	
High lipid content	+	+	+			
Proteases	+	+	+	+		+
Virus	+	+	+	+		
Endotoxins					+	+
Water				+	+	

Chapter 9 provides detailed information on the properties of biological molecules. The analytical methods developer can provide valuable tools for evaluating product related impurities during purification process development.

*Process-related impurities*

The upstream-derived process impurities are determined by the selected cell line (see Table 3.4), its culture conditions and the methods required to isolate the product, e.g. cell disruption. Some quality and safety risks are also dependent on the cell lines and the isolation methods. The lowest initial impurity levels are generally achieved with secretion



systems grown in chemically defined, protein-free culture media. The highest levels are present when production cells need to be disrupted and cellular debris contaminates the target product during the initial recovery steps.

A high-level of process-related impurities and cell debris requires greater efforts during recovery and purification. All impurities potentially reduce the capacity of separation or purification steps to handle the target product quantities. Potential cost advantages of production systems with such features are compromised, sometimes significantly so, by these disadvantages. For example: cell debris, lipids and antifoams are sometimes added in fermentation processes. When they are still present after product isolation and cell-removal steps, they may clog membranes and chromatography columns.

Any production source has its own native proteins, which will contaminate the target product to a certain extent. In cellular production systems one refers to them as host cell proteins (HCP). When human plasma is the starting material, the target protein is in solution with all the other plasma proteins, which represents the maximum host-related contamination scenario. For example, plasma contains large quantities of human serum albumin, which can be a target product, but which is also the key contaminant for all other plasma proteins of interest. In cellular production systems, most of the time only a fraction of the cellular proteins end up in the starting material for the downstream process. For example, CHO HCP are released when the cells are damaged during separation, or naturally as a consequence of cell death towards the end of cell culture. In both cases, contamination can be limited through selection of processing methods or process controls. With *E. coli*, the isolation of inclusion bodies and extensive washing steps allow removal of a majority of the bacterial HCP despite their significant release upon cell disruption. One particularly important category of HCPs are proteins with enzymatic activity such as proteases or glycosidases.

Production sources of mammalian origin such as cells, tissue, human plasma and transgenic milk can be contaminated by exogenous virus. Mammalian cells often carry endogenous virus, e.g. CHO cells inherently contain retroviral particles. There is also a possibility that transmissible spongiform encephalopathy (TSE) agents can contaminate mammalian production sources as well as in-process raw materials that are of mammalian origin or manufactured with materials of mammalian origin.

In *E. coli*, and other Gram-negative bacteria, endotoxins are located in the cell wall and are released during cell disruption—one reason why there has been an effort to design the cells so they can secrete product, rather than sequester it in inclusion bodies. For mammalian cultures, endotoxin should not be an issue, or at least it is one that is controlled by compliance with good manufacturing practices. In cell culture, endotoxins are considered a contaminant not an impurity derived from the host organism. Endotoxins can be introduced into purification processes by contaminated water, buffers, additives and resins.

Cellular nucleic acids, such as genomic DNA, are released in the same way that HCP are released. The amount of nucleic acid in the starting material is also dependent on the degree of cell death during late cell culture or the disruption of producer cells during isolation of the product.

Finally, in many cases, water is considered an unwanted impurity. The product feed-stream may be highly diluted after cell culture or following renaturation from inclusion bodies. Removal of excess water can be essential for a cost-effective process.



3.3.3 Knowing the target molecule and its stability window

Features that are relevant to process designers include the physico-chemical and biological properties of the target molecule and its stability under the chemical and physical conditions it comes in contact with throughout the process (see Table 3.5).

Chromatographic techniques are based upon the controlled interaction between the solute and a sorbent (resin). The critical binding properties of the solutes may be exposed on the surface of the molecule or hidden in the interior parts and only available for interaction after modification, e.g. denaturation. This exposure of interior parts can be used to achieve a high degree of separation (e.g. as in reversed-phase chromatography) but may also result in irreversible loss of material and/or activity. Another solute property that may be used for purification is the molecular size of the whole molecule (e.g. as in size exclusion). Parts of the molecule may be employed to fit into ‘pockets’ (e.g. as for steric orientation in affinity chromatography). Keeping the molecule in its natural environment will, in most cases, preserve activity. Sometimes, care must be taken to prevent removal of essential co-factors in the purification step.

Knowledge of the conditions, such as pH, salt concentrations, additives, etc., that preserve the product will be crucial to a cost-effective separation strategy. Some of this information will be obtained during preliminary testing of the starting material.

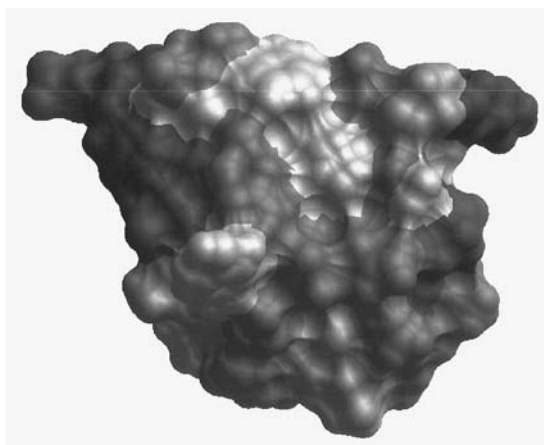
Note that the two sides of Table 3.5 are not directly related to each other. However, the factors affecting biological activity can influence the stability of the native configuration of a biological substance through a combination of mechanisms. Such effects can range from small conformational changes to complete denaturation and loss of the desired biological activity.

Solute properties commonly used in process scale purification are size, charge, hydrophobicity and affinity. These properties are illustrated for a protein in Figure 3.5. It should be noted that a protein exhibits several of the surface properties mentioned and that the relative proportion varies with the conditions. This fact illustrates an important conclusion about the chromatographic behaviour of complex macromolecules—whereas a rough

Table 3.5

Target molecule properties relevant for the design of the process and for the stability of the biological activity of the product

Properties relevant to purification	Factors affecting biological activity
Molecular weight	Protease presence
Molecular shape	Protein concentration
Net charge	pH
Surface charge	Temperature
Surface hydrophobicity	Co-solvents
Specific binding sites on surface	Salts, conductivity
	Co-factors
	Redox potential



**Figure 3.5** Molecular model of insulin in which a different grey scale relates to different surface properties (increase of blackening in the order aromatic, hydrophobic, polar, negatively charged and positively charged areas). The different areas are visualized in colour in Chapter 9. The image was created using the ICM program (MolSoft LLC, Metuchen, USA) by entry 9INS in the Protein Databank [22, 23]. By courtesy of Martin Norin, Pharmacia & Upjohn.

characterization is necessary to gather information about the macroscopic properties of the solute, it will be very difficult and laborious to obtain enough detailed information to totally predict chromatographic behaviour. Therefore, experimental method scouting is an important tool for screening suitable purification conditions. An assay for the product of interest will be required to monitor the progress of the purification. The properties of some important biological macromolecules, with references to suitable characterization methods are found in Chapter 9.

### 3.4 RISK ANALYSIS AND RISK MITIGATION

Over the last 5 years or so, greater emphasis has been placed on risk management for biotechnology processes. Risk management requires that risks are first assessed and then mitigated. Purification steps mitigate risks by clearing them from the feedstream. Utilizing each step to remove multiple risks is part of the process developers' challenge.

Several documents on risk management are available from both industry and regulatory agencies [24–26]. Some of the more commonly used risk analysis tools include HACCP (hazard analysis and critical control points), FMEA (failure mode and effect analysis) and FTA (fault tree analysis). The application of FMEA to biotechnology process characterization has been described by Seely and Haury [27]. Regulatory risks should also be assessed in early development [28]. A good strategy for designing a purification process that will produce a biological product with appropriate quality, safety and consistent purity is to consider the risk factors, some of which are discussed here.

### 3.4.1 Categories of risks, prioritization and mitigation strategies

Risks that can be mitigated by the purification process may arise from source materials, process impurities and product impurities. Depending on the host organism and upstream process, a process developer will design a multi-step purification process to remove those risks. Assays for some of these risks are discussed in Chapter 5.

Certain impurities have the ability to modify the product through enzymatic activity, typically protease and glycosidase. Others carry a risk for the safety of the final drug product, e.g. endotoxins, immunogenic proteins, virus or genomic DNA from the producing cell. A third important category of risks arise from process variability, which may lead to process failure as a result of a process step operating outside its limits and delivering product out of specification.

The process should be designed for immediate or very fast removal of impurities that can potentially alter or damage the target product during the process. Damage to the product from enzymatic activity increases starting material variability, may negatively impact process robustness and reduces product yield. High product yield is a key driver of process economy (see Chapter 8).

Cell debris, lipids and antifoam additives from cell culture represent a risk for robust performance of subsequent separation and purification steps. Residuals from these impurities can reduce capacity of chromatography columns or flux through membranes, cause unpredictable cross-contamination of intermediate products from batch to batch, require significant extra cleaning and may reduce the life time of valuable chromatography resins or ultrafiltration membranes. Ideally, the recovery process needs to be designed for quantitative removal of these risks prior to the start of the purification process.

Product-related or process-related protein impurities, DNA, endotoxin, virus, mycoplasma and TSEs all represent potential safety risks to the patient. Purity, including absence of immunogenic effects, and biosafety (absence of infectious risks) can therefore not be compromised. The purification process needs to deliver a product with such risks reduced to acceptable levels correlated with clinical studies. While individual steps may have specific objectives and different capabilities related to different risks, the overall design must take all of the risks into account.

Process variability is in a risk category of its own. Variability is caused by the biological production system, but also by post-cell culture effects, such as the impact of process conditions on the biological molecule, incompletely removed enzymatic activity or inadequate process control. Chromatographic resins, filters and membranes have a certain batch-to-batch variability. Process design includes the definition of a window of operation for each step that ensures consistency of product quality and accommodates the 'natural' variability as well as that of raw materials and their performance. In practice, the initial design of an individual step may need to be adjusted to the capability of both previous and subsequent steps, and all downstream steps may require adjustment once changes are implemented at the cell-culture level. This incremental optimization is performed as part of the process integration phase of development (Table 3.6).

**Table 3.6**

Overview of risks associated with process- and product-related impurities, risk-mitigation strategies and priority in downstream process

Impurity	Risk feature	Mitigation/method	Priority in process
Protease	Damage/alteration of product	Minimize release, genetic engineering, pH inactivation, removal	Very high, address in first purification step
Glycosidase, other enzymatic activity	Damage/alteration of product	Prevention of release from cell, fast inactivation/removal	High, address in first purification step
Lipids, cell debris	Performance alteration of purification resins/membranes	Recovery process, filters, centrifuges, flocculation	High, address prior to purification
Antifoams	Performance alteration of purification resins/membranes	Avoid use, filters	High, address prior to purification
Host cell proteins	Safety risk for patient: immunogenicity	Orthogonal removal steps: IEC, HIC, multi-modal chromatography	Medium to high, address throughout process
Cell-culture media	Safety risk for patient: antibiotics, immunogenicity of protein additives, potential adventitious agents	Culture without antibiotics and use of protein-free cell-culture media	Low, addressed prior to downstream processing
Nucleic acids	Safety risk for patient: low risk of carcinogenic effect of nucleic acids	Anion-exchange chromatography, cation-exchange chromatography to bind product	Medium, address in polishing steps
Endotoxins	Safety risk for patient: fevers	Anion-exchange chromatography cation-exchange chromatography to bind product	High, address in polishing steps, avoid introduction into cell culture by compliance with GMP
Virus	Safety risk for patient: infections	Cell line testing, virus clearance by two or more steps with independent mechanisms	Very high, address throughout process
Mycoplasma	Safety risk for patient: infections	Cell line selection, raw material testing	Low, addressed prior to downstream processing

(Continued)

**Table 3.6** (Continued)

Impurity	Risk feature	Mitigation/method	Priority in process
TSE	Safety risk for patient: infections	Donor screening (human plasma), raw material selection (animal free)	Medium, mostly addressed prior to processing
Product-related impurities	Safety risk for patient: altered potency, immune response	Orthogonal removal with polishing steps: IEC, HIC, multi-modal chromatography, RPC, SEC	Medium to high, dependent on type of drug and medical indication
Any	Process variability	Design robust process, apply design space concept	Very high, address in all steps

*Abbreviations:* IEC, ion exchange chromatography; HIC, hydrophobic interaction chromatography; RPC, reversed-phase chromatography; SEC, size exclusion chromatography

### 3.4.2 Mitigation of safety risks

General risk mitigation strategies have been discussed in the previous section. Some more specific guidance is warranted for mitigation of safety risks.

If the host organism's proteins are co-purified with the target molecule, the immunogenicity of the final product may pose a risk to the patient. HCP are typically removed by an orthogonal, multi-step purification process to very low levels (e.g. 10–50 ppm)<sup>2</sup>. Their removal is one way of measuring consistency of manufacturing. As with other impurities, they can take up valuable capacity in chromatography, so specific binding of the product as a first step is often the preferred strategy.

The risk associated with DNA has been downgraded for today's highly purified biotechnology products [29, 30]. Nevertheless, DNA needs to be cleared to acceptable levels. In the European Union, for example, the allowable limit has been stated as 10 ng/dose [31]. With gene-therapy vectors, however, there is much more concern. As noted in the CPMP Position Statement of 1997, there are cases, such as previously non-approved continuous cell lines and transforming sequences from viral vectors, in which it is necessary to routinely control elimination of host cell DNA.

In process design, the first step is to determine how much DNA is in the feedstream from the initial recovery, and then select robust methods for its removal. One or more steps may be required. High nucleic acid content increases viscosity. Treatment with DNase will usually effectively reduce the viscosity but will add one extra impurity to be removed by subsequent steps. Nucleic acids are negatively charged and loading an anion exchanger at high salt concentrations will, in most cases, allow for binding of nucleic acids without binding the proteins. Adsorption of the product to a cation exchanger, hydrophobic resin or an affinity resin will also enable DNA removal. Other techniques, such as precipitation with polyethyleneimine, can be used, but introduce another impurity and the need for clarification of

<sup>2</sup> ppm, parts per million, concentration of impurity relative to the target molecule concentration.

**Table 3.7**

Virus clearance in a typical platform for monoclonal antibody purification

Unit operation	Viral clearance mechanism
Protein A	Removal: product adsorbed, virus in flow through post-elution: low pH inactivation
Virus filtration	Removal by size: product in flow through virus retained by filter
Anion exchange	Removal: virus adsorbed, product in flow through
Hydrophobic interaction	Removal: mechanism variable

the target solute from the precipitant. In-process measurements to quantify DNA removal indicate manufacturing consistency. Consistency of DNA clearance has been used to predict virus removal capability of an anion exchanger in lieu of end-of-resin lifetime studies [32].

As described above, the most commonly used cell substrates for production of biotechnology products today are *E. coli* and mammalian cells such as CHO. The greatest difference in terms of downstream processing after product isolation is the need to remove endotoxins from *E. coli* and known or potential viruses from mammalian cells. Even in bacterial and yeast substrates, animal-derived media components have raised regulatory concerns over viral safety.

Viral safety issues must be addressed prior to the start of clinical trials in humans. Precautionary measures include screening cells and unprocessed bulk, as well as performing viral clearance studies. Surface properties of virus vary and a combination of techniques is often required for effective clearance. Viral clearance is achieved by removal and/or inactivation. Table 3.7 shows a typical purification process for a monoclonal antibody and the unit operations that provide viral clearance. Viruses tend to have a pI between 3 and 7 and can often be separated from monoclonal antibodies on anion exchangers.

The availability of polymerase chain reaction (PCR) has enhanced the process developer's ability to understand early in development which steps will be effective in virus removal. Viral risks can be mitigated by evaluating which potential viruses might infect the cell substrate; using PCR to quantitate endogenous retroviral particles, which are inherent in mammalian cells such as CHO; and evaluating the efficacy of each removal step in an exploratory study. The use of PCR is discussed further in the Chapter 5.

One other risk that must be addressed is TSE agents. For highly purified biotechnology products, the risk can be mitigated by using non-animal derived materials, or if animal-derived materials are necessary, by using those that are sourced from countries in which BSE is not considered to be a risk. TSEs are caused by infectious prion proteins but, in fact, the risk of transmission may be very small [33]. There has been concern over the use of animal-derived serum in cell culture, with the theoretical risk of co-purification of the infectious prion protein with a biotherapeutic. If the risk is not defined, then for some regions of the world it is necessary to re-bank the WCBs [34]. One estimate of the risk for variant Creutzfeldt Jakob Disease (vCJD) from contaminated foetal calf serum (FCS) used to prepare a viral working seed was conservatively calculated at 1 case in 2 billion doses of vaccine [35].

The source material itself should be considered. The risk from vCJD is one that must be mitigated for production of human blood-derived products. This risk is usually mitigated by donor screening and performing clearance studies using a model prion protein, e.g. the sheep scrapie agent [36]. Such studies are very expensive and although clearance can be achieved by chromatography, filtration and precipitation methods, cleaning becomes a significant issue (see also Chapter 7).

Endotoxins cause fevers in humans. Endotoxin limits are set by USP (U.S. Pharmacopoeia) and other Pharmacopoeias based on endotoxin units per dose and patient weight. Endotoxins are highly negatively charged macromolecules and may be adsorbed on an anion exchanger. As with nucleic acids the alternative is to adsorb the product to a cation exchanger, hydrophobic or affinity chromatography resin and allow the endotoxins to pass through the packed column. Although there are commercially available chromatographic resins designed solely to remove endotoxins, their use is likely to increase the number of steps in a process, with concomitant potential for product loss and increased costs.

Finally, when designing a new process consider current industry standards. New technologies may offer better economy. Furthermore, regulatory agencies expect current technologies that can enhance patient safety to be used. Issues that can minimize patient safety risks include using newer resins that tolerate harsher, more effective cleaning agents and using improved analytical methods to assess removal of impurities. Current technologies may also require controls and testing methods that were not in place prior to their implementation. For example, when disposables are used to minimize cleaning efforts, the potential for extractables must be evaluated.

### 3.5 DOWNSTREAM PROCESSING

The downstream process consists of recovery steps that isolate the target product from the production system and purification steps that remove all impurities to an acceptable level. The type and sequence of steps depend on the target molecule and the production organism.

In this section, we describe generally applicable process-design strategies and the logic leading to them. Details of step design and features of the methods used are provided in Chapter 4 on Separation Technologies.

#### 3.5.1 Recovery process

The recovery process design depends largely on the way the host organism produces the product. It prepares the product for purification and protects the purification steps from damage or performance and robustness issues due to impurities originating from the cells or the culture media. A summary of recovery methods is described in Table 3.8. This table lists production sources, recovery steps and technologies. Further information on recovery methods is provided in Chapter 4.

**Table 3.8**

Summary of typical recovery operations (see also Chapter 4)

Production source/product location	Typical recovery process steps	Typical technology selection
Mammalian cells, secreted	Cell removal	Centrifugation, filtration cascades, filter-aids
<i>E. coli</i> , intracellular inclusion bodies	Cell disruption, inclusion body isolation, renaturation, clarification	Chemical or mechanical methods for disruption
<i>E. coli</i> , periplasmic (soluble)	Cell disruption, clarification	Mechanical disruption, lysis, filtration, centrifugation
<i>E. coli</i> , secreted	Cell removal	Filtration, centrifugation
Yeast, intracellular (soluble)	Cell disruption, clarification	Mechanical disruption, lysis
Human plasma	Capture of target protein from raw material	Ethanol fractionation (Cohn), chromatographic capture
Transgenic milk	Clarification	
Tissue	Cell disruption, clarification	

### 3.5.2 Purification process

Ideally, the purification process starts with a clarified, particle-free intermediate product transferred from the recovery process. Chromatography, tangential flow filtration (TFF) and virus filtration are the main methods currently used for purification of proteins. Virus-based vaccines are purified using centrifugation, filtration and chromatography. Plasmid DNA methods include precipitation, extraction and chromatography.

At the core of purification process design is the selection of methods and the development of the most appropriate sequence of steps. Each of the methods use raw materials (resins, membranes, buffers, etc.) and requires a number of associated activities, which need consideration in developing the most economical design and in adapting it to the facility and routines available for manufacturing.

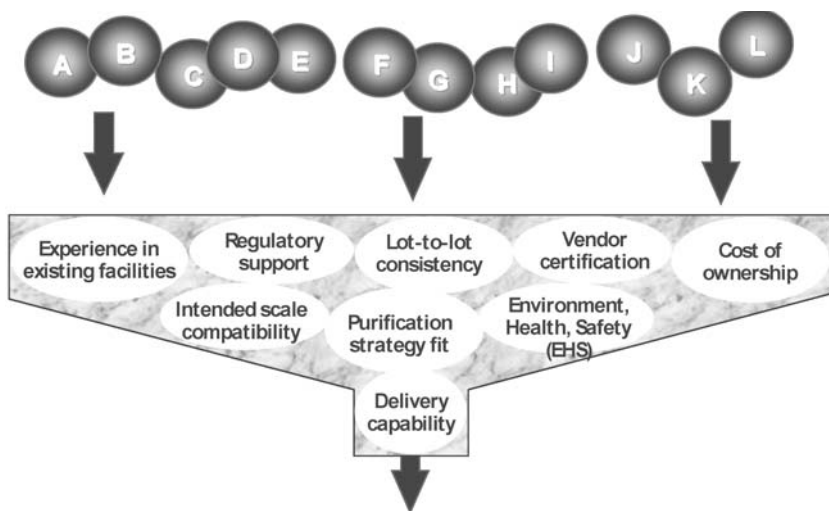
This section describes key activities to be performed by the process developer for the design of a robust purification process. The number of options available for the design is seemingly endless. However, heuristic approaches allow significant reduction of those options without compromising science and the outcome.

#### *Selection of industrial raw materials*

Experimental work is best reduced to evaluating methods and raw materials that fulfil criteria required for compatibility with manufacturing scale, the need for a robust process and regulatory compliance. When time allows, promising technical alternatives will be investigated even if they do not (yet) fulfil the criteria used for pre-selection.

However, an essential part of the success of the heuristic approach comes from limiting the options to those established by experience. Figure 3.6 illustrates the pre-selection





**Figure 3.6** Pre-selection of chromatography resins for manufacturing, heuristic approach to robustness and regulatory compliance. The illustration shows key selection criteria to be applied prior to or independent of experimental performance evaluation. The same or a similar set of selection criteria can be used for filters, TFF membranes or virus filters. Ideally, the development master plan describes acceptance criteria to be used for this selection.

criteria that can be applied to both membranes and filters and also to chromatographic resins. Ideally, the general acceptance criteria for selecting processing tools are documented in the company's development master plan.

Procurement teams accountable for purchasing materials are often in charge of conducting systematic vendor audits. Assessment criteria include delivery capability under normal and worst-case conditions in line with the intended production scale, compliance with key regulations, regulatory documentation support and compliance with environment, health and safety regulations (EHS) applicable to the location where the purchased materials will be used.

Manufacturing staff supports this audit with information on existing facilities and their potential constraints related to the use of various technologies. Preferences based on existing handling experience are often provided, and the strategies used to reduce the number of raw materials used at the facility are usually included.

Process-development teams support the pre-selection with information on the latest technology developments that should be included, the requirements of the specific purification strategy, testing of lot-to-lot consistency of selected candidates and a cost-of-ownership estimate. This term describes the cost for buying and using or storing a product over the time it is in ownership of the company that applies it in its operations. It is a better selection criterion than just price because it takes all activities and investments into account that are needed as a consequence of selecting the product.

The iterative process of gaining experience through development projects, ongoing manufacturing and supplier audits allows identification of preferred vendors, preferred production technologies for most steps and preferred purification or separation products.

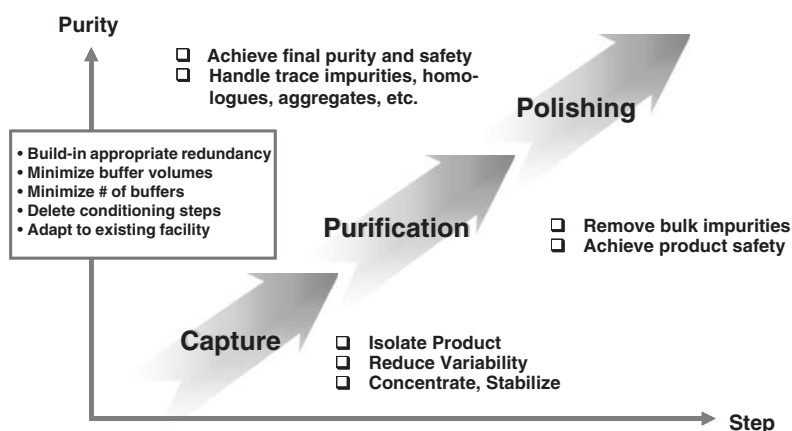
Using a pre-selection strategy significantly increases the chances of producing a successful design and usually provides reliable development data in a shorter time. Whether the selected raw materials are used in a largely standardized process platform or in a *de novo* design of a manufacturing process, this strategy significantly increases the probability that the process will be robust and validatable.

### 3.5.3 The capture, purification, polishing (CPP) concept

Following a risk analysis, the purification process is typically designed in three stages, each with a distinct purpose: capture, purification (sometimes referred to as ‘intermediate purification’) and polishing. Steps achieving viral safety as well as steps required to concentrate the intermediate product or to change the process buffers are woven into the three main purification stages.

Together with the pre-selection of raw materials, the capture, purification and polishing concept provides assurance that the process developer will deliver robustness and more straightforward validation of the resulting process. Figure 3.7 provides an overview of the concept and Table 3.9 gives more detailed information on the whole process, the scope of each step, technologies recommended and the specific challenges that often confront the process developer. More details for each technique used in purification are provided in Chapter 4.

Typically, in the initial process-development phase the selectivity of alternative resins for each step will be investigated. Dynamic binding capacity will be determined. Next, the conditions for each step will be optimized, often as the best compromise between



**Figure 3.7** Capture, purification and polishing. This three-stage design of purification processes enables a focused approach to remove identified risks. Each stage can use the best suitable technology to achieve a limited number of key objectives. Each stage builds on the others, and together they achieve the most cost efficient and robust production of the target substance. The concept is applicable to essentially all biopharmaceutical products and has been established successfully throughout the industry.

Table 3.9

Process design, each step in a modern production sequence is dedicated to a well-defined objective

		Process step	Key objectives	Technology applied	Key challenges
Upstream		Mammalian cell culture	High product titer	High producer cells:	Achieve robust synthesis of target features at high titers
		Microbial fermentation	Product folding and post-translational modifications correct for intended function Product produced in native form, easy transfer to DSP	<ul style="list-style-type: none"><li>• Mammalian: CHO, NS0</li><li>• Microbial: <i>E. coli</i>, <i>S. cerevisiae</i>, <i>P. pastoris</i></li></ul> Protein-free, chemically defined culture media	Achieve shorter batch time  Simplification of down stream processing
Downstream process	Recovery	Product isolation Cell separation	Isolate and prepare product for purification Remove producing cells and/or cell debris Protect purification steps from performance issues	Cell disruption methods	Achieve quantitative product recovery
				Protein re-folding, renaturation	Maintain biological activity
				Centrifugation	Control release of impurities from production cells
				Membranes: normal flow filtration (NFF), microfiltration (MF) Aqueous two phase separation (ATPS), precipitation, flocculation	Minimize generation of additional product related impurities

Purification	Capture	Secure process robustness & economy: • Transfer to stable environment  • Reduce volume	Affinity chromatography  Ion-exchange chromatography (IEC)  Multi-modal chromatography	High binding capacity for affinity resins Capacity in combination with fast handling of large volume Achieve very high product yield
	Purification	Removal of the bulk of process-related impurities  High log reduction for virus	Ion-exchange chromatography  Multi-modal chromatography	Find best selectivity for broad range of impurities, e.g. HCP, DNA, aggregates Find step(s) that contribute high level of virus clearance
	Polishing	Removal of remaining traces of process-related impurities Removal of product-related impurities	Hydrophobic interaction chromatography (HIC) IEC, HIC  Reversed phase (RPC)	Reproducibly remove wide variety of impurities at very low concentration  Minimize generation of additional product related impurities
	Virus clearance	Biosafety, control of risk for infection	Size exclusion (SEC) Inactivation and removal methods	Flux and cost of virus filters
	Formulation	Transfer to formulation buffer	Ultrafiltration/diafiltration	Long-term product stability

*Note:* Technologies applied to achieve the objective are selected based on experience and thorough understanding of their capabilities. For each process step, there are some key challenges.

capacity, purity and time required for the step. One important constraint is that quality and process robustness can never be compromised in the design. The cleaning-in-place (CIP) regime will be developed taking into account any known contamination risks and life-time targets. Finally, the steps will be linked to each other in the 'process integration' phase (see Section 3.5.4).

Platform technologies often incorporate this three-stage design. The capture, intermediate and polishing steps for a monoclonal antibody are described in Section 3.6.

#### *Capture stage*

This step isolates the product from the bulk of the impurities. These impurities may include water, proteases, DNA, endotoxins, culture media components and cellular debris and lipids remaining post-recovery. A several-fold reduction in volume can be achieved by capture steps, which results in significant time and cost savings further downstream. Shorter time for loading the feed and smaller hold tank volumes are examples of such savings. Ideally, the capture step transfers the intermediate product into controlled conditions that no longer carry a product-stability risk. The step yield should remain high, e.g. considerably above 90%.

In order to purify the product and stabilize and concentrate it in this one step, techniques are utilized that can specifically bind the product and let impurities flow through. In principle, an affinity resin that is highly specific for the product serves this purpose best. However, resins should be chosen, not only for their selectivity, but also for capacity and economy of use. The best cost–performance relation should be sought. Affinity and ion exchange chromatography are the most commonly used purification techniques at this stage.

This is the purification step in which the most complicated feedstream is applied. Column fouling can be an issue, depending on the nature of the feedstream and its interaction with the resin. Rather harsh cleaning agents may be required to maintain performance and enhance column lifetime. A well-designed capture step is a key contributor to good process economy, which depends on process yield and reduction of process failure (see Chapter 8).

#### *Purification stage*

Impurities are already significantly reduced after the capture step. However, there is usually one (intermediate) purification step that is designed into the process to remove the bulk of remaining impurities such as HCP, DNA, or other process-related impurities. This step may also provide viral clearance, if needed. In some cases, the product is so pure after the capture step, that this purification step becomes a polishing step, i.e. one designed to remove traces of impurities. Most Mab purification processes work in this way, since capture with Protein A affinity leaves only minute amounts of impurities for the subsequent steps to remove.

Typically, ion-exchange or hydrophobic interaction chromatography serve as purification techniques for this intermediate stage. In many cases, this step is designed so that the product does not bind to the resin, but the impurities do.

This operational mode is referred to as ‘flow-through’ in contrast to the ‘bind-elute’ mode in which the product is adsorbed by the resin. Flow-through steps offer the advantage of increased capacity to process the target product, especially when impurities are only present in low concentrations. Smaller columns than those needed for the corresponding bind-elute mode can be used. Small anion exchange devices can be used in this stage, either columns or membrane based (so called membrane adsorbers that allow high volume throughput but have relatively low total protein binding capacity). Even a few litres of such adsorbents can bind the impurities from a large volume of intermediate product.

#### *Polishing stage*

Polishing steps are generally used to remove traces of impurities, e.g. modified target molecules that are deemed to be undesirable in the final product. Modifications such as oxidation, incorrect glycosylation and aggregation can produce variants that alter product efficacy, potency and safety. Deamidation is an issue to be addressed when purifying some insulins.

Polishing steps often necessitate a very high degree of resolution, which may require resins with smaller particles and concomitant higher cost. However, the volumes and total loads are reduced by the capture and intermediate steps, so smaller columns can be used. Ion exchange, size exclusion and reversed phase are techniques that are typically utilized for polishing steps.

The term polishing is also applied to the, typically two, post-Protein A steps in Mab processes where the emphasis is on product aggregates and also on the removal of the remaining traces of HCP. In these processes, resins with relatively large particle sizes, i.e. 60–90 µm, are often used. Ion exchange, hydrophobic interaction chromatography, ceramic hydroxyl apatite and more recently multi-modal chromatography are the techniques used in these steps.

### **3.5.4 Process integration, combining steps for an efficient process**

In a manufacturing process for biologics, the result from each step can influence the performance of the others. However, the steps are not necessarily linked to each other in an optimal fashion in the first version of the design. Sub-optimal links can be time-consuming and costly; they may even force the introduction of additional steps or adjustments to the intermediate product (associated operations). Process integration is the phase of development where these issues are resolved (see Table 3.10).

The difference in batch time between mammalian cell culture (10–20 days) and the downstream process (2–5 days) leads to low utilization of the DSP line, if not more than one fermentor is producing product in. Many facilities have three to four, sometimes six to eight fermentors operating in a parallel, staggered mode so that the next upstream batch is delivered once the downstream line is ready with the previous batch, e.g. every 2nd day.

Batch sizes from large fermentors operated at high product titer can be inconveniently large for processing on the capture column in a single cycle. Many processes

**Table 3.10**

Process-integration challenges, typical links between process steps or associated, non-productive activities that should be optimized during process integration

Issue with link or associated operation	Process-integration strategy
Difference in batch time between upstream and downstream processes (mammalian cell culture)	Run several upstream batches in staggered mode against one DSP line
Very large product batches require inconveniently large and costly capture step	Divide each batch and process in a number of cycles, process on smaller column, develop method to store product post-recovery
Large numbers of buffers, different for each step and for load, wash, elution and CIP	Optimize steps to use the same buffer or just a few standard buffers, use NaOH for CIP
Hold times between steps, adjustment of intermediate product for loading on next step	Automatic, in-line buffer adjustment, optimize sequence to minimize adjustment
Frequent column packing	Optimize CIP method to reduce need for re-packing, use automated packing and slurry preparation (see Chapter 12)
Long change-over between production campaigns	Use disposables, keep devices for the next campaign prepared in advance

are designed to process the batch in several cycles and thus allow using a smaller capture column. Since batch time between the two main parts of the process is so much different, another approach can be to ‘de-couple’ the two parts. This may be possible, if a method is available to store the intermediate product without compromising stability, e.g. post-recovery.

If process steps have been developed independent of each other, it is likely that they use different buffers and elution conditions of one step may not be adjusted to allow direct loading onto the next step. This can be changed so that each step uses only one or two basic buffers and elution conditions match loading conditions for the next step. If the latter is not possible, one may at least design for the possibility to switch buffers with in-line adjustments, i.e. without hold time between the steps. For the whole process, the number of different buffers and cleaning solutions can be minimized to three or four, and sodium hydroxide can be made the standard for cleaning columns and filters in many processes.

Packing of large columns takes 1–2 days including testing. Therefore, the need for column packing shall be reduced. One important route to achieve this is to make sure that nothing accumulates on the packed bed or the column parts that disturbs performance over time. The cleaning in place method for the column can be developed to prevent this, if one systematically looks for the issues and does not just implement a method in the assumption that there will be no issues.

Once the process is designed and transferred to the facility, a few additional aspects of integration appear: more than one product may have to be produced in campaigns following tightly after each other. Change-over between campaigns takes time away from

the available productive time in manufacturing. The use of disposables as well as timely preparation of devices for the next campaign, e.g. columns, are typical approaches to reduce the change-over time. Another such aspect arises when the timing of the individual steps needs to fit into work shifts, or when the facility has other limitations such as limited buffer preparation and storage capacity. This is an aspect of process integration that ideally is addressed through intensive communication between development and manufacturing well in advance of the transfer. However, the same issues may come up when a process needs to be run at a contract manufacturer. From an efficiency perspective some of these adjustments may be counterproductive. However, since facilities are very expensive to build, it may not be an option to use all of the improvement opportunities listed in Table 3.10. (For more details see Chapter 2 and Chapter 8.)

### 3.6 SELECTED DOWNSTREAM PROCESSING PLATFORM EXAMPLES

A platform technology is built on a foundation of knowledge. Experience in purification of similar products, e.g. from the same class of proteins such as IgG antibodies, provides that knowledge for downstream processing platforms. Platform technologies facilitate rapid and economical process development and scale-up, which potentially lead to an increase in the number of product candidates that can be evaluated, rapid market entry, and even a reduced validation effort. Familiarity with a process can result in more robust processes and better technology transfer from development to manufacturing. As noted during an oral presentation, platform technologies create predictable activities and durations that result in a generic timeline [37]. A few other potential advantages are the use of established vendors for raw materials and established waste disposal. The use of platform technologies for cell culture and cell clarification provides a greater likelihood of success for the downstream platform since the process impurities as well as the majority of host cell impurities will be very similar. There are also platform analytical approaches that are applied to development of monoclonal antibodies.

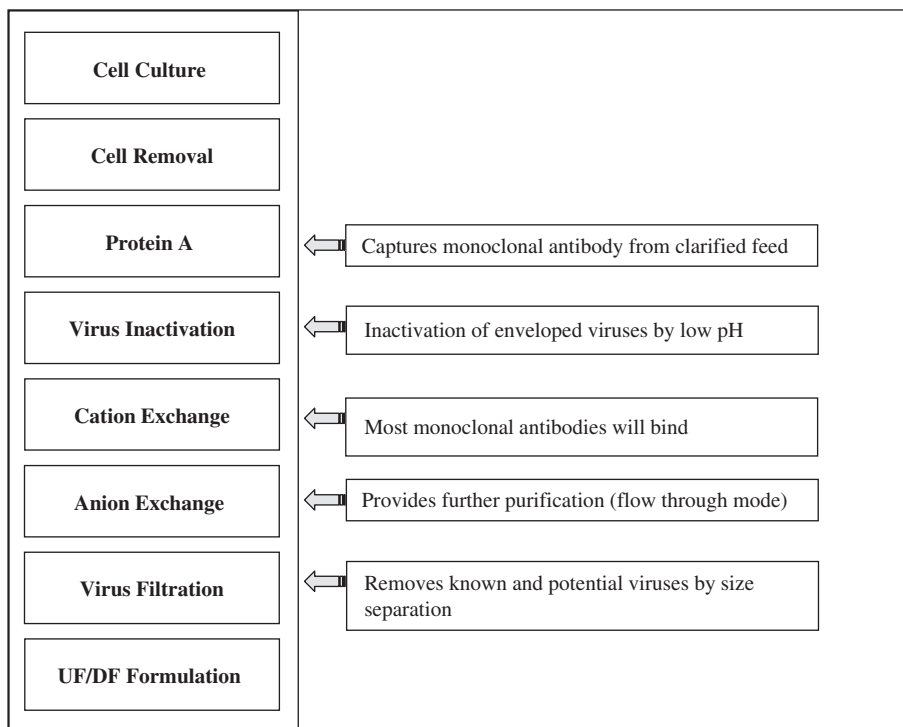
This platform approach has reduced the time to toxicology and first-in-human (FIH) studies, reduced the number of protocols and complexity of multi-site operations, eased setting of preliminary specifications for INDs and built more robustness into FIH methods and testing [38].

Platform technologies are composed of a number of unit operations and methods. Not only the order and type of steps can be templated, but also many process conditions (e.g. buffer, flow rate) can be fixed. There are two classes of biotechnological products for which such technologies exist—namely, monoclonal antibodies and DNA plasmids.

#### 3.6.1 Monoclonal antibodies

An example of a platform technology for a monoclonal antibody processing is shown in Figure 3.8.





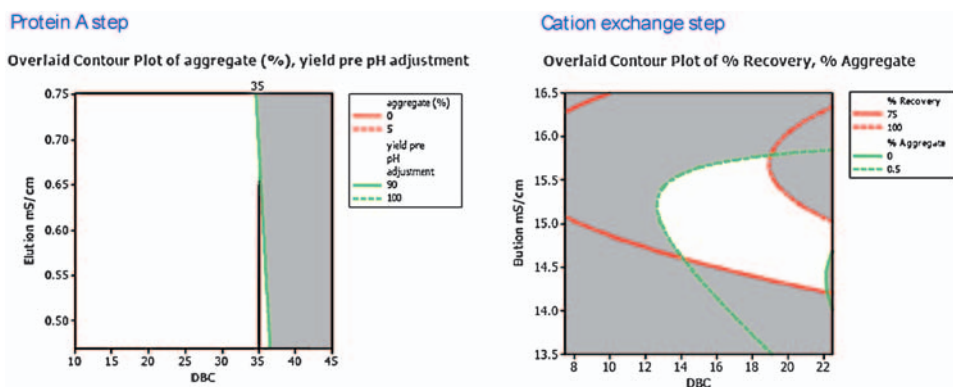
**Figure 3.8** A platform technology for monoclonal antibody purification.

IgG monoclonal antibodies consist of two heavy and two light chains. They have a molecular weight around 150 kDa, are glycosylated and have an isoelectric point between pH 6 and 9.

The main success of the downstream platform technology arises from the capability of Protein A to selectively bind human IgG of sub-class 1, 2 and 4 at the Fc region, which facilitates the isolation of monoclonal antibodies from cell-culture harvest. Purity levels are commonly 99% or greater after this one unit operation. True affinity steps that bind their target always through the same mechanism and at the same binding site support a platform concept in two main ways: a predictable recipe can be used with very little need for adjustments between different projects and the outcome is also predictable. Very few surprises are to be expected in terms of appearance of new impurities or significantly increased impurity levels post the affinity step. Figure 3.9 illustrates this as a wide window of operation relative, e.g. to a cation-exchange step used in the same position.

Protein A is one example of an affinity method, which has established itself in industrial processes. Very few other affinity steps are used in large-scale manufacturing, but more may be expected if new molecule classes find a similarly wide spread interest as monoclonal antibodies and an attempt to develop a platform concept for them begins to make sense.

Monoclonal antibodies usually differ from one another in surface charge and glycosylation. This makes it necessary to evaluate the suitability of the other platform unit operations,



**Figure 3.9** Contour plots illustrating the window of operation for a typical Protein A affinity step and a cation-exchange step, both in purification of a monoclonal antibody (courtesy: Julian Bonnerjea, Lonza Biologics plc, Slough, UK).

i.e. post-affinity chromatography. In some cases, the order of anion and cation exchange is reversed, hydrophobic interaction or hydroxyapatite columns can be used. Prior to adding or substituting a chromatography step in a platform, it is advisable to first evaluate column load, wash and elution buffers and pH to optimize the existing step. Notably, some antibodies are prone to aggregation and will only be stable within certain pH and conductivity ranges.

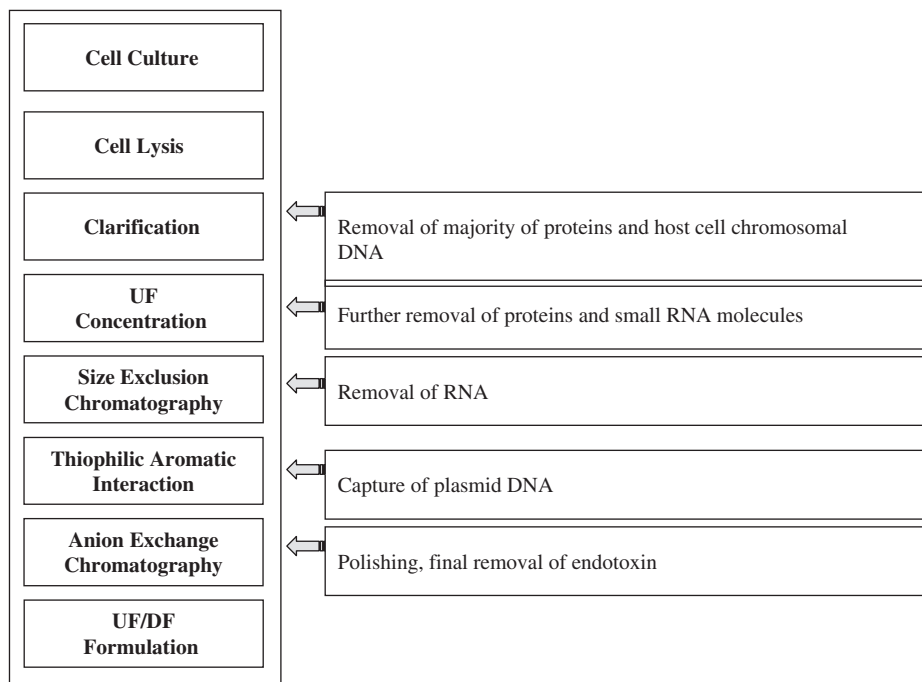
### 3.6.2 Plasmid DNA (pDNA)

Plasmid DNA technology allows the use of platform technologies not only in purification and analysis, but also in production of the target molecule.

Plasmid DNA production can be dependent on the *E. coli* cell line and the plasmid backbone. The creation of *E. coli* cell lines with high expression levels and plasmid DNA backbones has drastically increased the output from fermentation in recent years [39, 40]. This stresses current downstream purification processes and requires an adaptation of the purification strategy. The relatively consistent physico-chemical properties of plasmid DNA support the attempt of the development of a true purification platform. However, the use of plasmid DNA is by far not as established as for monoclonal antibodies. Platform concepts for these target molecules are only beginning to emerge.

The typical process contains the following steps (see Figure 3.10): first, the pDNA is extracted by lysing the cells. Although different means of lysis have been investigated thoroughly, by far the majority of lysis procedures make use of SDS–detergent lysis at alkaline pH [41, 42]. Typically, at larger scale, the SDS–protein complex is removed by filtration, while at small-scale centrifugation is the preferred technology. After filtration or centrifugation, a clarified starting material for downstream processing is obtained.

The clarified material is first concentrated using hollow fibre ultrafiltration to decrease volume and allow for faster downstream processing. Besides concentrating the sample, this also removes part of the RNA, the main impurity. The next step is size exclusion



**Figure 3.10** A platform technology for pDNA purification.

chromatography (SEC) in group-separation mode, which removes the bulk of RNA impurities and conditions the sample for loading on the third purification step, thiophilic interaction chromatography. In this step, the specific isoform of interest, supercoiled plasmid DNA, is captured. In an alternative approach, this type of chromatography is replaced by hydrophobic interaction chromatography. Finally, the plasmid DNA sample is polished on an ion-exchange chromatography column before an ultrafiltration/diafiltration step formulates the final bulk product.

Different platforms can be developed, if the same major objectives are fulfilled: volume reduction, RNA removal, supercoiled plasmid DNA capture and final formulation. In one alternative platform, ion-exchange chromatography is used to capture the plasmid DNA and selective elution contributes to RNA removal [43]. Another approach adds an orthogonal step to increase product purity. In this approach, a lyotropic salt is added to the clarified alkaline lysate to permit application of the sample to a HIC column. After capture of supercoiled plasmid DNA by HIC, the product is polished by purification on an anion exchange resin [44].

Comparing the different processes will reveal advantages and disadvantages for all of them, but they are all based on increasing experience with large-scale plasmid DNA purification and can significantly reduce process-development times and allow production of a large number of different pDNA in a short timeframe.

### 3.7 CHARACTERIZING THE PROCESS, PROCESS UNDERSTANDING

Every process needs to be characterized in order to understand the relation between process performance and product quality and in order to develop the process-control strategy for manufacturing scale. This section will discuss three aspects related to this development of process understanding: first the concept of ‘design space’ established by the International Conference on Harmonization (ICH), next ‘design of experiments (DoE)’ as a key tool in characterizing the process and finally ‘process characterization’ as the last step in process design leading to validation and approval.

#### 3.7.1 Design space concept, ICH Q8

The importance of process development cannot be overemphasized. Process development is now addressed by ICH Q8, entitled Pharmaceutical Development [45]. This document defines the ‘design space’ as ‘The multidimensional combination and interaction of input variables (e.g. material attributes) and process parameters that have been demonstrated to provide assurance of quality. Working within the design space is not considered a change....’. When working within the design space, process changes in manufacturing can be made much faster to enhance productivity, improve yield or provide other process improvements. The data for the design space come from early development, characterization studies, validation and manufacturing experience correlated with clinical data. The principles of process development and its role in supporting product-development optimization are described for bacterial fermentation [46], but the same principles can be applied to downstream processing.

Documentation of development and characterization studies is critical, in part because it enables technology transfer, trouble shooting and investigations of out-of-specification (OOS) results. Furthermore, development reports are requested by some regulatory agencies. Development reports are also critical for future process changes. As noted in ICH Q8, inclusion of relevant knowledge gained from experiments giving unexpected results can be useful. Quite often development personnel move to another company. When a process change is made and there are no development data, it may not be clear what effect the change will have on product quality. In some situations, no one knows why a specific step was incorporated and process change necessitates repeating some clinical studies. A comparison of the costs of documenting development with those of clinical trials will provide evidence as to the importance of documented process development.

#### 3.7.2 Statistical design of experiments (DoE)

Once a suitable process has been identified based on heuristics, the next step towards achieving a validated state (i.e. a well-controlled and understood process) is to perform studies to quantify cause–effect relationships from the critical process parameters (CPPs) to the CQAs of the product produced with it. DoE is a powerful tool from a statistical perspective in quantifying these relationships, but it is important to point out that any DoE study should be built on a foundation of process know-how and empirical knowledge whenever available. With increasing importance being placed on process understanding,

process-development tools such as statistical DoE are seeing more widespread use. As implied by the name, statistical DoE is a method of selecting experimental conditions to maximize the potential for statistical analysis of the results. This statistical analysis can be used to determine the impact of process variables on the process output, provide model equations to predict the results of process changes and define the robustness of the process through analysis of normal process variability [47].

The final goal of many development studies of chromatographic unit operations is to establish ranges for CPPs within which the process outputs meet acceptance limits, i.e. the design space and control limits.

Statistical DoE provides several advantages over the traditional process-development approach of examining the impact of each process variable, or factor, individually. The first is the ability to assign a numerical value to the impact of each factor on the process. This significance value can be used to focus development efforts on factors that can be manipulated to optimize the process, or increase process robustness. Because the factors are varied concurrently, the potential impact of one factor on another is examined, as well as its individual contribution. This reduces the risk of neglecting to characterize the impact of a process variable that, on its own, does not significantly impact the process, but when combined with variation of another factor could lead to unexpected results. It also increases the likelihood of finding the true optimum for process conditions. In determining the significance and interactions of factors, model equations are developed. The predictive ability of these equations can be statistically determined, and increased through further experimentation. These equations can then be used to predict the results of untested experimental conditions and model even complex process behaviour. One important aspect is that with multiple CQAs, several model equations have to be developed and combined to determine the control limits for the CPPs.

While there are many software packages that can assist in statistical design of experiments, DoE is used most effectively when combined with knowledge of the target solute, impurities, contaminants, the chromatography resin and process limitations. This information is key in building a DoE that will provide the most relevant information. What factors are examined, and over what ranges those factors will be tested will depend on how those factors are likely to affect the target solute, what you are separating it from and the agent of separation, as well as the ability to achieve those conditions in a manufacturing environment. For this reason, it is generally desirable to obtain this information before setting up a DoE by conducting experiments, researching literature, contacting the chromatography resin vendor and discussing the capabilities of your facilities at final scale.

Once preliminary information has been gathered, DoE can be utilized in three distinct stages of process development. The first stage of process development is generally the selection of factors to be used in the first definition of the process. This can be facilitated by conducting a 'screening' DoE.

A list of all factors that could potentially impact the process should be compiled. To reduce the number of experiments necessary, one may use heuristics to exclude those factors that is known to have a significant impact on the process, as they will be examined in the next stage of process development. A low-resolution, fractional factorial design can then be selected to screen the remaining factors for significance in order to identify the CPPs. The range over which the factors should be tested should be rather broad at this stage. Once the statistical significance is determined for each factor, the practical significance should be examined.

The next stage of development in which DoE is useful is the optimization of process conditions. In this stage, the identified CPPs (significant factors from the screening design and previously known factors) are varied over a more narrow range, and the optimum conditions are determined. Information from the screening DoE, preliminary experiments and knowledge of production capabilities can be useful in setting appropriate ranges for these experiments. The goal of this stage is to have a clear understanding of the impact that each factor has on the process over the range examined. This understanding can be measured in the ability of the resulting model equations to explain the experimental results through various statistical tests. High-resolution factorial designs may be sufficient to provide good model equations, but more complex process responses may require additional experiments to refine the model equations, e.g. by including quadratic terms to explain curvature. Once the effects of the factors are sufficiently defined by model equations, those equations can be used to determine the optimum conditions for the process.

When optimum process conditions have been selected, the robustness of that process must be examined. Once again, DoE can be utilized to build model equations that will define process robustness. In some cases, the model equations used in process optimization will be sufficient to define process robustness. They can be tested over factor ranges slightly larger than the process specifications, with the target conditions at the centre. If the optimization model equations do not suffice, a new DoE with much smaller variation intervals compared to screening and optimization studies can be performed to build more accurate model equations. The purpose of the robustness study is to ensure that the CQAs remains under control when the process is subjected to variations within the design space.

DoE studies can be performed in any scale, but due to time- and cost-restraints larger screening studies are commonly performed at laboratory scale, whereas optimization and robustness studies are performed at lab- and/or pilot scale, in some rare cases even production scale. More recently, development labs have started to use high-throughput experimental setups with robots and microtiter plates allowing hundreds of experiments in the same time where the classic sequential workflow would only allow a few.

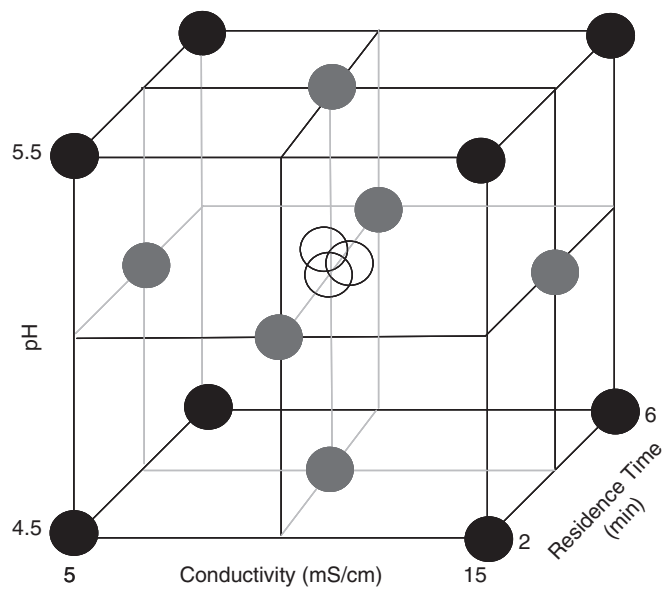
Data from a DoE study<sup>3</sup> on a recently introduced cation exchanger will be used to illustrate the use of DoE from an optimization and robustness assessment perspective. The effect from residence time (2–6 min), conductivity (5–15 mS/cm) and pH (4.5–5.5) on dynamic binding capacity at 10% breakthrough (QB10%) for a monoclonal antibody was studied. A total of 17 experiments, illustrated in the figure below, were performed to fully quantify the effect from the three CPPs on dynamic binding capacity.

Figure 3.11 is a typical illustration of a DoE setup spanning the investigated design space. This particular design is a so called central composite face-centred (CCF design), that enables quantification of all main effects, interaction effects and curvature effects from the studied CPPs.

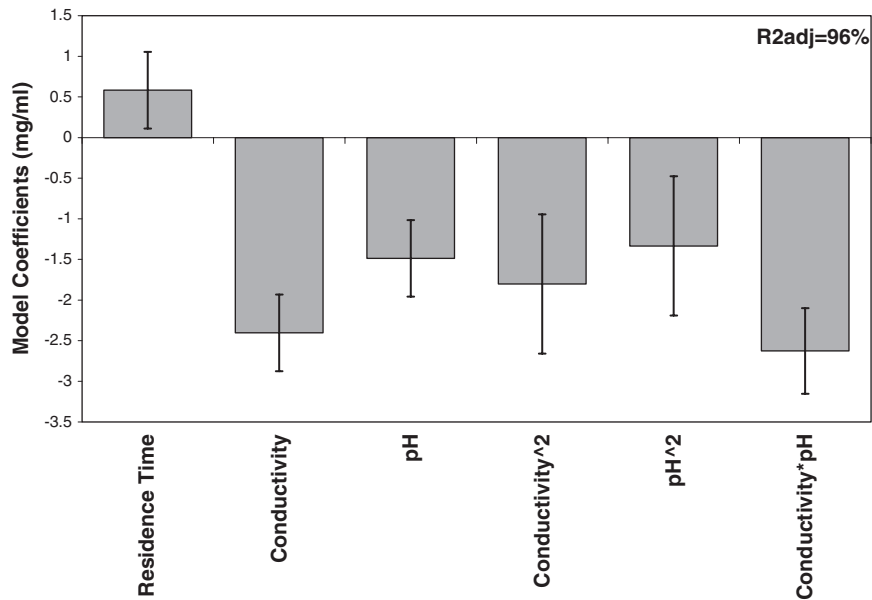
As illustrated by Figure 3.12, it was found that within the investigated ranges, residence time had a small effect compared to conductivity and pH, whereas both conductivity and pH were shown to have significant linear as well as second degree curvature effects on the QB10% for the Mab. In addition, a significant interaction effect between pH and conductivity was found.

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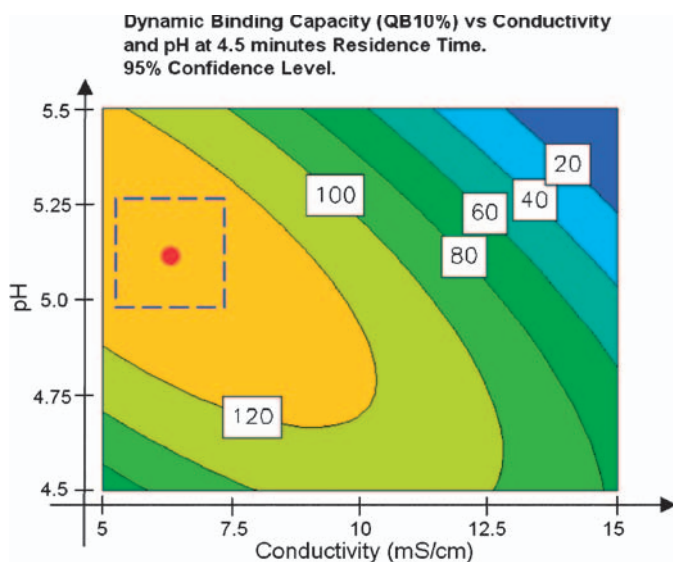
<sup>3</sup> Application Note 28-4078-17 AA, Capto S Cation Exchanger for post-Protein A Purification of Monoclonal Antibodies, GE Healthcare, 2006.



**Figure 3.11** Illustration of a typical design of experiment (DoE) investigating critical performance parameters of a chromatographic step.



**Figure 3.12** Model coefficient plot showing the relative size of the significant effects from residence time, conductivity and pH on the dynamic binding capacity for the Mab. The DoE model represented by these coefficients explains approximately 96% of the observed QB10% variation.



**Figure 3.13** Example of response surface plot for a monoclonal antibody on cation exchanger Capto™ S illustrating the effect from variations in conductivity and pH at a constant residence time of 4.5 min. Possible target settings (red dot) and control limits (blue line) would ensure dynamic binding capacities above 120 mg/ml with a 95% confidence level.

The rather complex model coefficients for the effects from conductivity and pH on the dynamic binding capacity translate into an easily interpretable response surface, as illustrated in Figure 3.13.

Figure 3.13 shows the combined effect from variations in conductivity and pH on the QB10% for the studied Mab at a 95% confidence level. Assuming that a dynamic binding capacity of at least 120 mg/ml is always wanted from this process step, it would be reasonable to set the target for pH at 5.1 and the target for conductivity at 6 mS/cm (as illustrated by the red dot) in order to give some room for variation (illustrated by the blue lines) in these parameters and still be able to have a dynamic binding capacity of at least 120 mg/ml.

It is also important to note that, as with any study, some additional runs should be performed in the region of greatest interest to verify the indications from the study. In this example, a robustness test centred on the indicated set point (red dot) with narrow variation ranges that are still practical in manufacturing (the blue lines) could be the final test before proceeding to conformance runs.

### 3.7.3 Process characterization

During process design and process development a certain degree of information on important characteristics of the process are already collected. Process characterization is a dedicated and more systematic effort to determine the operating limits that enable



production of a defined product with defined quality. Process characterization is the natural end point of a process-design project in that it provides both a confirmation of the design and a deeper understanding of the mechanisms the design uses.

This activity takes place once the process is designed, generally not until after clinical phase 2, and is usually discussed in conjunction with process validation (see Chapter 7). The CQAs of the product should be determined from earlier development studies linked to clinical trials before process characterization is performed. The effect of CPPs on those CQAs within the design space is formally established in the characterization studies. These studies further the understanding of what each process step does—i.e. what impurities it removes and what product quality results from each step. Characterization studies provide the process understanding that enables process control in manufacturing such that batch failures are minimized. A step-wise approach to process characterization has been described [48]. This approach addresses timing of the studies and the requirement for pre-characterization work, which includes data mining and risk assessment, qualifying a scale down model and development of characterization protocols. A detailed description of process characterization studies is also described in a book chapter [49]. In the previous section, we presented DoE as one important method used in process characterization studies today, c.f. the concept of robustness testing. Analytical methods that are applied are discussed in Chapter 5.

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## Separation Technologies

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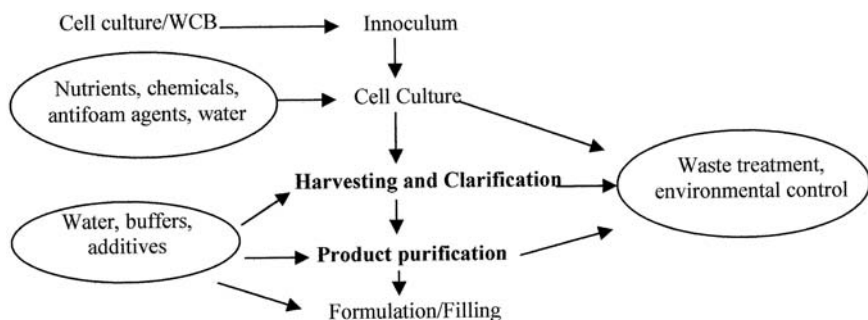
### 4.1 INTRODUCTION

As discussed in Chapter 3, biopharmaceuticals are today mainly produced by genetically engineered cell culturing based processes. Such a process may be divided into the actual cell culture, separation and formulation (see Figure 4.1). The separation step is typically divided into two distinct operations—recovery and purification. Recovery steps usually include product isolation and feedstream clarification, which also provide a certain level of purification. Following recovery steps, purification steps enable production of high-quality biopharmaceuticals. In this chapter, current technologies for recovery and purification are addressed, with a focus on purification for cell-culture processes. However, the strategy and methodology may be equally applicable to other sources of raw material, e.g. blood plasma, tissue homogenate or plant extracts.

### 4.2 RECOVERY

Recovery steps isolate the product and prepare the process feedstream for purification. Depending on the host cells, the product may be intracellular, located in the periplasmic space or extracellular. The location of the product will dictate which recovery methods are most appropriate (see Table 4.1 and Chapter 3).

After harvest of bacterial cells that do not secrete product, cell disruption is carried out using either chemical or mechanical methods [1, 2]. The product is captured from the disrupted cells and cellular debris is removed prior to further processing. Cellular debris is typically removed by filtration or centrifugation. When mammalian cells are used to produce a recombinant protein or monoclonal antibody, the majority of product is secreted and cell-removal systems may even be designed into the bioreactor unit operation. Recovery steps are also used to reduce volume and stabilize products by removing harmful substances such as proteases. Regardless of product source, removal of cells, cellular debris, aggregates and precipitated material is usually necessary prior to purification. In some cases, two clarification steps may be necessary—the first to remove large particulates and the second to remove residual lipids, nucleic acids and any remaining cellular debris. A comparison of methods for harvesting a protein from yeast fermentation has



**Figure 4.1** Outline of a cell-culture based production process WCB = working cell bank.

**Table 4.1**

Location of product is dependent on production cell type	
Host	Product locations
<i>E. coli</i>	Inclusion body, periplasm or secreted
Yeast	Secreted
Mammalian cells	Secreted

been described [3]. Centrifugation followed by depth filtration, centrifugation followed by filter-aid enhanced depth filtration, and microfiltration were compared (see below).

#### 4.2.1 Centrifugation

Centrifugation can be thought of as a settling tank driven by controlled centrifugal forces. It is used to separate bacterial cells prior to their disruption and product-containing liquids from cells, cellular debris and other particulates [4–6]. Centrifugation can be run either intermittently or continuously. Large-scale centrifugation is carried out as a continuous process to enable handling of large volumes. Decanter and disk stack centrifuge designs are used in bioprocessing. Decanter centrifuges are typically used with the highest solids concentrations and when the largest particles need to be isolated. Disk stack centrifuges are more commonly used in biotechnology processes to handle relatively high concentrations of insoluble materials, but the resulting effluent may still contain some particulates that need to be removed prior to purification. This is particularly relevant when separating fragile cells that require lower  $g$ -forces. Therefore, centrifugation is often followed by another clarification step such as normal flow filtration (see below).

At large scale, centrifugation requires a significantly large capital investment. Factors to consider when selecting a centrifuge include potential for product loss due to shear forces; hygiene; compatibility with feedstream chemistry, which is especially important

for centrifuge seals; and minimizing processing time when product is in contact with harmful proteases or other substances that may cause degradation or aggregation.

### 4.2.2 Filtration

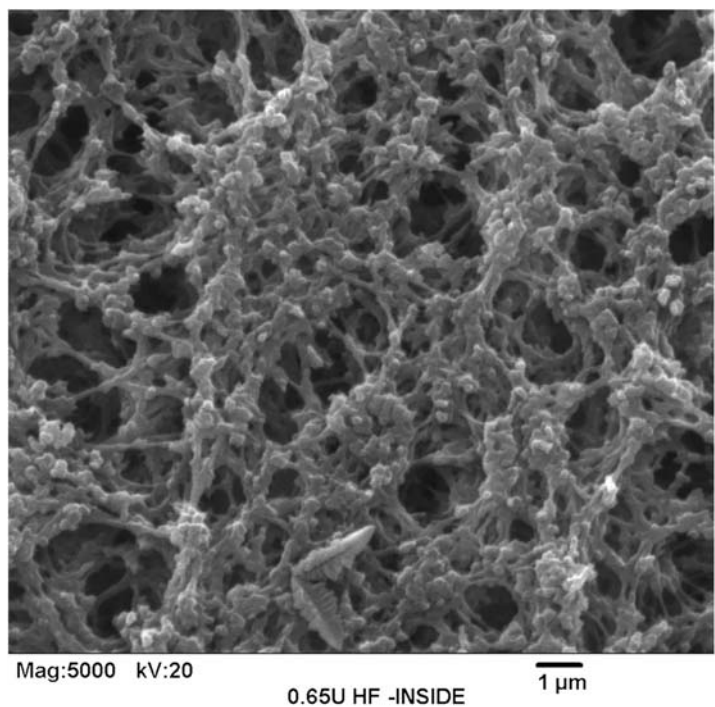
Depth filters and membrane filters are constructed differently, but both can separate product-containing fluids from particulates or be used to harvest cells prior to lysis. There are two flow modes—normal flow (also called dead-end) and tangential flow filtration (TFF). In normal flow filtration, the fluid flow path is perpendicular to the filter surface; whereas, in TFF (also called cross-flow) the flow path is parallel to the filter surface.

Depth filtration is relatively easy to operate and capital costs are low. It is operated in a normal flow mode and typically employs filters made of materials such as cellulose. Depth filters are constructed so that particles in a defined size range are trapped by a combination of mechanisms, including size exclusion and adsorption within the spaces of the internal structure of the filter. Filter aids are sometimes used to decrease clogging of these depth filters and often to enhance the overall separation—in some cases by adding a positive or negative charge [7, 8]. The reference by Arnold lists manufacturers of depth filters. Depth filters comprised of multiple layers have been shown to provide the highest capacity for clarification of yeast cell suspensions. Properties of the layers were investigated and it was found that the optimal construction consists of an upper layer that allows significant yeast cell penetration, while still protecting the retentive layer [9]. Depth filters may also be combined with membrane filters.

Membrane microfilters operated in a tangential flow mode are used to separate particles and produce relatively clear broths. Alternatively, they can be used to isolate particles, i.e. virus or plasmid DNA [10]. Tangential flow minimizes the formation of a gel polarization layer on the membrane surface. Both TFF cassettes and hollow fibre modules for cell harvesting have been discussed [11]. Unlike the depth filters, membrane filters are manufactured in such a way that pore size is well controlled. The construction is intended to prevent distortion of the membrane structure. Flow through the filter is tortuous [12]. The structure of the inside of a 0.65  $\mu\text{m}$  microporous hollow fibre membrane is shown in Figure 4.2.

Optimization of filtration will depend on its intended use. Table 4.2 compares microfiltration for cell harvesting with protein solution clarification. For both operations, high yields and high flux are important for optimal performance. Avoiding fouling by controlling transmembrane pressure (TMP) is particularly important for clarification.

A comparison of three different strategies for recovery from a high cell density yeast fermentation broth has been described. The options included centrifugation followed by depth filtration, centrifugation followed by filter-aid enhanced depth filtration and microfiltration. The options that utilized centrifugation and depth filtration also incorporated a 0.2- $\mu\text{m}$  filter. The authors report that all three options can deliver the desired product recovery, harvest time and clarification targets. The differences arose in process performance, which included recovery, processing time and development time; costs; scalability and process robustness [13]. Microfiltration provided the highest yield, lowest capital cost and highest ease of scalability, but the microfilter was more costly than the depth filters. However, the options incorporating centrifugation had higher capital costs and scale up of centrifugation was noted to be challenging.



**Figure 4.2** Scanning electron micrograph of a 0.65-micron microfilter used for recovery. 5000× magnification. Courtesy of Craig Robinson, GE Healthcare. Reproduced with permission from GE Healthcare Bio-Sciences Inc.

**Table 4.2**

Optimizing microfiltration depends on intended use		
	Clarification	Cell harvest
Product in	Permeate	Retentate
Channel diameter	Larger with larger proteins	Larger with high-cell densities
Path length	Shorter path lengths	Shorter with high-cell densities
Pore size	Typically 5–10 times larger than target protein	Tightest (most dense membrane) that allows full passage of largest media contaminant

**4.2.3 Other techniques**

Other techniques that are being investigated, and may have already been implemented in some cases, include two-phase systems, such as liquid–liquid extraction and liquid-affinity techniques. Affinity precipitation and other precipitation methods have been also



investigated in the past. While future studies may enable these technologies to be more widely applied for recovery and purification of biological molecules, at the moment, they tend to entail a lot of development work.

*Fluidized beds*

One technique that has been successfully applied is expanded bed adsorption (EBA), employing a stable fluidized bed [14]. EBA entails the use of chromatography resins that are initially maintained at a fixed bed height with large spaces between the beads that allow debris to flow through. The product is loaded in an upward direction and the debris flows out the top while the product binds. While suitable for some feedstreams, others may be too viscous and require an additional viscosity-reducing step, e.g. the removal of DNA, so that a stable bed is formed. Product elution is usually carried out after the bed is packed, but there are situations where companies prefer to maintain the stabilized expanded bed instead and accept a dilution of the desorbed sample zone. Cleaning becomes a critical issue when debris-containing feeds are applied to chromatographic materials and fouling may lead to break down of the stable fluidized bed. Direct coupling of EBA with a downstream purification step has been described [15]. In another case, *Pichia pastoris* cultivation was interfaced with EBA [16]. In the production of recombinant human serum albumin by *P. pastoris*, the use of EBA reduced the number of clarification steps from three or four to one [17]. Whereas EBA generally works well for *Pichia*, it has not proven to be generally successful for mammalian cell cultures.

*Protein crystallization*

Crystallization of proteins is a simple and low-cost operation. By combining control of temperature and co-solvent addition, process-scale crystallization can be used to create a stable, purified product. Although not possible for all proteins, crystallization has been described as an alternative purification method, but requires a considerable development effort [18].

Deciding which recovery method to use requires consideration of many factors and empirical work using feedstream representative of manufacturing at full scale (see Table 4.3). The recovery process must be capable of being integrated with other unit operations such as cell culture/fermentation and downstream processing. The outcome should be a consistently clarified feedstream in which the product is stable and ready for purification.

**Table 4.3**

Some considerations for recovery operations

Volume to be processed	Scale-up capability
Amount of insoluble material	Integration with other unit operations
Available equipment	Containment
Cleanability	Multi- or single-use
Economics	Facility capabilities



4.3 PURIFICATION

Purification steps remove process- and product-related impurities from the product. On average for biotherapeutics delivered parenterally, three purification steps are incorporated. For difficult separation problems more steps may be needed. Chromatography remains the most commonly used purification method. As noted in a recent publication on downstream processing, ‘chromatography is still king’ [19]. The use of orthogonal methods (e.g. affinity chromatography and ion exchange) in a logical sequence enhances purification efficiency and reduces manufacturing costs. When selecting recovery or purification tools, the design of those tools should be considered. Design qualification is a concept originally introduced in the medical device arena, but is today applied to biotherapeutics’ manufacturing as well.

4.3.1 Design principles

The increased importance placed on the design of equipment and processes is intended to ensure that pharmaceutical products will be made consistently and meet predetermined specifications. The value of speed to market has led to the use of reliable, vendor-produced chromatography resins and platform technologies that simplify the process developer’s choices.

4.3.2 Chromatography resins

Chromatography resins are also called gels, media and matrix—in this book we use the term chromatography resins for the functionalized micro particles used for separation. Properties of these chromatography resins include particle size and particle-size distribution, chemical stability, rigidity, ligand density and distribution, pore size and pore-size distribution, and hydrophilicity or hydrophobicity (see Table 4.4). Cell-culture productivity

Table 4.4

Chromatography resin properties and the factors they influence

Property	Influences
Mechanical properties	Throughput, potential scale of manufacturing, maximum operating velocity
Ligand density and distribution	Binding capacity, selectivity, recovery
Pore size and pore size distribution	Dynamic-binding capacity
Particle size and particle size distribution	Resolution, product purity, removal of impurities, dynamic capacity
Chemical stability	Lifespan, sanitizability and cleanability
Hydrophilicity/hydrophobicity	Product recovery, cleanability

improvements dictate using high-flow velocities and high-binding capacities in order to purify the required amounts of products in a short timeframe. With a cell-culture productivity target of 10–20 g/L for the next 3–5 years, design of chromatography resins to accommodate those levels is essential for a competitive biopharmaceutical industry [20].

Most process chromatographers first evaluate selectivity and capacity. The selectivity determines which impurities can be removed from the product and the degree of purity obtained by a minimum number of steps. How does a user decide what other properties are important for a given application? Prior to designing a purification process, process developers should address the following questions that will lead to an understanding of important design principles for chromatography resins:

- What is the required purity?
- How much pure product needs to be delivered?
- How much time is totally acceptable and feasible for any given step?
- What is the cost constraint?

When producing a biotherapeutic that will need to be delivered in large doses for a large patient population, consider volumetric throughput requirements. Will the chromatography resin allow rapid pumping through large volumes without column bed compression? This will require a fairly rigid chromatography resin (or special column construction, e.g. multiple columns in series), in other words one with good mechanical properties. Small-scale pressure versus flow curves can provide valuable information on large-scale column flow properties. Although the curves may be non-linear due to chromatography resin compressibility, wall effects and bed rearrangement, they can provide valuable information by applying appropriate theoretical models and statistics (see Chapter 12) [21].

The chromatography resin surface chemistry can be an important design consideration. When processing very large quantities of product, a high dynamic-binding capacity may be required to allow for realistic demands on column and system design. In this case, ligand density and distribution can be important factors for adsorptive chromatography resins.

The hydrophobicity and/or hydrophilicity of the functional group, linker and chromatography resin backbone are also important considerations in chromatography resin selection. These factors are linked to product recovery and cleanability. When working with a labile protein with hydrophobic pockets, product can be lost due to irreversible binding and unfolding on a very hydrophobic material. Hydrophobic interactions often make cleaning of chromatography resins more difficult. The ability to use sufficiently harsh cleaning and sanitizing agents is one of the most important chromatography resin parameters for long lifespans that reduce costs by minimizing the need to unpack, clean/sanitize and repack columns. (See Chapters 7 and 12 for further information on column packing and reuse.)

Once a chromatography resin is selected, the appropriate analytical methods optimized and validated, and manufacturing validated, it is essential that the chromatography resin manufacturing is maintained in a validated state so that consistency is achieved from lot to lot for all the relevant properties. That consistency is a key element in ensuring that biopharmaceutical products will be manufactured reproducibly. Nevertheless, the user must

assure that the chromatography resin used for the process fulfills all criteria that are critical to quality.

Over the last few decades, chromatography resin development has resulted in improved capacity, faster mass transfer, higher operating velocity, better chemical resistance and larger selectivity. However, it is not possible to achieve all improvements in one general resin for all applications. Rather, we have seen a development towards designing resins optimized for a specific substance class or application.

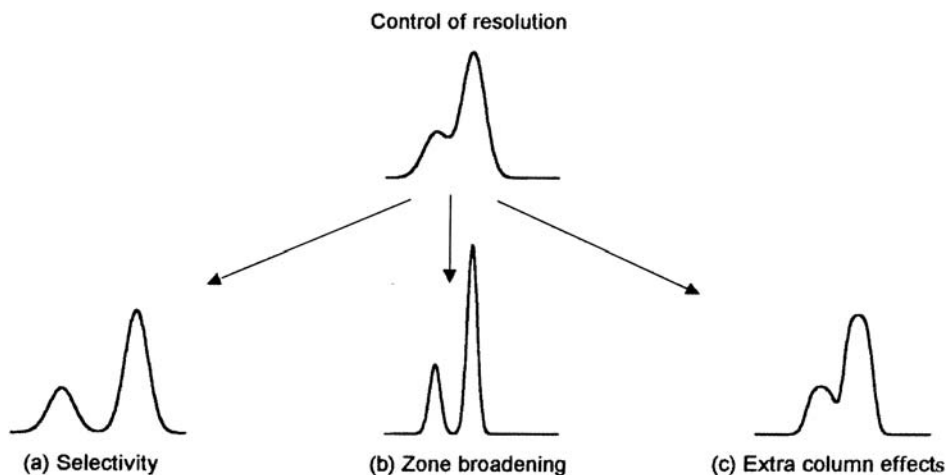
### 4.3.3 Selectivity and productivity of some popular chromatographic techniques

Design of the purification step includes the arrangement of the order of techniques used and optimization of the running conditions to yield a product of sufficient purity, while dimensioning the sub-steps to cope with the processing volumes of earlier steps, e.g. the recovery step. The building blocks commonly used for chromatography are based upon separation by size (SEC), charge (IEC), hydrophobicity (HIC), lipophilicity (RPC) and bio-recognition (AC). The discriminating power increases in the order  $SEC < IEC, HIC, RPC < AC$ . Using a resin of high-discriminating power early in the purification scheme will result in a process of fewer steps, which is advantageous. The important objectives for process design are high purity, high recovery and high capacity, leading to high productivity.

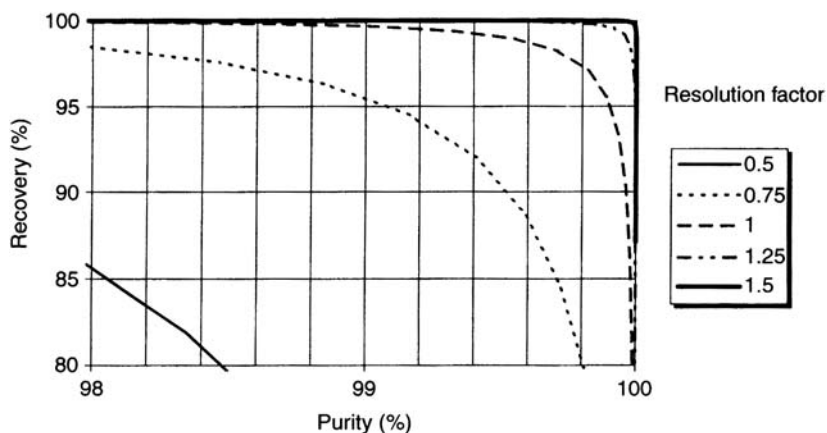
- *Purity*

To achieve high purity, the most important parameter of the chromatographic step is selectivity. A high-selectivity chromatography resin is employed to give a high resolution between the product and closely eluting impurities. Resolution is defined as the distance between two peaks divided by the mean of the peak widths (see Chapter 10). Resolution is achieved by a large separation factor (peak-to-peak distance) and/or a low-dispersion factor (peak width), as shown in Figure 4.3. The peak-to-peak distance is affected by the inherent selectivity of the chromatography medium and the column length for isocratic elution and the slope of the gradient for gradient elution. The dispersion factor is affected by the flow rate, particle size of the chromatography resin, retention time and the diffusivity of the solute for isocratic elution. Extra-column effects (e.g. large dead volumes, mixing chambers, etc.) will also cause broadening of zones. Step elution, e.g. as often applied in AC, is very favourable as it creates a large separation factor between bound and non-bound material. The dispersion during the elution step is as for isocratic elution.

Since resolution is such a fundamental measure of degree of separation, the meaning of resolution for some different cases, and the effect on purity and/or yield, is illustrated in Figure 4.4. It is seen that a purity of 99.9% at 95% yield requires a resolution of 1.0 between the product and an impurity composing 10% of the start material, provided that the peaks are symmetrical (this is seldom the case at high loads—*cf.* Chapter 10). The resolution factor 1.5 is used as a reference value here, since it corresponds, in practice, to a complete separation of peaks (i.e. a purity of 100% at 99% yield of a 50/50 mixture) and provides a certain safety margin in case of variation of the composition of the feed



**Figure 4.3** The achievement of resolution in isocratic elution. Resolution may be increased by (a) large peak-to-peak distance (selectivity) and/or (b) small zone broadening (column efficiency). Resolution will be decreased by (c) extra-column effects (e.g. large sample volume, see Chapter 10). Sample volumes are 0.1 and 10% of column volume for chromatograms (b) and (c), respectively. (Figures are not drawn to scale.)



**Figure 4.4** Recovery as a function of final purity at various chromatographic resolution factors for a sample containing 10% impurities.

stream and sample volume. However, it must be noted that a resolution factor of 1.5 is no measure of purity—the purity of the fraction collected needs to be confirmed by complementary assays (see Chapter 5). The resolution factor needed is related to the purity and yield required, and the type of impurities that are present. The goal for optimization of each individual step is to achieve sufficient resolution of the product from impurities

at the working conditions. What counts is the total result of the process, which means that the requirement for resolution in different steps may differ considerably, and also that the separation strategy may be dictated by the relative ease of achieving high resolution using different techniques, i.e. a resolution factor of 1.5 is clearly not the ultimate goal for every step.

- *Recovery*

Recovery of active product is the prime goal of the separation. The recovery is dependent upon the resolution attained and the requirement for purity. In some cases, material may be irreversibly lost on the chromatography resin or denatured, leading to a low recovery of active material even though the purity and the capacity is high. Thus, control of recovery during optimization is essential. It should also be noted that loss of active product may be due to long hold-up times in the system or the bed, and this parameter may therefore need to be controlled, especially when system configuration will vary (e.g. due to scale-up).

- *Capacity*

The dynamic-binding capacity of the chromatography resin for the target solute may be estimated by determination of the breakthrough capacity (see Chapter 10). This will be the maximum applicable amount of material the chromatography resin can adsorb before material leaks through the bed under the running conditions used, e.g. pH, ionic strength and concentration of interfering substances. The equilibrium capacity per unit bed volume is limited by the surface area of the resin that is sterically available for the solute and the ligand concentration.

The association constant will affect the degree of utilization of the capacity (i.e. a low-association constant in AC will result in low dynamic-binding capacity, *cf.* Figure 4.21). This effect may also result from an inappropriate choice of adsorptive buffers in IEC, HIC or RPC, which causes the solute to be retarded and not fully retained.

The operational level of capacity utilization will be dictated by the amount and type of impurities that will bind more strongly to the chromatography medium than the target solute. Therefore, binding conditions that will favour a higher proportion of adsorbed target solute as compared to impurities may be optimal, although this may correspond to lower degree of utilization if the target solute was to be adsorbed from a pure solution [22]. Thus, using a realistic feed for the optimization is very important.

It should also be noted that many successful purification processes contain ‘negative chromatography’ steps, i.e. where the product is designed to elute with the flow through fraction while critical impurities are adsorbed.

Material may be lost at very high loadings, and it is therefore recommended to check the recovery of active material as a function of loading. Also, since peak shape generally becomes more asymmetrical at high loads, i.e. showing tailing (see Chapter 10) that will reduce the resolution, it may be advantageous to operate at a slightly lower loading. However, sample displacement effects may sometimes sharpen the solute zones. Symmetrical peaks were found at column loads up to 30% of the maximum capacity [23].

In summary, the preferred way to determine the capacity is to use a representative feed (including impurities) to obtain the 5% breakthrough level, check the recovery of active product and calculate the material balance to assure quantitative recovery at the selected conditions. Also, the variability of feed properties needs to be taken into account. Of course, only a fraction of the dynamic-binding capacity is utilized, e.g. 80%, in the real production run to avoid losses of valuable material in the flow through.

- *Productivity*

The throughput is equal to the amount of purified product per unit time and the productivity is the throughput per volume of chromatography resin (i.e. g/(L × h)). The amount of purified product per unit bed volume is given by utilized capacity times relative recovery times purity, i.e.

$$\text{productivity} = \frac{\text{amount purified product}}{\text{bed volume} \times \text{time}} = \frac{\text{capacity} \times \text{recovery} \times \text{purity}}{\text{time}}$$

It is tempting to optimize this relationship in different steps, i.e. (a) select a chromatography resin that maximizes the capacity per bed volume, (b) choose conditions that maximize the relative recovery and (c) maximize the resolution per cycle time. However, maximizing the utilized capacity (by increasing the sample load, i.e. sample volume times sample concentration) will, in non-trivial cases, have detrimental effects on the relative recovery and also on the resolution per cycle time (and vice versa). Finding the best compromise of these parameters is the essence of the optimization step. The strategy will differ slightly depending upon separation technique employed but primarily involves selection of a high-selectivity resin (to provide maximum resolution between target solute and impurities), having large capacity (to cope with high loads before the column gets overloaded) and matrix properties to provide high recovery of active product. The running parameters involve conditions for solute adsorption (e.g. ionic strength and pH), maximum applicable sample load and influence of flow rate and gradient volume in the desorption step. As noted by Janson and Hedman [24], increasing the selectivity of the chromatographic process will increase the throughput dramatically as opposed to an increase in column efficiency, which only has a minor influence on the throughput. Times for sample application, wash, column cleaning and regeneration will reduce the productivity. In the examples given below, productivity for the elution step only is calculated.

Evidently, the purpose of the step, i.e. early recovery, initial purification or final polishing, will dictate which of the capacity, recovery or purity is the critical objective.

In order to optimize the different separation steps, a basic understanding of the different techniques commonly used and their practical advantages and limitations is essential (see Chapter 10 for a detailed discussion).

The most important parameters for optimization of preparative chromatography are summarized in Table 4.5.

**Table 4.5**

Important factors to consider in optimizing different preparative chromatographic step

Molecular characteristic	Chromatographic technique	Features	Limitations	Important factors
Size	SEC	<ul style="list-style-type: none"> <li>• Insensitive to buffer composition</li> <li>• Uncomplicated</li> </ul>	<ul style="list-style-type: none"> <li>• Dilutes the sample</li> <li>• Limited resolution and sample volume</li> </ul>	<ul style="list-style-type: none"> <li>• Pore size and pore volume of resin</li> <li>• Bed height and flow rate</li> </ul>
Charge	IEC	<ul style="list-style-type: none"> <li>• Concentrates sample</li> <li>• High-sample capacity</li> </ul>	<ul style="list-style-type: none"> <li>• Low salt for adsorption</li> <li>• Sample eluted in high salt</li> </ul>	<ul style="list-style-type: none"> <li>• pH</li> <li>• Gradient slope</li> <li>• Sample load</li> <li>• Contact time</li> </ul>
Lipophilicity	RPC	<ul style="list-style-type: none"> <li>• High resolvability, especially for small solutes</li> <li>• Concentrates sample</li> </ul>	<ul style="list-style-type: none"> <li>• May denature the sample</li> <li>• Medium sample capacity</li> <li>• Organic solvents needed</li> </ul>	<ul style="list-style-type: none"> <li>• Resin backbone</li> <li>• Gradient slope of modifier</li> <li>• Sample load</li> </ul>
Hydrophobicity	HIC	<ul style="list-style-type: none"> <li>• High resolvability</li> <li>• Sample eluted in low salt</li> <li>• High-sample capacity</li> <li>• Concentrates sample</li> </ul>	<ul style="list-style-type: none"> <li>• High salt for adsorption</li> <li>• Sample solubility</li> </ul>	<ul style="list-style-type: none"> <li>• Hydrophobic ligand type</li> <li>• Salt concentration</li> <li>• Gradient slope</li> <li>• Contact time</li> </ul>
Biospecific sites	AC	<ul style="list-style-type: none"> <li>• High-discrimination factor</li> <li>• Step elution</li> <li>• Concentrates sample</li> </ul>	<ul style="list-style-type: none"> <li>• Preparation of ligand</li> <li>• Medium sample capacity</li> </ul>	<ul style="list-style-type: none"> <li>• Association constant</li> <li>• Contact time</li> <li>• Elution conditions</li> </ul>

*Size exclusion chromatography*

The difference in size and shape between molecules is the basic property utilized for separation by size exclusion chromatography (SEC), frequently known as gel filtration when applied to biomolecules. The SEC mechanism was observed first in the mid-1950s by a number of researchers [25, 26]. The technique rapidly became popular due to the gentle separation mechanism preserving biological activity and also because suitable macroporous resins (i.e. Sephadex™) soon became commercially available [27].

Preparative SEC may be arbitrarily divided into buffer exchange and fractionation. Buffer exchange refers to the situation where low-molecular weight components of the sample (typically salt molecules) are exchanged for another buffering substance (e.g. for conditioning prior to next step) or solvent (i.e. desalting). In fractionation, the solute of interest is to be separated from other solutes of similar size and this separation puts higher demands on the choice of chromatography resin and selection of running conditions.

- *Size exclusion chromatography resins*

Resins for SEC may be composed of porous natural polymers, such as agarose or dextran, or synthetic polymers, such as polyacrylamide, that have been cross-linked in order to increase the rigidity. Macroporous silica has also been employed for SEC. To prevent denaturation of e.g. proteins on the silica surface, it is coated with a hydrophilic layer. The mechanism in gel filtration does not, ideally, involve any surface interaction (other than steric hindrance), and therefore the actual matrix structure has little influence on the functional performance [28]. The decisive factor for the function is the pore size and pore-size distribution of the chromatography resin (see Chapter 10). However, surface interactions may occur and differences between different types of resins are noticed, some types being more hydrophobic than others.

- *Optimization of resolution in SEC*

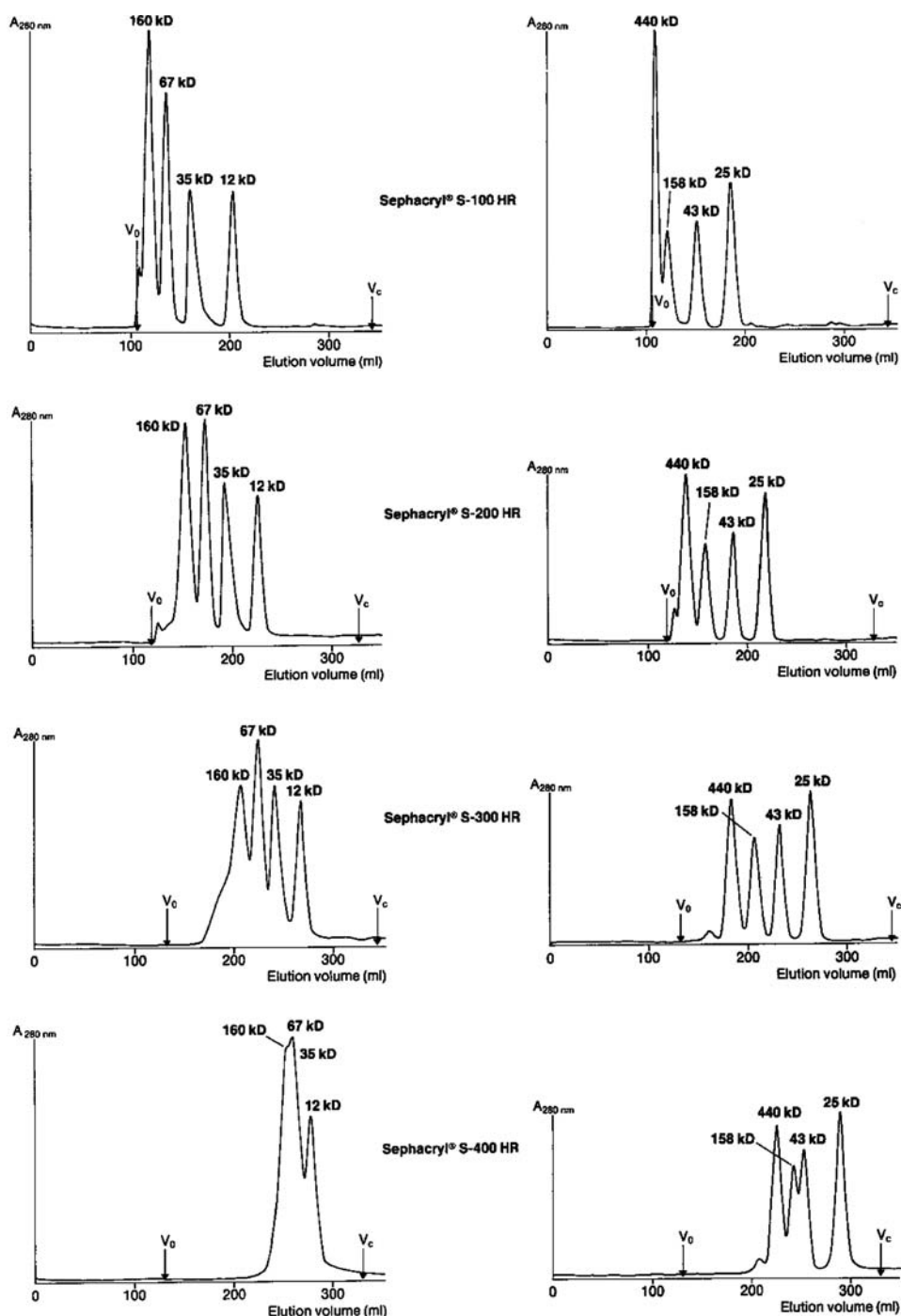
The resolution of solutes in SEC is determined by the size differences between the solutes to be separated and the selectivity of the chromatography resin. It is influenced by parameters such as flow rate, sample volume, particle size and column dimensions.

The separation range in SEC is regulated by the pore dimensions of the chromatography resin. This is illustrated in Figure 4.5, where a protein mixture is separated on Sephacryl™ having different apparent pore dimensions. The selectivity of the chromatography resin, expressed by the relative difference in elution volume as a function of solute size (or molecular mass) is related to the pore size distribution and pore volume of the material (see Chapter 10). It may be estimated that the pore size needs to be two times the solute size in order to allow for partial permeation (see Figure 4.5).

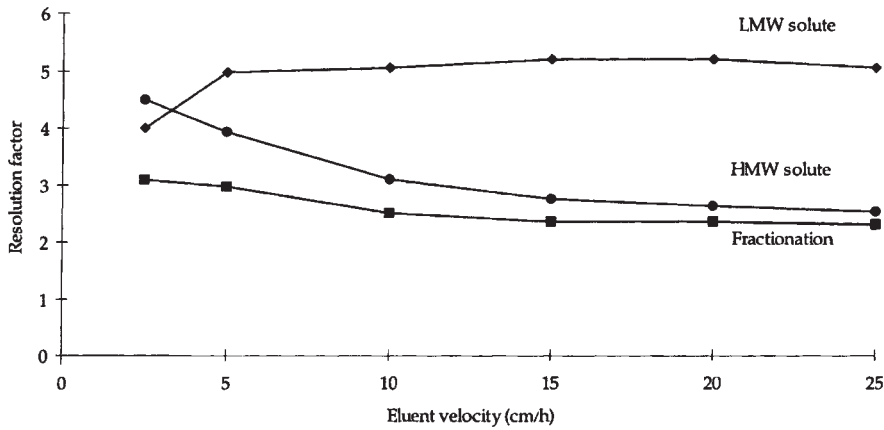
One of the most important operational parameters regulating the resolution in SEC is the flow rate (or velocity if expressed in distance per unit time). The resolution generally decreases with increasing flow rate, as shown in Figure 4.6. This is caused by slow mass transfer of macromolecules leading to broadening of the zones (the elution volume in SEC is not affected by the flow rate). The figure also illustrates the reverse behaviour for the resolution of a medium-sized molecule and a small solute. This is caused by dispersion of the zone for the small solute due to axial diffusion at low-flow rates. The effect may be predicted from the van Deemter equation (see Chapter 10). The degree of zone broadening is also related to the particle size, and the reduction in resolution with increased flow rate is less for resins of smaller particle size (due to shorter diffusion path lengths).

Flow rate is one factor that determines the throughput; the other is the sample load. The limit to sample concentration in SEC is, as opposed to adsorptive techniques, only set by physicochemical restrictions and high-solute concentrations, up to approximately 70 mg/ml for a protein such as BSA may be applied (above this concentration, the sample plug gets too viscous and hydrodynamically unstable). This means that the sample volume is the productivity-determining factor. However, increasing the sample volume will produce large sample zones (since the solute is not adsorbed), leading to a decrease in resolution as illustrated in Figure 4.7. It may be noted that the inherent low zone broadening of small particle-sized chromatography resins will be sacrificed when large sample volumes are processed, and in this situation a medium-sized chromatography resin of large pore volume may be optimal (see Chapter 10). As indicated by Figure 4.7, the resolution

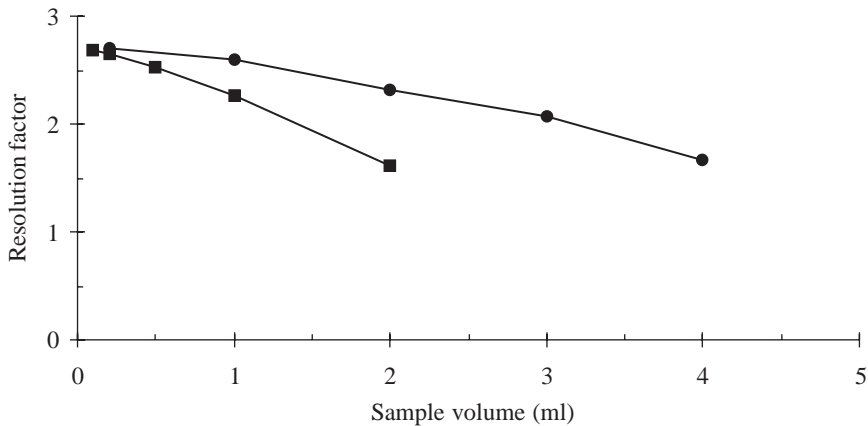




**Figure 4.5** Selectivity in SEC, influence of pore dimensions. Apparent average pore dimensions (from Hagel *et al.* [29]): Sephacryl™; S-100; 6.6 nm, Sephacryl S-200; 7.7 nm, Sephacryl S-300; 13 nm, and Sephacryl S-400; 31 nm. Solute size (calculated from  $R = 0.81 M_r^{1/3}$ , Hagel [30]): (left panels) IgG 4.4 nm, human serum albumin 3.3 nm,  $\beta$ -lactoglobulin 2.6 nm, and cytochrome *c* 1.9 nm; (right panels) ferritin 6.2 nm, aldolase; 4.4 nm, ovalbumin 2.8 nm, and chymotrypsinogen A 2.4 nm. Reproduced from Hagel *et al.* [31] with permission.



**Figure 4.6** Resolution as function of flow rate in SEC. Separation of thyroglobulin from BSA; ●, BSA from myoglobin; ■, and myoglobin from cytidine; ◆, on Superose™ 6 prep grade. Calculated from Hagel and Andersson [32].



**Figure 4.7** Resolution as a function of sample volume in SEC. Separation of thyroglobulin and BSA on Superose 6 prep grade. Column dimensions (●) 510 × 16 mm ID (3 ml sample = 2.9% of bed volume), (■) 530 × 10 mm ID (1.2 ml sample = 2.9% of bed volume).

may be kept constant by increasing the bed volume in proportion to sample volume (i.e. a plot of resolution versus sample volume as percent of bed volume makes all the data in Figure 4.7 fall in one line).

Thus, since processing a large volume in one cycle at low-flow rate or splitting the feed and running many cycles at high-flow rate both will result in large zone broadening and subsequently low resolution (but for different reasons), there exists an optimum with respect to sample volume and number of cycles for processing a fixed volume of feed.

This optimum can be calculated, and is found in the range of 2–6% of the column volume processed per cycle, depending upon the initial resolution and the purity and yield required [31].

- *Productivity in SEC*

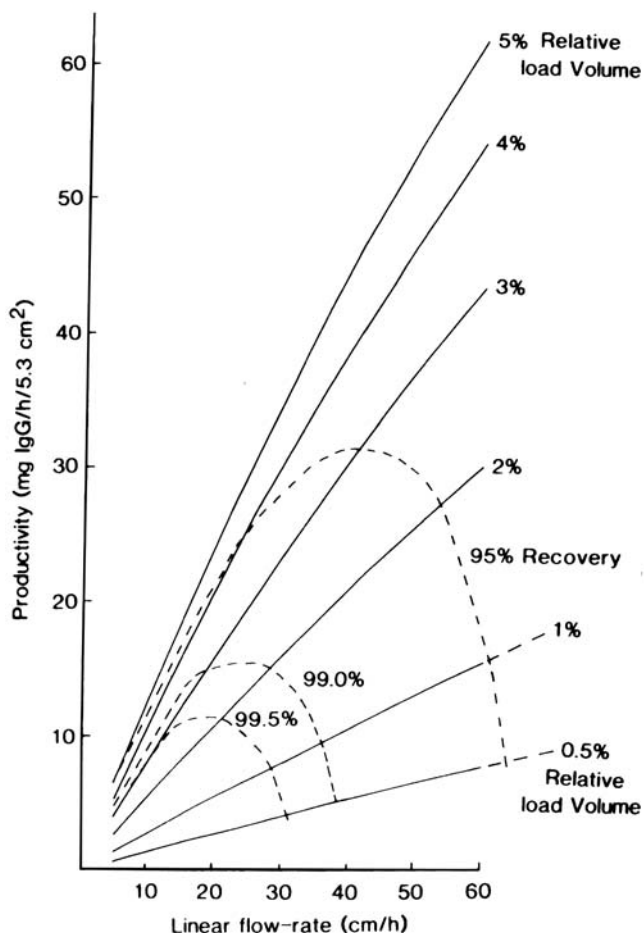
The productivity of SEC is governed by the maximum sample volume, the maximum sample concentration and the cycle time. The maximum applicable sample volume is proportional to the bed volume, and the maximum sample concentration is related to the viscosity effects of concentrated samples. The cycle time may not be as high as for adsorptive processes since an excessive band broadening cannot be compensated for by regulating the spacing of proteins by the running conditions (i.e. the selectivity is fixed). In order to process a certain volume in a certain time, there exists an optimum with respect to sample volume and cycle time. Calculations verified by experiments on Sephacryl support the general rule of processing a volume equal to 2–6% of the column volume each cycle at cycle times of 5–1 h, respectively [31]. The productivity is drastically influenced by the recovery, as demonstrated by the calculation of the purification of IgG to 99.99% purity shown in Figure 4.8. The highest productivity was found for a sample load of approximately 3% of the bed volume (as anticipated, a smaller load is required for a higher purity, i.e. resolution).

Viscosity effects have been noted at sample concentrations exceeding 75 g protein per litre with a protein such as albumin. Thus, on a 100 L gel filtration column, up to 300 g of a protein product may be purified each cycle. The cycle time varies with the particle size of the resin and the diffusivity of the solute. However, on a 30  $\mu\text{m}$  preparative grade material the productivity may be anticipated to be approximately 1 g/L of chromatography resin and hour in fractionation mode.

In desalting, the sample volume may be very large, and in theory equal to the pore volume of the chromatography resin since the large solute of interest will be eluted at the void volume and the low-molecular weight impurities will be eluted at the total volume (i.e. if a proper chromatography resin is selected). Thus, a sample volume of 30–40% of the column volume may be applied for desalting, and a maximum of 3 kg of protein may be purified in each cycle on a 100 L column. For a chromatography resin of low-pore fraction, this figure will probably be around 1 kg of purified protein per cycle. One example of productivity is given by the removal of ethanol from human serum albumin where 12 L of 9% albumin solution was de-ethanolized in 13.5 min on a 75 L column of Sephadex G-25 Coarse. This corresponds to a productivity of 64 g/L of chromatography resin and hour, or 1.44 kg/ cycle at a sample volume of 16% of the bed volume [33]. Calculations show that the productivity in desalting mode may in favourable situations approach 150 g/h and litres of bed volume [34].

### *Ion exchange chromatography*

The charges or, rather, charged patches on the solute surface will be attracted by opposite charges attached to a chromatography matrix provided the ionic strength of the surrounding buffer is low. This interaction is the basic phenomenon in ion exchange



**Figure 4.8** Productivity in fractionation with SEC. Calculation of productivity of purification of IgG from transferrin at various sample volumes and recoveries as a function of eluent velocity. Calculations based on experimental data on Superdex™ 200 prep grade 600 × 26 mm ID column and a reduction of transferrin content from 16.7 to 0.01%. Courtesy of B. Arve. Reproduced from Hagel and Janson [33] with permission.

chromatography (IEC). The chromatography resin may be regarded as an SEC resin to which charged groups have been attached via a spacer arm. Elution is frequently carried out by increasing the ionic strength of the buffer to compete with the solute for the charged sites of the matrix. The change may be gradual (gradient elution) or stepwise (step elution). This type of elution will have a sharpening effect on the solute zones as opposed to isocratic elution in which the ionic strength is kept constant (see Chapter 10). Changing the pH and thus the charge of the solute will also promote elution.

Ion exchange has, as opposed to SEC, a long history. The first example of ion exchange purifications is attributed to Moses, who purified acrid water with the aid of a special type

of wood (2 Mos. 15:25). In chromatography, ion exchange has been employed for several decades for the separation of small inorganic ions. However, it was not until hydrophilic materials of large pore size were introduced in the late 1950s, that ion exchange chromatography of biological macromolecules became a useful separation tool [23].

- *Ion exchange resins*

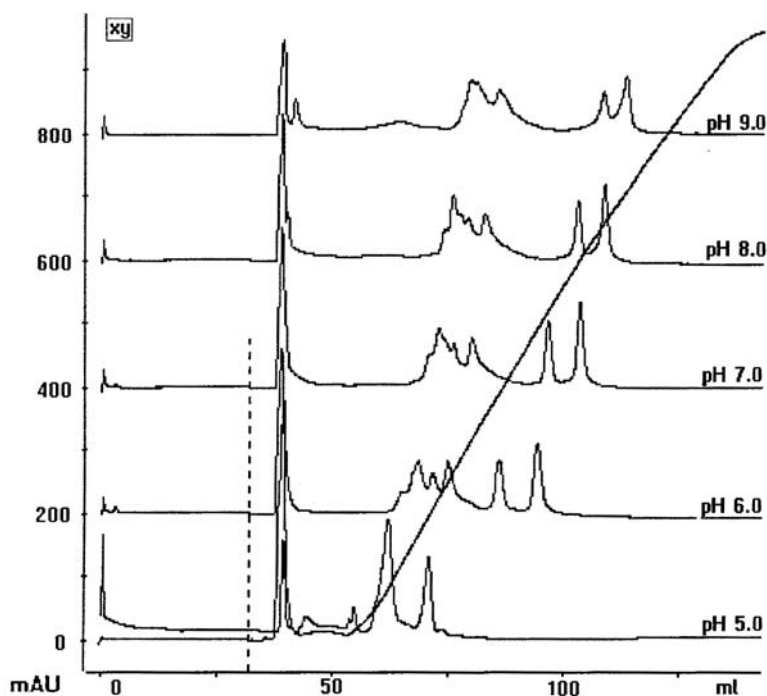
There are basically two different types of resins used for IEC. The most frequently used type is anion exchanger, which has positively charged (often amino) groups attached to the chromatography matrix. This type of chromatography resin will retain polynucleotides (due to the negatively charged phosphate groups) and proteins, and peptides at a pH above the isoelectric point where these solutes are negatively charged. If the charged groups on the ion exchanger are titratable, e.g. secondary or tertiary amines as DEAE (diethylaminoethyl), the ion exchanger is said to be 'weak' whereas if the charge of the ion exchanger is independent of pH over the range commonly used, as QAE (quaternary aminoethyl), the exchanger is said to be 'strong'. Thus, the ion exchanger being classified as weak or strong has nothing to do with the strength of the interaction. The other type of ion exchangers are cation exchangers for which the negative charge will attract proteins and peptides below their isoelectric point. Common-charged groups for cation exchangers are CM (carboxymethyl), giving a weak ion exchanger, and SP (sulphopropyl), giving a strong ion exchanger. To enhance the availability of the ion exchange ligand for larger macromolecules, the group is often attached to the matrix through a spacer arm.

- *Optimization of resolution in IEC*

The discrimination power of different anionic or cationic-charged ligands is generally not very different and therefore the most powerful way to alter the selectivity is to change the charge of the solute, i.e. by varying the pH. This is illustrated in Figures 4.9 and 4.10. As expected, the retention time increases with increasing pH (and negative charge) for proteins above the isoelectric points being chromatographed on an anion exchanger. The opposite is true for proteins below their isoelectric point chromatographed on a cation exchanger. However, there is no one-to-one relationship between the net charge and the retention time in IEC of proteins due to the complex distribution of charges over the molecular surface (see Chapter 9). The variation of charge with pH is most powerfully exploited in isoelectric focusing or chromatofocusing (e.g. see Janson and Rydén [35]).

Changing the speed with which the mobile phase composition is changed (i.e. the gradient) will result in a change of the spacing of solutes, and thus the resolution as shown in Figure 4.11. The gain in peak-to-peak distance is larger than the loss in zone broadening, which will result in a net gain in resolution.

This is one of the most common parameters used for regulating the separation in adsorption chromatography, and best results are expected to be found in the retention range of 5–20 column volumes as evident from Figure 4.11 (*cf.* Chapter 10). The actual chromatogram, reproduced in Figure 4.12, shows that Peaks 2 and 3 are not separated at the conditions used. One way to improve a separation is to decrease the gradient slope, i.e. increase

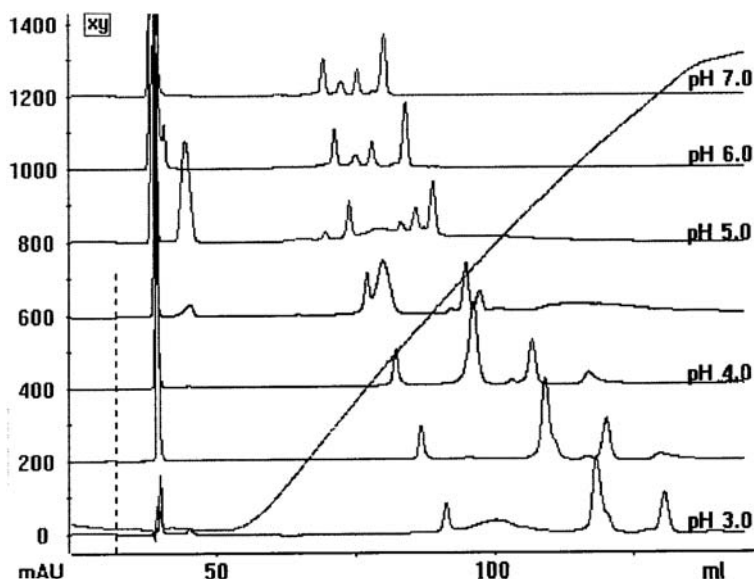


**Figure 4.9** Selectivity change as a function of pH for an anion exchanger. Separation of chymotrypsinogen A (isoelectric point 9.0), cytochrome *c* (9.4), lysozyme (11.0), transferrin (6.0), ovalbumin (4.7), and  $\beta$ -lactoglobulin (5.1) on Mono Q<sup>TM</sup> (a quaternary ion exchanger). Work from GE Healthcare Bio-Sciences AB, reproduced with permission.

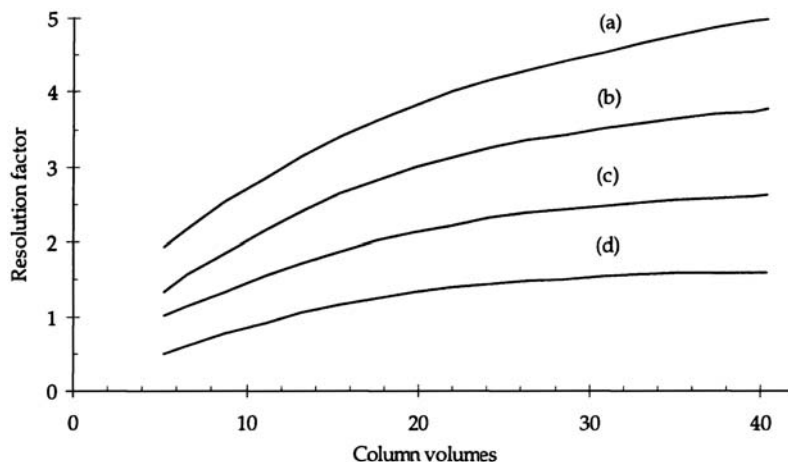
the number of column volumes in the gradient and/or reduce the gradient range (i.e. if Peak 1 is uninteresting one might test a gradient from 45% B to 85% B in 10 column volumes).

Another way will be to decrease the sample load (*cf.* Figure 4.11c,d). In this case the best solution would probably be to vary the pH (provided that the pH was not optimized for this separation in the first place) or select another chromatography medium (e.g. test a DEAE ion exchanger).

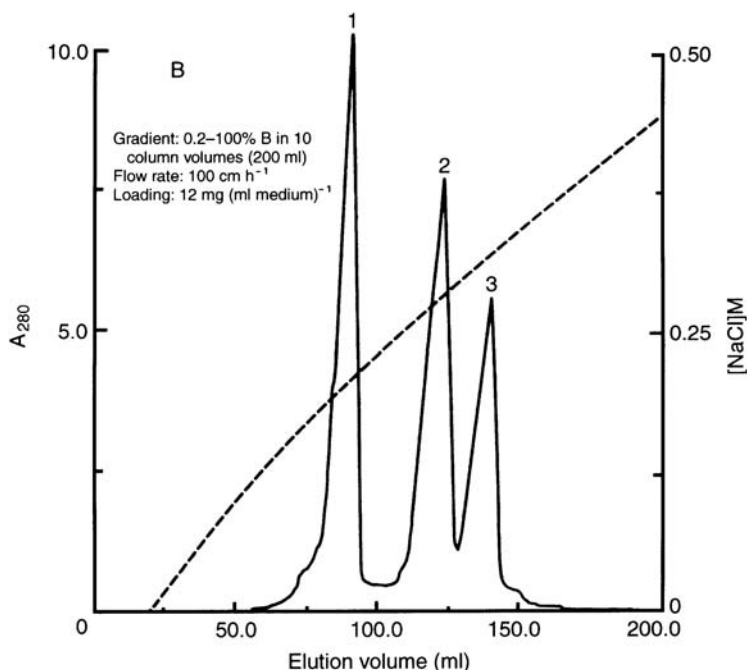
The third important parameter is the sample load. A large sample load will occupy a large zone in the bed leading to a broader peak. Under overload conditions, the elution volume will decrease with load and the load will also produce pronounced tailing of the peak leading to contamination of successive peaks (see Chapter 10). At very high loads another phenomenon called sample displacement will occur, which may sharpen the solute zones, but overlap between zones may reduce yield and/or purity. Working at overload conditions (and sacrificing yield) is sometimes suggested in process chromatography in order to maximize productivity. However, overload effects may be avoided by restricting the load to approximately one-third of the maximum load (illustrating the importance of using chromatography resins of high capacity for the product). Under these conditions the concentration of solutes adsorbed will vary approximately linearly with



**Figure 4.10** Selectivity change as a function of pH for a cation exchanger. Same protein mixture as used in Figure 4.8. Chromatographic resin: Mono S<sup>TM</sup> (a strong cation exchanger). Work from GE Healthcare Bio-Sciences AB, reproduced with permission.



**Figure 4.11** Influence of gradient slope on resolution in ion exchange chromatography. Gradient: 0.2–100% B in 5–40 column volumes, corresponding to 1.0–0.125% B per 1 ml of sorbent). Separation of wheat germ isolectin on S Sepharose<sup>TM</sup> High Performance (see Figure 4.12): (a) resolution of Peaks 1 and 2 at 3 mg sample load and (b) at 12 mg sample load, (c) resolution of Peaks 2 and 3 at 3 mg sample load and (d) at 12 mg sample load. Adapted from Sofer and Nyström [36].

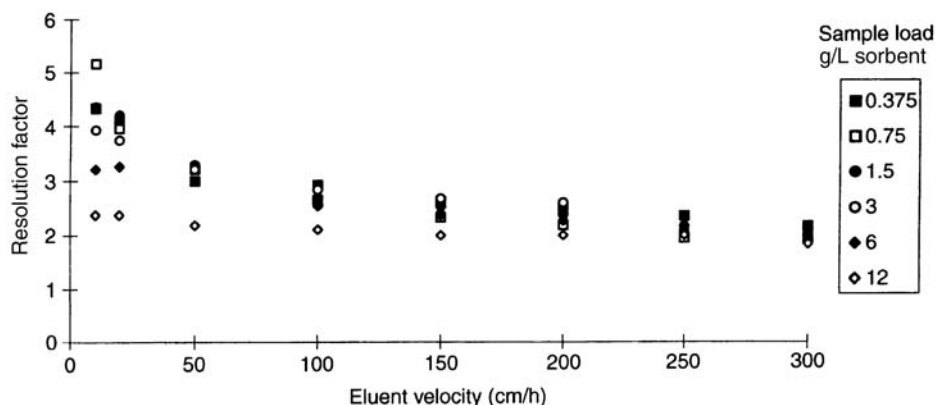


**Figure 4.12** Separation of wheat germ isolectin at high sample loading, 12 mg sample per 1 ml of chromatographic medium, S Sepharose™ High Performance (a total load of 0.24 g). Column:  $10 \times 1.6$  cm ID. Buffer A, 20 mM HCOOH, pH 4.0. Buffer B, 20 mM HCOOH in 0.5 M NaCl, pH 4.0. Sample eluted in 10 column volumes of gradient. Reproduced from Sofer and Nyström [36] with permission.

the concentration of solutes in the mobile phase (working under these conditions is called linear elution chromatography, *cf.* Chapter 10) and peaks will be fairly symmetrical, as shown by Figure 4.12 where 12 mg of protein per 1 ml of sorbent is loaded. Still, the zone broadening will decrease with load, leading to increased resolution as illustrated in Figure 4.13. This figure also shows the influence from the flow rate and, as found for SEC, the relative influence from zone broadening due to high-flow rate is low when running at high-sample loads.

In the most favourable case the conditions chosen will allow selective binding of the target molecule or of the impurities only. In difficult separations careful optimization is required and typically involves, first, choosing pH to achieve the most favourable selectivity (getting the solute of interest first in the gradient reduces the risk for contamination but increases the risk for the solute being displaced by more strongly retained solutes). The second parameter to optimize is the salt gradient of the mobile phase, which should be within the range of 5–20 column volumes to get favourable resolution. Third, utilize this resolution to increase the sample load to the resolution limit that is acceptable with respect to purity, yield and robustness.





**Figure 4.13** Influence of sample loading on resolution in IEC at various eluent velocities. Resolution of Peaks 1 and 2 of wheat germ isoelectin chromatographed on S Sepharose™ High Performance (see Figure 4.12). Calculated from Sofer and Nyström [36]. Note that a velocity of 50 cm/h corresponds to a residence time of 12 min and 300 cm/h to 2 min.

It is assumed that the contact time of the sample, i.e. the time allowed for mass transfer and the sample to equilibrate with the sorbent, is sufficient for quantitative binding. The required contact time is a function of solute pore diffusivity, solvent viscosity and particle size, and is regulated by the eluent velocity during sample load. Convection will contribute to mass transfer (see Chapter 10).

Gradient elution may sometimes not be feasible for large-scale processes. The optimized gradient conditions need, in this case, to be transferred to a series of steps to first elute less-retained solutes and then elute the product, and finally desorbing all tightly bound solutes in a wash step. The resolution of complex samples cannot be expected to be as good in a step elution as in a gradient run, but may be sufficient to yield a product of required purity.

- *Productivity in IEC*

The productivity, expressed as amount product per unit time and unit chromatography resin, is determined by the breakthrough capacity of the ion exchanger,  $Q_B$ , the recovery of active product and the cycle time. The operational capacity of the chromatography resin for the solute of interest in the sample mixture, i.e. the breakthrough capacity, is determined by frontal analysis as described in Chapter 10.

One needs to distinguish between two different situations where ion exchange is employed. One being in the early purification stage, where capacity is of major concern and purity is of less concern. In this case, applying sample close to the maximum load is possible, depending upon feedstream variability. The sample volume and concentration may be very large since the entire product is adsorbed, unless the solution contains a high concentration of competing ionic substances (e.g. has a high-ionic strength). As a practical rule, it is recommended to keep total sample loading below 80% of the dynamic-binding capacity of the ion exchanger. However, at high concentrations the system is operated in

the non-linear region of the isotherm, and severe tailing of the peaks will be observed, something that may decrease the purity and/or yield of product (see Chapter 10). The risk of losing material due to aggregation or precipitation at high concentrations must also be considered. It may be interesting to note that the maximum capacity for densely packed albumin molecules in the pore space of an ion-exchanger was calculated to be 200 g/L [37]. Of course, only a fraction of this capacity is available for real cases.

The other situation occurs when quantitative recovery and high purity are the objectives. In this case, the load may be limited to 30% of the capacity of the chromatography resin, as shown in Figures 4.11 and 4.12. The productivity may, at this load, be calculated to be 1–25 g protein per litre of chromatography resin an hour, depending upon the difficulty of the separation (e.g. Peaks 1 or 2 in Figure 4.12).

After the breakthrough capacity has been determined for the actual feed and eluent conditions, it is wise to apply the intended load and check the purity, yield and activity of the collected fraction.

#### *Reversed phase chromatography*

In reversed phase chromatography (RPC), hydrophobic substances dissolved in a polar solvent are separated due to their preferential interaction with non-polar ligands attached to a chromatography matrix. The liquid phase is composed of an aqueous buffer containing a water-soluble organic modifier, and this modifier will also constitute the liquid interface at the non-polar ligands.

The model presently proposed is a solvophobic model, where the solute is forced into the stationary phase due to the strong mutual interaction of the molecules in the mobile phase (thus ‘excluding’ the solute from the mobile phase). However, this model has been challenged by one with a more direct surface-to-surface interaction between the solute and the chromatography resin, [38] and the dominating mechanism in RPC is probably different for small organic molecules, polar compounds and macromolecules (see Chapter 10).

The first RPC separations appeared in the late 1940s when polar solutes were separated on chemically modified soft polymeric gels [39]. The use of RPC was applied to the purification of polypeptides in the late 1970s and has since then achieved considerable interest due to the high-resolving power of the technique [40]. For biomolecules, RPC has been applied primarily for the separation of peptides, including large-scale preparative use [41]. The high-surface coverage of the stationary phase in RPC will cause strong interactions with the solute. This may cause disruption of the tertiary structure of proteins, which leads to denaturation and loss of activity. Therefore, the application of RPC for preparative protein purification is generally limited to stable solutes or substances that may be renatured. The use of organic modifiers in the solvent at levels of up to 80% will also put restrictions on the applicability to large-scale purifications (e.g. explosion-proof equipment is needed and disposal costs for solvent may add significantly to the production cost).

- *Reversed phase resins*

Reversed phase resins are composed of a base matrix to which organic ligands, commonly *n*-alkyl chains, are attached. In some cases, the base matrix is sufficiently non-polar to provide the lipophilic environment required for interactions, and no substitution is made.

The base matrices used includes silica, zirconium oxide, polystyrene/divinylbenzene and other types of organic polymers. It is generally found that the type of base matrix used will influence the separation, probably due to the influence of surface interactions (see Chapter 10). Whereas silica has been successfully used as a base matrix for analytical RPC, the limited pH stability may restrict the use of silica for process scale purifications when neutral or basic pH is required. The strong influence of the silica base matrix may create problems with lot-to-lot reproducibility and scale-up, and it has been recommended to avoid frequent change of chromatography resin batches [42]. Accordingly, there is currently a large interest in evaluating the properties of polymer-based resins for large-scale purifications.

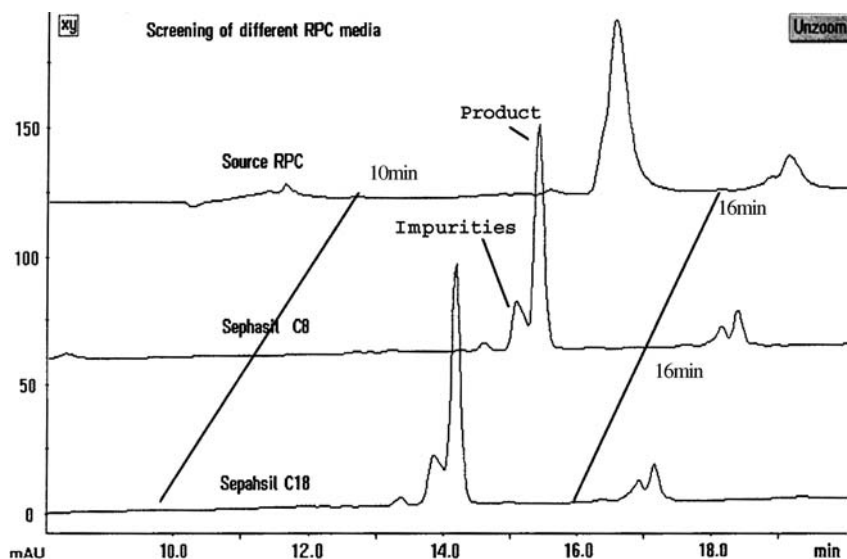
The lipophilic surface or coating will attract organic modifier molecules that will form the adsorbed liquid phase. Different ligands for coating silica are used, and butyl, octyl or octadecyl groups (denoted C4, C8 and C18, respectively) are common. For larger molecules, the ligand chain length *per se* has only a small influence; the retention will be primarily governed by the ligand density and properties of the matrix. The shorter ligands are commonly used for protein separation (to improve recovery) and the longer for peptide separation.

- *Optimization of resolution in RPC*

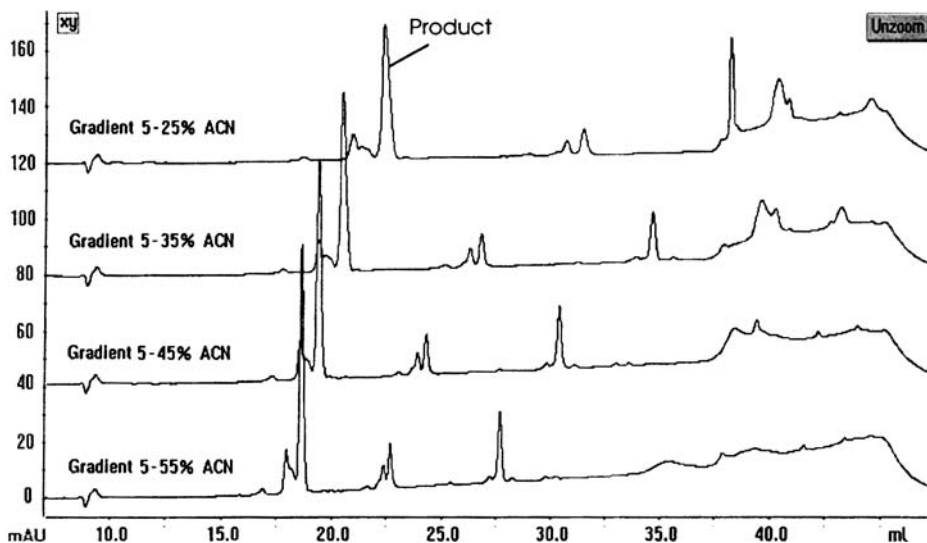
Since the retention in RPC is due to surface interactions between the solute and the chromatography resin, the premier action for achieving selectivity is to screen different resins. The choice of ligand is made from the following prerequisites: the interaction should not be so strong as to denature the solute (meaning that shorter lengths are used for, e.g. proteins), and to avoid extreme elution conditions. It may also be noted that the base matrix may affect the separation due to the close contact with the surface. In an evaluation of RPC of proteins, Pearson *et al.* found that the most determining factor for resolution was the type of silica used [43]. The empirical influence of the type of chromatography matrix is illustrated in Figure 4.14, where the separation of a synthetic peptide mixture on two silica-based materials and one polymeric material is shown.

The resolution of a solute mixture using RPC may be regulated by the composition of the mobile phase, as for IEC. Changing the pH will affect the degree of ionization and may be used for regulating the selectivity of small solutes; however, the effect for larger solutes, e.g. proteins, is unpredictable and the general recommendation has been to run at low pH to keep peak shape symmetrical [39], though the pH may cause special selectivity effects as reported by Builder *et al.* [45]. These authors reported that human IGF-1 was separated from the methionine-sulphoxide variant by RPC at pH 7 but not at pH 3. The charge of the solute may be masked by adding an ion-pairing reagent (e.g. trifluoroacetic acid) to the mobile phase. Acetonitrile is often used as an organic modifier, though other modifiers such as propanol, methanol or ethanol may also be suitable.

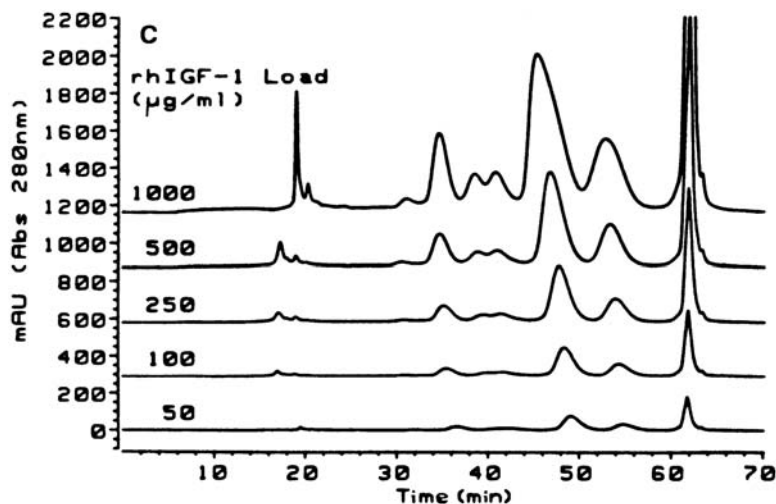
Elution in RPC predominantly achieved by a gradient. The number of column volumes is often in the range of 15–20, and the resolution regulated by the steepness of the gradient. This is illustrated in Figure 4.15, which shows that the general rule for IEC, i.e. the largest effect is obtained in the range from 5 to 20 column volumes of gradient, is also applicable to RPC. It may be noted that the effect is different for large solutes, e.g. proteins, as compared to smaller solutes such as peptides. The difference is probably due to the larger interacting surface area of proteins as compared to peptides (see Chapter 10).



**Figure 4.14** Selectivity in RPC, effect of chromatographic resin. SOURCE<sup>TM</sup> RPC (15  $\mu$ m, polymer based), Sephasil<sup>TM</sup> C8 prep grade (12  $\mu$ m, silica based), Sephasil C18 prep grade (12  $\mu$ m, silica based). Column length 10 cm. Eluent velocity 480 cm/h. Gradient 0–60% acetonitrile (in 0.05% trifluoroacetic acid) in 20 column volumes. Sample: crude synthetic decapeptide. Reproduced from Karlson and Renlund [44] with permission.



**Figure 4.15** Influence of gradient steepness on resolution in RPC. Chromatographic resin: Sephasil<sup>TM</sup> C8 prep grade. Column: 10  $\times$  0.4 cm ID. Eluent velocity: 480 cm/h. Gradient as indicated in figure corresponds to 5–25% acetonitrile (with 0.05% trifluoroacetic acid) in, from the top, 20, 13, 10, and 8 column volumes. Sample: crude synthetic decapeptide. Reproduced from Karlson and Renlund [44] with permission.



**Figure 4.16** Effect of sample load on resolution in RPC. Chromatographic resin: Waters C4 silica, 15  $\mu\text{m}$ , 300  $\text{\AA}$ . Column: 30  $\times$  0.39 cm ID. Flow rate: 0.7 ml/min. Gradient: 23–24% acetonitrile (100 mM sodium phosphate, pH 7) in 40 mm. Sample: different loads of recombinant human IGF-1 as indicated in the figure. (By courtesy of S. Builder, Genentech Inc. Reproduced from Olson *et al.* [41] with permission.)

The effect of a large sample load is illustrated in Figure 4.16. It is noticed that as the load is increased from 0.05 to 1 mg of solute per 1 ml of this chromatography medium, the peak retention time decreases and the tailing of the main peak increases, something that is characteristic of sample overload (*cf.* Chapter 10).

- *Productivity in RPC*

The productivity of RPC may be illustrated by the industrial purification of human IGF-1 shown in Figure 4.16. The optimized process, which involves running at 50  $^{\circ}\text{C}$  to achieve the required resolution, produced approximately 1 g rhIGF-1 per litre of chromatography medium an hour [41]. This productivity is of the same order as for SEC or a ‘difficult’ separation by IEC. However, results showed that considerably higher loads are possible for the purification of synthetic peptides [46]. Up to 5 mg of a 13 amino acid residue peptide was loaded per 1 ml of sorbent (Sephasil<sup>TM</sup> C8 preparative grade), with adequate resolution from a major impurity and without observation of tailing. The upper limit for sample load was set by sample displacement forcing the front of the peak to be eluted close to the start of the gradient.

#### *Hydrophobic interaction chromatography*

Molecules exposing hydrophobic patches on their surface may be separated due to their interaction with a non-polar ligand. The interaction is mediated by a polar solvent and is enhanced by a high-ionic strength of the mobile phase. The elution is normally carried out

by decreasing the ionic strength of the mobile phase to increase the solvability of the molecule in the mobile phase (though other means of decreasing the polarity of the solvent exist—see Chapter 10). The basic principle is similar to RPC; however, the conditions are much milder, generally preserving tertiary structure and thus activity of proteins (though strong hydrophobic interactions may induce alterations in conformational structure).

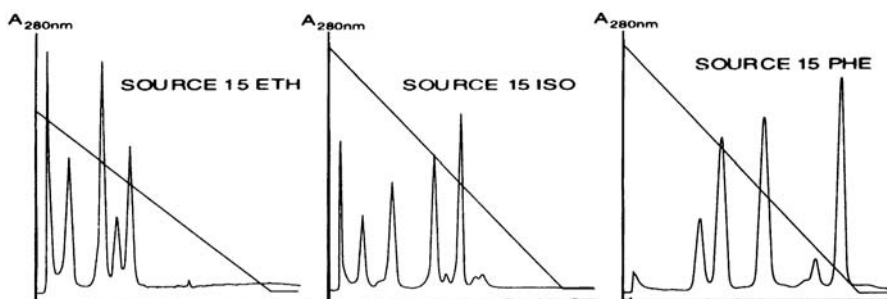
Hydrophobic interaction is a phenomenon that may also influence the separation in other modes of chromatography, e.g. SEC and IEC, and is suppressed by reducing the ionic strength or adding an organic modifier to the buffer, i.e. the conditions used for elution in hydrophobic interaction chromatography (HIC). Thus, although the phenomenon had been noticed for some time (e.g. salting-out chromatography) it was not until the early 1970s that resins for HIC became available [47].

- *Hydrophobic interaction resins*

Chromatography resins for hydrophobic interaction basically consists of a SEC matrix to which hydrophobic groups are attached. Some common groups, in order of increasing hydrophobic interaction strength are: *butyl* < *octyl* < *phenyl*. Results from the characterization of different resins indicated that other parameters (e.g. type of base matrix, spacers, etc.) might also influence the selectivity [48]. The interaction increases with ligand density. The degree of substitution is one to two orders of magnitude less than for RPC resins, and is commonly in the range of 10–50  $\mu\text{mol/ml}$  of chromatography medium, which gives typical protein-binding capacities up to 60 mg/ml (*cf.* Figure 4.20).

- *Optimization of resolution in HIC*

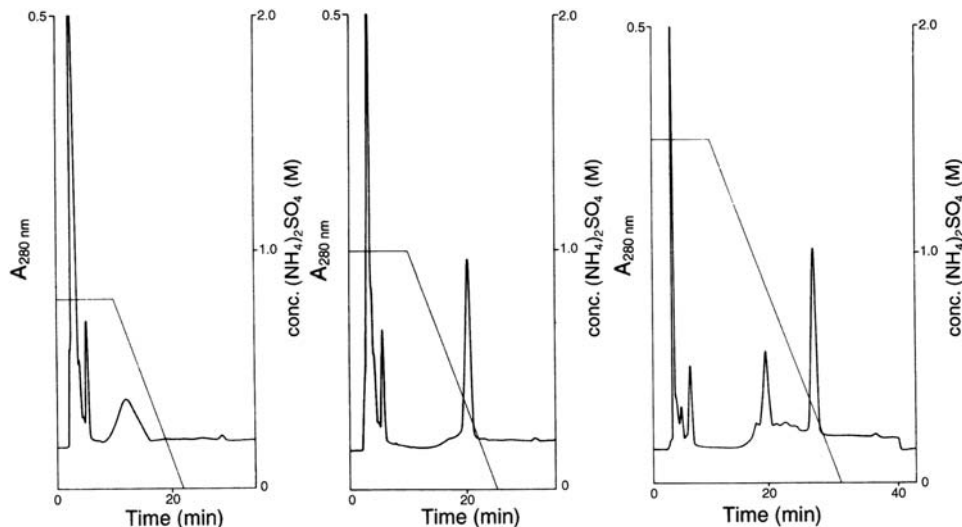
The degree of interaction in HIC is decisively influenced by the type of ligand that is attached to the sorbent. The interaction increases generally in the order of increasing carbon chain length and aromatic content as seen in Figure 4.17. The interaction may be



**Figure 4.17** Effect of the type of ligand on the selectivity in HIC. Sample: myoglobin, ribonuclease, lysozyme,  $\alpha$ -chymotrypsinogen. Column: RESOURCE<sup>TM</sup>, 30  $\times$  6.4 mm ID. Resins: SOURCE<sup>TM</sup> 15 ETH (ether), SOURCE 15 ISO (isopropyl) and SOURCE 15 PHE (phenyl). Gradient: from 2 M ammonium sulphate in 0.1 M potassium phosphate, pH 7 to 0.1 M potassium phosphate, pH 7 in 20 column volumes at 1 ml/min. Work from GE Healthcare Bio-Sciences AB, reproduced with permission.

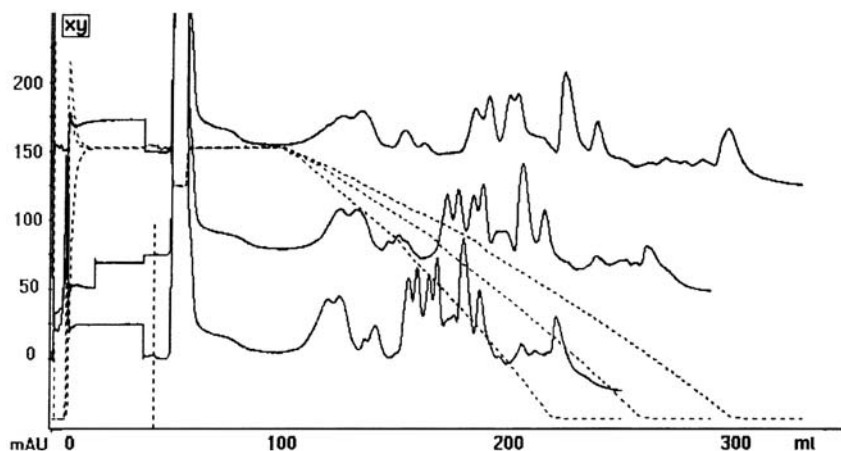
enhanced by salts that are known as cosmotropic salts (promoting an ordered water structure—see Chapter 10); however, no large shifts in selectivity are obtained for different salts and the effect may equally be obtained by changing the gradient volume [49]. The cooperative effects between salt type and ligand type lead to the general conclusion that the desired separation would be obtained by keeping one parameter constant while varying the other. However, in order to avoid ionic strengths that are too high (which might cause loss of sample), the use of the most hydrophobic ligand that is compatible with the protein (i.e. does not cause denaturation) is generally the best choice.

The best purification situation is achieved when conditions where the impurities are not adsorbed may be chosen. This is possible only for impurities that are more weakly adsorbed than the target solute. The ionic strength of the adsorptive buffer may be chosen to promote adsorption of the product (and more hydrophobic solutes). This is illustrated in Figure 4.18, which shows that at 1.0 M ammonium sulphate the contaminating albumin does not bind to the column while the product, IgG, is retained. However, it is important to assure that the product is retained and not only retarded on the chromatography medium, since the latter condition will not yield a robust purification method. As for other adsorptive techniques, the resolution may be regulated with the gradient volume. As seen in Figure 4.19, the best resolution in this case is obtained for gradient volumes in the range of 20 column volumes.



**Figure 4.18** Influence of initial salt concentration on the retention in HIC. Sample: 100  $\mu$ l anti-CEA MAB (IgG1). Column: Alkyl Superose<sup>TM</sup> HR 5/5. Flow rate: 0.5 ml/min. Buffer: 0.1 M phosphate, pH 7.0 with decreasing gradient of ammonium sulphate as indicated in the figure. Sample applied in 0.8, 1.0 and 1.5 M ammonium sulphate. Results show that albumin does not bind and IgG is merely retarded at 0.8 M, IgG binds at 1.0 M, and at 1.5 M albumin is also binding (and consuming column capacity). Thus, optimum conditions in this case is an initial concentration of 1.0 M ammonium sulphate. Reproduced from Sofer and Nyström, [36] with permission.





**Figure 4.19** Influence of gradient volume on resolution in HIC. Sample: alkaline phosphatase in partly purified *E. coli* homogenate. Column: SOURCE<sup>TM</sup> 15PHE packed in  $10 \times 1$  cm ID column. Flow rate 2.0 ml/min. Buffer: 0.1 M sodium phosphate, pH 7.0, gradient: 1.2–0 M ammonium sulphate. Gradient volume (from the top): 25, 20 and 15 column volumes. Work from GE Healthcare Bio-Sciences AB, reproduced with permission.

- *Productivity in HIC*

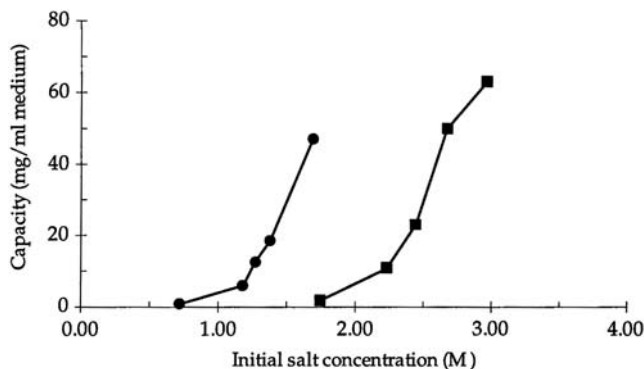
The productivity of HIC is, as for other modes of chromatography, equal to the throughput per unit resin. The throughput is equal to the capacity times the yield per unit time. The capacity of HIC is high, more than 30 mg/ml for a protein, and the yield may be quantitative. However, the sample load in HIC is dependent upon the solvability of the sample in the high-ionic strength buffer used for adsorption and also the contact time for the sample. The influence of the ionic strength of the adsorptive buffer on the dynamic capacity is shown in Figure 4.20, and it is seen that the effect may be dramatic. On the other hand, the capacity for the product may be drastically reduced by co-adsorbing components from the feedstream and the recovery of active product may also be compromised by inappropriate conditions causing precipitation of solutes on the column [22].

From the experiments shown in Figure 4.20, the productivity of HIC was estimated to be 180 g/h and per litre of HIC resin for a 'pure system' (allowing 95% of the breakthrough capacity to be applied). However, 50 g/h and per litre of HIC resin is more realistic for a real-life system (e.g. purification of monoclonal IgG from ascites [50]), where perhaps only 30% of the capacity is available for the product. In addition, the use of the total equilibrium capacity can only be realized under conditions where the contact time for the solute is sufficient for quantitative adsorption.

#### *Affinity chromatography*

The highest degree of selectivity is obtained when several complementary interaction mechanisms are operating simultaneously and at specific locations. This is the basis for





**Figure 4.20** Influence of the initial salt concentration on the capacity of HIC. Sample: (●)  $\alpha$ -chymotrypsinogen and (■) RNase. Column: Phenyl Sepharose™ High Performance. Salt: ammonium sulphate. (Work from GE Healthcare Bio-Sciences AB, reproduced with permission.)

affinity chromatography (AC), which requires not only certain surface properties of the chromatography resin but also a well-defined location of these properties. By definition there are no general affinity resin, but each resin is designed for the substance or class of substances to be purified. Conditions for adsorption must nevertheless be selected to favour interaction, whereas elution may be carried out either by competing with the molecule for the ligand or by changing the mobile phase composition (e.g. pH) to alter the properties of the solute or ligand. It can also be noted that AC may not be expected to differentiate between molecules differing slightly in parts other than the binding site, unless the binding site is affected by the difference.

Affinity between matrix and solutes has occasionally been observed, e.g. the selective adsorption of  $\alpha$ -amylase onto insoluble starch in 1910 [51]. With the introduction of coupling chemistry, the possibility of producing purposely designed affinity resins made the technique a valuable separation tool [52].

Also, this has provided the opportunity to couple affinity ligands that are more or less selective in the molecular recognition process, and therefore AC encompasses a broad range of ligand types. This also makes the term AC quite broad, and while some authors have reserved the term to describe biologically functional interactions only, we tend to use the term more freely to incorporate any interaction that is due to the occurrence of specific groups on the surface (in this context single ionic or hydrophobic groups are not classified as specific). Very high degrees of purification may be achieved, e.g. 1000-fold with high recovery of active material.

In the search for new selective separation methods, affinity-based techniques have received renewed interest. This is due to the progress in molecular biology and combinatorial chemistry, which has provided tools for producing a large variety of ligands at moderate cost. In addition, the increased research in structure–function relationships has shown that it may be possible to drastically reduce the size of, e.g. protein ligands, which in turn will provide a more chemically stable ligand.

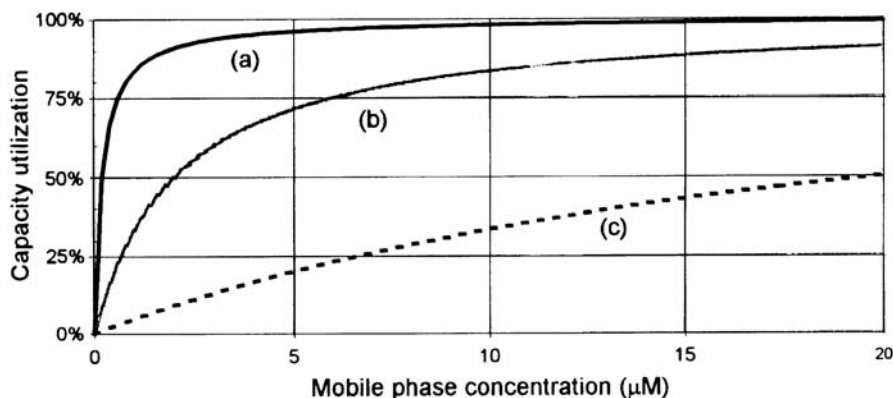
- *Affinity resins*

Affinity resins are composed of a macroporous chromatography matrix to which the specific ligand is attached. The pore size of the matrix must be large enough to provide room for the, often bulky, ligand and to provide free access to the ligand for the interacting macromolecule. Ligands may be divided into two groups according to the specificity of the interaction, i.e. group-specific and mono-specific. The group-specific ligands have affinity for a group of related substances. This type of ligands includes Protein A and Protein G for the purification of immunoglobulins, lectins for purification of glycoproteins, dyes for purification of NAD<sup>+</sup>- and NADP<sup>+</sup>-dependent enzymes, polynucleotides for purification of oligonucleotides containing complementary sequences, etc. Other types of ligands that may be incorporated into this class are chelating groups (e.g. iminodiacetic acid) used for sorption of metal ions, which in turn will attract certain amino acids exposed on the surface, e.g. histidine and cysteine. This technique is called immobilized metal affinity chromatography (IMAC) [53]. IMAC has been frequently employed for the purification of recombinant proteins tagged with a polyhistidine tail.

Mono-specific ligands bind to a very small number of solutes. Examples are lysine that binds plasminogen, and biotin that binds avidin. However, the binding may be very strong and may require very harsh conditions for elution of the solute.

Antibodies have very high specificity and have become very popular as ligands (i.e. the solute of interest is purified, antibodies toward the solute are raised, purified, and finally attached to a matrix). This type of affinity resin is called an immunoabsorbent. Single-step purification factors of several thousand folds with more than 90% recovery may be achieved [54]. Attempts are being made to reduce the size of the immunoabsorbent ligand and incorporate only the active fragment. However, there is probably a limit to this reduction, i.e. to preserve a degree of ordered structure of the ligand, which may be essential to maintain sufficient adsorption strength. The effect may be seen by the 100-fold decrease in association constant of a synthetic ligand AC medium designed for mimicking a protein receptor [55]. These types of affinity resins, originally introduced by Porath and co-workers [56], can withstand harsh chemical and thermal cleaning of the chromatography medium. However, the low-association constant may be disadvantageous in preparative purifications from a dilute feed (*cf.* Figure 4.21).

Another way to identify solute-specific ligands is by searching collections ('libraries') of a large number (e.g.  $10^6$ – $10^{10}$ ) of variants of a species, e.g. proteins, peptides or oligonucleotides, for the selective interaction with the target molecule [57, 58]. The selection process may be carried out in the presence of impurities, e.g. in the feed solution, or with other conditions that are desirable to use as selection criteria. Phage display technology has proven to be very useful for rapid selection of very stable microprotein, i.e.  $M_r = 800$ – $7000$ , domains [57]. Nord *et al.* described a new family of affinity ligands, called affibodies, based upon random mutations of amino acids in 13 positions of the stable three-helix bundle 7000 g/mole Z-domain derived from staphylococcal Protein A [59]. Another approach where a traditional antibody response is created in Llamas and a phage display library of the VHH-region (see Chapter 9) constructed and used for further optimization was



**Figure 4.21** Influence of the association constant on the degree of utilization of capacity of AC resins. Association constant: (a)  $5 \times 10^6$  per M, (b)  $5 \times 10^5$  per M, and (c)  $5 \times 10^4$  per M. A concentration of 20  $\mu\text{M}$  corresponds to 1 mg/ml for a solute of  $M_r$  50,000.

recently described for isolation of an ice-structuring protein [60]. One of the advantages of using the VHH-region is the small size, e.g.  $M_r = 12,000\text{--}15,000$ , and hence stable ligand with high affinity.

Stability versus harsh cleaning conditions, e.g. 0.5 M NaOH, is a special issue for affinity ligands being composed of proteins or peptides. One way to reduce this problem is to use small stable peptides and further increase the stability by selectively replacing alkaline sensitive aminoacids, e.g. asparagine, with more stable ones [61]. In this way it was possible to produce a new Protein A type of resin (MabSelect SuRe™) with significantly improved alkaline stability allowing cleaning with 0.5 M NaOH for short cycles [62]. Another option is to replace NaOH as cleaning agent with a solvent mixture. However, so far no general solvent mixture to replace NaOH has been found.

An optimal orientation of ligands may be assured by a proper ligand design, e.g. with the aid of genetic engineering techniques. One example is given by an affinity Protein A chromatography medium (rProtein A Sepharose Fast Flow) where the coupling to the matrix is directed to the C-terminus by incorporation of an extra cysteine at this site. This results in a more efficacious chromatography medium with an 80% increase in breakthrough capacity of human IgG as compared to random multi-point attachment.

Though there exists a wealth of affinity resins (e.g. see Carlsson *et al.* [54]) also suitable for large-scale purifications, the purification situation at hand may call for a novel ligand, e.g. developed as described above. The ligand may be coupled to a suitable chromatography resin by the vendor of the chromatography resin or in-house. This is facilitated by the availability of pre-activated chromatography resins. A good base matrix for an AC medium shall provide suitable coupling points, have a large pore size (to accommodate bulky ligands, promote fast access to sites), have a large surface area (to get high capacity) and be chemically inert to coupling chemistry.

- *Optimization of resolution and productivity in AC*

To achieve a quantitative adsorption of solutes, the binding constant needs to be sufficiently high (i.e. the association constant should be larger than  $10^5$  per M, which is  $10\ \mu\text{M}$  affinity). At lower association constants, the solute is only retarded on the column and the sample volume must be reduced, and a long column may be needed to obtain the desired separation (systems where the association constant is less than  $10^4$  per M are referred to as weak affinity systems). It is important to note that the association constant affects the degree of utilization of the sorbent capacity as illustrated in Figure 4.21. Thus, a low-association constant and a low-solute concentration are very unfavourable in preparative AC, as pointed out by Chase [64]. It was recommended that the affinity step would be preceded by e.g. an IEC step, to increase the concentration of solute prior to a weak affinity step.

At high-association constants (i.e. low-dissociation constants) the solute is quantitatively adsorbed, impurities washed away and the pure solute eluted by a stepwise change of the mobile phase composition.

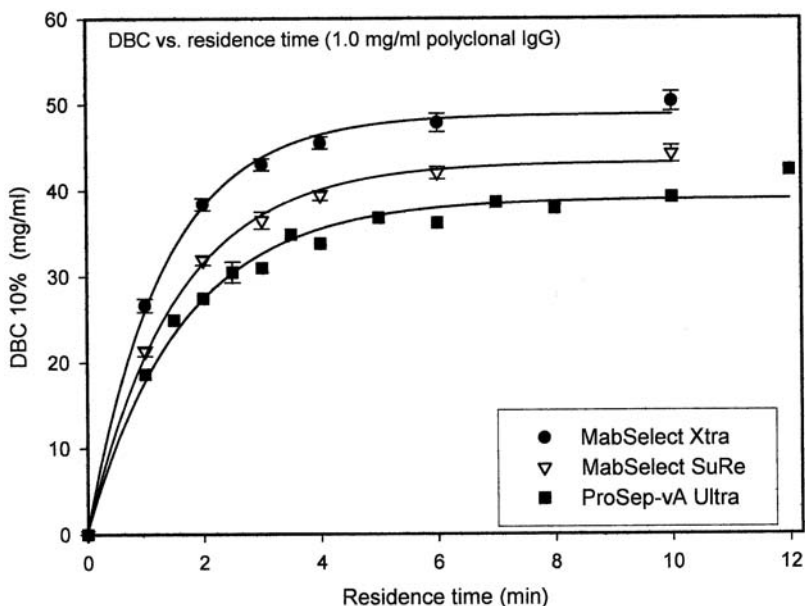
Adsorption of large solutes may be expected to be slower in AC than other modes of adsorptive processes since the interaction requires a correct orientation of the solute. Therefore, low-flow rates for adsorption have generally been recommended. However, the kinetics of modern affinity resins are comparable to those of IEC and, for process purposes, no special consideration needs to be given to flow rate in most affinity purifications. However, as for other adsorptive modes, the capacity increases with increased contact time as shown in Figure 4.22 (and contact time is influenced by flow rate).

Desorption in AC is achieved by increasing the dissociation constant (e.g. desorption of solutes requires that the dissociation constant of the ligand–solute complex is larger than  $10^{-10}$  per M). This may be achieved by decreasing the pH, increasing the ionic strength or by competitive elution, where an agent will compete with the solute or the ligand for the affinity sites. Contaminating agents introduced (e.g. leachables, eluting agents) may need to be removed by a subsequent step, e.g. size exclusion chromatography [65]. For example, desorption in IMAC involves reducing the pH, increasing the ionic strength or stripping the metal ion by adding EDTA or another powerful complexing agent.

The theoretical-binding capacity of a protein with  $M_r$  60,000 is approximately 80 mg/ml on a 4% agarose-based affinity resin [54]. Commercial resins differ substantially in sorbent capacity; however, as illustrated by Figure 4.22, the capacity of modern resins approaches the theoretical limit.

#### *Other modes of chromatography*

The chromatographic techniques mentioned above have the advantage of being relatively well understood, and hence the design of a purification process is fairly straightforward. In addition to these techniques, purifications steps based on e.g. hydroxyapatite, dye chromatography and, lately, multi-modal chromatography has been successfully used in an empirical mode [62].



**Figure 4.22** Influence of sample residence time on dynamic capacity at 10% breakthrough in AC. Sample: polyclonal human IgG, 1 mg/ml. Chromatography medium: MabSelect Xtra™, MabSelect SuRe™ and ProSep-vA Ultra™. Buffer: 20 mM sodium phosphate in 0.15 M sodium chloride, pH 7.3. Reproduced from Hahn *et al.* [63] with permission from Elsevier.

#### 4.3.4 Scale-up of chromatographic purifications

Knowing the basic principles of the chromatographic steps employed and verifying these during the design phase would result in the scale-up of a well-designed purification process that should be straightforward. Guidelines for scale-up of chromatographic processes are relatively simple. Of course, non-chromatographic factors must also be considered to ensure that the entire process is scaled up efficiently with no loss of product activity.

##### *Guidelines*

After the purification scheme is optimized on a laboratory scale, the first scale-up is usually of the order of 100-fold. Depending upon the quantity of the product required and recycling capabilities versus additional cost for testing, etc., this may in fact be the final scale. For example, to scale up 100-fold, parameters such as sample load, volumetric flow rate and chromatography resin volume will be increased 100-fold, whereas the column bed height, eluent velocity, sample concentration and ratio of sample volume to bed volume will be kept constant (Table 4.6). If a gradient is used for elution, the ratio of gradient volume to bed volume will also be kept constant, and, therefore, the time required for the gradient to develop will also remain essentially constant. Further scale-up, from pilot plant to production, is usually 10–30-fold to ensure reproducibility.

**Table 4.6**

Guidelines for scale-up of chromatographic purifications

Maintain	Bed height Eluent velocity Sample concentration Gradient slope/bed volume Sample residence time
Increase	Column diameter Sample volume in proportion to column cross-sectional area Volumetric flow rate in proportion to column cross-sectional area Gradient volume in proportion to column cross-sectional area
Check	Reduction in supportive wall effects (increased pressure drop) Sample distribution (i.e. zone broadening) Piping and system dead volumes

**Table 4.7**

Scale-up of RPC purification step of rhIGF-1

Scale	Column size (cm)	Bed volume (ml)	Scale factor	Velocity (cm/min <sup>-1</sup> )	Purity (%)	Recovery	
						mg	%
Analytical	30 × 0.39	3.58	1	5.86	>99	3.5	97
Pilot	30 × 4.7	520	145	5.76	>99	500	96
Production	60 × 10	4710	1305	5.73	>99	4500	96

Source: From Olson *et al.* [41].

Normally, volumetric scale-up is performed by increasing the column diameter. However, sometimes this may not be feasible, e.g. due to lack of commercially available column dimensions for the intended scale. Rather than taking the extra cost of over-dimensioning a process one may use the empirical guideline proposed by Yamamoto *et al.* [66], which states that resolution is kept constant provided that the column length increases in proportion to the eluent velocity times the gradient slope per unit bed volume. Following this guideline, it may be recommended that the sample volume will be increased in proportion to the bed volume, the eluent velocity kept constant, while the gradient slope per unit bed volume will be increased in proportion to the column length (i.e. the gradient time will increase in proportion to the increase in column length).

One example of the combination of the two approaches was given by Olson *et al.* [41]. They scaled up the reversed-phase purification of recombinant human insulin-like growth factor 1 roughly 1300-fold in two steps (see Table 4.7). The first step was a 145-fold scale-up in accordance with the guidelines given in Table 4.6, and the second step was a ninefold scale-up combining an increase in both column diameter and length. Separation was preserved by keeping the residence time constant, while increasing the gradient time. It is seen that the recovery follows the scale factor quite nicely.

### *System factors*

If a deviation from the laboratory scale results is seen in the larger column, there are several factors to check.

- *Broad peaks*

Larger equipment may cause extra-column zone broadening due to different lengths and diameters of outlet pipes or tubings, connections and volume of monitor cells, etc., which may act as mixing chambers. If the larger column has a less-efficient flow distribution system than the analytical column, greater axial dispersion in the bed as well as extra zone spreading in the end pieces will occur. Although the effect on the separation may not be detrimental, it may yield low-plate counts of a sharp solute zone used for control of column zone broadening. Even though this may indicate a difference in performance due to system effects, the impact of the difference on the actual purification must be evaluated (e.g. the extra zone broadening may be insignificant compared to the peak width of the product).

- *Narrow peaks*

Sometimes it may be noticed that larger scale columns yield better performance than laboratory columns. This may be due to positive effects from sample application, column packing or even reduced non-specific adsorption to column walls, inlet and outlet flow distributors, tubing, pipes, etc. (since the relative surface area exposed to the solutes will decrease).

- *Low-flow rate*

An increase in column diameter will reduce the supportive wall force. This may be noted as a decrease in flow rate at a constant pressure drop over columns packed with semi-rigid resin. For example, a decrease in flow of 30–45% was noted for a column packed with Sepharose 6 Fast Flow when the diameter increased from 2.6 to 10 cm [36]. Other system effects, such as pressure drops over connectors, valves, monitor cells, etc., will also reduce the flow rate achieved at a constant pressure.

- *Peak cutting*

The monitoring system should be examined to verify that transport distances between column outlets, monitors and fraction-collection valves do not introduce time delays or volume changes, which make the process controller switch the fraction-collector valve position at the wrong time. The sampling rate of the controller needs to be sufficiently high to avoid delaying the collection of signals and execution of e.g. valve control.

### *Examples of scale-up*

The simple solution to avoid system factors is to select optimal equipment and use the optimal system configuration for the purpose at hand (e.g. when the column plate height is to be determined all sources of extra zone broadening are to be bypassed). In fact, quite

impressive results have been obtained using process columns. Sephamic stainless steel columns with a diameter of 100 cm have been packed with Sephacryl S-200 SF to yield height equivalent to a theoretical plate (HETP) values (see Chapter 10) corresponding to those found for laboratory columns (Table 4.8).

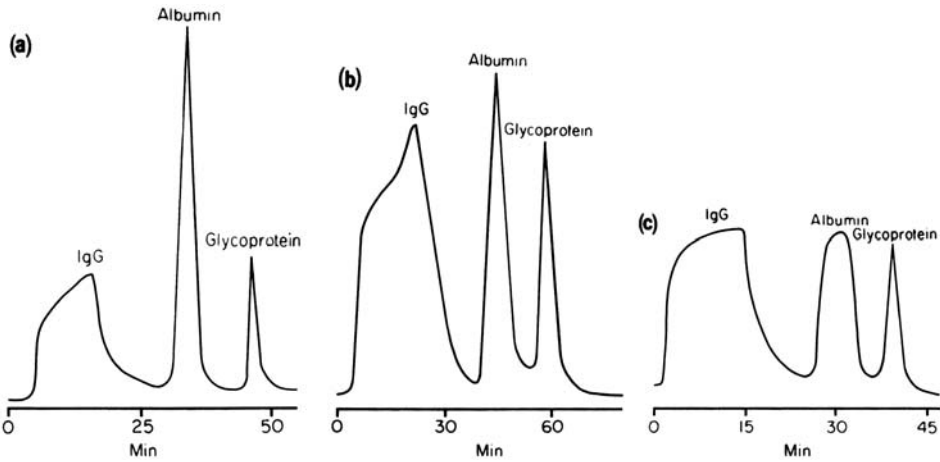
On the laboratory scale, chromatographic steps are easily and rapidly optimized using high-resolution (e.g. 10  $\mu\text{m}$ ) resins. If the particle size is increased to achieve better throughput and reduced operating costs at the pilot stage, further optimization may be required. For some types of resins, e.g. ion exchangers, established guidelines may be used for this transition (see Chapter 10).

The 250-fold scale-up of an ion exchange step, used in the chromatographic fractionation of desalted plasma, is shown in Figure 4.23. The bed height (15 cm) and the eluent velocity (120 cm/h) remained constant as the process was scaled up from columns of 30 ml to 1.5 L and to 7.5 L. As can be seen from the figure, resolution between IgG, albumin and glycoprotein is maintained reasonably well throughout the scale-up procedure.

**Table 4.8**

Plate height of pilot and process columns for SEC

Column dimensions (cm)	Bed volume (L)	Sample volume (L)	Eluent velocity (cm/h)	HETP (cm)
30 $\times$ 36	32	0.8	10	0.030
30 $\times$ 100	236	5.9	10	0.034



**Figure 4.23** Scale-up of IEC. Sample: desalted plasma. Ion exchanger: DEAE Sepharose™ Fast Flow. Eluent velocity 120 cm/h. Bed height 15 cm. (a) Column K16 (16 mm ID), 1.2 g of protein loaded, (b) column BP 113 (113 mm ID), 90 g protein loaded, and (c) column BP 252 (253 mm ID), 330 g protein loaded. Reproduced from Sofer and Nyström [36] with permission.



### *Non-chromatographic scale factors*

The scale-up of chromatographic processes is mostly straightforward. However, non-chromatographic factors can alter the purification result during scaling up of the entire process. These factors include changes in sample composition and concentration that occur as the fermentation scale increases, precipitation in the biological feedstock due to longer holding times when large volumes must be handled, non-reproducibility of large-scale buffer preparations and microbial growth in the buffer due to increased handling and longer holding times [67]. Additives may affect the  $pK_a$  values of buffer and thus change the pH [68]. Changes in temperature, pH and ionic strength may alter the sample properties and, as a result, the chromatographic purification result. Even though these factors should ideally have been considered in the design of the purification process, reports of these types of effects are not uncommon in meetings on preparative chromatography. This illustrates that it may be difficult to foresee all possible sources of problems during scale-up. However, a thorough understanding of the chromatographic process and scale-up guidelines will aid in determining if the cause of the problem is the chromatography step or not.

### **4.3.5 Ultrafiltration**

Ultrafiltration is commonly used in downstream processing for buffer exchange (i.e. diafiltration) and product concentration. It is also used for fractionation or purification. Membranes are rated by molecular weight cutoffs, called nominal molecular weight cutoffs (NMWC). Membrane structure can influence separation consistency. Minimizing macrovoids enables the use of higher pressures and higher temperatures, and gives better separations due to sharp NMWC. Cassettes are the first choice for most protein processing. Hollow fibres are more appropriate when the product is sensitive to shear or a high-viscosity solution needs to be processed. Hollow fibre devices have been used to concentrate adenoviral vectors and purify influenza vaccines. The conditions for the purification of an influenza vaccine are shown in Table 4.9. Ultrafiltration was tested as an alternative to sucrose or CsCl gradient centrifugation. Ultrafiltration provided a closed system

**Table 4.9**

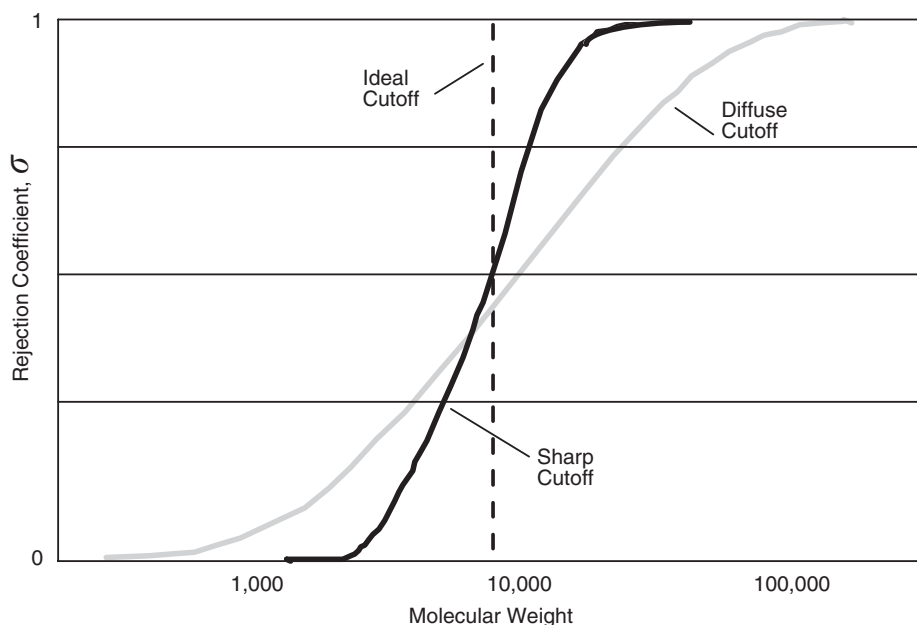
Influenza vaccine purification	
Membrane pore size rating	750,000 NMWC
Membrane surface area	420 cm <sup>2</sup> (0.45 ft <sup>2</sup> )
Solution volume	0.5 L
Concentration	6 g/L
Inlet pressure	1.4 psig
Outlet pressure	0 psig
Average productivity	20 l/mh
Process time	3.5 h
Product recovery	Close to 100%

that could be autoclaved prior to operation and used at low-operating pressure to maintain shear-sensitive virus. This operation was later scaled up 20-fold using the same path length. Diafiltration, and not concentration, was used to remove a protein contaminant, which was removed below the detection limit.

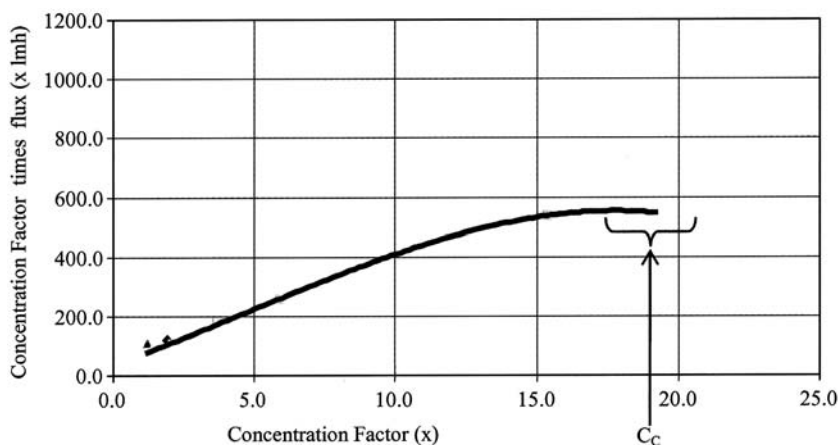
#### *Optimization of ultrafiltration*

As seen in Figure 4.24, even a membrane with a sharp cutoff deviates from the ideal indicating that membrane filtration is generally not optimal for high-resolution separations. At least a 10-fold difference in size between the product and an impurity is needed to achieve a good fractionation. Even with this size difference, a 10-time wash is recommended for complete removal of the impurity. Selectivity of the membrane is enhanced by a uniform pore size distribution. If the target molecule is passing through the membrane both yield and selectivity may decrease due to build up of a gel layer on the membrane.

In general, the membrane pore size should be 3–5 times smaller than the target molecule for an efficient concentration process. The process will be optimized by increasing the transmembrane pressure (TMP), while keeping the cross-flow constant and repeating this for e.g. three different cross-flow rates. Under the same TMP, an increase in cross-flow generally improves flux due to reduction of the concentration/polarization layer. The TMP and cross-flow can be increased until it adversely affects the process efficiency and yield, but the system itself can be limiting. The minimum amount of membrane area that achieves the concentration goal is used to minimize the costs.



**Figure 4.24** Typical rejection characteristics of ultrafiltration membranes.



**Figure 4.25** Optimization of diafiltration. The figure illustrates the maximum concentration factor of the feed, here denoted  $C_c$ , after which the processing time is adversely affected by the concentration. Courtesy of Yujing Yang, GE Healthcare. Reproduced with permission from GE Healthcare Bio-Sciences Inc.

For diafiltration, it is necessary to determine the volume required to exchange buffers. Generally 3–5 volumes are sufficient for buffer exchange, but this must be optimized for each application. If consumption of the new buffer is a concern then the sample should be concentrated as much as possible before diafiltration. On the other hand, too high-sample concentration will have a detrimental effect on processing time. A plot of the product concentration factor times flux against concentration factor will provide a curve showing a relationship that indicates when the diafiltration processing time will be strongly influenced by product concentration. This can be seen in Figure 4.25.

When combining concentration and diafiltration, the flux rates and permeate volume impact the total processing time. Table 4.10 shows the beneficial effect of performing the diafiltration step when the feed is sufficiently diluted to avoid reduction in flux rate due to protein solution viscosity and gel layer formation. The importance of using this strategy will be application-dependent. A strategy for optimization and scale up of ultrafiltration/diafiltration has been described for high-concentration monoclonal antibody preparations [69]. Transmembrane pressure and feed flux were optimized in total recycle mode. Optimum diafiltration concentration and minimum process time were also evaluated. Since monoclonal antibodies are often needed in a high dose and dosage is volume limited, the ability to optimize both diafiltration and ultrafiltration at high concentrations is critical for manufacturing success.

#### 4.3.6 Virus filters

Viral safety is one of the most often discussed topics at meetings on bioprocessing. Virus filters, incorrectly called nanofilters, are often incorporated into downstream processes to enhance viral safety [70]. Intended to operate predominantly by size exclusion, these filters

Table 4.10

Concentration and diafiltration

	Batch volume (L)	Protein concentration (%)	Flux rate (L/h)	Average flux (L/h)	Permeate volume (L)	Process time (h)
A. Starting volume	1000	5	940			
1. Concentrate 5×	200	25	140	460	800	1.7
2. Diafilter 5×	200	25	160	150	1000	6.7
Case A totals					1800	8.4
B. Starting volume	1000	5	940			
1. Diafilter 5×	1000	5	1060	1000	5000	5.0
2. Concentrate 5×	200	25	520	520	800	1.5
Case B totals					5800	6.5
C. Starting volume	1000	5	940			
1. Concentrate 2×	500	10	600	740	500	0.7
2. Diafilter 5×	500	10	680	640	2500	3.9
3. Concentrate 2.5×	200	25	160	370	300	0.8
Case C totals					3300	5.4

*Note:* The beneficial effect of performing diafiltration before excessive concentration is clearly noted. Case C takes the least time.

have been demonstrated to consistently remove large viruses (>80–120 nm diameter). Other mechanisms such as adsorption can also effect a separation of virus from protein solution, but are less of a consideration in the selection of the most suitable virus filter. Details of virus filter selection and characterization, physical and mechanical characteristics, virus filter evaluation studies, integrity testing and sterilization can be found in a PDA Technical Report [71].

4.4 EQUIPMENT

Equipment is addressed in detail in Chapter 11. In most cases, off-the-shelf equipment is used for research and development; whereas, custom systems are often selected for production scale. Catalogue shopping is not an option for custom systems, which require increased involvement for both system user and supplier when custom systems are needed. Some major factors that should be considered when purchasing equipment include

- Ability to be cleaned and sanitized
- Capability to deliver the necessary output (e.g. flow/pressure)
- Compatibility of wetted materials with product and intermediates
- Acceptable footprint for facility in which equipment is to be used
- Transparency to scale
- Flexibility for multiproduct facilities
- Cost
- Reliability

## 4.5 SELECTING TOOLS FROM R&D TO PRODUCTION

It is important to understand manufacturing requirements when designing a process. Manufacturing capabilities may dictate the use of specific types of analytical and production equipment. When a process is developed using tools that are not scalable, the process will need to be re-designed if the product is successful in the early clinical trials. Process redesign, however, can impact product quality and necessitate the repeat of costly toxicology and even clinical studies. Depending on the timeframe, this is also likely to delay market entry.

The selection of appropriate separation tools during research and development stages and final scale manufacturing can positively impact product quality and cost. Using technologies that are scaleable from the start is advisable. For example, stirred cells, commonly used for ultrafiltration at laboratory scale, cannot be scaled up. Changing to a plate and frame or other type of ultrafiltration device for pilot scale to manufacture clinical trial material may lead to variability that affects other subsequent steps. Using CsCl gradient centrifugation is another example of an inappropriate purification tool for large-scale manufacturing.

Membranes and chromatography resins should be selected from the wide range that is commercially available and suitable for scale-up. Processes that utilize very soft, compressible chromatography resins will be very difficult, if not impossible, to use at high-flow rates. Chromatography resins with fragile ligands also pose potential future problems, e.g. leakage. Filters, e.g. are evaluated for extractables using real production feedstreams. Considering the potential final scale early on is advisable to predict future equipment and system needs as well as suitable chromatography resins and membranes.

Raw materials should also be considered. There is no real cost savings gained by using poor quality reagents in development of a process. The impurities may affect the process and make determination of relevant process control parameters difficult.

However, streamlining the development process is becoming common practice in today's biotechnology industry. By using defined and high-quality raw materials and separation resins (i.e. chromatography resins and membranes) that are made by highly controlled and validated processes, process developers can design a process that consistently produces product of suitable quality for its intended use. By using scaleable materials and equipment (see Chapter 11), those processes are easily transferred to the next stage of manufacturing.

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## Analysis

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### 5.1 INTRODUCTION

Biological molecules are often highly complex, and multiple analytical methods are required to demonstrate that final products are consistently produced and remain stable. Characterization of biological products involves analysing physicochemical properties, biological activities, immunochemical properties, product purity and impurities [1].

Over the last decade, vast improvements have been made in analytical tools used to characterize biological products, process intermediates and impurities. New and improved methods allow enhanced characterization, which can minimize, and even eliminate, the need to repeat clinical trials when process changes are made. As observed by one FDA speaker at a meeting in 2006, ‘How can you afford not to do this? The expense of product characterization is small compared to the cost of re-running clinical trials’ [2].

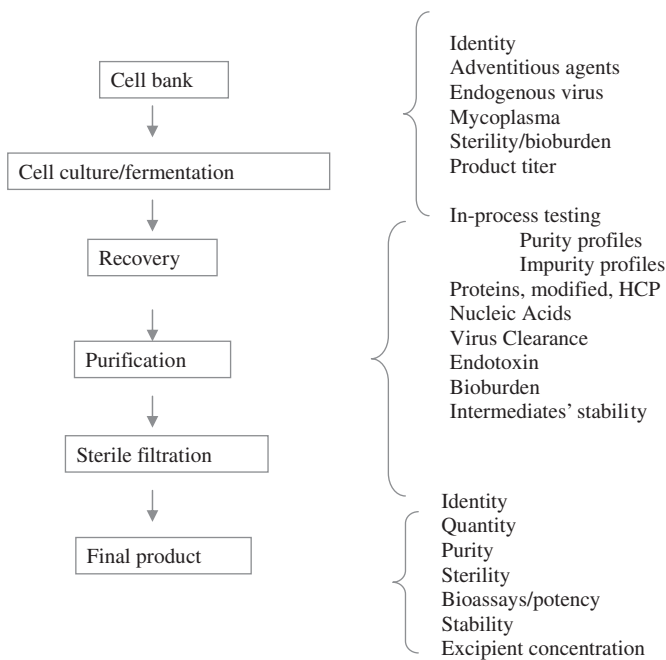
In this chapter, we address analysis of protein and nucleic acid products.

### 5.2 PROTEINS

The success of the biotechnology industry is due in large part to the tremendous advances in analytical protein chemistry [3]. For therapeutic proteins made by biotechnological processes, consistency can be evaluated by demonstrating genetic stability of the host, combined with in-process and final product analysis. Methods used for product and process intermediate analysis include those that monitor and define identity, purity, potency and stability. Assays are needed throughout a biotherapeutic production process. Some of these are depicted in Figure 5.1.

#### 5.2.1 Identity

Identity tests are performed for each lot of product. Commonly used identity tests for biotechnology products are shown in Table 5.1. The assays used for identity are also used to evaluate other parameters, such as purity. Traditional gel electrophoresis and more recently adopted capillary electrophoresis (CE) techniques are also used for determining molecular weight (e.g. sodium dodecyl sulphate–polyacrylamide gel electrophoresis, SDS–PAGE)



**Figure 5.1** Commonly needed assays for a mammalian cell culture process used to produce therapeutic proteins.

**Table 5.1**

Commonly used identity tests for biotechnology products
Peptide mapping
N- and C-terminal sequencing
Mass spectrometry (MS)
High-performance liquid chromatography (HPLC)
Isoelectric focusing (IEF)
Carbohydrate analysis
Immunoassays
Bioactivity

and heterogeneity (e.g. isoelectric focusing, IEF). Particularly noteworthy is the increased use of mass spectrometry (MS) during the last 5 or so years to provide verification of amino acid sequences.

**5.2.2 Purity**

Purity is often defined by product structure consistency and absence (or defined levels) of both product-related and process-related impurities. Product-related impurities include

aggregated, deamidated or otherwise modified product forms. Process impurities are those that are either inherent in the source material, e.g. host cell proteins (HCP) or endotoxins from *E. coli*, or added during processing, e.g. cell culture media, leachables, additives. Purity levels for biotherapeutics are quite high. Today's analytical methods make purity evaluation more sensitive than in the past. Application of new methods to existing products and processes may turn up some surprises. Some protein products may exist in multiple, biologically active forms, but others that are chemically equivalent may not be suitable for therapeutic usage. For example, chemically equivalent aggregates may be immunogenic and cause more harm than benefit. Protein modifications that can produce product-related impurities include proteolysis, deamidation, oxidation, carbamylation in the presence of urea, phosphorylation and aggregation. Several analytical methods are usually combined to detect these impurities. As noted in Ref. [1], classification of a variant as a product-related impurity depends on whether that variant has comparable activity, efficacy and safety.

The presence of aggregates does raise concerns, since they are considered to be potentially immunogenic. Assays that are routinely used in QC to detect aggregates and those that can be used to characterize aggregates and demonstrate comparability when changes are made have been reviewed [4]. Methods used routinely are size exclusion-HPLC (SEC-HPLC) and SDS-PAGE. Characterization and comparability-demonstrating assays include analytical ultracentrifugation, field flow fractionation, dynamic light scattering and MS. Other methods include spectrophotometric (fluorescence, circular dichroism and infrared), ultrasonic resonance and structural signature technologies [5]. As noted in an oral presentation SEC and OD A410 measurements can determine soluble and insoluble aggregates, respectively, and are commonly used to monitor monoclonal antibody aggregation [6].

SDS-PAGE combined with scanning laser densitometry is used for quantifying recombinant proteins and their degradation products. Since the impurities in feedstreams do not interfere with this method, it can be used during purification. Specific method protocols are provided in a recent publication [7].

Although gel electrophoresis is still widely used, many companies have replaced it with CE due to the latter's ability to be automated and provide quantitative, reproducible information while minimizing the need for toxic reagents. CE methods include CE-SDS, cIEF (capillary isoelectric focusing) and CZE (capillary zone electrophoresis). CZE with laser induced fluorescence (LIF) detection is used for glycan analysis [8]. CE is used today for lot release testing and in process development.

Amino acid analysis and N- and C-terminal sequencing were the work horses of the 1990s but their limitations spurred on the application of MS combined with HPLC for analysing glycoproteins. The different MS modes and their advantages and disadvantages are reviewed in Ref. [3], which also describes the application of this technology for solving a difficult characterization problem and for analysing oligosaccharides isolated from a monoclonal antibody.

### *Structural analysis*

Different levels of structural characterization, including primary, secondary, tertiary and product/ligand conjugate have been described by Ritter and McEntire [9]. Structural analysis is important to ensure lot-to-lot consistency. Structural changes can be caused by

modifications made during cell culture/fermentation, during processing, formulation and storage. Changes may occur in primary, secondary, tertiary and even quaternary structures. MS, which is both selective and sensitive, is used to detect structural changes in peptides, proteins and carbohydrates. MS is typically used in conjunction with HPLC methods such as reversed phase HPLC for peptide mapping. 'Top-down' characterization of protein pharmaceuticals by HPLC combined with MS has been used to demonstrate comparability of a recombinant Factor IX product after a manufacturing site change [10]. The top-down approach provides the option of gas-phase ion fragmentation of the target molecule. Tandem MS/MS was also used to enhance the resolving capability.

Nuclear magnetic resonance (NMR) is now being developed for structural analysis of larger proteins. In the past, this technique was typically limited to proteins with masses of 25 kDa or less. Isotope labelling of amino acids has lead to simpler spectra that can now be used for characterization of at least 41 kDa proteins. It is expected that this technique will, in the future, provide more information about protein dynamics and structure [11]. NMR spectroscopy is also being used for characterization and quality control (QC) of carbohydrate-based vaccines [12]. This application has been reviewed by the National Institute for Biological Standards and Control (NIBSC) in the UK. The integrity of glycan chains that are incorporated into glycoconjugate vaccines has been demonstrated using one and two-dimensional NMR spectra. Manufacturing intermediates and polysaccharide degradation in final bulk conjugate vaccines can also be assessed by NMR.

One method for elucidating glycoprotein carbohydrate structure involves enzymatic removal of the oligosaccharides from a protein, followed by fluorescent labelling of the oligosaccharides and high performance anion exchange chromatography (HPAEC) combined with MS. IEF in gels and cIEF are often used to assess consistency of charged glycoforms during purification. Carbohydrate moieties can influence potency—in particular, sialylated oligosaccharide modifications must be assessed as they have been shown to alter clearance and effectiveness of biotherapeutics. A 2001 FDA license approval included instructions for the manufacturer to set quantitative limits for N-glycosylated sialylated oligosaccharides for the first 30 commercial lots.

Correct secondary and tertiary structures are required to ensure consistent performance of complex biotherapeutics. Incorrect disulphide formation is likely to alter product function and stability, but can be detected by multiple methods including HPLC, differential scanning calorimetry and SDS-PAGE (see Ref. [1]). Circular dichroism is used to evaluate secondary structure; NMR and X-ray crystallography are used for tertiary structure analysis; and analytical ultracentrifugation and light-scattering methods can be applied to quaternary structure analysis. Potency assays can confirm proper protein conformation. The use of Fourier-Transform Midinfrared spectroscopy for screening liquid protein formulations has been described [13].

### *Process impurities*

Process impurities can be derived from the host cell and processing agents, materials and equipment. An extensive list of impurities that are of concern to regulators is provided in a summary from the Well Characterized Biotechnology Pharmaceutical (WCBP) Chemistry, Manufacturing and Controls (CMC) Strategy Forum. Impurities that come from media

components, cell components, chemical additives and leachables may be associated with patient-safety problems, including immunogenicity, hormonal activity, toxicity and genotoxicity, transmissible spongiform encephalopathies (TSEs) and irritants [14].

Clearance studies are often appropriate to demonstrate removal of process impurities. These studies can reduce the number of lot release tests, thereby reducing QC costs. It should be kept in mind, however, that it is necessary to maintain the assays so they can be used for process changes and out-of-specification investigations.

The issue of how much evaluation of impurities is necessary is an issue that requires application of a risk assessment. However, it has been suggested that most substances that account for at least 0.1% of the product should be detected and monitored. All substances that account for over 1% should be detected and monitored [15].

The most common impurities include HCP, host cell DNA, leachables such as Protein A from affinity columns, extractables from plastics and antifoam agents and other additives.

#### HOST CELL PROTEINS (HCP)

HCP assays present a significant challenge. Antibodies are typically raised in goats or rabbits against a mixture of HCP. The HCP are usually obtained by removing the gene for the product, then growing the cells as if for production of the product. The problem is that some HCP will be less immunogenic, even non-immunogenic, and the antibodies that are generated may not detect all HCP. Another problem is that when culture conditions are changed, HCPs can change so that the antibodies are no longer suitable. The polyclonal antibodies that are generated from the goats and/or rabbits are used to develop enzyme-linked immunosorbent assays (ELISAs). SDS-PAGE and 2D-PAGE are also used, and Western blotting with antibodies to product and antibodies to HCPs can be used to distinguish product from HCPs. This can be important if there are any HCPs that are co-purifying with the product. It has been recommended that in this case, the purification process should be re-engineered to remove that HCP.

ELISAs are more sensitive than gels and measure HCP mass. Although there are no established limits for HCP, FDA has found that most biotechnology products contain ELISA-based HCP levels between 1 and 100 ppm [16]. Companies are recommended to base acceptance criteria for HCP on data obtained from lots used in pre-clinical or clinical studies [17]. A comparison of product-specific and multiproduct HCP immunoassays is provided in Ref. [16], which also discusses the applicability of commercially available generic immunoassays.

#### HOST CELL DNA

Methods applied to analysis of nucleic acids include sequence analysis, hybridization, restriction mapping, quantitative PCR (Q-PCR) and total DNA measurements. Several methods are typically used to demonstrate genetic stability [18]. Q-PCR assays, with sensitivities ranging down to the femtogram level, allow manufacturers to avoid scaled down spiking studies with host cell DNA. In the event that a Q-PCR assay is unavailable, slot blot hybridization assays with species-specific probes can be used. The Threshold® system for total DNA is also an option [19]. The latter is not applicable for DNA plasmid or viral products. In one instance during validation of a Q-PCR assay against the Threshold system, the level of DNA detected by the Threshold system kept increasing; whereas, the

levels detected by Q-PCR remained constant. Investigations lead to the discovery that the samples were contaminated by bioburden, which, as expected, were not detected by the specific probes.

#### LEACHABLES AND EXTRACTABLES

Leachables such as Protein A from affinity columns are typically assayed by ELISAs. Other methods for detection of leachables from chromatography resins are described in Chapter 7. ELISAs can be used to validate removal of any leached Protein A or used to establish a final product release specification. In early development, it is sometimes easier and more relevant just to test final product. Specifications of 10–12 ppm Protein A in the final product are usually considered acceptable [17].

Testing for extractables is now a more widely discussed topic than ever before. While extractables have always been addressed by filter companies, the increased use of disposables in biopharmaceutical manufacturing has raised the importance of assays for extractables. In some cases, vendor information is sufficient, but it is usually necessary to evaluate extractables with the actual solutions that will be in contact with the disposables. Several references provide the reader with extensive information on extractables [20, 21]. Methods and results for evaluating extractables from plastic materials have been described [22].

#### ANTIFOAMS AND OTHER ADDITIVES

Additives, such as antifoams, detergents and stabilizing agents, are sometimes needed in a process. Assays should be, but are not always, available from the vendor. The assays should be capable of being validated. Even if they are validated by the vendor, they will have to be qualified to demonstrate they are providing useful information in the presence of the product or process intermediates.

#### *Contaminants*

Contaminants are controlled by adherence to current good manufacturing practices (cGMPs). That control requires the use of validated assays for virus, bioburden and endotoxin and other pyrogens—all of which have the potential to cause harm to patients.

#### VIRUS

Details for the performance of viral clearance studies are provided in the ICH guideline Q5A [23]. Assays for virus can be divided into two categories: namely, those that detect infectious virus and those that detect both infectious and non-infectious viral sequences. Infectivity assays are performed either *in vivo* or *in vitro*, with *in vitro* being performed routinely for cell culture unprocessed bulk. The *in vitro* assays are based on the fact that certain cell types are susceptible to certain viruses and a microscopic examination will enable detection and quantification of replicating virus. The TCID<sub>50</sub> (tissue culture infectious dose) and PFU (plaque forming unit) methods are the most common assays.

The use of both infectivity and PCR assays has enhanced the industry's understanding of viral clearance mechanisms in some process steps, such as in the purification of monoclonal antibodies on immobilized Protein A resins. By using Q-PCR, the removal of viral particles can be quantified; while, infectivity assays demonstrate if the particles are inactivated. This has enhanced the overall virus clearance factors by enabling companies to claim both

physical removal by chromatography and inactivation by low pH elution buffers. Q-PCR has enabled some companies to apply generic and matrix viral clearance studies and gain an understanding of the impact of cell culture processes on endogenous retrovirus expression [24, 25]. The details of applying real time Q-PCR to evaluate virus clearance during purification are presented by Shi *et al.* [26].

#### BIOBURDEN

Bioburden include bacteria and fungi. Compendial sterility and bioburden tests are described in pharmacopeias, and there are other methods described in regulatory guidance documents. These are culture assays that are lengthy, taking weeks to complete, but must be performed to comply with current regulations. In addition to being time-consuming, culture assays have other problems that include inability of some microorganisms to replicate and others that cannot be isolated due to confluent growth [27]. Rapid microbiological methods (RMM) are being implemented by some companies for monitoring but at this writing they can not replace product release assays for protein biotherapeutics. (An exception is made for certain types of products, such as cellular therapies, with short shelf lives.) RMMs include, among others, technologies based on detection of growth, viability and nucleic acids. New RMM are reviewed by Miller [27] and Moldenhauer [28].

Mycoplasma are bacteria-like organisms without a cell wall. Although rarely a problem for downstream processing, mycoplasma can be introduced into cell cultures and literally take over those cultures and alter their output. Mycoplasma can also be introduced from raw materials. In common with testing for bacteria and fungi, the assays that are described in regulatory documents (e.g. FDA's Points to Consider on cell lines) are lengthy—28 days [29]. A PCR method has been able to detect all known mycoplasma species and requires only 5 h [30].

#### ENDOTOXIN AND OTHER PYROGENS

Pyrogens are substances that can produce a fever. The most common pyrogens are endotoxins, which are lipopolysaccharides (LPS) produced by Gram-negative bacteria such as *E. coli*. The limulus amoebocyte lysate (LAL) test is used to detect endotoxins. Another assay that detects endotoxins is the recombinant factor C assay [31, 32].

The advantage of the latter is lack of positive tests for substances containing glucans—a problem sometimes observed in downstream processing when trace amounts of carbohydrates are released from chromatographic resins. In the US, the rabbit pyrogen test is still required for licensed products (21 CFR 610.13(b)) to test for intrinsic pyrogenic substances other than endotoxin and may even be recommended for products, such as those for gene therapy, in clinical trials [33].

Other methods used for detection of pyrogenic materials are now developed and consideration is being given for them to replace the rabbit test. One *in vitro* assay that uses monocytoïd cells has been validated against the rabbit assay [34].

### 5.2.3 Quantity

Determination of the amount of product that is in the final vial is performed using methods such as UV A280 absorbance and HPLC. Total protein can also be measured using



dye-binding assays such as Bradford and Lowry methods. A comparison of rapid methods that measure protein titer was made [35]. Methods included ELISA, optical biosensor, chemiluminescence, rapid chromatography and nephelometry. The methods were compared for many parameters including ease of assay optimization, time, calibration, validation and costs.

### 5.2.4 Potency

Potency (biological activity) assays are often delayed during development. They are time-consuming, costly and difficult to validate in many cases. However, they must be fully validated before a product license application will be considered complete. If a potency assay is not in place by the start of phase 3, a clinical hold is likely to result [36]. These biological activity assays (bioassays) must measure an activity that correlates with clinical function. The components of a bioassay include dose, biological subject and response compared to a reference standard. These assays are a measure of correct tertiary structure and should also be stability-indicating (see Section 5.2.5). In some cases, binding assays can be correlated with an animal- or cell-based assay. *In vitro* bioassays include, for example, immunoassays and cell-binding assays. *In vitro* assays have often been found to be more sensitive to product changes, but not as physiologically relevant.

### 5.2.5 Stability

Genetic instability can lead to production of a modified product, new impurities, or a higher percentage of impurities. If a significant amount of genetic instability were to occur, there is a chance that the purification process would not be able to provide consistent product. Single point mutations can be detected by peptide mapping; N- or C-terminal sequence analysis can detect genetic changes that increase the expression of proteolytic enzymes; and charge-related post-translational modifications are usually detected by IEF. The ICH guideline on analysis of expression construct provides details of the methods used for assessing genetic stability (see Ref. [18]). Although genetic analysis gives useful information, the application of analytical tools that specifically evaluate protein instability are usually much more informative and relevant for a final protein product.

The ICH guideline on stability testing of biotechnology products recommends focusing on degradation products, which include those caused by deamidation, oxidation, sulfoxidation, fragmentation and aggregation [37]. Analytical methods used include SDS-PAGE, IEF, Western blot, immunoelectrophoresis, HPLC and peptide mapping. A 1998 paper illustrates degradation pathways in proteins [38]. A new approach for stability testing has been presented. It includes a determination of rate of decay by measuring slope rate against slopes from specification-setting lots [39]. Stressed or accelerated studies are usually performed and are especially useful for demonstrating comparability when process changes are made. Real-time stability data, however, do need to be accumulated. Assays used to demonstrate stability must, in fact, be stability indicating. All assays that can be used to show a change under recommended storage conditions should be used to determine stability [40]. Stability testing of final products also includes sterility and container integrity testing.

Examining a few FDA Form 483s (a form left after an FDA inspection, which requires that corrections be made) provides some insight into where firms make mistakes:

- Re-worked lots not placed on stability.
- Stability testing does not include analysis of re-constituted products.
- Moisture content has been identified as impacting stability, but there is no specification for moisture.
- Stability determined in container of different chemical composition from that used for shipping.

### 5.2.6 Assays for monoclonal antibodies

Table 5.2 lists many of the current assays used for characterization and demonstration of comparability, evaluation of stability and release of monoclonal antibodies. Some of these assays can be used for multiple purposes. For example SDS–PAGE or SDS–CE can be used to assess both impurities and size of target molecule.

**Table 5.2**

Analytical methods used to characterize protein and carbohydrate structures, evaluate stability and release monoclonal antibodies

Method	Detection
SDS–PAGE	Impurities, size
IEF	Carbohydrate, impurities, identity
HPLC	Impurities, purity
CE	Impurities, purity
Protein A HPLC	Product titer
Immunoassays: Western blot and ELISA	Protein quantitation, purity, potency, identity, impurities: HCP, BSA, Protein A
Ligand binding assays, e.g. Biacore®	Identity, potency
Bioassays: <i>in vitro</i> , <i>in vivo</i>	Identity, potency
UV A <sub>280</sub>	Concentration, purity
Total protein assays	Concentration
Mass spectrometry	Identity, protein quantitation
Peptide mapping	ID, purity, primary structure, deamidation, methionine oxidation, disulphide shuffling
N- and C-terminal sequencing	Identity
Amino acid analysis	Identity, concentration
Circular dichroism	Secondary structure
NMR, scanning calorimetry	Tertiary structure
Analytical ultracentrifugation, light scattering (dynamic/static)	Quaternary structure

5.3 NUCLEIC ACID PRODUCTS

Nucleic acid products include both virus-based gene therapy products and DNA plasmids. In this section, we focus on DNA plasmids. The reader is referred to two publications summarizing the Well Characterized Biotechnology Pharmaceutical (WCBP) CMC Strategy Forum held in January 2005, in which assay choices for viral vaccines and virus-based gene therapy products were addressed. The assays used for characterization, lot release, sterility, purity and safety are described in these publications [41, 42].

Much of the information already discussed in this chapter is relevant for DNA plasmids. Potency of DNA plasmids is difficult to measure but some *in vitro* potency assays, such as ELISA, FACS and RT-PCR, have been discussed [43].

5.3.1 Identity

Similar to the release of protein batches, identity tests are performed for each lot of nucleic acid product. Commonly used identity tests for nucleic acid products are shown in Table 5.3. The assays used for identity are also used to evaluate other parameters, such as purity. Traditional electrophoresis techniques such as agarose and capillary gel electrophoresis (CGE) are typically used for determining molecular weight and heterogeneity (isoforms, monomers, multimers). CGE for plasmid DNA is not yet a fully automated procedure, but requires in-depth knowledge and the detailed protocols are often considered company trade secrets [44].

5.3.2 Purity

As the major application for plasmid DNA is vaccination of healthy individuals, the purity requirements for nucleic acid products are very high. The development of new analytical tools pushes the limits of the allowed impurity levels to previously undetectable amounts. Furthermore, the physiological effect of the different isoforms or aggregates of plasmid DNA is still subject to debate. Although the demands on purification are trending towards less stringency with regard to these forms, it is currently considered good practice to regard

Table 5.3

Commonly used identity tests for nucleic acid products
Optical density measurement
Nucleic acid sequencing
Restriction digest analysis
Agarose gel electrophoresis
High-performance liquid chromatography (HPLC)
Capillary gel electrophoresis
Bioactivity

the less abundant isoforms like linear or open circular plasmid DNA as impurities, thus requiring either removal of these isoforms, or preferably avoiding their formation during fermentation or downstream processing. The presence of the different plasmid DNA isoforms (linear, open circular and supercoiled plasmid DNA) as well as aggregates is routinely determined by agarose gel electrophoresis. However, because of the limitations of this technique with respect to resolution and linearity in product concentration, CGE is now considered to be the state-of-the-art technology. In addition, a multitude of HPLC techniques, predominantly based on ion exchange and hydrophobic interaction chromatography, are used in today's QC departments.

Aggregates are still a source of concern, as their biological significance is not determined. Plasmid DNA, including aggregates, are generally considered to be very safe, and, to date, no immunological reactions against plasmid DNA have been detected [45]. However, aggregate formation is anticipated to have a dose-lowering effect on the final product. The presence of aggregates is usually analysed by CGE or chromatography techniques such as SEC.

In contrast to protein products, very little is known about product modifications of plasmid DNA. Although plasmid DNA molecules are very stable, oxidative processes are generally considered detrimental for long-term storage of plasmid DNA, and presence of any catalysts of such reactions in the final product should therefore be avoided [46].

The technical advances made in DNA sequencing during the last decade now allow for a reliable determination of the plasmid DNA sequence in the final sample before batch release. This technique has become a standard analysis method for confirming plasmid DNA in the final product is identical to that in the master cell bank.

### *Process impurities*

When plasmid DNA is produced in a Gram-negative bacterial system, the most common impurities include host cell DNA, RNA, HCP and endotoxins. As in any process for the production of biopharmaceuticals, levels of leachables from columns, extractables from plastics, antifoam agents and other additives should also be determined in the final product.

#### HOST CELL DNA

The bacterial systems used for plasmid DNA production are considered to be genetically stable [47]. Genetic stability need only be demonstrated one time, using either the master cell bank or a working cell bank. Remaining host cell genomic DNA in the final plasmid DNA product is quantified. Methods based on nucleic acid hybridization, i.e. Southern blot and the more quantitative Q-PCR, are commonly used. Alternatively, fluorescence-based methods are used for host cell DNA detection. Because of their limited specificity for different nucleic acids (e.g. DNA vs. RNA), and the presence of the plasmid DNA, extra caution is required when interpreting the results, and a more thorough validation of the methods within their limits is necessary.

#### RNA

RNA is the major impurity present in the production of plasmid DNA. It is estimated that more than 75% of all nucleotides present in the *E. coli* bacterial lysate are RNA [48].

Traditionally, RNA presence is shown by agarose gel electrophoresis. Currently, this technique is considered to be inaccurate because of the high rate of RNA hydrolysis, especially during extensive sample treatment and under elevated temperatures during gel electrophoresis. More recently, chromatographic techniques such as ion exchange and hydrophobic interaction chromatography are being used. Q-PCR assays are considered the most accurate and sensitive, and are therefore deemed to be the most appropriate analytical tool, even if major investment in equipment and personnel training is required.

#### HOST CELL PROTEINS

HCP assays for plasmid DNA products are easier to develop than those for protein products, since the plasmid DNA itself does not elicit antibodies against proteins. However, plasmid DNA is typically produced in *E. coli*, and different *E. coli* strains have a different protein profile. This requires the development of cell strain specific assays. Furthermore, the HCP profile is strongly affected by the fermentation conditions and the stress the production of plasmid DNA creates in the cell. ELISAs are developed by using polyclonal antibodies, specific to different *E. coli* strains.

Chemical protein assays based on the Biuret, Bradford, BCA or Lowry methods, SDS-PAGE and 2D-PAGE are also used, though the resolution and quantification with these methods is usually of a lower degree.

#### ENDOTOXIN AND OTHER PYROGENS

LPS are naturally occurring components of the Gram-negative *E. coli* cell wall. The methods used for analysis are the same as those used for protein products.

#### LEACHABLES AND EXTRACTABLES

For plasmid DNA purification, traditional non-protein based chromatography media are used. Possible extractables from resins are in general investigated by the vendor, and the required information is provided to the biopharmaceutical manufacturer (see also leachables and extractables above).

#### *Contaminants*

In the production of plasmid DNA-based biopharmaceuticals, it is strongly recommended to test both master and working cell banks for the presence of bacteriophages and other adventitious agent contamination.

#### BACTERIOPHAGES

Similar to virus assays for mammalian cell culture, bacteriophage assays have to be developed for *E. coli* cultures. Plaque assays have long been the standard for assaying bacteriophage, but improvements in Q-PCR during the last decade, together with the commercial availability of specialized primers, have contributed to the increasing success of this method for bacteriophage determination. By using more than one pair of primers, multiplex PCR allows simultaneous amplification of many targets of interest in one single reaction.

#### BIOBURDEN

Bioburden assays estimate the total viable aerobic count of microbial contamination, typically conducted before final sterile filtration. Usually, plasmid DNA sample is plated on agar plates and microbial growth is monitored after a 48 h incubation period (see also Bioburden above).

### 5.3.3 Quantity

Determination of the amount of product that is in the final vial is performed using methods such as electrophoresis and HPLC. Other methods are based on absorbance, such as UV A260 absorbance and the use of fluorescent dyes that specifically bind to ds DNA (e.g. PicoGreen or Hoechst 33258), or the dimeric cyanine dyes (TOTO and YOYO). Current DNA vaccines often consist of mixtures of different plasmid DNAs. This puts an extra strain on the quantification of the different plasmid DNAs in the mixture, especially if all of those consist of the same plasmid DNA backbone and only differ in their gene insert. Quantitative PCR-based methods have the advantage that, even in such mixtures, they are able to determine the plasmid DNA concentration of each of the plasmids separately [49].

### 5.3.4 Potency

Potency assays are addressed in Section 5.2.4 above.

Specifically for plasmid DNA, quantitative potency assays are still a major source of debate. Plasmid DNA potency assays include *in vitro* measures of transfection efficiency that monitor the transcription and/or translation of the encoded gene(s). Correlation between *in vitro* and *in vivo* potency should be demonstrated, while immunogenicity of the final plasmid DNA product is preferably monitored *in vivo*.

### 5.3.5 Stability

Plasmid DNA-based biopharmaceutical products have a distinct advantage over protein-based products in that their biological activity is not affected by changes in tertiary or quaternary structure, apart from precipitation or aggregation, two conditions that can be relatively easily avoided during plasmid DNA processing. Chemical modification, however, can result in a loss of biological activity. This suggests that high-resolution chemical analysis can be used to verify the stability of the final product.

While electrophoresis of a restriction enzyme digest on an agarose gel can quickly give an indication of plasmid size and identity, more accurate DNA sequencing can detect single nucleic acid mutations and is, therefore, used to confirm the DNA sequence of the master and working cell banks, as well as that of the final product.

Chemical modification at the nucleic acid level, caused by superoxide or transition-metal ions are important sources of DNA damage *in vivo*. Presence of free radicals and metal ions should therefore be avoided when storing the final purified product. Depurination and

$\beta$ -elimination of the plasmid DNA can potentially result in conversion from supercoiled plasmid DNA to its open circular or, in worst case, linear isoform [46]. Such chemical modifications are consequently monitored indirectly, using, for example, CGE to determine the relative ratios of the different plasmid DNA isoforms [50].

## 5.4 COMPARABILITY

The ICH guideline Q5E addresses comparability of biotechnology products subject to manufacturing process changes that are made by a single manufacturer or their contract manufacturer [51]. ICH notes that the tests used to demonstrate comparability should be carefully selected and optimized to maximize the potential for detecting relevant differences in product quality attributes before and after a manufacturing change. To demonstrate comparability, it is recommended that multiple methods be applied to evaluate a single quality attribute. Examples of these quality attributes include those related to product, i.e. molecular weight and secondary/tertiary structure, as well as impurities.

Due to the enhanced ability of analytical methods to define biological products and intermediates, in many cases the process no longer defines the product. This has led to greater acceptance of biogenerics, also called biosimilars, similar biological medicinal products and follow-on biologics. The arena is still fraught with conflict, as evidenced in a recent commentary [52]. But, the first similar biological medicinal product, Omnitrope, a recombinant growth hormone, has been approved. The EMEA has produced a guideline on similar biological medicinal products along with specific guidance for recombinant proteins including somatotropin (growth hormone), granulocyte-colony stimulating factor, insulin and erythropoietin. (All of these documents can be accessed at [www.emea.eu.int](http://www.emea.eu.int).) The guideline on quality issues states that the analytical techniques used should represent state-of-the-art, should be validated and, if available, standards and reference materials should be used for method qualification and validation [53].

## 5.5 SETTING SPECIFICATIONS AND REFERENCE STANDARDS

The ICH Q6B guideline describes test procedures and acceptance criteria for biotechnology products [54]. Methods used for monitoring quality attributes and process endpoints during process development are likely to provide extremely important information that enables setting of specifications once the process is finalized. Specifications should be established using those analytical methods that provide the most useful information on identity, purity, potency and stability. As discussed at a recent California Separation Society (CaSSS) meeting, degradation should be considered when establishing specifications. In-process specifications include step yields in purification processes. The methods that are most appropriate for making this determination are established during development. Unfortunately, there are very few global standards and specifications for biotechnology products and associated impurities. With the exception of the WHO DNA specification, companies determine their own specifications based on knowledge of their process and product, any tidbits of guidance they can obtain from the industry and assay capability. It is clear standards are needed.

Standards and reference materials for biotechnology are increasing as biogenerics are entering the picture. ‘Working’ reference standards should be established during early development, and specifications developed as the process develops. Reference standards should be finalized by the start of phase 3 clinical trials and should be stored aliquoted. Degradation of reference standards must be addressed and it has been suggested they be stored at a different temperature than product lots to prevent the same pattern of degradation [55]. Comparability of reference standards must be demonstrated when the standards are changed.

## 5.6 METHOD VALIDATION

Analytical methods used to make in-process decisions and release final product are performed in GMP-compliant laboratories. The ICH guideline on validation of analytical methods describes the parameters that must be validated based on what the assay is claimed to demonstrate [56, 57]. For example, in ICH Q2A, the parameters for validation of limit tests for impurities include specificity and detection limit; whereas, an impurity assay claimed to be quantitative should be validated for accuracy, precision, specificity, quantitation limit, linearity and range. Although ICH provides guidelines, the analyst should always use a scientific approach when deciding which parameters to validate. FDA observations on method validation include:

- The method validation procedure described several tests to be done, but did not describe when each test would be used.
- The test descriptions lacked details necessary to perform the tests, e.g. concentrations for accuracy determination.
- There were no acceptance criteria for tests, so acceptance/rejection of method validation could not be assessed

Some non-routine assays may be qualified rather than validated [58]. For example, HCP assays that are used in process validation should be qualified; but those used for drug substance testing should be validated (see Ref. [16]). Validation of analytical methods is required prior to performing process validation.

## 5.7 PROCESS ANALYTICAL TECHNOLOGIES (PAT)

PAT is defined as ‘Systems for analysis and control of manufacturing processes based on timely measurements of critical quality parameters and performance attributes of raw and in-process materials’ [59, 60]. In research and development, PAT can provide real-time information that is transferable to manufacturing. In manufacturing, the application of PAT can prevent processing at risk while waiting for the results from a remote laboratory. One company has described how they applied PAT to purification of a biotechnological product (see Table 5.4) [61].

In another application, on-line HPSEC with differential refractometry and multi-angle laser light scattering is being applied to control manufacturing of an HIV viral vaccine.



**Table 5.4**

Application of PAT to biotechnological process

Application	Analytical methods
Gradient feedback control	NIR, UV, conductivity
Product collection	HPLC, UV
Ultrafiltration monitoring	UV sensors: product concentration in retentate stream, product loss in permeate
Centrifugation monitoring	Suspended solids sensor: control of concentration in feedstream and centrate

These analytical methods determine the oligomeric state of the antigen, which is critical for its immunogenic properties [62].

PAT can provide better process control and result in better process understanding. It may also alter the approach to setting in-process specifications, e.g. measuring constant purity instead of yield. Although it is not likely to eliminate validation, the effort spent to incorporate PAT, especially for a new product or for a process change, can enhance manufacturing consistency and reduce failed batches—providing an economic advantage.

Sophisticated analytical methods have enabled the biotechnology industry to produce safe and efficacious products. As even more improvements are made in areas such as tertiary structure analysis and feedback mechanisms, even better control may be possible. As always, there will be a balance between what is technologically feasible and costs, especially as biogenerics have the potential to reduce profitability of biotechnology therapeutic products. The costs of quality control are high, but as noted by one prominent biotechnology expert, ‘without analysis, you have nothing [63]’.

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# – 6 –

## Cleaning and Sanitization

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### 6.1 INTRODUCTION

Cleaning is defined as the physical removal of soil, organic debris and particulates from surfaces; whereas, sanitization is defined as removal or elimination of vegetative bacterial cells. Disinfection is the application of a chemical or physical agent, which may or may not be sporicidal, onto a surface or substrate to kill or inhibit microorganisms. A disinfectant is usually considered to be a sanitizer when it is used in a more dilute state. Sterilization implies the absence of microorganisms [1]. In purification processes, both cleaning and sanitization must be addressed. To minimize problems not related directly to the feedstream and its interaction with resins, filters and equipment, the use of pyrogen-free, high quality water, e.g. water for injection (WFI), filtered air, and high-quality grade solvents and buffers, is recommended.

The selection of cleaning and sanitizing agents will be dictated by effectiveness, compatibility, costs, disposal issues and experience. Cleaning and sanitizing reagents, method of preparation, concentration, volume, contact time and temperature (and even column flow rate) must all be defined, and their acceptable ranges written into standard operating procedures (SOP). Holding times prior to cleaning are of particular importance as proteins may denature or aggregate during storage and exacerbate cleaning requirements. It is also important to address cleaning issues in ancillary downstream operations, e.g. buffer preparation equipment.

Demonstration of the removal of both cleaning and sanitization agents is necessary. In the case of sodium hydroxide, measurement of the pH and/or conductivity of the effluent may be sufficient. If using a detergent for cleaning, it is best to select one that absorbs UV light. With some cleaning agents, other, more sophisticated, and more expensive analytical tools may be needed. Finding easy-to-measure cleaning reagents with existing assays that can be validated is well worth the extra time and effort.

### 6.2 CLEANING

#### 6.2.1 Resins

Cleaning and sanitization are part of a complete column maintenance programme (see Table 6.1). It has been estimated that approximately 20–30% of a chromatography cycle

**Table 6.1**

## Regular column maintenance procedures

Procedure	Explanation	When	Example	Comments
Regeneration	Returns resin to its original functional state	After each separation cycle	2 cv <sup>a</sup> of 1 M NaCl for ion exchangers	Should remove impurities strongly bound to functional groups
Reequilibration	Returns resin to starting conditions for next cycle	After each separation cycle	3–4 cv of start buffer	Check that UV, pH and conductivity signals return to steady baseline levels. Important for reproducibility
Cleaning in place	Eliminates material not removed by regeneration and prevents progressive build-up of contaminants	After every 1–10 cycles	Protocol must be designed according to feedstream, resin and wetted materials stability	Reverse flow <sup>b</sup> and low flow rate will usually give the best results
Sanitization	Inactivates vegetative cells	Between batches	0.5–1.0 M NaOH, contact time 30–60 min	Prevents microbial growth and build-up of endotoxins

<sup>a</sup>Column volumes.<sup>b</sup>Not recommended for gel filtration columns as it may disturb bed.

is devoted to cleaning. And cleaning can account for 60–80% of the total buffer and WFI consumption. In some cases, the use of disposable chromatography columns is advocated to avoid costly cleaning validation efforts. As noted earlier (see Chapters 1 and 3), disposables are valuable for early clinical trial manufacturing and for multiproduct facilities. For large scale manufacturing, resin reuse is often essential to achieve an economic process, and routine cleaning and cleaning validation are essential components of resin lifetime studies.

One of the main concerns of regulatory agencies regarding chromatography is the risk of carryover from one cycle to the next. That carryover may be target molecule, i.e. protein, plasmid or viral product. It may also be degraded or otherwise altered product that changes final drug product potency and immunogenicity. In addition, there are potential carryover impurities such as host cell proteins, nucleic acids, retroviral particles and processing additives, e.g. detergents. Claiming there is no carryover is usually problematic. It has been shown that for protein products trace amounts of protein material can often be carried over. This is especially true for columns used early on in a downstream process. But carryover can be minimized and controlled by developing a suitable cleaning protocol.

Cleaning has the potential to cause degradation of resins and wetted components of columns and systems. Certain precautions can reduce the stringency of column cleaning that is necessary to ensure controllable carryover from run to run. These measures include treating the feed to remove non-solubilized proteins; low-molecular weight,

**Table 6.2**

Influence of two major product sources on impurities

Source	Product examples	Key impurities
Mammalian cells	Monoclonal antibodies, recombinant proteins	Host cell proteins, DNA, cell culture media
Microbial fermentation	Recombinant proteins from <i>Escherichia coli</i> and yeast	Intracellular or periplasmic systems: high lipid content from membranes, endotoxins from <i>E. coli</i> , proteases from yeast. Anti-foams

charged substances; and lipids. Desalting by diafiltration or chromatography can be used to provide a feedstream that is less likely to foul chromatography columns. The influence of product source on cleaning of chromatography columns is considerable. Table 6.2 shows a summary of the likely impurities associated with the two most commonly used source materials in biotechnology.

Cleaning frequency and cleaning methods data should be generated and documented during process development. Since the proportions and types of impurities may vary at different production stages, it is best to use production feedstream to obtain the most relevant data. Temperature should also be the same as in used in manufacturing. Blank runs (also called ‘mock’ or ‘sham runs’) can be analysed for carryover after multiple cycles. Longer contact times and increased concentrations of cleaning agents can be used to determine if more material will be removed under harsher conditions. Evaluating heavier than normal soils will also enable establishment of the optimal cleaning protocol. When an increase in back pressure, loss of resolution and/or yield or a decrease in other performance measurements is observed, try cleaning routines that restore performance. Determine a safety margin: e.g. if performance is restored by a cleaning agent contact time of 20 min, use a contact time of 25 min, provided all components are compatible for this length of time. Once the cleaning protocol is established, one can run a small scale automated system and evaluate repetitive cycles to determine useful lifespan. At full scale, routine monitoring is required. One additional approach has been to analyse small amounts of resin from around the circumference of the column, from its center, and from any area that appears discoloured. Some resin was also analysed after it was slurried for further cleaning. Assays included small ion capacity, a product-sensitive assay, endotoxin and bioburden.

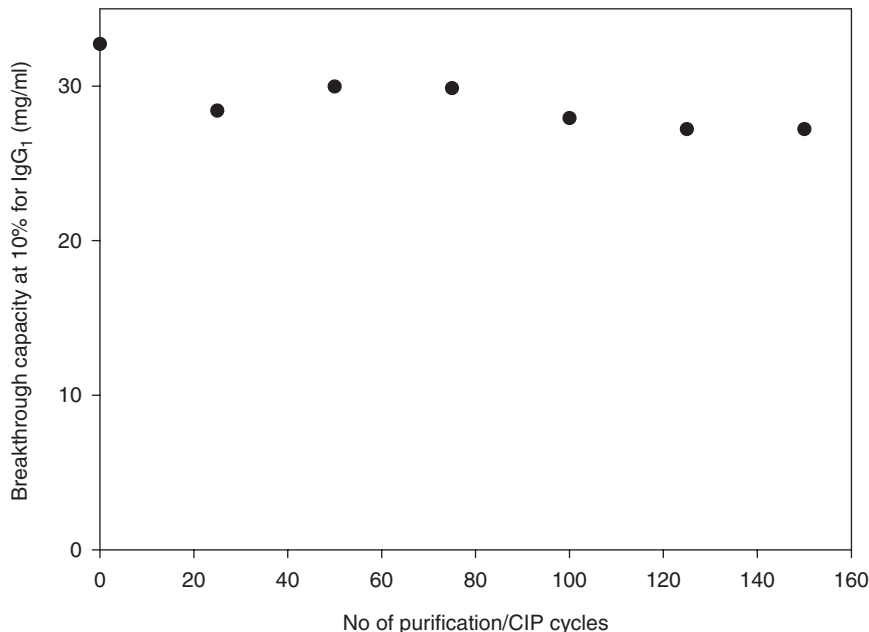
It is worthwhile noting that cleaning agents may have unusual effects on chromatography resins. For example, quaternary amines with an unpleasant odour can be released from Q anion exchangers during treatment with sodium hydroxide; but no loss of capacity is observable, and the released material is very difficult, if not impossible, to detect using gas chromatography. With proteinaceous ligands, cleaning can be more of an issue, as the ligand may not tolerate harsh cleaning agents. In some cases, guanidine hydrochloride has been an effective cleaning agent for affinity resins. The affinity of immobilized Protein G for antibodies was found to increase after exposure to alkaline cleaning conditions. This change necessitated the use of a potentially harmful low pH for elution of the antibodies.



A further study determined that cleaning with a combination of urea and acetic acid did not change the binding affinity for at least 50 cycles [2].

Suppliers can provide useful information for the design of a cleaning protocol. Ignoring relevant information can cause problems. During an FDA pre-approval inspection, it was observed that a company used acidic conditions for routine cleaning, but those conditions were known to cause resin degradation. The company was required to perform an expensive leakage study [3]. Keep in mind that a suitable cleaning agent for one mode of chromatography may be unacceptable for another. For example, in cleaning precipitated proteins from hydrophobic resins, high salt, commonly used with ion exchange resin, may cause irreversible binding of precipitated proteins. Not only must the resin and column be compatible with the cleaning agent, but the auxiliary equipment compatibility must also be taken into account. For example, a filter left in line during cleaning of columns with sodium hydroxide can be problematic if solvent compatibility of the filter is unknown. If there are no data on the filter extractables, all of the resin would have to be replaced, since filter extractables might contaminate the column.

Over the last decade, the manufacturers of chromatography resins have put considerable effort into making materials more resistant to cleaning and sanitizing agents, especially sodium hydroxide. Sodium hydroxide has become the 'gold standard' for sanitizing columns (see Section 6.4). Alone, or in conjunction with sodium chloride, it can provide both cleaning and sanitizing in one step. Figure 6.1 shows the consistency of dynamic



**Figure 6.1** Dynamic-binding capacity of humanized IgG<sub>1</sub> after purification on MabSelect SuRe™ and cleaning after each cycle with 0.1 M NaOH for 15 min. Data points represent mean of duplicates.

**Table 6.3**

Examples of impurities and CIP agents

Soluble proteins	NaCl, low ionic strength buffer, water
Precipitated proteins	NaOH, HAc, NaCl, water
Hydrophobic proteins	NaOH
Lipids	Non-ionic detergents, ethanol, isopropanol, acetonitrile
Nucleic acids	NaOH, NaCl, DNase
Endotoxins	NaOH
Viruses	NaOH

binding capacity of a newer immobilized Protein A resin over 149 cycles. The Protein A was designed for increased stability using protein engineering techniques, in which a number of alkali-sensitive amino acid residues were replaced [4]. In other studies, it was shown that while conventional Protein A resin loses dynamic binding capacity when treated with 0.1 M NaOH for 15 min, capacity remains constant (up to 90%) with the new stability-enhanced Protein A up to 60 cycles even with 0.5 M NaOH.

Appropriate cleaning methods will also be determined by evaluating the nature of the impurities. These impurities include soluble hydrophilic proteins, hydrophobic proteins and lipoproteins, precipitated proteins, lipids, nucleic acids, endotoxins and viruses. Examples of commonly used cleaning-in-place (CIP) agents are shown in Table 6.3. Such data may be useful in preliminary cleaning protocol development, but often cleaning protocols must address a mix of impurities and interaction with different surface chemistries. Manufacturers of biopharmaceuticals must ultimately design and validate their cleaning methods.

### 6.2.2 Filter media

Filter media that are to be reused require the same type of studies as those used for chromatography media. Harsher agents, such as sodium hypochlorite (bleach) and acids, can often be used with membranes. For feedstreams with particulates, it may be necessary to implement a backwash operation. Otherwise, the practice is to apply a crossflow cleaning velocity 1.5 times that used for the process. As the effectiveness of cleaning is most dependent on the chemical breakdown of the fouling layer, prolonged contact time and elevated temperatures are used as well. Typical water flux recoveries, a measure of cleaning efficiency, for crossflow filtration cassettes is shown in Table 6.4. The water flux for new membrane filters should be determined to establish a baseline value. A slight decrease with the initial use is often observed, however complete reinstatement of the flux is required for long term service and evidence of removal of any residual material to avoid lot-to-lot cross contamination.

**Table 6.4**

Typical water flux recoveries for cross-flow filtration cassettes

Cleaning method	Conditions time, temperature	Typical water flux recovery
0.5 M NaOH	30 min, room	>62%
1.0 M NaOH	120 min, 50 °C	>65%
0.5 M NaOH–300 ppm NaOCl	30 min, room	>80%
0.5 M NaOH–500 ppm NaOCl	30 min, room	>90%
0.5 M NaOH	30 min, room	
Followed by 0.5 M H <sub>2</sub> SO <sub>4</sub>	30 min, room	>84%

### 6.2.3 Equipment

Whereas chromatography resins and membranes are dedicated to one product, columns, filter housings and systems may be used for more than one product, provided that the cleaning and its validation are acceptable.

The design of the equipment should take into account the fact that rough surfaces may allow proteins to become deposited, and protein deposits are ideal sites for microbial growth that may lead to product contamination (see Section 6.4). Threaded connections should be avoided and sanitary fittings employed wherever possible. Wherever plastics are used, their compatibility with cleaning agents must be documented.

Challenge studies can sometimes provide useful information about the efficiency of cleaning methods. These studies point out those parts of a system to which special attention should be paid during cleaning. The use of coupons, i.e. cut out pieces of the equipment usually available from the supplier, can be used for challenges with agents that should not be introduced into a manufacturing facility. New directions in cleaning technologies include using less water and less detergent for faster and more efficient cleaning [5]. Remote visual inspection using boroscopes and cameras is another approach that is being implemented. Rather than have human entry for inspection after spraying riboflavin, these remote visual inspection devices can reduce the time it takes to verify that vessels and tanks are cleaned. Process analytical technology (PAT) is also being applied. On-line TOC can be used for carbon-containing molecules. For small molecules, current methods include ion-mobility spectrometry (IMS) and ion-trap mobility spectrometry (ITMS).

After being used for one product, systems are usually disassembled and thoroughly cleaned and sanitized. Removal of cleaning reagents must be demonstrated. The manufacturer must determine the acceptable residual limit for any cleaning agent, and care must be taken to properly store clean equipment. Much has been written about cleaning equipment and a great deal of information is available from equipment suppliers [6, 7]. Each manufacturer, however, must ensure that the cleaning is documented and appropriate for their application. Some recent (2005) common FDA Form 483s related to cleaning are shown in Table 6.5.

**Table 6.5**

FDA 483s related to cleaning issues

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No product-specific or other validated test methods for cleaning of multipurpose equipment
No study to demonstrate removal of residual from final purification column
Firm failed to follow SOP for cleaning of manufacturing area
No routine monitoring following cleaning of dedicated product contact parts and equipment
No verification that major manufacturing equipment has been cleaned prior to use
Equipment not always labelled as to its cleaning status
Written procedures not established for cleaning and maintenance of production equipment
Procedures do not assign responsibility for the cleaning process
Procedures do not explain frequency of cleaning procedures
Procedures do not describe inspection of cleaned equipment prior to use
Procedures do not detail protection of cleaned equipment prior to use
Procedures do not require documentation of the cleaning process
Process tanks routinely reused prior to cleaning with no justification

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*Source:* Courtesy of BioQuality, Idyllwild, CA.

**6.3 DECONTAMINATION OF TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHY AGENTS**

The resistance of transmissible spongiform encephalopathy (TSE) agents is legend. Stories, such as maintenance of infectivity after a year of being buried in the ground, abound in the industry. In biotechnological processes, the TSE issue can be obviated by proper sourcing of ruminant raw materials. A recent review by FDA-CBER describes the methods that have been used to decontaminate equipment surfaces and chromatography columns [8].

Some chemicals have been demonstrated to effectively reduce or eliminate TSE agents. Table 6.6 summarizes methods that appear to be effective. As noted in Ref. [8], the duration of exposure and concentration of decontamination agent will influence effectiveness. Empirical data must be provided for each specific regimen.

**6.3.1 Resins and filter media**

Chromatography resins and filters have been used to remove TSE infectivity. However, it is likely that the infectious agent, prion protein, is retained on the media since it is known to be rather ‘sticky’ and readily aggregates. A BSE agent was shown to be retained by an ion exchanger but infectivity was eluted by a 2 M NaCl wash [9]. Although Scott and Asher noted that the additional use of 1–2 M NaOH during sanitization provides some reassurance, there is no way to accurately measure resin- or filter-bound infectivity.

Table 6.6

TSE decontamination agents

Sodium hydroxide
Sodium hypochlorite
Sodium hydroxide plus heat
Sodium hydroxide plus steam sterilization
Sodium hydroxide followed by proteinase
Sodium hydroxide after detergent treatment
Acidic SDS
Phenolic disinectant
Detergents and proteinase
Proteinase and heat

Source: Adapted from Ref. [8], Scott and Asher.

6.3.2 Equipment

When dried onto the surface of equipment, TSE infectivity becomes even more difficult to remove. There is an extensive list of chemicals and physical treatments that do not work. Sodium hydroxide and some other treatments have been shown to be effective (see Table 6.6). But, the ability to make general conclusions from inactivation studies is limited at this time due to assay variability, TSE agent strain variability and operating conditions.

6.4 SANITIZATION

In many cases, sanitization and cleaning protocols are combined to reduce the number of operations and minimize costs, e.g. lower water consumption and disposal issues. In fact, we often see cleaning protocol targets listed as proteins and microorganisms, as well as endotoxins. An FDA warning letter in 2002 advised the manufacturer that ‘your cleaning procedure for product contact equipment surfaces has not been shown to be capable of reducing microbial and endotoxin contamination to acceptable levels’. A review on disinfectants points out that sanitizers may clean dirt and kill microorganisms. In this case, one can say that the sanitizer works as a cleaning agent with antimicrobial properties [10].

Sanitization is defined as reducing the numbers of microbial contaminants to acceptable levels. Sanitization is the removal or elimination of vegetative bacterial cells. Sterilization is the killing or removal of all forms of life, especially microorganisms [11]. Sterility connotes an absolute absence of microorganisms yet considers the probabilistic nature of sterilization processes [12].

Sources of contamination include input material, components in contact with product flow, product collection and storage, and the environment. Probably the greatest source of contamination is from personnel working in the manufacturing areas. Bioburden (i.e. bacteria and fungi) measurements are problematic. There may be assay interference from the

sample itself; therefore, bacteriostasis and fungistasis testing is necessary. The number and type of microorganisms found are relevant. Speciation is important as a change in the type of organism can present an untested challenge that requires process validation. If there is a very low level, which is accepted, there is a concern that the organism might grow during processing. One must also consider that even though bacteria are killed or filtered out, toxins (e.g. endotoxin, enterotoxins), spores and proteases can remain.

The biotech industry has found bioburden control to be a target during regulatory inspections. Some regulatory comments include:

- No established bioburden specifications for rinse samples from UF/DF filters and purification column resins
- Hold time after sanitization has not been validated
- No data to support lack of expiration dating on sanitizer
- Absence of bioburden monitoring of storage solutions to demonstrate storage buffer routinely maintains bacteriostatic effect
- Bioburden method for chromatography column storage solution has not been validated.

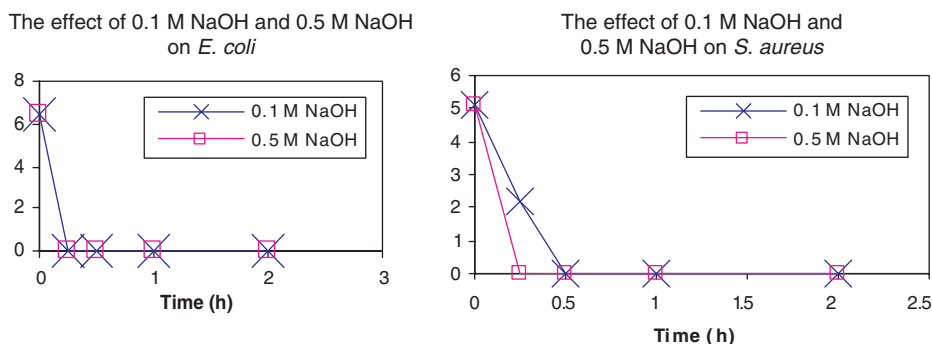
The PIC/S Aide Memoir on Inspection of Biotechnology Manufacturers instructs inspectors to evaluate if bioburden and endotoxin are measured in buffers, and if those buffers are sterilized [13].

#### 6.4.1 Resins

Resins are usually sanitized, not sterilized. When processing feedstreams contain particularly hazardous agents, such as infectious human viruses, the user may choose to sterilize and discard the resin after each run. In some cases, resins can be supplied so that they are sanitized and ready to use, but this comes at an additional cost.

Bioburden challenge studies on resins, systems and columns provide useful information about the effectiveness of sanitizing agents, which are typically performed by suppliers. Generally, these studies do not need to be repeated by the user; rather, continual monitoring is performed in the actual environment in which the process is performed. This appears to be more meaningful. However, some companies have chosen to perform microorganism-spiking studies. In these studies, the worst-case conditions were sought. But it is not always easy to determine what the worst case is. Although, a high protein load is considered a worst case, it is possible that in the absence of a high protein load, microorganisms have more binding sites and are thus more difficult to inactivate and remove. The PDA technical report No 42 on process validation of protein manufacturing states that ‘During process validation, routine bioburden monitoring should be included in the protocols’ [14]. The technical report also states that control and monitoring of bioburden is expected throughout the process.

Today, in-process, rapid microbiological methods (RMM) are being slowly implemented for bioprocessing and may be able to replace the longer traditional methods that require processing at risk. A review of RMM describes the currently available technologies [15].



**Figure 6.2** Spiking studies with a gram-negative (*E. coli*) and a Gram-positive organism (*S. aureus*) into MabSelect SuRe™.

Sodium hydroxide is the most commonly used sanitizing agent for resins. A typical challenge study uses the American Type Culture Collection (ATCC) microorganisms that are used to test WFI. These include Gram-positive and -negative bacteria as well as yeast and molds. An example of a limited microorganism challenge study is shown in Figure 6.2. In this study, the tested microorganisms, *Escherichia coli* and *Staphylococcus aureus*, were reduced from the original levels of  $2.6 \times 10^6$  colony forming units/ml (CFU/ml) or  $1.2 \times 10^5$  CFU/ml to below the detection limit of the method within 30 min. The bacteria were each spiked in duplicate into 70% slurries of MabSelect SuRe™, and NaOH, either 0.1 or 0.5 M. After thorough mixing, the containers were placed in a shaker at room temperature. Samples were taken as seen in Figure 6.2, spread on TSA plates, and incubated [16].

In other studies, we have found that *E. coli*, *S. aureus*, *Candida albicans* and *A. niger* can be reduced to below the detection limit of less than 3 microorganisms/ml. Spores from *B. subtilis*, however, remained at a level of about 10 organisms/ml even after treatment with 1.0 M NaOH for 48 h at 22 °C. Information on the validation of sanitization is provided in the PDA technical report on bioburden recovery validation [17].

For those resins that cannot tolerate sodium hydroxide at high concentrations, it is possible to add ethanol to enhance the sanitization effectiveness. In one challenge study with *C. albicans*, we found up to 1000 CFU/ml remained after exposure for 7 days to 0.01 M NaOH. But when 20% ethanol was added to the 0.01 M NaOH, no organisms could be detected after 7 days. In another study with Protein A Sepharose™ 4 FF, it was found that even *B. subtilis* could be reduced to below the detection limit with 0.1 M NaOH and 60% ethanol within 50 h. With 40% ethanol and 0.1 M NaOH, it took about 150 h to achieve the same result. The antibacterial action of acetic acid is also enhanced by the addition of ethanol. However, when operating large-scale chromatography systems, it is usually desirable to avoid the use of ethanol as it may result in the need to use explosion proof areas depending on concentration and volume of ethanol. Other methods that have been used for labile ligands include hibitane digluconate in benzyl alcohol. The capability of resins to be sanitized should be assessed during process design.

The selection process for sanitizing agents must take into account compatibility not only with resins, but also with column components such as O-rings and system-wetted surfaces.

### 6.4.2 Filter media

Filter manufacturers provide specific instructions for their products and usually specify concentrations, temperatures, contact times and other conditions that are appropriate for their products. Steaming-in-place can be used to sterilize some membrane filter cartridges *in situ*, provided all the lines and components are steamable. This is typically done prior to use. Superheated steam can damage cartridges and result in altered membrane performance, and certain precautions must be taken. Sodium hydroxide is also used for sanitizing reusable membranes, but not all membranes can tolerate 1 M NaOH. As with chromatography resins, suppliers provide valuable information on potential leachables/extractables. If vendor recommendations cannot be used, leachable/extractable studies will need to be performed by the user [18]. Further information may be found in a book chapter on validation of a filtration step [19].

### 6.4.3 Equipment

The CGMPs require that equipment and utensils shall be cleaned, maintained and sanitized at appropriate intervals to prevent malfunctions or contamination that would alter the safety, identity, strength, quality, or purity of the drug product beyond official or other established requirements [20]. A recent review notes that there is a lack of understanding of how sanitizers work (see Ref. [10]). This article points out that antimicrobial agents can be either sanitizers or disinfectants, depending on whether they are used to reduce bio-burden or kill potentially infectious agents. Chlorine dioxide, which can also be a sterilizing agent, has been proposed as a sanitizing agent. It is effective as both liquid and vapor and has been used for sanitizing a polysulphone UF membrane system and is being evaluated for packed chromatography columns and sterilization of disposable systems after their use [21]. Agarose-based resins, however, are unlikely to withstand this treatment.

Microorganisms become more resilient against removal and more resistant to antimicrobial chemicals and conditions, once they are located on a solid surface. A discussion on microbial adhesion on stainless steel is presented in a publication [22]. A recent review article, however, states that surface roughness is not as much of a contributing factor to bacterial adhesion as was previously thought [23].

The results of a sanitization study of a production chromatography system are shown in Tables 6.7a–c [24]. The system was infected with solutions containing three bacterial strains and one yeast strain. The system was sanitized by first rinsing with sterile water, followed by pumping 1 M NaOH through the system for one and a half hours. There was at least a reduction of  $10^6$  CFU for the four test organisms.



Table 6.7a

Organisms chosen for the microbial challenge tests		
<i>Escherichia coli</i>	ATCC 8739	Gram-negative bacteria
<i>Pseudomonas aeruginosa</i>	ATCC 9027	Gram-negative bacteria
<i>Staphylococcus aureus</i>	ATCC 6538	Gram-positive bacteria
<i>Pichia pastoris</i> <sup>a</sup>	GS 115	Yeast

<sup>a</sup>The yeast, *P. pastoris*, was selected instead of the USP XXV recommended yeast, *Candida albicans*, as it is more frequently used in process and production conditions.

Table 6.7b

Sanitization procedure			
Function	Solution	Volume	Time
Rinsing	Sterile water	5 L	12.5 min
Rinsing	1 M NaOH	5 L	12.5 min
Sanitization	1 M NaOH	4 L with circulation	75 min
pH neutralization	Sterile physiological saline solution	10 L	25 min

Table 6.7c

Results				
Viable count (CFU/ml)	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>P. pastoris</i>
Inoculum sample	$1.5 \times 10^6$	$2.1 \times 10^6$	$4.1 \times 10^6$	$0.8 \times 10^6$
Post-infection flow through	$8.8 \times 10^5$	$1.8 \times 10^6$	$1.6 \times 10^6$	$0.2 \times 10^6$
Pre-sanitization flow through	$1.2 \times 10^8$	$2.0 \times 10^8$	$1.2 \times 10^7$	$6.7 \times 10^6$
Post-sanitization effluent	0	0	0	0

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## Validation

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### 7.1 INTRODUCTION

In 1997 when the first edition of this book was published, validation was considered to be ‘just another damned regulatory obstacle. It costs us a great deal of time and money but we never see any return on that effort’ [1]. Some may still feel that way; whereas others are applying a practical and scientific approach that leads to better in-process controls rather than just aiming for the typical 3–5 successful, consecutive batches (known as conformance, consistency or validation batches). The biotechnology and biologics industry has a much better understanding today of process development and characterization studies that lead to successful process validation and continued manufacturing success. Progress has also been made in establishing commonly accepted terminology.

#### 7.1.1 Validation terminology

If ‘incomprehensible jargon is the hallmark of a profession’, then we have indeed had a validation profession [2]. Today, however, the International Conference on Harmonization (ICH) has provided relatively straightforward definitions that can be applied consistently within any organization in any part of the world. These definitions, from ICH Q7A, are reproduced in Table 7.1. For further information on definitions related to process validation of protein manufacturing, see PDA Technical Report No. 42 [3]. The entire validation conundrum can be simplified when everyone is using the same terminology.

As seen in Table 7.1, the term ‘qualification’ is used for equipment and systems. Qualification must be performed prior to process validation, and requires an understanding of equipment design, how the equipment or system will be used and its operating capabilities.

Prior to process validation, critical product quality attributes (CQAs) and critical process parameters (CPPs) are established. The understanding of the quality attributes comes from correlation of clinical results with product analysis (see Chapter 5). The CPPs are determined primarily in characterization studies. The definition of a characterization study from the PDA Technical Report No. 42 is: ‘*A late-stage study that evaluates the process to increase process knowledge and examines proposed operational ranges and their individual and/or combined impact on target protein quality*’. During characterization, statistical

Table 7.1

Validation definitions from ICH Q7A [4]

Design qualification (DQ)	Documented verification that the proposed design of the facilities, equipment or systems is suitable for the intended purpose
Installation qualification (IQ)	Documented verification that the equipment or systems, as installed or modified, comply with the approved design, the manufacturer’s recommendations and/or user requirements
Operational qualification (OQ)	Documented verification that the equipment or systems, as installed or modified, perform as intended throughout the anticipated operating ranges
Performance qualification (PQ)	Documented verification that the equipment and ancillary systems, as connected together, can perform effectively and reproducibly based on the approved process method and specifications
Process validation (PV)	Documented evidence that the process, operated within established parameters, can perform effectively and reproducibly to produce an intermediate or API meeting its pre-determined specifications and quality attributes

approaches such as Design of Experiments (DoE) are applied. Many companies perform characterization studies under formalized protocols so that the data can be used for investigations and process changes.

7.2 WHAT TO DO WHEN?

‘Anyone who isn’t confused here doesn’t really understand what is going on’ [5]. This quote does seem to apply to deciding what to validate and when to do it. Inexperienced companies often feel overwhelmed by the tasks at hand. The U.S. FDA has provided some guidance that makes it a bit clearer what needs to be done and when [6]. When perusing such documents, however, it becomes clear that there is no exact recipe, nor should there be one given the diversity of products and their intended therapeutic and diagnostic uses. Furthermore, documents such as FDA’s 1997 ‘Points to Consider’ are somewhat outdated. Even more problematic are the different opinions of worldwide regulatory agencies. On the one hand, Good Manufacturing Practices (GMP) compliance for manufacturing of clinical materials is a requirement in Europe [7]. At the same time that this requirement is being enforced in the EU, the U.S. FDA is attempting to loosen formal requirements for early clinical studies [8, 9].

There has been increased focus on the application of risk/benefit analysis to dictate what needs to be validated and when. There is always a balance between getting a potentially life-saving medical product to critically ill patients and trying to minimize safety risks that may even be hypothetical. Table 7.2 provides typical staged validation activities. These and other important activities are discussed here.

Table 7.2

Typical validation activities required for various stages Efforts and cost			
Toxicology studies	Phase 1 and 2	Phase 3	License
Assays: bioburden endotoxin	Aseptic processing Assays: sterility, endotoxin  Specific (safety-related) impurities removal (e.g. Protein A); Viral clearance; Release assays	Enhanced assay validation (all non- characterization assays)   Revalidation of assays and viral clearance as needed due to process changes	Stability Cleaning Process

7.2.1 Toxicology studies

Prior to entering toxicology studies, there should be a documented process and defined raw materials. Assays for bioburden (or sterility) and endotoxin should be validated. When toxicity studies fail due to impurities or contaminants, an entire project may be cancelled. Some companies even use full GMP compliance for toxicology studies to prevent this scenario.

7.2.2 Human clinical trials

For the manufacture of early clinical materials, compliance with GMP is required, but a graded approach is accepted as long as patient safety is taken into account. The product structure should be defined, the activity measured, testing for adventitious agents carried out and cell lines characterized. Written production and test procedures should be employed. There should be a working reference standard. Equipment, other than that used for sterilization, virus inactivation and decontamination, may not need to be fully qualified at early stages of clinical production, but it is essential to determine if the design criteria are met and have measures in place to ensure equipment performance. IQ/OQ/PQ should be performed for critical equipment. In some cases, determination of system suitability is adequate.

For product release, sterility is required, and aseptic processing should be validated. The product must be demonstrated to be stable for the entire time it is in the clinic. Validated assays related to safety should be performed, and where viral safety is an issue, viral clearance studies carried out.

Viral clearance is one area where there is still a great deal of confusion. Depending on the product and patient population, one or two viruses are usually required to be used in a viral clearance study for a biotech product. In Europe, a concept paper has been issued that will, hopefully, lead to clarification of requirements for viral clearance studies for biotech products used in clinical trials within the EU [10]. For blood and other product sources with known or potentially higher viral safety risk factors, the study requirements are more stringent [11].

When process changes that potentially impact viral safety are made during clinical trial manufacturing, viral clearance studies may need to be repeated. For example, a process change made to improve product purity might decrease viral clearance. In addition to viral clearance, the effect of process changes on the composition of the samples to be assayed may necessitate revalidation of assays (e.g. sterility assays).

Assay validation, other than for those assays related to safety, will be a work in progress during phase 2 and early phase 3 studies. There must, however, be some qualification to ensure the data are reliable and informative. Assays used solely for characterization studies do not have to be completely validated, but should be qualified to ensure the data provided by the assays are accurate, precise, linear within the range of use and show no interference from process stream components [12].

During phase 2, a working cell bank should be established if this has not already been done. Purity and potency assays should be validated. Prior to phase 3, in-process release tests should be validated.

By phase 3 clinical trials and no later than the end of those trials, full GMP compliance will be required [13]. Validation is a component of GMPs. All aspects of manufacturing and testing should be taken into account. For example, cleaning validation and validation of the stability of in-process intermediates, API (active pharmaceutical ingredient, also called drug substance or purified bulk) and final product must also be addressed.

Throughout the progression from toxicology studies to license application, comparability must be demonstrated.<sup>1</sup> If this can not be done, then earlier studies may have to be repeated. For a more comprehensive summary of activities for biopharmaceutical production from genetically engineered mammalian cells, see Appendix C.

## 7.3 VALIDATION OF DOWNSTREAM PROCESSES

### 7.3.1 General considerations

Successful validation of downstream operations requires that one consider all facets—from raw materials to API. In addition, the facility, air and water quality, equipment, maintenance programs, documentation requirements, technology transfer, change control and personnel training all have the potential to impact the process and, ultimately, final product quality. A book chapter on facility design addresses purification and support areas, water and heating, ventilation and air conditioning (HVAC) systems, facility cleaning and environmental monitoring [14]. The impact of upstream and recovery unit operations on downstream

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<sup>1</sup> Comparable is defined by the ICH Guideline Q5E as 'A conclusion that products have highly similar quality attributes before and after manufacturing process changes and that no adverse impact on the safety or efficacy, including immunogenicity, of the drug product occurred'.

processing can not be overlooked. A ‘little’ tweak in cell culture or a slightly greater disruption of cells in recovery has wreaked havoc with downstream process validation on more than one occasion. One example is the addition of antifoam in cell culture that caused increased resin leakage. Another is the increased release of DNA during a recovery operation that increased viscosity and decreased capacity of the first anion exchange chromatography step.

Downstream in-process assays, stability testing and thorough characterization of product and process intermediates are essential for understanding the process parameters that should be validated. Staying within the defined forward processing criteria when progressing from one unit operation to the next will prevent validation failures, provided enough is known to adequately define those criteria. (Forward processing criteria are those criteria that must be met to move from one process step to the next.) As processes are developed, it is important to keep in mind that it will be necessary to validate removal of anything added to the downstream process unless what is added is considered part of the product. Raw materials and equipment components should be evaluated for quality and consistency as well as any potential impact on the process and product.

### 7.3.2 Raw materials and process tools

Raw materials and process tools used for downstream processing may include buffers, salts, detergents, stabilizers, organic solvents, cleaning agents, filters and chromatography resins. In some cases, process steps include the use of enzymes or other substances from natural sources. In the event that these are derived from animal sources, it is necessary to evaluate the potential for contamination by transmissible spongiform encephalopathies (TSEs) (see also Chapter 3). Certificates of suitability are required for European markets and this applies to ovine and caprine species as well as bovine [15]. The U.S. FDA requests that even for early clinical studies, for human and animal-derived materials, documentation should include information on sourcing and/or test results for adventitious agents (see Ref. [8]). Each raw material should be evaluated to determine its criticality to the process. According to CGMPs, raw materials must be quarantined, identified and released by an authorized person—usually from a quality assurance department. Identity testing methods are often available from the supplier. It is advisable to select materials for which there exist validatable assays. Excessive testing should always be avoided; no company can afford the time or money to develop or use unnecessary tests, especially since those tests will require validation. Certificates of analysis should be received for each lot of raw material. Often these certificates provide valuable information that relates to the performance of the raw material and can reduce the amount of testing, provided that supplier audits are routinely performed.

#### *Acceptance criteria for chromatography resins*


During process development, it is useful to evaluate the specifications provided in certificates of analysis for chromatography resins. For example, it may be found that lots with particle size distribution at the lower limits specified by the resin manufacturer will not meet the user’s throughput requirements for manufacturing. Or it may be found that the degree of substitution must be in the upper range of the specifications to achieve the capacity anticipated for production. The most efficient and cost-effective strategy is to design a



robust process that will be accommodated by resins meeting the resin manufacturers' acceptance criteria. A typical certificate of analysis is shown in Figure 7.1.

Resin manufacturer testing can be broken down into physical, chemical and functional tests [16]. Physical tests include particle size distribution, flow measurements, bead structure, porosity, colour, packing and label inspection. Flow measurement, colour, packing and labelling inspection are often performed by the end-user for each lot. It may be cost-effective to rely on the supplier to perform more tedious tests such as particle size distribution, especially since the measurement of the flow rate indicates a practical impact of the particle size distribution. Generally, bead structure and porosity measurements can be omitted by the end-user. The effective porosity will be measured indirectly by a function test.

Chemical tests may include those for foreign materials (e.g. heavy metals), amount and type of functional groups, homogeneity and contaminants such as microorganisms. The

 <b>GE Healthcare</b>		<b>Certificate of Analysis</b>	
<b>Product:</b>  <b>Q Sepharose™ Fast Flow</b>		<b>Code Numbers:</b> 17-0510-01 17-0510-04 17-0510-05 17-0510-10 17-0510-11 17-0510-60 17-0510-99	
<b>Lot No:</b> 10001757			
<b>Test/Characteristic:</b>	<b>Limits:</b>	<b>Results:</b>	
<b>1 Function</b>			
Retention volume; mL			
- GammaBind™ G type 2	40 - 50	43	
- β-Lactoglobulin B	59 - 79	67	
- β-Lactoglobulin A	72 - 92	80	
<b>2 Total capacity</b> mmol Cl <sup>-</sup> / mL packed gel			
	0.18 - 0.25	0.21	
<b>3 Flow rate at 0.1 MPa</b> cm / hour Bed height: 14 - 16 cm			
	400 - 700	557	
<b>4 Particle size distribution</b> Volume share within 45 - 165 µm; %			
	min. 95	98	
<b>5 Microbial contamination</b> microorganisms / mL suspension			
	max. 100	0	
Manufactured in compliance with our ISO 9001:2000 certified quality management system.			
Approval date: 2006-06-29		Expiry date: 2011-06	
		Manufacturing date: 2006-06	

**Figure 7.1** Certificate of analysis for a chromatography resin.

user may choose to rely on some of the manufacturer's chemical tests. Many users titrate charged chromatography materials, i.e. ion exchangers, to determine the type and quantity of functional groups. (This is easily accomplished with a two-pump automated chromatography system by filling one pump with acid and the other with base.) Other tests may be used for different chromatographic resins, e.g. affinity and hydrophobic interaction resins. Homogeneity testing ensures the product does not contain materials other than those specified, e.g. the solvent for packaging. Microorganism contamination is usually determined by the supplier and repeated by the user prior to use. It is important to keep in mind that if an immobilized protein such as a monoclonal antibody is employed in the purification, the chemical tests required are quite extensive. For example, an immobilized monoclonal antibody must be of a similar quality to those used for therapeutic purposes [17].

Function tests are generally performed by the supplier with standard proteins under narrowly defined conditions. Resolution, recovery of test proteins and non-specific adsorption are frequently made measurements. These tests may or may not be relevant for the end-user.

Some of the physical, chemical and function tests may be employed to identify chromatography resins. Specific identity test protocols are usually available from the supplier. A general strategy for identity tests is to determine the nature of the matrix, its porosity and the ligand for substituted resins. For example, to determine the identity of agarose resins supplied in a suspension for gel filtration and ion exchange, the following approach is employed. Infrared spectroscopy is used to determine if the matrix is agarose and not for other matrices supplied in suspension. The content of dry substance is measured to determine the degree of cross-linking. A density determination may be employed to determine porosity. A pH colorimetric test is used to determine if the resin is unsubstituted or if it is positively or negatively charged. This test also determines the type of charge, i.e. strong or weak ion exchanger. In some cases, a function test is used to confirm identity.

Chromatography resins are obviously critical material. As discussed, many tests are performed by the resin manufacturers, but the most logical user testing strategy includes the identity test plus, if necessary, performing a function test at small scale that relates to the user's application. Often, a small column can be run with production feedstream to ensure proper flow properties, capacity, product purity and recovery and removal of specific product- and process-related impurities are obtained with each new resin lot. Dynamic capacity studies performed during process development can provide useful information for the development of the function test. From the user's perspective, the function test is a valuable measurement for each lot. Function test conditions should be established during development but production feedstream should be used for testing, as soon as possible, since feed from production runs usually differs from that of laboratory or pilot runs in the nature and amount of product and impurities.

Leachables from resins should be addressed. Information on potential leachables is generally provided by resin manufacturers. Assays for leachables and data from tests under extreme conditions are usually supplied in a regulatory support file or drug master file. However, assessing leachables under actual conditions of use may be required for some resins, i.e. those with potentially harmful leachables. Leachables are addressed below in more detail.

It is important to evaluate the conditions under which the resin manufacturer is operating, and having a system in place for evaluating suppliers is a requirement (see Ref. [4]).

For example, the user might want to determine if the manufacturer employs validated cleaning protocols, if production equipment is routinely maintained and calibrated and if the reporting structure complies with GMP. A site visit is generally requested by the user's quality assurance department to ensure that basic good manufacturing principles are used.

### 7.3.3 Equipment

Stainless steel is commonly used in the pharmaceutical industry, but many plastic materials are also used, especially for relatively small-scale operations. With increased usage of disposables, there are potentially even more polymeric materials in contact with process feedstreams. Many polymers have extractables and/or leachables (see definitions below), and the conditions of actual use should be evaluated to make sure there are no deleterious effects on the product. Filter extractables have been addressed, but apparently are sometimes overlooked, as evidenced by this FDA observation: 'filter extractables have not been performed for the filters used for the in-process bulk and buffers. Filters are not flushed to remove potential extractables prior to use'.

Some years ago a leachate that acted as an adjuvant was released from uncoated stoppers when the product stabilizer was changed from human albumin to polysorbate 80. A few patients responded by making antibodies to the product and its endogenous form, which resulted in severe adverse events. The investigation, although not totally conclusive, took 4 years and more than 100 investigators [18, 19]. In another case, a start-up company was purchasing WFI, which was supplied in a plastic container. The supplier changed the filling protocol to allow filling while the water was warm. The warm water extracted a chemical from the container that co-migrated with the product. Fortunately, it was observed in an HPLC assay of the product. Further information on extractables and leachables can be found in publications, just a few of which are referenced here [20–22].

*Extractable:* A chemical that can be released from a component under exaggerated conditions, such as harsh solvent, extremes of pH or temperature. Extractables have the potential to contaminate the dosage form of a drug.

*Leachable:* A subset of extractables. A chemical that has migrated from a component under normal conditions of use. Leachables are likely to come out under regular conditions.

Suppliers can provide useful information about the composition and potential for extractables, but extended use and cleaning and/or sanitization may cause deterioration of plastics over time. Stainless steel is not without problems, which include corrosion in the presence of high salt concentrations and the need for periodic re-passivation. A tool for deciding whether to use stainless steel or disposables for early clinical trial material manufacturing has been published [23].

#### *Equipment qualification*

IQ, OQ and PQ must be performed prior to process validation. In some cases, certain aspects of these qualifications can be combined to expedite the work. There are no specific requirements for the format of qualifications, and every company can design a form that fits in with their documentation system.

An initiative from ASTM's Committee E55 on Pharmaceutical Application of Process Analytical Technology has provided a potential opportunity to use new approaches to qualification of biopharmaceutical and pharmaceutical manufacturing systems. The resulting standard guide will provide a science and risk-based approach that will, among other issues, address incorporating traditional installation qualification (IQ)/OQ into the commissioning stage [24].

### *Installation qualification*

An IQ for chromatography equipment may include the following: detailed description of the equipment and utilities; examination of equipment design; review of calibration, operating and maintenance procedures and spare parts lists; flow chart of the process (liquid and signal flow); and documentation listings, i.e. name and location of operating and calibration log books and standard operating procedures (SOPs). Many of these items are now electronically created and maintained (see the Section 'Automated equipment qualification'). Specifically, in examining the equipment design, it is necessary to check electrical connections, piping, welds, material certificates and verify absence of dead pockets. Monitors (e.g. conductivity, pH, UV), sensors (e.g. level, pressure, air, temperature) and flow meters must be calibrated. An IQ for a production chromatography system often goes beyond just evaluating a chromatography column and skid. For simplification, other items, such as utilities, that must be qualified are often addressed in separate protocols, but all the items shown in Table 7.3 should be demonstrated to be complete prior to the OQ for

**Table 7.3**

Installation qualification checklist for a production chromatography system

Utilities	Equipment features	Sanitization program	Maintenance	Installation drawings	Lubricants
Electricity	Manufacturers specifications	SOP title and number	Preventive maintenance program		Product contact
Compressed air	Purchase order	Location of sanitization SOP	Maintenance manuals		Non-product contact
Water	Mechanical drawings	Date of development	Spare parts		
	Electrical drawings	Approval date	Logbook		
	Materials in product contact		Filters		
	Materials not in product contact				
	Instrumentation: critical and non-critical				
	Calibration SOPs				

Table 7.4

An installation qualification form for chromatography column valves

Description, valve function	Mark	Default position	Tag number	Visual inspection (approved/not approved)	Installation, MIP and default position according to P&ID (yes/no)
Column top, in	9	NC	XV-031.A		
Column bottom, in	10	NC	XV-031.C		
Column top, out	9	NC	XV-032.A		
Column bottom, out	10	NC	XV-032.C		
Column bypass		NO	XV-033		

Comments	
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Performed and recorded by		Date	
Approved by		Date	

chromatography columns and skids. An example of documentation for an IQ for production chromatography valves is shown in Table 7.4.

Operational qualification

For chromatography equipment, OQ testing includes confirming pump reliability, alarm signals, leakage of liquids and column performance (the latter is discussed below). The function must be verified for alarms, valves, monitors, flow control, air sensors, recorders, indicators, pressure transmitters and computer control (see below). For example, in an alarm function verification, alarm conditions might be simulated for incorrect valve position, high system pressure, low airtrap level, air in system, high and low conductivity, high and low pH and circuit breaker failure. A flow path alarm test is shown in Table 7.5. In some cases, a description of operator training is included in the OQ. The overall conclusions from the qualifications should be documented.

Automated equipment qualification

The good and the bad news is that ‘It is no longer necessary for mankind to scribble on pulverized trees with graphite sticks. The electronic age is here’ [25]. ICH Q7A addresses validation of automated systems and states that appropriate IQ and OQ should demonstrate suitability of computer hardware and software to perform assigned tasks. Software testing

Table 7.5

Operational qualification for a flow path alarm

Procedure			
Ensure that the flow path alarm is enabled under/SYSTEM/SETTINGS/ALARMS			
Set an open flow path except for the inlet valves, they shall all be closed			
Try to start a pump by setting a low flow rate			
The pump should not start and the message 'All inlets closed' appear			
Change the set flow path so that an inlet is open and instead close all outlets			
Try to start a pump by setting a low flow rate			
The pump should not start and the message 'All outlets closed' appear			
Record the test results in the table below.			
Alarm	Test successfully completed, system responded as expected (according to test procedure) (yes/no)		
Flow path alarm			

Attachment number	Attachment name	Number of pages	Attached (yes/no)
7	Flow path alarm test		

Comments

Performed and recorded by		Date	
Approved by		Date	

includes structural testing, which ensures code is developed according to accepted standards by qualified programmers and that it performs reliably. Structural testing may be performed by an audit of the software supplier but requires a considerable amount of expertise. The user must test the software as installed to ensure it is able to consistently perform the required actions.

Most activities related to downstream processing are automated and the records maintained electronically. Changing software is often fraught with concerns. A guidance for industry and FDA staff, published in 2002, points out that ‘Whenever software is changed, a validation analysis should be conducted not just for the individual change, but also to determine the extent and impact of that change on the entire software system’ [26]. Maintenance of the validated state of the system is critical to the successful production of

biotherapeutics. At least one regulatory warning letter notes that there was inadequate software version control [27].

Good Automated Manufacturing Practices (GAMP) are addressed in the ISPE guide for validation of automated systems [28]. Although this guide has no regulatory authority, it provides valuable information on user and supplier responsibilities and process control system validation. Risk assessment and the validation life cycle are explained in detail for such systems.

While we can still read the works of William Shakespeare, we often find that electronic data created a mere 10 years ago can no longer be read because the software is outdated and even if it could be located, it can not be run on the current PC. Regulatory agencies recognized this problem and now electronic records can be converted into other types of records (e.g. microfiche or paper) for long-term storage. Some of the initial concerns related to the U.S. FDA's 21 CFR Part 11 have been alleviated [29].

Common regulatory citations related to automated systems include deletion of electronic records, absence of audit trails, inadequate password protection, inadequate functional and structural design, code containing 'dead' and unused code and lacking annotations and inadequate validation of networked computer systems.

Given the advantages and complexities of automated systems, there is much to be said for using systems that are transparent to scale. As noted elsewhere in this book, platform technologies can expedite development times and implementation of manufacturing strategies.

### *Column packing and qualification*

Column packing at large scale can be a labour-intensive endeavour, requiring relatively large, specified areas in the manufacturing facility, large amounts of buffer, mechanical lifts and drains—to say nothing of downtime if a qualified packed column is not available when needed. In some cases, column packing is quite important to the success of a purification unit operation; in other cases, packing quality may have little, if any, influence on the separation. For size exclusion chromatography and other polishing techniques, column packing is very important. However, for an early capture step, packing is generally not nearly as critical (see Chapter 12).

Column packing procedures should be validated and correlated with performance. HETP, asymmetry and/or transitional analysis determinations are used to demonstrate that the packed bed integrity is maintained. Transitional analysis use is increasing since it is easy to measure and uses breakthrough under normal operating conditions [30]. These methods of qualifying column packing are also employed periodically after column use, cleaning and storage, and are usually included in the validation of column storage.

## **7.3.4 Process validation**

Once equipment (including packed columns) and systems have been qualified and analytical methods validated, process validation can take place. This is, as noted before, typically performed during phase 3 clinical trials. The equipment used during process validation should be equipment that will be used for licensed product, if possible. If equipment is of a smaller scale, then process validation will have to be repeated at the production scale. Validation protocols and reports will be needed.

In order to write the validation protocol, one has to understand the process and its expected outcome. Acceptance criteria must be established and met, or else the validation will need to be repeated. As noted above, process acceptance criteria and control parameters that enable them to be achieved are derived from development and the characterization studies.

Today, process validation is documented evidence that three to five consecutive batches can reproducibly produce product meeting its pre-determined specifications and quality attributes. The problem that sometimes arises is that after licensure failed batches occur in spite of successful validation. In addition, through the use of control charts, trends that indicate potential for failures may be observed over time, necessitating the need to make process modifications before the negative drift impacts product quality. Apparently, the success of three to five batches does not guarantee successful long-term manufacturing. In an effort to have better process control, the concepts of the design space and process analytical technologies (PAT) are being applied by many companies. (Design space is explained in Chapter 3 and is described in ICH Q8 on pharmaceutical development.) Establishing the design space may well be worth the time and effort to prevent future failures, but often companies are in a competitive race to get a product license and, especially with new products and new companies, there is insufficient time to establish the 'space' where performance is locked in. PAT is defined as 'Systems for analysis and control of manufacturing processes based on timely measurements of critical quality parameters and performance attributes of raw and in-process materials' [31].

PATs has been applied to downstream processing. An example is provided in Chapter 5. In fact, the biotechnology industry has been using this in-process control approach on a less grand scale for a long time. In chromatography, the use of pressure indicators and pH, conductivity and UV monitors is the norm. But, as noted by one FDA spokesperson, for biotechnology there is a need to focus on CQAs before establishing PAT [32]. CQAs for biotechnology products include some that are relatively easy to measure such as isoelectric point, aggregation, size and formulation components. But there are also some that are more complex including potency, post-translational modifications, adventitious agents such as microorganisms and impurities such as host cell proteins. Assays for some of these CQAs are addressed in Chapter 5.

In purification, the CQAs are specific to each process and product. For each downstream processing step, it is important to understand why it was implemented. For example, if removal of DNA and host cell proteins is an expected result from an anion exchanger, then the quantities that can be loaded and removed in that step will need to be defined. Parameters such as total load, flow rate, processing time, pH and conductivity are likely to influence the performance. But not every parameter is critical for each purification step. Those that are critical are defined during the characterization studies.

Performance can be determined by measuring parameters such as product purity, impurities' profiles and product recovery. These performance determinants are influenced by many factors, including resin properties, column packing and operational conditions as shown in Table 7.6.

In addition to the factors listed in Table 7.6, contact time must always be kept constant within pre-determined limits. Contact time usually affects resolution, cleaning and sanitization effectiveness and in-process intermediate stability. (Stability is addressed in Chapter 5 on Analysis.) Column storage, leachables, cleaning and sanitization, resin lifespan and scale changes need to be addressed as part of process validation.



**Table 7.6**

Factors influencing chromatographic performance

Resin properties	Column packing	Operational conditions
Resin porosity, particle size, size range, hydrophobicity/hydrophilicity	Column design	Flow rate ranges
Immobilized functional group, coupling stability	Packing consistency	Sample load ranges
Degree of substitution	Importance of packing on performance	Dynamic capacity

*Storage*

Storage of packed chromatography columns is an area to which regulatory authorities frequently pay considerable attention during inspections. The key issues are proper labelling (identity and status), storage conditions, inhibition of microbial growth, removal of storage solutions and maintenance of column packing integrity. Among the storage conditions that must be defined and then validated are time, temperature, pH, buffer and concentration of antimicrobial agent and its stability. In some companies, 0.2  $\mu\text{m}$  filters are placed at the column inlet and outlet during long-term storage to prevent entry of microorganisms.

It is necessary to demonstrate that the storage conditions are sufficient to inhibit microbial growth. Most companies test for bioburden and endotoxin after storage during validation and also during routine manufacturing. The problem is that the results for bioburden testing, which is the more meaningful of the two assays since it is broader in scope, are unlikely to come back from QC and processing continues at risk. The use of rapid microbiological methods may be a solution because results can be obtained in a few hours in the best case, instead of days or weeks for the traditional compendial methods. New methods, however, may pick up more organisms than the previously used tests, and this, of course, makes it difficult to implement this technology for an already licensed product. Speciation is possible with some of the methods. This is important knowledge since a change in the type of microorganisms present during processing can pose a challenge to the production of an acceptable product. Rapid microbiological methods have been recognized by FDA as being suitable for in-process control testing [33]. In fact, these methods are considered to fall into PAT, which is being encouraged by regulatory agencies. There are several technologies available for rapid microbiological testing [34].

Leaching (see below) may occur during storage and the storage agent may act as a cleaning agent, further removing previously undetected residual contaminants bound to the resin. For this reason, effluent should be collected and analysed after storage. It is also necessary to define and validate the conditions required for complete removal of storage solutions. For example, it has been shown that up to 10 column volumes can be necessary to remove 20% ethanol used as a storage solution. The contact time as well as volume of flushing solution also needs to be defined and validated. The selection of a storage agent that is bacteriostatic, compatible with the resin and associated wetted column components and is also easily removed and detected, should be part of development. Sodium hydroxide is the preferred storage agent because of its low cost, lack of significant disposal problems and

ease of measurement. Resin manufacturers usually provide information on appropriate storage solutions and conditions, but users must validate that those conditions work in their processes.

### Leachables

When addressing leachables from chromatography resins, it is important to perform a risk assessment and determine where in the process leakage can occur and at what level it is acceptable. Does it occur during storage, during cleaning and sanitization or does it occur during product elution? The nature of the leachate is also an important issue. Certainly if the leachate has the potential to be immunogenic or toxic, there is more concern. Anytime new chemistries are employed, information should be available about potential toxicity and immunogenicity. If leakage occurs during storage, then it is essential to validate the number of column volumes and contact time required to displace all leakage products. Table 7.7 shows that leakage from Protein Sepharose™ Fast Flow was highest during start-up when an unwashed resin was used for the first time. In this experiment, a 5 ml column was cycled three times. During each cycle, 50 ml of adsorption buffer, pH 7.0, and 25 ml of desorption buffer, pH 3.0, were pumped through the column at a flow rate of 5 cm/h. Protein A leakage in the eluate was determined by a radioimmunoassay (RIA). The amount of leakage decreased with each cycle until an almost steady level was reached.

Lasch and Janowski studied leakage of a protein, azocasein, coupled by either single- or multi-point attachment [35]. The results show that monovalently bound ligands leak extremely slowly, but double cross-linking with dialdehyde enhances stability. They conclude that there are two types of leakage: splitting of the bond between the protein and the matrix and slow desorption of ligands bound by multiple non-covalent interactions. A recent report shows that when Protein A fragmentation occurs during loading of harvested cell-culture fluid, the removal of the leached fragments on a subsequent cation exchange step is more difficult than removal of the intact molecule [36].

The most likely time in the process to incur leakage is during cleaning and sanitization, when the harshest chemical conditions are used. But, leakage may also occur during storage. Therefore it is important to determine the contact time and number of column volumes of a defined eluent that is necessary to reduce the leakage level to an acceptable, measurable quantity. Generally, the equilibration buffer is used to wash out any residual leachables.

**Table 7.7**

Leakage from Protein Sepharose™ Fast Flow during three cycles

Leakage of Protein A (ng/ml)				
Fraction	pH	Cycle 1	Cycle 2	Cycle 3
1	7	341	29	18
2		457	17	2
5		9	30	3
10		20	16	2
11		18	32	2
12	3	78	59	38
15		19	42	18

In the worst case, chromatographic materials leach during product elution. If they bind to the product, even if not toxic they may be immunogenic, and a subsequent step must be designed into the process to remove them. For example, it has been shown that when Protein A coupled to a chromatography matrix is used for monoclonal antibody purification, some Protein A is likely to bind and coelute with the monoclonal antibody. However, it has also been shown that a second chromatographic step, such as ion exchange, can remove all Protein A–monoclonal antibody complexes [37, 38]. For some antibodies, the best step to employ after Protein A affinity is cation exchange. The conditions employed for cation exchangers tend to cause dissociation of Protein A–antibody complexes. Dissociated Protein A binds more weakly to a cation changer than most IgGs, and can be removed by salt gradient elution [39]. If cation exchange is used, it is essential to ensure that the antibody is stable under the acidic conditions employed.

Fortunately, large amounts of chromatography materials generally do not leak under product elution conditions. Resin manufacturers usually provide data on studies that involve static bulk experiments under extreme conditions, such as high and low pH. Leakage products are collected and often concentrated, assays are then developed and optimized and data provided in regulatory support files. Some of the assays that can be used for leakage detection include HPLC, GC-MS, NMR, fluorescence spectroscopy, immunoassays, flow injection analysis, TOC and elemental analysis.<sup>2</sup>

Just about anything has potential leachables (e.g. silica resins may leach siloxanes, and agarose may leach carbohydrates). The level of leachables detected will depend on the assay sensitivity and the suitability of the assay for the sample being analysed. The acceptable level of leachables depends on the risks. Immobilized Protein A columns have become a standard in the purification of therapeutic and diagnostic monoclonal antibodies. For an *in vitro* diagnostic, a consistent amount in the final product that is demonstrated not to interfere with the assay might be acceptable. For an *in vivo* diagnostic or therapeutic, there should be no patient risk. The extensive experience in purification of monoclonal antibodies has resulted in some consensus on the amount of Protein A that might be acceptable in the final product. At a conference in June 2004, it was stated by an FDA spokesperson that 10–12 ppm Protein A detected in final product by an immunoassay is a target release specification [40]. Companies have a choice of validating the release specification or performing a clearance study (see Section ‘Clearance Studies’ below).

Whereas with proteinaceous ligands, there is a demonstrable risk; with ion exchange, the most frequently used chromatography mode, there is very little, if any, risk. Detection methods for leachables include gas chromatography for amines and ion chromatography for sulphite and sulphate groups. It is noteworthy that the human sense of smell has exquisite sensitivity for detection of amines from anion exchangers. Elemental analysis is used to detect sulphur, reversed phase chromatography for sulphonic acids and GC-MS for trace analysis of volatile compounds. Carbohydrate leakage is assessed by the anthrone reaction and TOC.

Chromatography resins, as well as filters, should be used under conditions recommended by their manufacturers. Considerable cost-savings can be realized when the technical data

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<sup>2</sup> HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; NMR, nuclear magnetic resonance; TOC, total organic carbon.

from the supplier is adequate and the conditions known to maintain the resin integrity used. Otherwise, users might have to repeat all of the supplier's studies. The following comments illustrate this point.

'There are no studies to assess leakage from the chromatography resins for the purification steps beyond the technical data supplied by the supplier. The cumulative impact of the process stream materials on resin stability is not monitored. The routine cleaning of one resin used includes acidic conditions under which the resin is known to degrade'. These comments were made by an FDA regulator during a pre-approval inspection [41].

#### *Cleaning and sanitization validation*

Cleaning validation is an area that demands considerable attention and often leads to negative comments during regulatory inspections (see Table 7.8). It has been suggested that for biotech products cleaning validation should probably begin in phase 2 clinical trials [42]. Resins, membranes and equipment-cleaning validation need to be addressed (cleaning and sanitization basics are addressed in Chapter 6). In order to validate a cleaning procedure, it is necessary to know what items are being removed, how samples are taken and when cleaning validation will be performed [43]. Holding times for both dirty and cleaned equipment (including packed columns) should be validated.

#### CLEANING VALIDATION

**Resins** Chromatography resins have large surface areas to which process and product impurities and contaminants such as microorganisms can adhere. Detection and validation of carryover between batches is essential.

Cleaning validation of packed columns usually requires a combination of small-scale studies and manufacturing runs. Small-scale studies can be useful for cleaning method development and validation (especially for lifespan studies), but they are fairly meaningless for validation unless production feedstreams and manufacturing conditions are used. Repetitive cycles of sample application, elution and cleaning/sanitization can be run on an automated system to assess long-term effectiveness of the protocols. Keep in mind that storage solutions may also have a cleaning effect, and simulate storage conditions in the small-scale study.

The production scale cleaning should be validated and then monitoring becomes part of the production routine. Routine controls may include maintenance of cleaning agent

**Table 7.8**

U.S. FDA observations about cleaning validation

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No validation of detergent residue from production equipment
Cleaning validation and changeover issues not properly addressed
No written procedure for performing cleaning validation
Cannot determine if cleaning procedures are based on validated methods
No validation data to support cleaning of chromatography columns used for protein purification
No documentation that validations included critical specifications for pressure/flow rates, WFI flush volume and temperature

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contact time and measurements of product purity and impurity profiles. Blank runs (i.e. no sample applied) are expected to assess carryover. Blank runs are typically performed at production scale every five or more cycles. As analytical methods become more sensitive in the future, it is possible that a PAT approach would suffice, provided one could demonstrate assay sensitivity and absence of masking of impurities.

**Membranes** Ultrafiltration and diafiltration membranes can be reused provided the cleaning is validated. Blank carryover runs for impurities and product have been performed using assays for host cell proteins, DNA and a product EIA (enzyme immunoassay). This particular study was used to support a 10-lot campaign for a small volume product [44]. Recommended cleaning agents for membranes include alkalis, acids and surfactants. Removal of these cleaning agents must be validated. Validation of a TFF system cleaning protocol has been presented in a book chapter [45].

**Equipment** There are several publications that provide valuable information on equipment cleaning validation [46, 47]. As noted in a review article on risk-based cleaning in biopharmaceutical API manufacturing, PAT can now be used to complement cleaning validation and optimize equipment usage based on real time data. In this way, PAT can identify parameters that indicate equipment cleanliness [48]. In this article by Mollah and White, matrix and family approaches and bracketing for equipment cleaning validation are discussed. It is also noted that ‘worst case’ validation testing strategies reduce the total quantity of validation studies for a system or process. Cleaning validation for buffer tanks used in the manufacture of Betaseron® has been described [49]. Worst-case conditions were used during three cleaning runs. Final WFI rinse and fresh WFI were tested and compared for conductivity, pH, endotoxin and bioburden.

For some equipment, it is difficult, if not impossible, to reach all areas during cleaning. Coupons (i.e. cut out pieces of the equipment material usually available from equipment manufacturers or their suppliers) can be used and challenged with worst-case situations, such as letting a soil dry for an extended time. Another consideration is that the amount of time dirty equipment is held prior to cleaning needs to be validated. This also applies to packed chromatography columns. Not specifying holding time prior to cleaning can result in an FDA form 483 (a negative finding during an inspection), as evidenced by the comment: ‘Cleaning/sanitization hold times for UF/DF skids have not been established’. Recovery from different surfaces should also be addressed. In one example, a spike of purified protein was 89% recovered on stainless steel, but only 62% on polypropylene and 55% on glass. [50]

**Assays and testing techniques** By far, the most commonly used method for cleaning validation is total organic carbon (TOC). Newer TOC equipment can now be used in-line, providing rapid, economical monitoring and validation of cleaning effectiveness [51]. In 2005, FDA stated that TOC can be an acceptable method for monitoring cleaning effectiveness (see [www.fda.gov/cder/guidance/cGMPs/equipment.htm](http://www.fda.gov/cder/guidance/cGMPs/equipment.htm)). Since TOC does not distinguish among different carbon-containing materials, when performing a risk assessment, the carbon is assumed to come from the material of highest risk. For packed chromatography columns evaluated during blank runs, the TOC values may be too high due to the presence of carbon-containing buffers or additives. In this case, a total protein assay, SDS-PAGE or another suitable assay may be needed.

**Table 7.9**

Assays frequently used for cleaning validation

Method	Detection
TOC	Carbon-containing residues
HPLC	Variety
ITMS	Non C-containing residues
SDS–PAGE	Protein pattern
UV	Protein/nucleic acid
pH	Residual NaOH; variety; usually compared to WFI
Conductivity	Residual NaOH; variety; usually compared to WFI
LAL <sup>a</sup>	Endotoxin
Bioburden	Microorganisms
Total protein assay	Protein
DNA assay	Nucleic acid

<sup>a</sup>Limulus amoebocyte lysate.

Methods that are applied to cleaning validation include testing of rinse fluids, swab testing and visual inspection. All have limitations and usually a combination of methods is used. Disadvantages of swabbing and rinse water sample analysis are discussed by Zeller [52]. Some of the more commonly used assays and their detection capabilities are shown in Table 7.9. Assays should be chosen based on their detection capabilities and sensitivity. This can be problematic as newer, more sensitive methods are applied to cleaning validation. A recent publication describes the use of LC–MS–MS for cleaning validation in manufacturing equipment for traditional pharmaceuticals [53]. Another technology that can be used to analyse residues is ion trap mobility spectrometry (ITMS). Widely used in security applications for the detection of narcotics and explosives, ITMS measurements take less than 1 min and can measure swab samples directly without dilution. ITMS has the potential to facilitate at-line measurements of cleaning residues and reduce the amount of equipment downtime during residue analysis. Results that are commonly returned in 24–48 hr can potentially be generated in under 4 hr with ITMS [54].

Swab testing is useful for equipment, and is used to sample poorly soluble, insoluble or occluded residues. Operator technique and swabbing pattern must be standardized. Method development of swab sampling for cleaning validation can be extensive [55].

Rinse water sampling is useful for equipment, resins and membranes. In some cases, poorly soluble residues may not be detected. Rinse water, however, is really the best method for chromatography resins. Another sampling strategy involves actual removal of some resin during reslurrying, which is often done to enhance resin performance by affording a greater contact area with the cleaning agent. This, of course, must be part of an approved protocol. In one case for a very large stainless steel column, small aliquots of resin were removed around the circumference of the column, in the centre and from any area that appeared discoloured. Assays included bioburden, endotoxin, resin small ion capacity and a product-specific assay. There was a small amount of protein that was found to carryover from run to run but the performance was not impaired.

Visual inspection for large chromatography columns may not provide much useful information as very little of the surface area is actually visible. There is a perception that a

discoloured column may not be clean. However, there are many discoloured columns being used to produce very pure biotherapeutics. It is essential to attempt to determine what causes the discolouration and to evaluate the potential impact of the discolouration. There have been studies using a plethora of reagents (including bleach that destroys the resin) to remove discolouration only to find out that nothing worked. Consistent performance was demonstrated in spite of the unpleasant appearance. Visual inspection and application of visible residue limit for cleaning validation was recently discussed [56]. Although the examples provided in the article are for more traditional pharmaceuticals, some interesting factors essential for validating visual detection of cleanliness are presented. For example, it is essential to define the observer viewing position, observer viewing distance, light intensity, viewing angle. Observer to observer variability also affects the outcome.

**Defining acceptable residual levels** Defining the maximum amount of carryover is a difficult task and is best addressed by a risk assessment. There may only be trace amounts, and residuals after harsh treatments such as NaOH and steam may be undetectable by specific methods. Yet, acceptance limits should be practical, achievable and verifiable. For biopharmaceuticals, understanding the nature of the risk is essential for defining acceptable residue limits that will not affect product quality, i.e. safety and potency.

For multiproduct facilities, there is likely to be a greater risk—one of the reasons these facilities use more disposables. Equipment cleaning validation and common errors within a multiproduct facility have been discussed [57]. It is not always possible to apply acceptance limits that are used for traditional pharmaceuticals to biopharmaceutical cleaning validation. The potential immunogenicity of protein residues after cleaning has been discussed. It is acknowledged that this is a theoretical risk that needs to be tested using both specific and non-specific assays. This can be followed by performing a risk calculation and then implementation of TOC between campaigns [58].

#### SANITIZATION VALIDATION

Validation of sanitization is typically performed during process validation and followed up by routine monitoring. The assays for bioburden must be validated. A recent FDA 483 noted that a company had not validated the bioburden method for chromatography column storage solution. In some cases, the test article will inhibit the growth of microorganisms, requiring that interference tests (bacteriostasis and fungistasis) be performed to validate the assays. During process validation, routine bioburden monitoring should be included in the protocols (see Ref. [3]).

The performance of challenge studies for validation of sanitization does not necessarily provide relevant information since control of microorganisms in the actual manufacturing environment is the critical element. Challenge studies are, however, useful in providing information on the effectiveness of sanitizing agents and identifying any components that are difficult to sanitize. Chapter 6 provides further information on sanitization.

#### *Resin and membrane lifetime*

##### RESINS

Formal lifetime studies should be initiated after the process conditions are defined. These studies are usually performed prospectively, i.e. prior to licensing of a product; but in some cases, concurrent validation is appropriate [59]. Although records of usage should be



maintained during development, lifetime validation studies performed prematurely will not provide relevant data. Since these studies are time-consuming, consume valuable production feedstream and must use a qualified small-scale model, considerable planning is required. One U.S. FDA product reviewer noted that a representative finding is ‘validation study did not include evaluation that the scale down version was representative of the manufacturing process’. In some cases, it was found that lifetime validation studies for resin and membrane reuse were not even performed [60].

Concurrent validation of lifespan provides real data on the performance, much like PAT. Assays must be sufficiently sensitive, and it is likely that much more in-process analysis will be needed than when lifetime is established prospectively. There may be hold times or continued processing at risk while waiting for the analytical results to be compiled. A publication from Health Canada describes an occasional GMP observation for manufacturers using concurrent validation of resin or membrane lifespan, i.e. inadequate parameters and/or intervals for confirming the performance [61].

For columns used for viral clearance, surrogate parameters may be found and implemented. At this time, however, it is unlikely that this strategy is accepted by worldwide regulatory agencies. The U.S. FDA Division of Monoclonal Antibodies has noted that validation of virus clearance with used resins is an expensive requirement that has not previously been subjected to extensive scientific analysis [62]. Studies have now been performed on chromatographic steps in which viruses flow through during loading. In a study on immobilized Protein A resins, it was observed that step yield and breakthrough were performance quality attributes that decay prior to any decrease in retrovirus clearance. Eluate impurity content, on the other hand, was not found to be a surrogate for retrovirus clearance. It was proposed that virus removal validation studies be performed on new media only and the relevant quality attributes be monitored during Protein A unit operations [63]. In another study, this time using an anion exchange step so that virus bind and the monoclonal antibody flows through the column, it was found that performance attributes that could replace re-evaluation of viral clearance for used resins included band spreading (measured by HETP, see Chapter 12), DNA clearance and accumulating backpressure [64].

Trying to predict when columns will fail is not always feasible. For one company, it was found that dynamic capacity decayed with continued use of a Protein A column. With anion exchange, that same company found that removal of key impurities decreased. But with cation exchange, there were no identifiable sources that could predict column failure [65].

Some factors that affect resin lifespan are shown in Table 7.10. These are addressed in more detail elsewhere and are summarized here [66].

Chromatographic steps placed early in the purification process are subject to the crudest feedstreams, which are more likely to result in cleaning problems that decrease column lifetime. The mode of chromatography, i.e. flow through or binding, also impacts lifetime. With less contact time, impurities are less likely to become irreversibly bound. With affinity resins, lifetime tends to be shorter than with other types of chromatography. However, this is not always the case and the impact of the other factors will be critical. Columns that are cleaned well, stored properly and maintained with minimal bioburden are more likely to last a longer time. If system components are not compatible with cleaning and storage conditions, there may be leachables that decrease resin lifetime. The use of high-quality raw materials also enhances resin lifetime.



Table 7.10

Factors that affect resin lifespan

Position of step in purification process
Nature of feedstream
Mode of chromatography
Type of chromatography resin
Column maintenance
Column packing and attrition
System components
Quality of raw materials

Table 7.11

Measurable parameters to determine lifetime of chromatography resins

Product purity	Bed height
Impurities (e.g. host cell proteins)	Column packing integrity
Flow rate/pressure	Dynamic binding capacity
Elution profile	Ionic capacity for ion exchangers
Regeneration/re-equilibration profile	Leachables
Product yield	Appearance

There are many parameters that can be measured to validate lifetime. However, they may not all be relevant for a given separation. Some commonly used parameters for chromatography resin lifetime studies are provided in Table 7.11. An assessment can be made of when it is more cost-effective to replace the resin than validate its reuse. Such a study was performed by one company and they found that for their particular situation, they saved about \$15M USD per year when validating resin lifespan for use in 30 lots; but for 90 lots, the cost savings were less than \$1 MUSD (see Ref. [66]).

MEMBRANES

Lifetime studies for ultrafiltration and diafiltration membranes have much in common with the studies for resins. Filter integrity, normalized clean water permeability and transmembrane pressure are specific measurements for reused membranes. Analysis of membrane surfaces can be performed to understand if there is a build up of impurities. Further information on filtration media life time is presented by Rathore in Ref. [66].

*Small and manufacturing scales*

Both small and manufacturing scales are used for process validation. Small scale studies, as discussed above, are used for prospective lifetime validation. They are also used, in conjunction with full-scale studies, for intermediate stability and cleaning validation studies.

Small scale models are often essential for clearance studies (see below). As noted in the ICH guideline on viral safety, ‘the level of the purification of the scaled-down version should represent as closely as possible the production procedure’ [67]. Designing a small scale model that truly represents manufacturing can be problematic. Some differences between a scaled down model and full scale are unavoidable and must be understood and considered when interpreting results (see Ref. [3]). Wetted materials are often different, e.g. stainless steel is commonly used at manufacturing scale; plastics at smaller scale. Wall effects may alter chromatographic performance when scale is changed significantly. Scale down (and scale up) guidelines for chromatography are relatively straightforward (see also Chapter 4).

- Ensure system design reflects production scale
- Scale down column accurately
- Ensure consistency of contact time
- Follow SOPs for buffer preparation
- Equilibrate columns properly
- Use suitable resins and membranes
- Use correct pH, ionic strength and temperature
- Use production sample

Wherever possible, the chromatography or UF/DF skids should be mimicked at small scale. Scale down of columns usually entails maintaining bed height and decreasing column diameter, while maintaining linear flow. Contact time is the real determinant of consistent separation and, in some cases, columns with a different bed height can be used as long as comparability to production can be demonstrated. Volumetric flow has been used for scale up by calculating delay volumes and assessing extra-column effects [68]. Buffers and other solutions should be made by the same protocol, and with the same raw material quality, as in manufacturing. Equilibration of columns should be measured as in manufacturing, e.g. by measuring pH and conductivity for ion exchangers. Resins and membranes should be quarantined, identified and released. Calibration of monitors and temperature should be the same as in manufacturing. Production sample should be used.

Qualification of scale down is best performed at the user’s site, where all of the analytical methods are available to test comparability of product purity and recovery and impurities’ removal (see also Chapter 5). Sometimes trivial issues lead to small scale model failures. Variable distances from outlet to monitors and differences in calibration of monitors such as those for pH and conductivity can be significant. For filtration, it is always advisable to begin in development with a scalable system configuration.

At full scale, engineering runs are performed to demonstrate scale up accuracy prior to starting formal process validation. This is not a time for experimentation. As with scale down, there are often some minor (and sometimes some not-so-minor) modifications that need to be made (see also Chapter 4). If clearance studies have been performed prior to any final modifications at full scale, it is essential to re-evaluate the small-scale models to ensure they still reflect manufacturing.

### *Clearance studies*

In downstream processing, clearance studies are used to validate removal and/or inactivation of adventitious agents such as virus and impurities such as host cell proteins and DNA, and processing impurities such as Protein A and cell culture additives. As assay sensitivity has increased, it is now possible to perform some of the clearance studies at full scale and avoid spiking studies. DNA clearance using Q-PCR (Quantitative Polymerase Chain Reaction) at full scale is one example. If performed at full scale, the API is usually tested as well during conformance batches.

Performing a clearance study to validate removal or inactivation of impurities can provide cost savings as it allows for elimination of a lot release test. It is always worthwhile, however, to maintain the assays in the event there is an out of specification (OOS) finding. The assays may help in finding the root cause of the OOS.

When evaluating clearance of high-risk materials, such as viruses and TSEs, there is no choice but to use small-scale qualified models. For safety reasons, these studies are usually performed at contract laboratories. Virus- or TSE-containing solutions are spiked into the feedstream, but the spike may alter chromatographic or filter performance. By using a solution in which the virus is going to be spiked as a blank, it is usually possible to determine if the spike volume alters chromatographic and filter performance. Spike volume can then be adjusted prior to the actual study. Typically, yield, purity and impurities are evaluated with different spike volumes.

## **7.4 MAKING CHANGES**

Changes in downstream processes may be made to enhance product quality and/or patient safety; improve the process, final product stability and economics; increase scale and/or productivity; and comply with changes in regulatory requirements [69]. Change is inevitable and regulatory authorities expect state-of-the-art technologies to be applied where they can enhance product quality and/or patient safety. A formal change control system is a GMP requirement (see Ref. [4]) Manufacturers should evaluate relevant quality attributes of the product to demonstrate modifications do not adversely affect safety and efficacy of drug product. A change control system during clinical manufacturing can also prevent regulatory delays as it can clarify the differences, if any, compared to product used in clinical trials.

Whenever changes are made in clinical manufacturing, it is imperative that the impact on the final product and patient safety are addressed. Biophysical techniques used to assess comparability and case studies for protein therapeutics have been discussed [70]. If the changes can not be evaluated by analysing the product and performing a risk assessment, then earlier pre-clinical and clinical studies may need to be repeated.

The biotech industry's experience in process chromatography is now extensive. This experience has enabled companies to make changes in downstream processes without incurring unwarranted regulatory burdens, such as repeating full clinical studies for licensed products.

When companies work within the design space, changes to licensed products can be made without regulatory delays (see Chapter 3). For licensed products, PDA Technical Report No 38 addresses post-approval changes in chromatography systems and provides insight into assessing and making changes [71]. Directed toward US regulations, the principles should apply worldwide. The document addresses structurally well-characterized drug substances for which impurities can be monitored at the recommended levels.

Comparability protocols are accepted by the U.S. FDA to reduce the level of reporting for changes [72]. These protocols describe tests, validation studies and acceptable limits to be achieved to demonstrate the absence of an adverse effect from specified categories of changes. If a company meets their acceptance criteria during the validation of the change, then they can accelerate implementation of the change and realize a great cost saving.

For biotechnology-derived products, the following issues should be addressed when a change is proposed.

- What is the purpose of the original step (e.g. what impurities are removed)
- Type and mechanism of separation
- Impact of change on subsequent steps and on overall process robustness
- Potential impact on viral safety and control of bioburden
- Impact on in-process and final product stability
- Changes in storage conditions
- Effectiveness of cleaning and sanitization protocols
- Availability of appropriate analytical methods

A more extensive list of factors to consider when changing chromatographic systems is included as Appendix A in Technical Report No. 38.

## 7.5 SUMMARY

Validation is a tool that should enhance confidence in process performance. Process Analytical Technologies should further enhance that confidence. Confidence in a process is not possible unless there is an understanding of the CQAs and critical control parameters, which are derived from good process development. Over the last decade considerable progress has been made in understanding downstream processes. The enhanced understanding is due to experience and to improvements in analytical tools. The consistent production of highly pure biotherapeutics by downstream processing is ensured by good development practices, process validation and in-process controls.

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## Economics

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Economics is all about numbers. Ideally numbers are unbiased in representing the facts about a situation. Mathematical operations used for financial calculations are truly of the simplest kind and thus do not give much reason for error. Are numbers, therefore, the perfect guide for process optimization, technology selection or development decisions? Can process-cost related numbers in presentations or publications be trusted and transferred into your own situation? Do we understand the purpose behind a presentation of financial data and specific cost-saving opportunities? And do we understand all consequences of implementing a proposal, to reduce costs based on the data presented?

Each company has their specific economical context and business priorities. Therefore, everyone needs to evaluate what is being discussed in this chapter and adapt conclusions for their own situation. Numbers can be taken out of their context, and the published information may be incomplete for simple confidentiality reasons. Numbers can be compared against other unrelated numbers and put into a new context to promote new ideas that can be misleading. Numbers can be presented with a purpose that may not be relevant for you and the verbal messages that sound very attractive may be convincing but inappropriate for your needs. Understanding the context is key for success and thorough number verification is always needed. There are a few references that might be helpful [1–10], but the information published about economics of manufacturing is most often incomplete, and some reading between lines is often needed to reach the appropriate message and facts. As a general rule, your view on numbers should encompass as complete a picture as possible and ideally include both direct and indirect economical effects of a proposal or decision.

Recognize that our counterparts in business, e.g. suppliers of technology, research partners or simply colleagues in other departments, need to show profit to their owners and managers. It means that they will present ‘their product’ in the best light. It also means that the more you are (willingly) dependent on them, the less you will want them to take short cuts or make mistakes due to strong economical pressure.

You will be a ‘hero’ when you save your company millions every year! Until the hidden flaw in your calculation may surface one day. Having warned you of that risk, rest assured that there is always room for improvement, sometimes a lot! This chapter shall help you to find the best way to become the million dollar hero.

In this chapter, we will develop a hierarchy of positive economical effects that can be controlled via the decisions you may make both at a strategic level and at the detailed



technical and operational level. Most of the discussion focuses on monoclonal antibodies because the dosage regimes and total quantities make them the prime case for economical improvements. We begin this chapter with an educational excursion into some examples of how numbers are often presented and how the subsequent judgment may be either right or wrong.

## 8.1 ECONOMICS: AN EDUCATIONAL EXCURSION

The activities and products you work with are part of your company's operations and your company is part of the (bio) pharmaceutical sector of a global economy. We want to understand how costs generated by biopharmaceutical manufacturing processes and benefits from process improvements are related and how they relate to other costs and improvement opportunities your company may have [11]. We will look at this from three different perspectives: corporate management, manufacturing management and a more development-focused view.

### 8.1.1 Costs as seen from a corporate level

Numbers are often presented as 'ratios', i.e. relative to another number. Judging a cost as 'high' or 'low' also turns into a relative judgment and the competitive situation becomes relevant too. Table 8.1 provides a rough estimate of manufacturing costs for the biopharmaceutical industry in the form of the 'cost of sales' (CoS)<sup>1</sup> ratio, which seems to be at an average of 16% of revenue for companies focusing on therapeutic proteins. Manufacturing, as we discuss it in this book, comprises a significant portion of CoS even though not all of it. Company D in Table 8.1 has two products in the market, one vaccine and one Mab. Their CoS fall between 25 and 30% of revenue, i.e. is higher. For companies with different categories of products, CoS seems higher in general. For one large company with a portfolio of proteins, vaccines and classic pharmaceuticals, the CoS was also between 25 and 30% of sales. For another firm marketing one protein and a variety of large volume insulins, the CoS was in the same range.

Marketing and sales-related costs are above 30% of revenue for those two companies, also higher than the four businesses focusing on proteins (24% average, represented as the SG&A<sup>2</sup> ratio in Table 8.1). Most biotech drugs are parenteral drugs sold mainly to the hospital market, which may be one reason for the lower sales costs. Where a company needs more sales activities, SG&A costs increase. R&D expenditure seems to reach up to 20% of sales for the biopharmaceutical industry.

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<sup>1</sup> Cost of sales (CoS), also referred to as cost of goods sold or cost of revenue describes the direct expenses incurred in producing a particular good for sale, including the actual cost of materials that comprise the good, and direct labour expense in putting the good in saleable condition.

<sup>2</sup> Selling, General & Administrative Expense (SG&A) is the sum of all direct and indirect selling expenses and all general and administrative expenses of a company. SG&A is often referred to as 'cost of doing business' and includes, e.g. all Sales, Marketing and Executive Management costs.

**Table 8.1**

Profit & loss (p&l) summaries from annual reports 2005 for four established companies engaged in biopharmaceutical proteins [12–15]

2005/(MUSD)	A (%)	B (%)	C (%)	D (%)	Average (%)
Total revenue	100	100	100	100	100
Cost of sales (CoS)	17	15	10	27	16
R&D	19	19	23	31	20
Selling, general & admin (SG&A)	22	22	33	40	24
Other p&l items	13	15	38	5	16
Net income	30	29	−5	−3	24

*Note:* Two of the selected companies in the biopharmaceuticals field generate a strong net income; two generate a moderate loss (average net income ~25% of sales). The companies have CoS ratios averaging 16% of sales. R&D ratio averages 20% of sales. SG&A averages 24%. Ratios vary with size and complexity of product portfolio, and with routes for distribution

A relatively low CoS ratio for protein focused companies is unlikely to be due to ‘low manufacturing costs’. It is, instead, more reasonable to assume that the difference in ratio is related to the higher sales value of the protein therapeutics compared to a mixed portfolio with classic pharmaceuticals or to an insulin-dominated portfolio (see also Figure 2.1).

However, it seems fair to conclude that companies with a CoS ratio at ~16% of sales are not under immediate pressure to improve manufacturing costs. In fact, business analysts scrutinizing the performance of the biopharma industry at a high level are not normally focused on manufacturing issues; rather, they focus on the large increases in R&D expenses concomitant with stagnating new product output as its key challenge (see Section 1.3 of Chapter 1). If financial analysts are concerned about manufacturing, then it is usually related to the ability to produce for the market (the capability issue has been addressed in Chapter 2).

SG&A, roughly equivalent to sales and marketing costs, regularly seem to load ~50% more costs than manufacturing on biopharma companies and it may be assumed, with good reason, that this part of the P&L<sup>3</sup> is a priority candidate for cost-improvement programs initiated by management.

If key ratios alone cannot explain the significant pressure on manufacturing costs, what else is there? As described in Sections 1.3.1 and 1.3.2 of Chapter 1 where we provided industrial context, the most likely reason for management to be concerned about costs is found in the very general, strong pressure to realize the promise of biomedicines in a more and more competitive environment where the products can no longer be priced freely, and under the financial performance expectations of our global economy.

Not only do improved protein drugs enter the market more and more rapidly after a novel therapy has been launched, but now biosimilars (also called follow on proteins, biogenerics) are also coming up in the horizon to compete with many of the classic

<sup>3</sup> P&L is the acronym for a company’s profit and loss statement.

profit-generating biopharmaceuticals. Such competition can have significant impact on strategies to manage the product lifecycle including plans for manufacturing or views on costs. The national health care systems are critical about the price of medicines, and there is at least one example where reimbursement of a modern biopharma product might be refused: the 2006 German recommendation to ban certain fast-acting insulins from prescription as long as they have higher costs than classic insulins [16]. The regulatory agencies as well as business analysts express concern about the output from the R&D process. Finally, it seems that the distribution channels used by this industry might prevent the industry from harvesting a significant part of the value its products may represent (see also Section 2.2 of Chapter 2). Companies do not receive the full value patients are paying for the drugs.

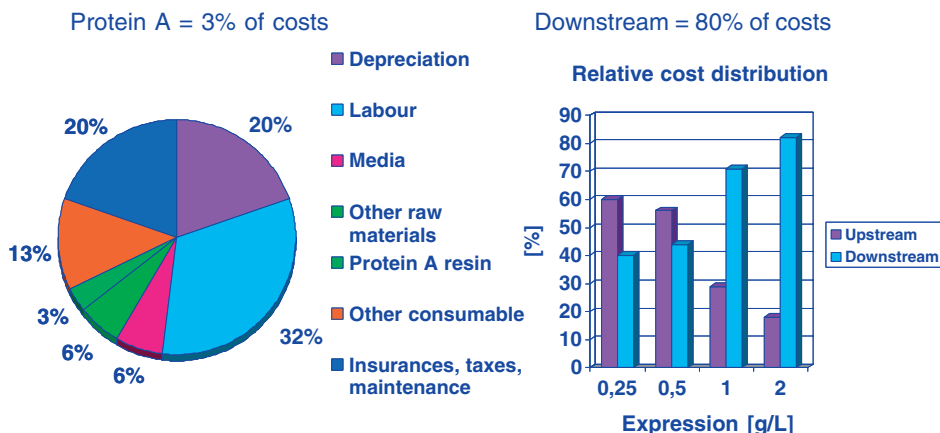
In summary, from a corporate perspective, there is simply no escape from cost consciousness in all parts of the operation and from always seeking the best manufacturing solutions. Excellent CoS ratios alone do not protect biopharma companies from competitive or political pressure on the business. Low manufacturing costs are also not a guaranteed protection, and the industry has many other challenges apart from making its products in an efficient manner.

### 8.1.2 Costs as seen from a manufacturing management level

Numbers are often presented with a purpose and formatted to carry a message. In some cases, this narrows the view significantly and covers up other important economic aspects. At the manufacturing level it is obvious that it is only manufacturing costs that are addressed. Other costs in the company do not serve as a way out. However, the degree of elimination of important economic elements can still be very significant. We use two examples (Figure 8.1) with a view on costs as found in many of the current presentations available to the public. The questions are: which aspect of manufacturing carries which costs and where would the focus point be for cost reductions? And of course, why? As a guiding statement, we use a comment made in a paper published by senior Genentech staff: 'A good process will be cost effective and alternatives to reduce costs should be evaluated. This is not to say, that the cheapest process is the best...(but)...extra efforts may not (always) be warranted' [17].

The headlines above the two parts of Figure 8.1 initially do not seem to make sense, so far away from each other are the percentage values, i.e. 'Protein A resin represents 3% of the cost' and 'Downstream causes 80% of the cost'. This is a fine example of how the presentation of numbers can sometimes be designed for a purpose. Neither is 3% a representative number for the whole downstream process, nor is 80% a meaningful number to use in decision making. From the graph on the left, one may conclude that the cost discussion about Protein A resins is not so important. From the graph on the right, one may justify a project looking into new, unproven technology. Both messages may be entirely misleading. Let us take a closer look at this and extract some meaningful facts:

Information obtained from the pie chart includes the main cost drivers being taxes and other costs related to 'owning a facility', plus depreciation and labour (fixed costs, even labour is not widely variable in proportion to the quantity of product produced).



**Figure 8.1** Two different relative views on costs for downstream processing: on the left a typical pie chart overview of annual costs incurred in a manufacturing operation. Cost drivers are taxes and other costs related to ‘owning a facility’, depreciation and labour. On the right: a bar graph comparing costs for upstream and downstream processing using a relative scale. This illustration is typically used to highlight a particular cost position and suggest cost improvement.

One immediate conclusion is that a facility with ‘fixed costs’ of almost 75% would cause a major financial problem if not very well utilized with production of marketed product.

Protein A affinity resin costs are part of the expenses and represent 3% of the annual cost. Other raw materials and consumables together represent 19% of the total. Cell-culture media represent 6%. Assuming ‘media’ are the only consumables for the upstream process, one may conclude that the ratio between consumables and raw materials for the upstream process versus the downstream process is just below 80:20 (22 vs. 6%). This confirms, just for expenses, the ratio presented in the bar diagram for higher product titers. Labour and facility costs, however, would normally also be divided between the two parts of the production process, i.e. as allocated costs. At this point the pie chart lacks detail to make a good estimate. There is no information on analytical costs either, which almost certainly are part of the facility described with the pie chart. If included in the total, analytical costs would be in the range between 25 and 35% of the total annual costs with a high labour component. Fill and finish is not mentioned either, nor is warehousing. The allocation between upstream and downstream processing would obviously only need to consider the rest. Myers [9] estimates downstream costs to one third of total costs.

Together with the pie chart, the actual dollar numbers and a few of the background facts were also published [18]. The data relate to a facility for monoclonal antibody production on the US West Coast with, at the time,  $8 \times 12,500$  L fermentation capacity intended for 1200 kg annual antibody production at an estimated annual operational cost of 120 MUSD. The 300,000 sq ft facility was FDA approved in April 2000. The numbers provided for this facility reveal the planned cost per gram of antibody to be 100 USD. Werner [8] calculated

an example with intended production of 250 kg per annum in one 10,000 L fermentor. Moving from a product titer of 0.1–1 g/L and a process yield of 40–70%, the cost to produce 1 g of antibody in this scenario fell from 1500 USD/g to 260 USD/g. As discussed in Section 2.1.2 of Chapter 2, a downstream line always remains significantly underutilized with just one fermentor delivering material to it. In other words, the numbers published by Werner probably represent a case based on existing reality but not fully optimized, whereas the facility example above does assume very good facility utilization. More recently, model calculations have been presented for very large-scale manufacturing of antibodies and high titer cell lines (5 g/L) [19]. The underlying process contains only two chromatography steps and plant utilization as well as all other aspects are well optimized. The cost of making 1 g of antibody under such ideal conditions is being pushed down to as low as 30 USD; the published range is 30–300 USD/g dependent on conditions [6, 20]. One may want to note that these cost calculations do not consider filling, but the cost is no longer very far away from the most ambitious published data [7] or the objective of a US government study aiming for 10 USD [21].

The bar diagram on the right-hand side of Figure 8.1 is an illustration of the sort typically used by accountants to point at a cost that might deserve attention. Some explanatory comments: total upstream costs remain constant, at least at a first glance, when the product titer increases as shown. At the same time, more product needs to be processed downstream, which, at first glance, leads to a proportional increase of scale and cost in the downstream process. As a result, the relation between upstream and downstream costs shifts and downstream costs dominate the picture once titers of 1 g/L are reached or surpassed. This has been published in two reference papers [22, 23]. However, the specific costs<sup>4</sup> calculated per gram of produced drug are going to be constant or declining even for the downstream process (see Section 8.4.2) and the bar diagram does not serve as an acceptable indication for cost increases in the downstream process without further explanation. The specific cost is really the one that counts, especially at the corporate level.

In fact, it is only now when cell-culture titers begin to reach 5 g/L that the productivity of cell culture begins to match what is available from a downstream line equipped with modern resins and an optimized process design (see Chapters 2 and 4). In that case, six to eight fermentors are needed for one downstream line to match its production capability, with significant increases for the costs of an intensified upstream operation. However, by far not all facilities can be operated to the best efficiency and not all processes are using latest technology. Problems to handle the product quantity per batch in an existing, sometimes old downstream process may first surface as a problem of rapid cost increase prognoses from manufacturing. These may in turn be based on the fact that the existing process cannot be linearly scaled with the upstream process output per batch because it reaches practical limits of scalability due to, e.g. specifications of the resins used in it or because the dimensioning of the downstream facility has not taken performance improvements upstream into account. However, such reasons for a cost issue or a scalability issue need to be properly distinguished from an issue of technology capability; they represent two

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<sup>4</sup> Specific costs is used as the term to describe costs per unit, in this case gram of produced product, in contrast to the general term ‘cost’, which does not relate to a constant quantity of product.

very different problems with similarly different solutions. If there is a way to upgrade a facility and use modern technology and process design, there should no longer be a capability or a cost issue.

One other issue with the bar graph (Figure 8.1, right) and its comparison of upstream and downstream costs lies in the fact that they do not include the same content since they are not loaded with a similar level of activities adding up to the costs. Downstream costs generally occur with an external supplier of technology and thus carry the full cost of that company's R&D (20% of revenue), SG&A (24%) and also a net income (25%) legitimately expected by the supply company's owners (percentages taken from Table 8.1). Upstream costs do not include any of these costs or profits as cells are normally entirely internal to the user. The very significant costs of developing cell lines over many years to the level of productivity achieved today are left out from the manufacturing cost presentation and are instead reported as R&D costs. Selling or marketing of the cell line does not occur (no SG&A). And since the cells are delivered from the internal cell bank no profit has to be paid for using the cell (an exception being royalty costs, e.g. for use of cell lines or cell biology/molecular biology methods involved in cell line development). In summary, to make the two cost positions comparable, downstream costs would first have to be lowered with the profits of the supplier and also their SG&A and R&D costs. With this correction, e.g. using Table 8.1 the comparable downstream costs might be up to 80% lower than they appear to the technology user. Next, it would have to be checked whether royalty costs for cell lines are included in manufacturing costs and properly allocated to the upstream costs. Since royalties are normally paid on revenue from the drug product even a small percentage of royalty will increase upstream costs very significantly (e.g. at 1% royalty payments for a 500 MUSD antibody the effect would be 5 MUSD every year). Before both corrections are applied the comparison as in the bar diagram does not compare 'apples and apples' and cannot be considered valid. This may be claimed notwithstanding the fact that the production manager may well be forced by his/her reality to make the comparison anyway. It may help though to put a perspective on cost issues and to make sure that the discussion leads to the best decision, i.e. to avoid the build-up of ill-justified prejudices against technology.

To conclude this discussion, the multitude of numbers and arguments contains a few simple facts: The cost for a gram of Mab in manufacturing is or will soon be down to ~30–100 USD/g for most producers. There is a strong relationship of this specific cost to product titer, process yield and facility utilization (additional aspects include pricing of the drug and coverage of global markets). These are the factors that have reduced the cost most and will continue to do so in the future, probably reaching a limit dictated by quality and safety aspects. Specific costs have gone down everywhere, and not up! At a cost of 30–100 USD/g and a sales value of at least 2000 USD/g (upwards towards several 10,000 USD/g), our brief manufacturing level analysis confirms the finding at the corporate level (Section 8.1.1) that there does not really seem to be an immediate cost problem in manufacturing as long as the scenarios are in line with those discussed here. Predicted trends look even more promising. In fact, the numbers look so good at this level that it seems difficult to imagine how, for instance, a biosimilar product could gain a competitive edge through manufacturing costs. Interestingly, biosimilars are often addressed with previous generation, relatively high cost processes in order to be 'as similar as possible' to an

original product. However, the reduction in development times including clinical trials (pending decisions by regulatory bodies), as well as a potential acceptance of lower profit margins by companies marketing biosimilars will put pressure on innovator companies to strive for the most cost-effective processes possible. Since everyone can move to low(er) cost locations, the location aspect does not really provide for a permanent competitive advantage for anyone. Location can be a temporary threat, however, for companies who do not have the flexibility of moving parts of their operations.

8.1.3 Costs as seen with an interesting novel technology in mind

Economics sometimes do not give the best rationale for selecting a new technology. A more extensive analysis is required to make a decision for or against a new technology. We will present the economic benefits of novel membrane-based chromatography devices to illustrate this point [24].

Membrane-based chromatography has been proposed by a number of authors for use in a post-Protein A polishing step, in this case, by applying anion exchange in flow-through mode to remove trace impurities and to support virus clearance [25, 26]. Technically, the established alternative is the use of an anion exchange chromatography column in the same position in the purification train and in the operational mode, i.e. flow-through. Both options do the functional job well. Reference [24] compares them with scientific rigour and in great detail, and arrives at the economical conclusion summarized and amended by us in Table 8.2.

The study seemed to indicate an economic advantage for the single-use approach using membrane adsorbers. Despite the eight times higher raw material costs over the calculated lifespan of 10 years, the buffer costs for cleaning and equilibration are so much lower that the membrane-based single-use device would appear to offer a long-term cost benefit. A specific benefit has correctly been included for the reduced efforts for developing and validating cleaning methods for the membrane, and the possibility to re-use the Q resin.

However, the Q resin used in the study is a material developed more than 20 years ago. While this is relevant to the extent that this resin is still in use in many existing processes,

Table 8.2

Two different economical comparisons looking at the same process; Ref. [24] looked at the Q membrane and the Q resin marketed since 1985

[USD]	Q membrane single-use	Q resin (1985) re-use, 100 cycles	Q resin (2005) re-use, 100 cycles
Cost of use (10 years)	4,722,134	6,109,200	4,320,083
Raw material portion	3,600,000	440,000	648,000
		Linear flow: 120 cm/h	Linear flow: 450 cm/h
		Capacity: 76.5 g/L	Capacity: 140 g/L

*Note:* We added data for a resin marketed since 2005 for comparison. All parameters used in Ref. [24] remained constant apart from linear flow and capacity, which are higher for the novel resin. Relevant comparisons must use current alternatives.



it would not be relevant today. We have therefore amended Table 8.2 with data from a modern Q resin, which, dependent on load challenge, allows up to five times higher capacity and four to five times higher linear flow under these operational conditions (Mabs, flow-through mode) [27]. In order to make a relevant amendment to the study all other conditions were left constant, e.g. four column fillings have been assumed and the benefits from lower development and validation efforts were maintained for the membrane device. In fact, the break even point with small advantage for the new resin for cost of use over 10 years is already reached at a capacity of 140 g/L or two times higher than used in [24], i.e. under conservative assumptions for the resin-based process variant.

It becomes obvious that the economics can be highly dependent on what is actually compared. In this case the message from development to management might have been different, if current technology had been used for both alternatives. It is, indeed, a well-known fact that a single-use approach is difficult to justify economically in a production scenario at very large scale and with regular production, i.e. many batches per year. Re-use is a powerful cost-reduction concept in regular large-scale manufacturing [28]. Whether or not the additional efforts for occasional column packing, cleaning between batches and the validation of re-use is an acceptable or even desirable effort in view of the potential savings will be very much dependent on the case. On the one hand, operation costs may be lower if cleaning and sanitization can be cut out. On the other hand, conversion to single-use places a different burden on the manufacturer in terms of the risk for inventory management and staying in control of consistency and quality over time. Material costs clearly go up as discussed in Ref. [24] (see extract of data in Table 8.2). Activities related to cleaning and cleaning validation, or buffer costs with savings potential have their strongest impact on campaign production with low batch numbers.

## 8.2 LEAN MANUFACTURING, REMOVAL OF UNPRODUCTIVE ACTIVITIES

One of the largely unsolved but very relevant issues in discussions about cost is how one can assess if costs can be justified by the value that is generated from the expense. Biased arguments can be heard when costs are discussed, often as a consequence of preferences for technology. Delegation and compartmentalization of tasks that include cost management may also lead to bias due to a lack of transparency and absence of a complete picture.

A more transparent approach could be very useful. In fact, we suggest that the risk-assessment methods and process-characterization strategies already in use to understand and mitigate the key risks to critical quality attributes of the product and key performance attributes of the process would provide useful tools upon which to base a value judgment. Costs associated with mitigation of risks identified as high could be considered as generating high value. Activities that do not contribute to risk mitigation or are not critical to process performance would automatically be of low value and associated costs would be prioritized for reduction or deletion.

An overriding approach to address these issues could be a LEAN Six Sigma program [29]. With common sense and internal agreement, but without turning the exercise into a scientific problem, one could assign values to certain issues to be dealt with during



**Table 8.3**

Value grid for a typical monoclonal antibody downstream process; values entered here are estimates and serve as an example of the concept for assigning value to different steps and activities in the process

Downstream process	Recovery	Protein A	Virus 1	Polishing 1	Polishing 2	Virus 2	Control (%)
Bulk impurity removal	0.2	15.8	0.0	0.0	0.0	0.0	16.1
HCP removal	1.0	4.2	0.0	0.6	0.6	0.0	6.5
Aggregate removal	0.0	0.0	0.0	2.9	6.8	0.0	9.7
DNA removal	1.3	1.9	0.0	2.9	0.3	0.0	6.5
Virus clearance	0.0	4.0	3.2	4.0	0.0	4.8	16.1
General robustness	1.9	13.5	0.0	1.9	1.9	0.0	19.4
Platform impact	0.0	4.5	0.0	0.3	0.3	1.3	6.5
Time to 1st in human	0.0	15.5	0.0	1.9	1.9	0.0	19.4
Relative value (%)	4	60	3	15	12	6	100

*Note:* Some value tags will vary dependent on the situation, e.g. virus clearance value will vary with the test virus in question. Each team designing a process would have to prepare their own version of this grid. Not all aspects are important in all cases.

the downstream process and create an appropriately detailed value grid (Table 8.3) for the different steps in the process.

Each step and/or activity in the process would receive a sum of value tags for contribution to the different issues, including a robustness and time-to-market value. The relative distribution of value generation would become clearly visible and cost discussions would have a basis transparent to everyone. Different groups going through this exercise could come to a different result. That is not a problem. The discussion would have a fact-based foundation and could not be driven by too many qualitative or personal aspects. Promoters or suppliers of alternative technology could provide their version as input. Still the team establishing a process design would have to develop a consensus based on only those facts accepted by the team and management could influence the consensus also based on the facts developed for their decision.

The numbers in Table 8.3 are based on normalized, weighted importance of the different issues: bulk impurity removal, virus clearance, overall robustness (see Chapter 3 on process design) and time to 1st in human have been given the highest weight (see control column). Reasons for the weighting can be summarized as follows: bulk impurity removal ensures purity, contributes to absence of immunogenicity and product stability all of them being key quality attributes. Virus clearance is a fundamental safety issue. Time to 1st in human has a strong impact on the payback from the development project. However, there is a certain level of judgment included as well, which needs to be agreed upon by the team

**Table 8.4**

Activities in a downstream process that may be of low value

Unproductive activity	Potential solution
Diluting, concentrating or re-buffering the (intermediate) product	Perform in-line or avoid through modification of the main steps
Intermediate pooling between steps	Move product directly between steps, consider (semi-) continuous processing modes
Buffer preparation, storage of dilute buffers	Prepare buffers in-line from concentrates
Column packing, preparation of fresh resin	Use automated devices, latest packing technology, consider disposable devices
Column equilibration, cleaning	Use of disposable or ready-to-process devices, use of concentrated buffers in equilibration
Hold times for testing	Use of PAT enabling sensors, direct probes for product and impurities
Change over between products in multi-product facility	Consider use of disposables, see column packing solution, optimize facility for higher flexibility (see Section 8.4.1)

performing the evaluation or be decided by management. The sum of values adds up to 100% to allow a simple comparison of step contribution.

Most processes contain activities that do not add significant, or even any, value to the product being manufactured. These activities may be considered necessary evils to circumvent technical difficulties, or they may be compromises for routes considered too expensive or unavailable at the time of the facility installation.

Each of the unproductive activities listed in Table 8.4 takes time in the downstream process. Some of the tasks may be a risk to product quality, e.g. hold times under conditions that are not optimal for product stability. Therefore, eliminating or reducing the time spent on these activities has the potential to shorten batch time and increase the number of batches that can be run in the facility, i.e. the amount of product that can be produced. As we will discuss in more detail later in this chapter, this is one of the best opportunities for providing real economical gains.

### 8.3 COST MODEL: MONOCLONAL ANTIBODY DOWNSTREAM PROCESS

Since the literature will not provide very detailed, complete or specifically relevant economic information for your particular situation (this text too can only serve as guidance), it may be useful to have tools that can calculate process scenarios for your specific design or choice of options [1]. In order to complement the review presented here we used a model process-design developed in our labs. Data generated with that process were fed into commercially available economical calculation software.

The process simulations were done using SuperPro Designer® software. SuperPro Designer is process-simulation software provided by Intelligen Inc. [30]. It is a modelling

tool that enables analysis of integrated processes by calculating material and energy balances, process scheduling and resource tracking, process economy, throughput analysis and de-bottlenecking, environmental calculations, etc. The results obtained such as capital investment, manufacturing cost, environmental impact, etc., can be used to directly compare different process designs. In addition the results can be used to estimate viability of purification process designs in comparison to set objectives, impact of new technologies and improvement of individual process steps. However, when such tool is used and output from the model is discussed, it is very important to be aware of all conditions entered into the model and the constraints under which the calculations are carried out. Without caution, the use of such advanced tools can be very misleading.

For the model process, calculations were performed on one batch of 10,000 L, containing 50 kg Mab, i.e. a titer of 5 g/L unless an analysis for different titers was performed. This is a titer not yet found in any commercial Mab processes, although it is predicted that such expression levels will soon be the norm in mammalian cell-culture systems (see Chapter 2). Apart from this, other assumptions are on the conservative side, e.g. for resin capacity, flow rates used and life-time achievable.

The following description of selected features of the model we used shall serve as illustration of the issues to be aware of when using software like this. Our purpose was to learn what the best result achievable with a given technical solution would be: the model calculates resin costs always on the basis of the assumption that the maximum lifetime of the resin can be used. For example, if costs for one batch were calculated with four processing cycles and the resin lifetime was assumed to be 200 cycles, the batch cost for the resin would be adjusted to 1/50 of the total purchase cost for the column filling at list price. Columns were sized as follows: the model automatically adjusted the column diameter to the column volume capable to handle the loaded product quantity. Column bed heights were held to 20 cm except when the effect of the column bed height was examined.

For other calculations than just one batch, it was assumed that the next batch could be loaded immediately once one batch was processed, i.e. automatically producing the best quantity. This is not always entirely in line with reality, where cell-culture and downstream process are not optimally tuned, and downtimes or hold times between batches may have to be accepted due to the practicalities of production routines and the particular process. Therefore, the model calculations may need adjustment to match relevant circumstances. SuperPro Designer can accomplish this adjustment, if the information is available.

SuperPro Designer provides built-in cost estimates for pricing of all equipment types. We have not included equipment costs in our calculations for this book. In many cases, equipment is already available or even written off and we wanted to have a more clear picture of process-design related savings. For the process discussed here, the cost for buffers was set at 2.0 USD/L.

SuperPro Designer defines labour cost so that it includes all the labour-dependent operating cost except the costs for the laboratory space, quality control and quality assurance. Labour cost in our model is defined at 69 USD/h. Many companies routinely and conveniently include the depreciation for laboratories in the labour costs and thus arrive at much higher hourly rates, such as 200 USD/h. We did not consider limited availability

of labour in our model. We assumed that four operators will be needed for the downstream process.

In the capability section of Chapter 2, it is pointed out that the use of current technology is essential to make the cost scenarios relevant. The process referred to in most of the examples use recently introduced chromatography resins and yields results as described in Table 8.6. The process step yields taken from Table 8.5 require careful optimization of the steps and may vary with the challenge from the specific production material to be processed.

Table 8.6 only lists some details on the chromatography steps. However the sum of process time, production cost and process yield reflects the whole downstream process including recovery and purification with the steps listed in Table 8.5.

**Table 8.5**

Model process steps with assumed yields for the different unit operations

Step	Process step yield (%)
Clarification	95
Depth filtration (Cuno)	100
Protein A (MabSelect SuRe™)	97
Virus (pH) inactivation	100
Cation exchange (Capto® S)	92
Diafilter	100
Anion exchange (Capto® Q)	98
Virus filtration	99
Formulation	97
Bioburden reduction	100
Overall yield (%)	79
mAb produced (kg)	39.4 kg starting from 50 kg in cell culture

**Table 8.6**

Purification steps in the reference process used in our model calculations

Chromatography resin	Load (g/L)	CV (L)	Yield (%)	Lifetime (cycles)	Cycles/batch	Total time (h)	Production cost (USD/kg)	Process yield (%)
MabSelect SuRe™	28	423	97	200	4			
Capto® S	96	478	92	50	1	26 <sup>a</sup>	12,138 <sup>a</sup>	79 <sup>a</sup>
Capto® Q	160	261	98	50	1			

<sup>a</sup>The summary of time, costs and yield in the framed box includes all downstream process steps shown in Table 8.5, not just the three chromatography steps listed here. If automated and optimized, the process can be operated in 2 days with a two-shift scheme as required for most of the scenarios in Chapter 2.

## 8.4 COST IMPROVEMENT OPTIONS

In this section we will provide a small library of options to reduce costs related to manufacturing, namely the specific cost to produce a gram of monoclonal antibody. We will mainly apply the model process described in the previous chapter, but in some cases other model calculations or published data will be used.

Depending on your starting point, you will find that some or all of this is highly relevant, e.g. because you can design the process from a blank page and use all options to create an effective manufacturing solution. Sometimes only a few aspects may be useful, e.g. because you have an existing process in an existing facility and the degree of freedom for changes is limited, or because you have already improved your previous generation process gradually over time.

In any case, here you will find all the high-gain strategies to reduce costs associated with chromatography. We will also discuss and calculate one or two low-gain strategies. In all cases, this will help to decrease costs and, at the same time, prevent subsequent problems such as lack of robustness.

### 8.4.1 Facility utilization

The new facility described briefly in Section 8.1.2 was completed in the year 2000 and had an investment cost of 250 MUSD. It was built with a fermentation capacity of  $8 \times 12,500$  L. As a second example, Genentech recently acquired an already built 470,000 sq ft fully functional facility with 90,000 L fermentation capacity from Biogen Idec for a price of 407 MUSD. These two examples prove that production capacity is a strategic asset and it is expensive. On an annual basis, such costs will be recorded as depreciation, i.e. as part of CoS. Facilities are typically depreciated over a period of 20 years. Much of the capital equipment in the facility would typically be depreciated over 5 years.

To get an initial feeling for the annual cost contribution from the facility investment (not differentiating between facility and installed equipment), we can use the double-declining balance method (see MS EXCEL) and a remaining value of 1 MUSD ('salvage value') after 20 years. The 250 MUSD facility has an annual cost from depreciation that is reduced from 25 to 15 MUSD during the first 5 years, and it is still 10 MUSD in the tenth year. This means that an idle facility is a very expensive thing to have on the profit and loss sheet, regardless of the method of calculation. More advanced methods may be used for depreciation, and these methods may differ between countries, depend on the drug product and the product's occupancy share of the facility.

In real life, during the plant start-up, as the demand rises over a few years to its eventual peak, the CoS will start high and then drop. Modelling of CoS is often at the steady-state of maximum demand, and in a plant that is the perfect size for this demand, a situation that in reality hardly happens. Werner has described the effect of facility utilization by stating that the gram cost for antibodies might be as high as 1500 USD/g at 20% utilization and might go down to 300 USD/g at 100% utilization [31].

The dilemma of knowing how much production is needed to optimize value generation for a company has been quantified by Levine [32]: excess capacity in a facility utilized to just 50% can cost 2–3 MUSD per month. Inadequate capacity that results in loss of sales can lead to operating profit losses of as much as 40–45 MUSD per month. In fact, as long as the manufactured product can be sold, management is much more likely to prioritize prevention of profit losses than reduction of costs from production. This is in line with the earlier finding that CoS ratios are favourable in this industry.

In the past, with low product titers, many of the larger volume antibody drugs required dedicated facilities, and facility utilization often managed by adapting the investment to the needs that were known at the time of the decision to build. The more products and the shorter production campaigns, the higher the risk that facility capacity is lost in change-over times and lack of flexibility; With much higher titers and often numerous antibodies in the pipeline, we see that the utilization problem is changing significantly from an expensive ‘guessing of how much will be needed’ to a ‘development of the best strategy of creating flexibility’ in multi-product plants.

During process design and optimization, what can you do to support the ‘flexibility issue’? First and foremost, it seems important to develop technology platforms that are ‘re-usable’ for different projects and protein drugs of the same category. We present a brief summary and some economics aspects of this strategy in Section 8.5, and we refer you to the process-design discussion in Chapter 3 for more technical aspects.

Details for some of the following concepts will be given later in this chapter: all of them support flexibility in a very general sense. The latest downstream processing technology should be used in order to enable a performance level with one batch every second day. Next, one may want to consider looking into shorter batch times for the cell culture too, instead of only focusing on high titers. Finally, it may be advisable to consider the opportunities of largely disposable processing and to adapt the process design accordingly. Disposable production, at least in principle, allows reduced change-over time and would help to maximize the potential batch numbers in a multi-product facility. From today’s perspective the key feasibility aspects to deal with seem to be scale of operation and batch frequency. A preliminary estimate is that if production needs allow cell-culture batches not higher than 1000 L, then entirely disposable manufacturing could well be a viable option. In order to support a management decision in favour of disposable processing, the risk balance would have to be presented. There is an underlying assumption with disposable use that batch-to-batch consistency and long-term availability will not be a problem and that the actual disposal will be easy and local regulations will not complicate it.

We have discussed process capability in Chapter 2 and seen that batch sizes of 50 kg are not unrealistic. Such a batch might have a sales value upwards of 100 MUSD. Therefore, any other risk for production shortages, namely risks for batch failure, will also have a very high priority in management considerations and one will most likely be adverse to any such risk when it can reasonably be avoided. The highest risk is in the early phase, in cell culture. One strategy that has been evaluated to reduce the effect of lost batches is to monitor sterility of cell culture with rapid microbial detection methods. In case of a sterility problem, the culture can be terminated and the bioreactor quickly made available for the next batch [33]. The risk of losing a batch is one of the obstacles to implement continuous

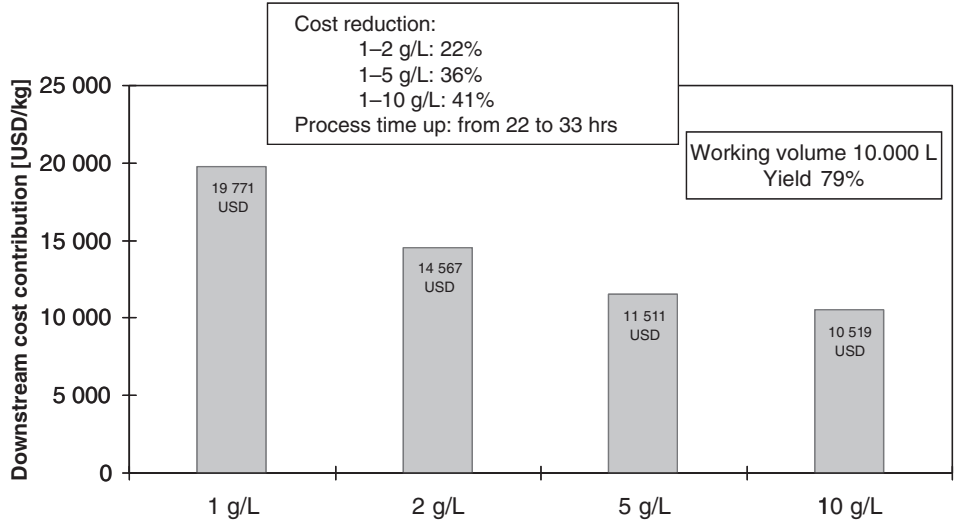
perfusion culture as the upstream process approach of choice. While the fed batch approach used by almost everyone usually includes that the inoculation train could always provide quick replacement of a lost culture batch, a failed continuous culture could not be replaced as easily.

In summary, utilization of the facility to put saleable product onto the market is top priority and by far the most-effective means for managing the specific cost we are looking at in this chapter, i.e. the cost per gram of a therapeutic grade monoclonal antibody. The value of this strategy is considered so high that companies have started to share their production capacity, even at the risk that this means allowing access for a potential competitor to ones own ‘holy grail’, the manufacturing facility [34].

8.4.2 Cell culture: product titer and culture time

The positive effect of product titers from cell culture has been mentioned and referenced several times already. Now, what is left is to confirm this within our model process and calculate the potential savings in the downstream process. Savings upstream are obvious with higher titers.

The process used in Figure 8.2 is the one listed in Table 8.5 with one 10,000 L fermentor and one downstream line. Recovery is achieved with a cascade of filtration steps, Protein A affinity chromatography is used for capture and two ion-exchange steps follow



**Figure 8.2** Effect of cell-culture product titer on specific downstream processing cost calculated per kilogram antibody. As part of the development of product titers from 1 g/L up to and beyond 5 g/L, the specific cost for the downstream process (except facility and equipment as well as QC-related costs) can be reduced by 36%.

for polishing. Virus safety is achieved with acid inactivation and virus filtration. The final step is an ultra/diafiltration step to formulate the final bulk product.

The cost for one batch is calculated at different product titers ranging from 1 g/L, which might even today be considered low, to 10 g/L representing definitely an outlook into the future at this point. Batch size varies from 10 to 100 kg at fermentation level. Overall yield is  $\sim 79\%$ . Modern resins are used for all titers. Column sizes and batch-cycling routines are automatically adjusted to batch size.

The trend displayed in Figure 8.2 represents the following opportunity: when total product need increases, e.g. from 100 to 500 kg, one option to accommodate the need is improving a first generation product titer from 1 to 5 g/L. This would reduce downstream processing costs per kilogram product by approximately 36% for the one fermentor scenario. Ten batches would have to be run, which is perfectly possible.

In savings per kilogram produced antibody, the greatest effect in going from 1 to 5 g/L in our model was coming from buffers and labour. Resins and filters need to be scaled roughly proportionally to the product quantity and did not contribute that much.

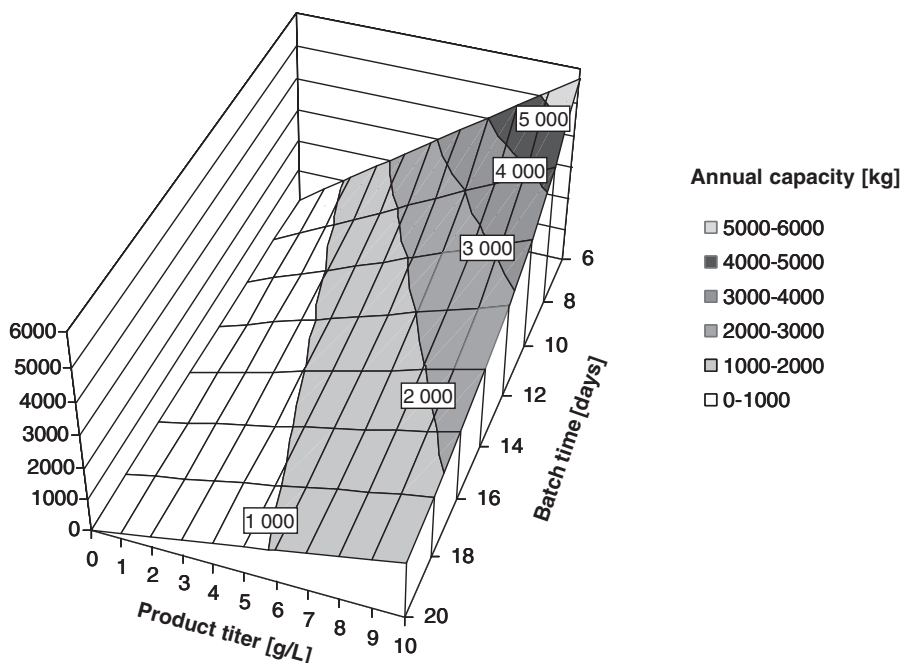
Since the model adjusts batch costs for the resins to their total lifetime, the cost savings can be read directly from the graph even if more batches than just one are run: one kilogram produced at a titer of 1 g/L costs 19,771 USD, the same quantity costs 11,511 USD at 5 g/L. Recalculated for the annual quantity of 500 kg, the savings amount to 4.1 MUSD from the downstream process as included in the model (see Section 8.3). Savings would be even greater, if moving from old to modern resins at the same time (see Section 8.4.4).

Turner [35] presented an overview of Mab cell-culture processes from different companies with regard to their product titers. Interestingly, all processes with titers higher than 3 g/L had cell-culture batch times between 14 and 20 days. At a given titer, it is obvious that a reduced cell-culture batch time could increase the productivity of the facility. Werner [31] has quantified this effect in one of his model scenarios: 260 kg antibody per year was the baseline production rate at a cell-culture batch time of 14 days.

With 10 days and the same titer of 1.2 g/L, the production rate went up to 327 kg (+25%) and the specific cost was reduced from 378 to 300 USD/g (−20%).

In general, longer batch time at cell-culture level reduces flexibility in the facility and increases risk. In effect, it will be more difficult to maximize the annual capacity or utilize a given capacity with very long batch times. Figure 8.3 really asks the question: is it always best to optimize for titer when it is really economics and flexibility that count? Currently, the public discussion seems to focus entirely on product titer. Lower product titer and shorter batch time may offer several advantages that include a higher degree of flexibility and potentially fewer issues related to product impurities, such as aggregates or degradation of the biologically active product by host cell enzymes at late-stage fermentation. Both effects result in difficult to clear product-related impurities and a risk for antigenic effects. However, costs from the upstream process would increase, since more culture media would be needed for the same product quantity. In order to support a management decision in favour of such alternative cell-culture strategy, one disadvantage of 'large batch numbers' would need to be addressed: higher analytical costs, proportional to the number of batches.



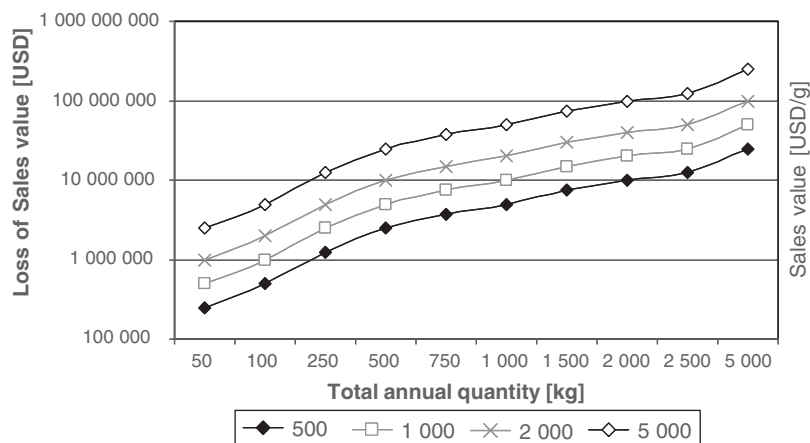


**Figure 8.3** Annual productivity of one 10,000 L fermentor as a function of product titer and batch time. One fermentor could—in a model calculation—produce 1000 kg per annum at a titer of 5.8 g/L and a 20-day batch time, at a titer of 3.5 g/L and 12 days batch time or at a titer of 1.7 g/L and 6 days batch time. 2000 kg are possible from titers of 3.5 g and 6 days batch time up to 10 g/L and 17 days. Higher annual rates from just one fermentor require titers above 5 g/L and shorter batch time than 12 days.

### 8.4.3 Process yield

In 1990, J. Asenjo edited a book on Separation Processes in Biotechnology containing a review on downstream process economics by Datar and Rosén [36]. This reference is now one of the classic papers in economics for biopharmaceuticals manufacturing. They stated: ‘Of primary concern is yield’. This observation is still valid, even though many strategies have been implemented for optimizing yields in the meantime. The model process used in the reference was designed for 1000 kg of annual insulin production. The specific cost they arrived at was 25 USD/g at a process yield as low as 28% (*E. coli* fermentation with 17 downstream steps, of which only two were chromatography steps). For most of today’s high-value protein drugs even a process yield of 50% would be considered low, and a good yield would probably be considered to be 70–80%.

Whatever the reason for an insufficient production rate, for a saleable product it is really the value of lost sales that has a negative financial impact on a company. The Enbrel® experience confirms the message about lost sales in Figure 8.4. Due to very positive market demand for Enbrel, additional facilities had to be built or rented to compensate for



**Figure 8.4** Loss of saleable product per % of process yield. For a product such as insulin with a sales value around 500 USD/g, 1% less process yield may mean 5 MUSD of lost sales if the annual quantity is 1 ton. For a high-value antibody at 5000 USD/g with the same annual need, 1% less yield may result in 50 MUSD of lost sales every year.

insufficient productivity of the first-generation process [34]. While they were not operational yet, the product could not produce revenue to its full potential. A lost sale from a lost percent of yield has exactly the same consequences.

The effect of process yield improvements on economy is very significant and second only to the gains realized from improved facility utilization. In fact, process yield may be viewed as a special aspect of facility utilization. For our model process, we have assumed an overall yield of good 79% for the downstream sequence. For an antibody with annual market needs of 1.5 tons this model requires production of approximately 1.9 tons with a 0.4 ton-surplus production in cell culture that is lost ‘between the cracks’ of the manufacturing systems. In comparison, at a process yield of 20% less, the cell-culture surplus would be an impressive 1.0 ton of antibody, which the facility and hardware installation would need to be over-dimensioned for in the initial steps before it gets lost in the process. This calculation illustrates the span of financial gains between non-optimized processes and a fully optimized process. Yield improvement gives process developers probably the best opportunity to win the hero’s gold medal in economics.

From the classic insulin example summarized above, one may conclude that the key strategy to improve process yield lies in the reduction of the number of process steps. Over the past 20 years, the average industrial purification process has lost one chromatography step (down to three steps from four) and several ‘conditioning steps’, such as UF/DF steps. This is, among other aspects, also due to the significant increase in the proportion of antibodies in the mix of processes evaluated, a statistical impact. Almost all of these Mab processes use an affinity step for capture (Protein A), which is very powerful in addressing a number of critical downstream issues in one single step.

In the same period of 20 years the downstream process became less complex through upstream process improvements: At the end of the eighties, the product at ~100 mg/L was

contaminated by a protein load at  $\sim 20$  g/L (e.g. from foetal calf serum). In the nineties product titers increased and culture media contained only a few defined proteins at maybe 0.5 g/L (typically BSA, Transferin, Insulin). Today chemically defined, protein-free culture media are used and the product concentration reaches several grams per liter. This development represents a radical change of complexity at the start of the purification process and is clearly enabling the achievement of higher yields too. Today, a clarified cell-culture supernatant prior to chromatography is already 90% pure as compared to maybe 20% pure when titers were around 1 g/L.

#### 8.4.4 Use of the latest resin technology

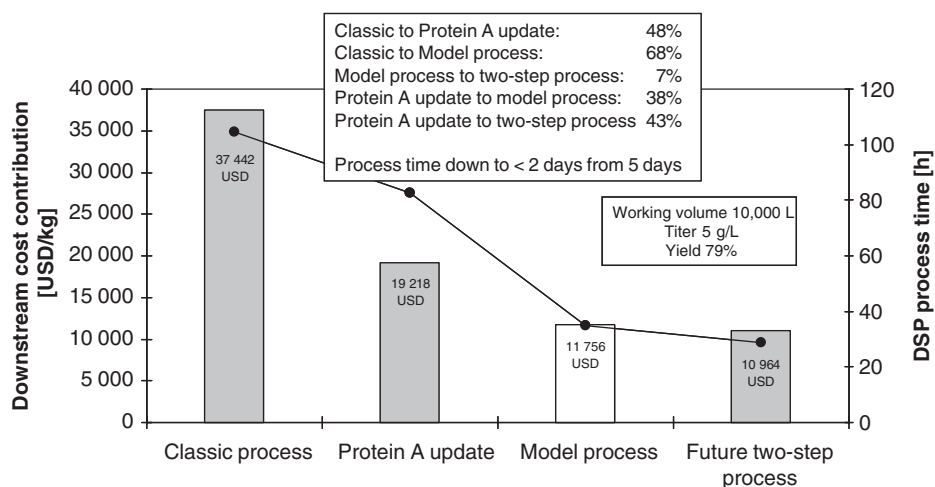
We stated in the process capability discussion (Chapter 2) that we applied specifications of the latest generation of chromatography products to estimate the capability of different approaches to the downstream process. We have also commented on the use of old technology in the educational excursion (Section 8.1.3). Many processes for drug products introduced to the market during the past two decades are, however, based on downstream technology that was developed and introduced 15–30 years ago. As an example, Sepharose® Fast Flow resins had assumed the role of wide-spread ‘industry workhorses’ from the early 1990s onwards. Anion and cation exchangers on that base matrix were introduced in 1985, and Protein A Sepharose Fast Flow resins were introduced in 1989. For the purpose of an economics discussion, these are still relevant to calculate possible cost reductions as part of the development of a second-generation process. However, for a process designed today, the use of modern replacements of these resins seems obligatory.

Figure 8.5 contains a ‘classic process’, which uses the Sepharose Fast Flow generation of resins in all chromatography steps (everything else is as in the model process (Table 8.5)). Faced with the update such first-generation process for monoclonal antibodies, one has the option to focus on just the cost-intensive Protein A capture step or to implement a full renovation of the process.

The ‘Protein A update’ in Figure 8.5 represents the former alternative: the Sepharose Fast Flow resin is replaced with MabSelect®, a modern resin. Even this limited upgrade can reduce annual costs by 9.1 MUSD in a 500 kg annual production scenario. The ‘model process’ in Figure 8.5 represents the latter option, a full upgrade to latest resin technology as listed in Table 8.5 and would reduce downstream costs for the same scenario by 12.8 MUSD annually.

When comparing improved chromatography resins that are available today with those used for first-generation products, the main factors contributing to the favourable economic effect include reduced process time through higher flow rates and increased capacity, i.e. more batches in the same timeframe, lower investments in resins and columns and lower buffer consumption. The lifetime, in particular for the Protein A resin, has also been improved (Figure 8.5). The related stability improvement of the chromatography resin against sodium hydroxide contributes through increased life time as well as through significantly reduced costs for the cleaning chemicals. Batch time in the model process is reduced to 2 days as required by current processes with high titer cell culture (see Chapter 2).

More recently, two different routes to purification of Mabs using just two chromatography steps have been presented. One uses a special approach to operate an anion



**Figure 8.5** Cost and batch time improvements resulting from the use of updated chromatography resins and the cost effect from reduction in the number of steps. The ‘classic process’ uses resins of the Sepharose Fast Flow family. The ‘Protein A update’ replaces the capture step with MabSelect. The ‘model process’ contains only recent resins (see Table 8.5). The ‘two-step process’ uses MabSelect SuRe™ and Capto® adhere, a novel multi-modal resin. The bars represent cost, the line plots process time. All processes run one batch at 10,000 L and 5 g/L titer.

exchanger [37] and the other one uses a novel multi-modal anion exchange ligand [27] to produce the required quality with one step less than the classic three-step scheme. The latter option is included in Figure 8.5 as the ‘future two-step process’. Yield increase, high productivity, simplicity of operation, reduced buffer consumption and product pool testing are among the expected advantages of this improvement once implemented in manufacturing. Savings in our model amount to 0.4 MUSD per 500 kg annual production. Process time is not reduced much further by this variant as the intermediate product is more dilute when processed by the last step (ultrafiltration/diafiltration). Note that equipment savings are not included in the model.

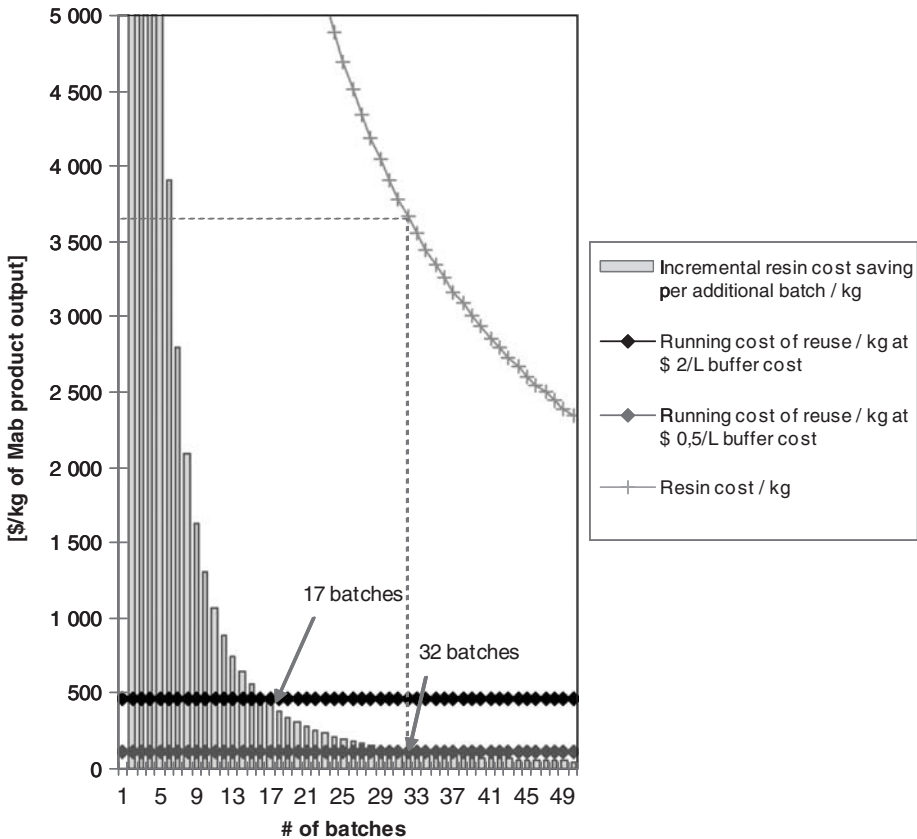
#### 8.4.5 Re-use strategies

Since the mid 1980s, chromatography resins with stability against sodium hydroxide have become the standard for the industry and have enabled cleaning-in-place (CIP) and re-use strategies to reduce costs. One may claim that affinity chromatography using Protein A resins only received its breakthrough because (a) prices for these products came down at least tenfold from small laboratory pack prices when large-scale use first boosted consumption and introduced a scale benefit to resin manufacturers and (b) because it could be demonstrated early that many chromatography runs were indeed possible even with a relatively sensitive proteinaceous ligand [38]. Today, there is a Protein A resin on the market, which has been specifically designed for sodium hydroxide stability (MabSelect SuRe™). The continued development of Protein A resins and its declining cost of use due

to these two main factors were a prerequisite for introducing affinity chromatography technology into commercially viable Mab processes and for enabling platform approaches with a specific total production cost of between 30 and 100 USD/g.

Re-use works in favour of process economics because the cost of a resin can be distributed over a larger quantity of protein drug product. The cost contribution of re-used Protein A resins has been reported to be between 1 and 3 USD/g antibody [Ref. 19 and Section 8.1.2] dependent, e.g. on scale of operation and product titer.

Figure 8.6 shows the decline of resin cost per kilogram of produced antibody with increasing number of batches (lifetime) with one column filling at list price and binding capacity as used in the model process. The bars in Figure 8.6 illustrate the incremental



**Figure 8.6** Simplified comparison: re-use cost benefits and buffer costs for re-use. The curved line plots MabSelect Sure resin costs per kilogram of monoclonal antibody product. The bars plot the incremental saving per kilogram of Mab for each additional batch. The straight lines illustrate specific buffer costs for cleaning and regeneration at two different buffer cost levels (2 and 0.5 USD/L). The dotted lines mark the specific cost of the Protein A resin at 32 batches.

savings each time one runs an additional batch. Incremental savings are smaller the more batches have been run already. However, there is also a cost to re-use, namely the cost of validating more re-use cycles [24] and a low but incrementally increasing risk of losing a batch. Therefore, there is most likely a limit where re-use no longer makes economical sense. Figure 8.6 illustrates that with a simplified approach using the break-even between buffer costs for cleaning and regeneration on the one hand (the straight lines in the graph) and incremental cost savings for re-use as an indicator point where further re-use should be carefully evaluated.

It is in fact likely that longer re-use makes financial sense, because the cost for the money required for the early upfront payment for new resin as well as costs for more frequent packing or even facility downtime suggest longer use. However, a more advanced financial calculation of re-use was performed by D. Lewis-Sandy (using NPV<sup>5</sup>) suggesting that a financial optimum of re-use for Protein A resins is found at ~30 batches [28] in good agreement with the simplified estimate in Figure 8.6.

Many companies use batch cycling to reduce the volume of the Protein A column. Four cycles per batch is not an unusual practice. Figure 8.6 suggests a lifetime of 120 cycles in the case (cross-over between cleaning costs and incremental savings). At a lifetime of 200 cycles, this would mean that the resin is used for 50 batches. Even longer lifetime has been reported with optimized CIP methods [39], i.e. the assumptions in Figure 8.6 seem entirely feasible.

#### 8.4.6 Buffer consumption and cleaning buffers

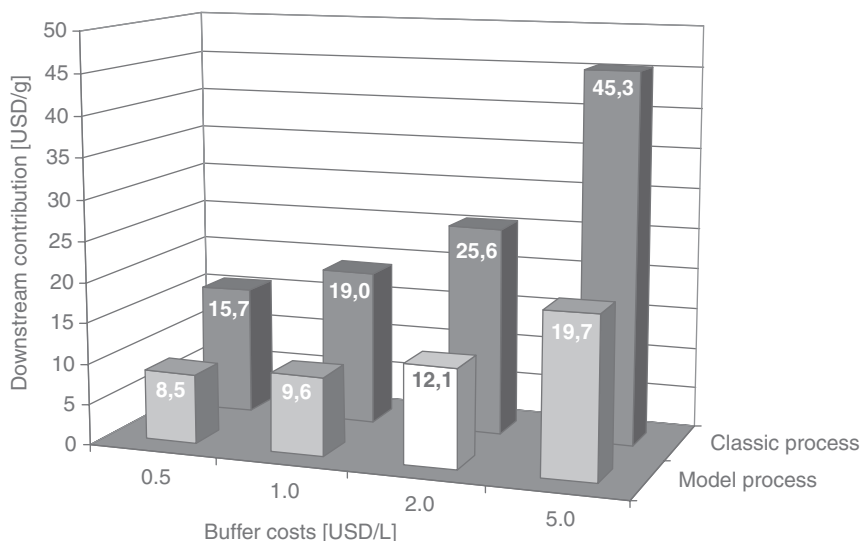
During the research for this book it became obvious that there is no true consensus in the industry about buffer costs, i.e. about the seemingly simple question: what does it cost to prepare a liter of typical buffer for use in downstream processing? Without digging deeper into the ‘why’, we have received numbers varying between 0.3 and 5 USD/L when asking collaboration partners or consultants. Zhou and Tressel [24] have found an average of 3.0–3.3 USD/L for equilibration, wash and storage buffers at contract manufacturing organizations or small and mid-sized biotech firms, and 0.3–0.5 USD for large biotech firms. Accounting practice in different companies, business practice in CMOs and a certain scale effect may provide explanations for the huge differences.

In our standard model, we used 2 USD/L as a compromise between the widely varying values. In Figures 8.6 and 8.7 we have varied buffer costs to demonstrate the point: the costs of buffers are key in downstream processing and should be carefully managed and optimized. The white bar in Figure 8.7 marks the conditions with our model process.

Reducing buffer consumption in the chromatography steps in our model by one-third, we see a specific cost reduction of 6%, i.e. at 2 USD/L. In the 500 kg annual production scenario, this would equal a saving of 400 KUSD every year.

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<sup>5</sup> NPV—net present value: an approach used in capital budgeting. NPV is used to investigate the profitability of an investment or project. NPV compares the value of a dollar today versus the value of the same dollar in the future after taking inflation and return into account. NPV analysis is sensitive to the reliability of future cash inflow predictions from the project.



**Figure 8.7** Effect from buffer costs on downstream process costs; the classic Mab downstream process and the current process (see Figure 8.5) are plotted with a range of different buffer costs provided from industry contacts and a few references. It is important to agree on the buffer cost for accurate comparisons of economy. The white bar marks the standard conditions in our model calculation. The effect from buffer costs is significant.

There are a few possibilities to reduce buffer volumes as part of a good process-design practice. To name just two: for equilibration, one may first use a buffer concentrate and then the actual equilibration buffer. Wolk et al. [17] reported a reduction from 15–20 column volumes down to just 6 column volumes of equilibration buffer with this approach. For elution, one may design the gradient to concentrate all unwanted impurities into waste and not use extra buffer to elute them separately.

Buffer costs in our model include buffer handling as well. Therefore, all means to reduce the handling costs would also have an effect. This could include use of concentrated buffers to reduce storage needs.

Protein A chromatography in its early days used (corrosive) 6 M Guanidine Hydrochloride (GuHCl) as the recommended cleaning agent. For the latest generation of sodium hydroxide stable resin, clearly NaOH would be preferred for cost and handling reasons. While 0.1 M NaOH would cost, e.g. 0.5 USD/L, the 6 M GuHCl solution costs above 50 USD/L and additionally incurs the same costs again for waste disposal. There are also other alternative solutions for cleaning of Protein A resin, that are cheaper than GuHCl, but one may need to scan the patent literature to be sure they can be used freely.

#### 8.4.7 Exchange of one step against a cheaper one

Once everything has been optimized and set within a process, one may still get the question whether it would be possible to exchange a whole piece of the process, e.g. one step

for another one with a cheaper raw material or one with a lower cost due to longer-term use capability. If the process performs to the satisfaction of manufacturing, quality assurance, etc. this question would typically first be based on an accountant's analysis: This is the most expensive step; could we not do something about it? As discussed already (Section 8.1.2), such simplified analysis can be misleading. However, it has its clear and obvious merits in questioning major expenses in principle. In a typical Mab downstream process, it is the Protein A step as well as the viral filtration step that are likely to catch the accountant's attention. The value grid in Table 8.3 suggests that, indeed, the virus filter may be the most costly investment in the whole downstream process relative to the value generated.

We will use the example of Protein A in a monoclonal antibody downstream process as an example to look a bit further into such a case. Technically, Protein A can be exchanged, e.g. by using a cation exchanger as the capture step and acceptable product quality can still be achieved. There is at least one known case of a major marketed monoclonal antibody, Humira®, where this concept has been implemented in manufacturing [40]. List price purchasing costs per liter differ by a factor of 8–10 between Protein A resins and ion exchangers (more toward the lower end of that range for the latest generation products). Ion exchangers tend to have roughly twice the useful capacity of Protein A resins. So why not just do it?

There is no very simple and straightforward answer to this question. The fact is that hardly anyone replaces Protein A with ion exchangers, not even in the several hundred projects currently in clinical trials. The main elements of the complex answer, in an abbreviated fashion, include technology platform value gains, risk management and general acceptance considerations and the size of the potential gains versus possible value losses or robustness issues.

First, look at a somewhat more accurate calculation of possible gains: there are references for a specific cost of the Protein A resin between 1 and 3 USD/g of antibody [20], however, relevant for very large scale, well-optimized processes and regular production with high batch frequency. A typical ion exchanger would range between 0.3 and 0.5 USD/g in the same scenario. Costs in a scenario with low utilization of resin lifetime would be multiples of these values. Therefore, a management decision to replace Protein A affinity chromatography is one of the most complex issues in designing an economic process. Economical benefits range is extremely wide on a pure cost basis, but very high-value risk aspects need to be balanced against those in any case. Protein A (or any affinity step) increases process robustness (in general) and permits greater flexibility in the operation of the downstream steps.

One issue that is related to the size of the upfront investment into the Protein A resin is a financial risk that might arise from an unlikely, but possible, event that causes damage to the resin early in production. That concern is distinct from the cost savings aspect. One may try to partly address this through payment terms and agreements between manufacturers and vendors under specific agreements, including possibly insurance agreements, i.e. instead of leaving this issue to a cost discussion only.

When replacing an affinity step with an ion exchanger, there would be no major economic benefit related to equipment costs. Most manufacturers use a Protein A column volume that is two to four times smaller than the volume used for the corresponding ion exchanger. The smaller column is feasible when using batch cycling. The column for



Protein A would thus be equal or smaller to one used for an ion exchange column handling the whole batch in one cycle. Therefore, no major savings can be expected from buffer costs when using ion exchange instead of affinity chromatography, since buffer use is proportional to column volume.

These considerations turn the decision to make such a change clearly into a strategic management consideration. Some more advanced companies seem to have left this discussion behind them, at least for now, obviously considering the gains to be too small [20]. Their main reason seems to be that the value of maintaining a robust manufacturing platform with high yields is too high to accept uncertainties and development re-work for each new antibody (see Section 8.5). At the end of the day, they would have to make a choice and may not want to spend resources on an issue worth at best 1 USD/g when the process is well optimized and resources could be used on other issues that offer greater cost benefits, such as those discussed in Sections 8.2 and 8.4.1–8.4.3. Just one lost batch as a result of decreased robustness (see Chapter 3, section on design of experiments) would negate all possible raw material cost differences for many years to come.

The Protein A case discussed here is probably the major example of a technology perceived as expensive in the toolbox of protein manufacturing today. Virus removal filters are also in this category of ‘expensive’ tools. Yet, when taking all aspects into account, both of these tools may afford the best bet for maximizing a company’s results.

A final comment about other step changes or modifications of an established process: economical gains would be very small if one technology performing a certain task is replaced with another that does no more, even if it could do so cheaper. Modern ion exchangers, for instance, contribute with no more than 0.3 USD/g of antibody in an optimized process. Economically, it is almost certainly not worthwhile spending resources to change such step. There must be other reasons for such a project, namely the general ability to produce a sufficient quantity, to improve quality or to reduce safety risks.

## **8.5 IMPACT FROM R&D, PLATFORM STRATEGIES AND TECHNOLOGY OUTLOOK**

We have already introduced the concept of technology platforms as part of the industrial context discussion (Section 1.4.4 of Chapter 1).

Technology platforms are typically preferred by top management of the development functions as well as the manufacturing management. This demonstrates the value of the concept as companies perceive it, very high indeed. Use and maintenance of technology platforms helps companies reach the market faster and make fewer mistakes on the way through development. This generates tens of millions of dollars in net present value for the development project as it progresses from early development to provide first-in-human (FIH) product (see Figure 1.5) and then again during scale-up and transfer to full-scale manufacturing [41, 42].

Technology platforms are a key vehicle for management to control and influence the profit and loss sheet with its R&D and Manufacturing (CoS) lines. The use of platform technologies explains why companies tend to be conservative about technology change.

**Table 8.7**

Short list of development priorities for downstream-related technology improvements

Feature	Comment
Capacity	Resins, in particular affinity resins with higher capacity with similar or higher volume throughput
Selectivity	Novel selectivities, specific for key impurities, enabling shorter or more robust processing, platforms similar to those for Mabs but designed for other protein or nucleic acid therapeutics
Flexible operation	Features for resins, devices or hardware that allow smooth operation in multi-product facilities with optimized productivity
Direct measurement	Sensors that allow direct measurement of product and impurities
Virus filters	Higher throughput and lower usage cost to reduce the specific cost contributions arising from viral safety requirements

*Note:* Improvements as listed here will have a guaranteed economical impact once implemented.

In addition to new biotherapeutics, R&D, or more correctly ‘D’ as the implementer of platform strategies, delivers multi-million dollars to the company’s financial results.

A common product lifecycle strategy includes moving rapidly to product launch, using a rapidly developed Phase I process first followed by an improved (but hardly optimal) Phase III process. After licensure, when capacity utilization of the plant becomes an issue, post-licensure process changes are developed and implemented. At this point, the opportunity costs of making another product in the plant drives a reduction in the time scheduled for the older product. The downstream process improvements are bundled with increases in upstream titer, and the latest in resin and membrane technologies are used to increase yield, throughput and reduce the step number if possible. Regulatory considerations are always an additional source of guidance or constraints for post-approval changes. More recently, the concept of ‘design-space’ has created new hope that such changes may become easier, i.e. as long as the process change does not result in ‘out-of-design-space’ situations.

Despite the conservatism and many good reasons to keep a proven technology, this approach will not work forever without strong efforts to improve the specifications of the existing technology and, from time to time, the introduction of novel separation tools. Key features high on the priority list for future improvements from vendors developing downstream processing technology are listed in Table 8.7.

Any of these technology improvements are likely to advance the economics of protein biopharmaceutical manufacturing.

## 8.6 CONCLUSIONS, THE IMPROVEMENT HIERARCHY

The summary of this chapter is short and is found in Table 8.8. A lot of money can be at stake for companies, both when planning to implement an improvement and when deciding against it.

**Table 8.8**

The ten commandments for improving economics in downstream processing

Rank	Commandment
1	Use technology platforms; install routines to develop and maintain updates for elements you consider to be the best combination of tools in manufacturing; control implementation tightly
2	Optimize facility utilization, consider even capacity sharing
3	Maximize facility utilization by creating flexibility to operate in multi-product facilities de-couple process steps, bring down change-over time
4	Use LEAN manufacturing; remove non-productive/non-value generating steps ('waste') to gain even more product from the process and the facility
5	Address process yield
6	Employ strategies to eliminate batch failures, measured as 'out-of-specification'
7	Use advanced selectivity and reduce the number of steps in your process, combine steps in a smart way
8	Use the most recent technology that combines the positive effects from highest volume throughput and protein capacity
9	Minimize buffer consumption and use inexpensive, but high-quality cleaning buffers
10	Bundle post-licensure changes with higher titer process introduction, make smaller changes to your process as part of your platform maintenance: e.g. upgraded resin, optimized column dimensions, optimized running conditions

The Ten Commandments suggest to consider the multi-million dollar aspects first, such as keeping to the established platform strategy, doing everything on different levels to support facility utilization and working with process yield, including the reduction of batch failures and its consequences. Over time, one will want to make sure to move to the most-recent technology. The rest is small gain! At any time, companies want to be sure the purpose of improvement is very clear both scientifically, and from the business perspective.

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## **Basic Properties of Peptides, Proteins, Nucleic Acids and Virus Particles**

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### **9.1 INTRODUCTION**

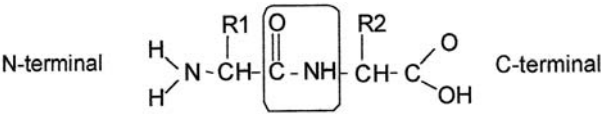
The basic properties of the solute to be purified as compared to the properties of the impurities has a decisive influence on the initial design of a purification protocol and the experimental design of the scouting and the optimization procedure. Therefore, some of the basic properties related to chromatographic purification of some important biomacromolecules are given here. Needless to say, the presentation is not exhaustive or complete and the reader is referred to other work for in-depth description of various biomacromolecules [1, 2]. The discussion will be limited to the four types of solutes presently of largest interest to industrial bioprocessing, i.e. peptides, proteins, nucleic acids (including plasmids) and virus particles. Methods for analysis of these solutes are briefly discussed and more information is found in Chapter 5. A discussion of platform technologies for purification of monoclonal antibodies and plasmid DNA is found in Section 3.6 of Chapter 3.

### **9.2 PEPTIDES**

Peptides have received renewed interest, one of the reasons being the search for small protein fragments with retained biological functionality. A majority of the peptides approved for human use are produced by chemical synthesis and only a few are extracted from natural source. Larger peptides (e.g. containing more than 25 amino acids) are also produced by recombinant techniques. The distinction between peptides, polypeptides and proteins is arbitrary. One definition being that proteins have a stable three-dimensional structure while peptides do not which limits the upper range of peptides to approximately 50 amino acids [3]. This will correspond to a maximum molecular mass of roughly 6000 g/mole for peptides calculated from an average relative molecular mass of 119 for an amino acid residue [1].

9.2.1 Amino acid composition

Peptides are composed of amino acids, being linked via a peptide bond as illustrated in Figure 9.1. One end of the peptide will contain the amino group and is called the N-terminal, the other end will contain a carboxylic acid group and is called the C-terminal. There are 20 amino acids occurring naturally in proteins (see Table 9.1). Depending upon the substitution the amino acids display acidic, basic, hydrophobic, aromatic or polar side groups. It is the properties of these side groups that generally are used for chromatographic



**Figure 9.1** Chemical structure of a dipeptide, illustrating the peptide bond. R1 and R2 denote side chains described in Table 9.1.

**Table 9.1**

Amino acids found in peptides and proteins

Amino acid	Designation	Side chain	$pK_{a3}$	$\lambda_{max}$ (nm)
Alanine	Ala, A	Non-polar, aliphatic		
Arginine	Arg, R	Basic, amino	12.0	
Asparagine	Asn, N	Polar, amide		
Aspartic acid	Asp, D	Acid, carboxylic	3.9–4.0	
Cysteine	Cys, C	Polar, thiol	9.0–9.5	
Glutamic acid	Glu, E	Acid, carboxylic	4.3–4.5	
Glutamine	Gln, Q	Polar, amide		
Glycine	Gly, G	Non-polar		
Histidine	His, H	Polar, imidazole	6.0–7.0	
Isoleucine	Ile, I	Non-polar, aliphatic		
Leucine	Leu, L	Non-polar, aliphatic		
Lysine	Lys, K	Basic, amino	10.4–11.1	
Methionine	Met, M	Non-polar, sulphur containing		
Phenylalanine	Phe, F	Aromatic, non-polar		257.4
Proline	Pro, P	Cyclic, aliphatic		
Serine	Ser, S	Hydroxylic, polar		
Threonine	Thr, T	Hydroxylic, polar		
Tryptophan	Trp, W	Aromatic, polar		279.8
Tyrosine	Tyr, Y	Aromatic, polar	10.0–10.3	274.6 293.0
Valine	Val, V	Non-polar, aliphatic		

*Note:* In addition to these acid constants the C-terminal group has a  $pK_{a1}$  of 3.5–4.3 and the N-terminal group has a  $pK_{a2}$  of 6.8–8.0, depending upon influence from neighbouring substituents. Data from Ref. [1].

purification (e.g. a peptide being rich in aspartic acid is a good candidate for purification by cation-exchange chromatography) unless the separation is based upon size. For a long peptide the thiol groups of cysteine may undergo internal reaction under mild oxidizing conditions forming a disulphide bond. If this is not desired, non-oxidizing conditions should be sought. Other reactions that may take place are oxidation of methionine and tryptophan and deamidation of asparagine and glutamine to form aspartic acid and glutamic acid.

### 9.2.2 Structure of peptides

Peptides having a length of a few amino acids may be regarded as having a globular shape. When the number of residues increases the molecule will gradually behave as a flexible coil. Peptides composed of more than 20 amino acid residues may fold to internalize non-polar residues and the structure may be stabilized by formation of internal disulphide bonds. The formation of coiled coils, e.g. two polypeptides wound around each other may also serve to internalize non-polar residues. Coiled coils between two or three  $\alpha$ -helices serve to stabilize the  $\alpha$ -helices of large polypeptides and proteins [4].

### 9.2.3 Surface properties of peptides

Since peptides generally lack three-dimensional structure all amino acids are exposed and may interact with the chromatographic resin. This leads to a very high relative interaction surface for peptides as compared to larger molecules (e.g. proteins) for which only a fraction of the molecule interacts with the chromatographic surface. Consequently surface-based separation techniques such as reversed phase chromatography have been shown to yield very high resolution of peptides and separations due to a change from isoleucine to leucine in a ten amino acid peptide is possible [3]. The surface properties have been characterized by hydrophobicity indexes, which may be useful for predicting the separation of peptides up to 50 amino acid residues [3]. Also the surface area of the peptide must be taken into consideration [5]. For larger peptides the possibility of formation of an internal structure will affect the exposed amino acids and the interaction with a chromatographic resin.

### 9.2.4 Characterization methods of peptides

Except for peptides containing aromatic amino acid residues, peptides do not absorb light above 220 nm. Generally the absorption due to the peptide bond may be used to follow the concentration of peptides in a column effluent, e.g. 206–220 nm. In special cases the absorption at 268–280 nm due to the aromatic substituents (see Table 9.1) or the fluorescence emission at 282, 348 and 303 nm from respectively phenylalanine, tryptophan and tyrosine residues can be exploited.

The Edman degradation procedure can be used for determination of the amino acid sequence of up to 70 residues, larger peptides are hydrolysed to shorter fragments prior



to analysis [1]. One amino acid, having a free  $\alpha$ -amino acid, is reacted and removed at a time from the N-terminal and identified by its elution position in a reversed phase chromatography step. Mass spectrometry may also be utilized for direct peptide sequencing up to approximately 20 residues. Longer peptides and proteins need to be fragmented before determination [6].

Qualitative characterization of peptides and peptide fragments (e.g. from proteolytic digests) may be obtained by reversed phase chromatography sometimes in concert with ion-exchange chromatography to give a two-dimensional separation followed by a selective detection, i.e. mass spectrometry [3, 6]. Capillary electrophoresis offers a different selectivity than liquid chromatography for peptide separations [7]. Combination of powerful separation techniques, such as high performance liquid chromatography or two-dimensional electrophoresis combined with a selective detection, such as electrospray mass spectrometry (ES-MS) or matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) of tryptic fragments to yield peptide mass fingerprints is still the current status of the art [6]. More recently fragmentation has been carried out by MS, i.e. as in MALDI-MS/MS.

## 9.3 PROTEINS

Proteins continue to be a very important type of biopharmaceutical. Even though smaller fragments, i.e. peptide mimetics, have been found to yield specific affinity to desired sites it is believed that tertiary structure and modifications (e.g. glycosylation) play an important role for the efficacy of biopharmaceuticals. The trend is towards recombinant production of proteins for human use to replace extraction of proteins from natural sources to eliminate the risk of lethal impurities (e.g. endogenous virus and prions) in the end product. Recombinant products includes large peptides, e.g. recombinant glucagon (29 residues), small proteins, e.g. recombinant insulin (51 residues) and very large proteins, e.g. recombinant factor VIII (2332 residues). This is still small as compared to the longest single-chain polypeptide chain reported of nearly 27,000 amino acids in the muscle protein titin [8].

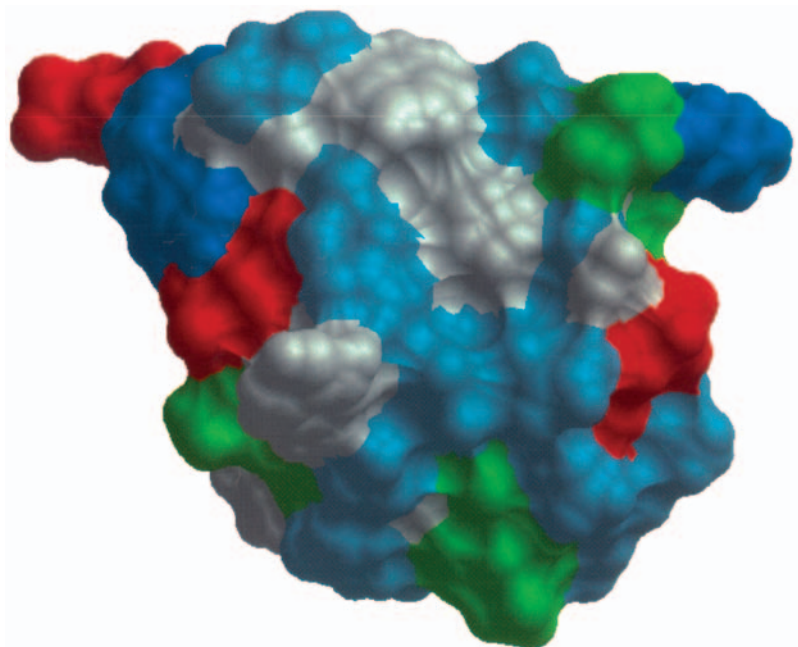
### 9.3.1 Structure of proteins

Proteins are composed of peptide chains sometimes linked together by disulphide bonds between cysteine residues. The sequence of amino acids composing the peptide chain is called the primary structure. The spatial arrangement of the peptide chain results in a minimum entropy (this will result in certain energetically favourable geometric structures such as alpha helix or beta strands held together by intra-molecular hydrogen bonds) and is called the secondary structure of the protein. These structural elements are further arranged to allow for a minimum of surface energy by promoting most of the hydrophobic patches of the protein to be directed to the core of the molecule for a hydrophilic protein and vice versa for a hydrophobic protein (e.g. the transmembrane part of a membrane protein). This will result in the, often compact, three-dimensional structure of proteins, which may be stabilized by intra-molecular covalent disulphide bonds. This is the tertiary structure of the protein. Individual protein or peptide molecules may interact to form multimers, this being the quaternary structure.

The structure of proteins may be disrupted by agents in the mobile phase, e.g. organic solvents, reducing agents, chaotropic salts, urea or guanidine hydrochloride etc. or inactivated due to losses of essential cofactors. It is important to take these effects into consideration to avoid losses of active protein. Proteins may also be denatured due to strong interactions with, e.g. hydrophobic surfaces such as displayed in reversed phase chromatography (see Chapter 5). Other types of surface interactions may be favourable, e.g. gel filtration has been found effective for refolding of proteins [9].

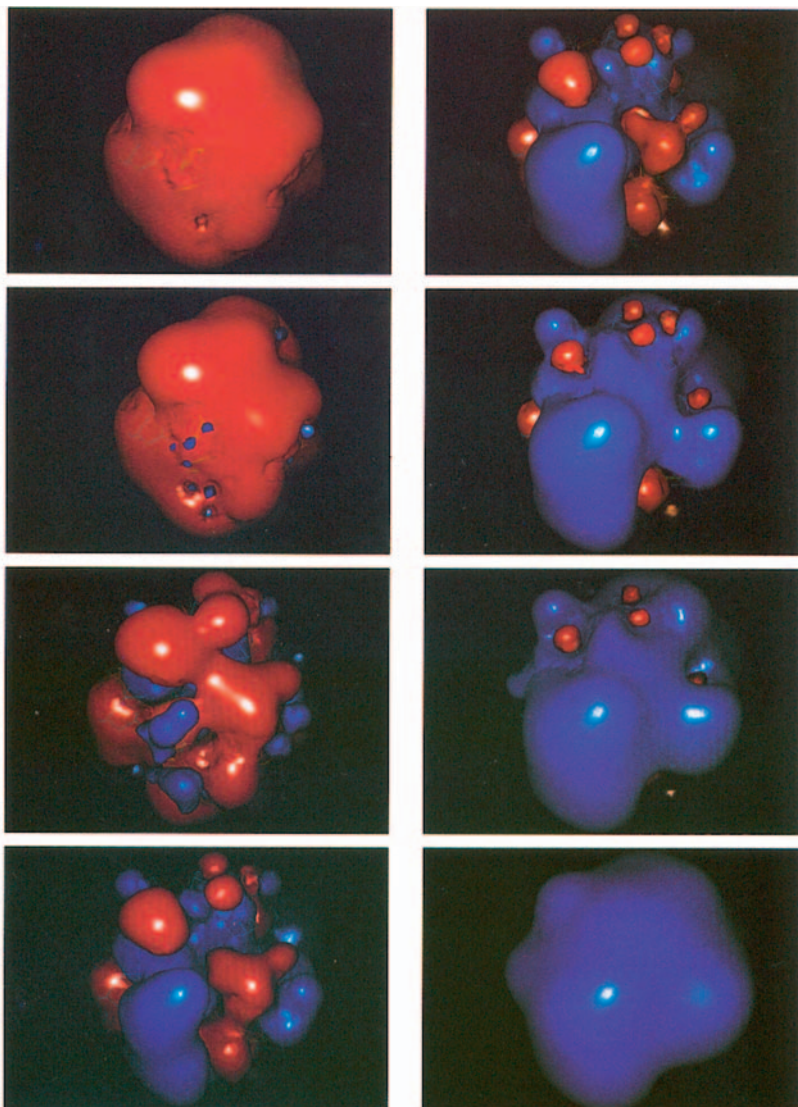
### 9.3.2 Surface properties of proteins

The surface properties of proteins are far more complicated than for peptides. One example is given by Figure 9.2 showing the surface properties of insulin. A conclusion from the complex surface properties shown by proteins was that a prediction of chromatographic behaviour *a priori* is not likely to be totally accurate and experimental method scouting is important for screening suitable purification conditions (see Chapter 3). However, as shown by Carredano *et al.* the use of protein descriptors is a powerful tool to predict surface properties of proteins [10]. On the other hand, surface properties of proteins are not



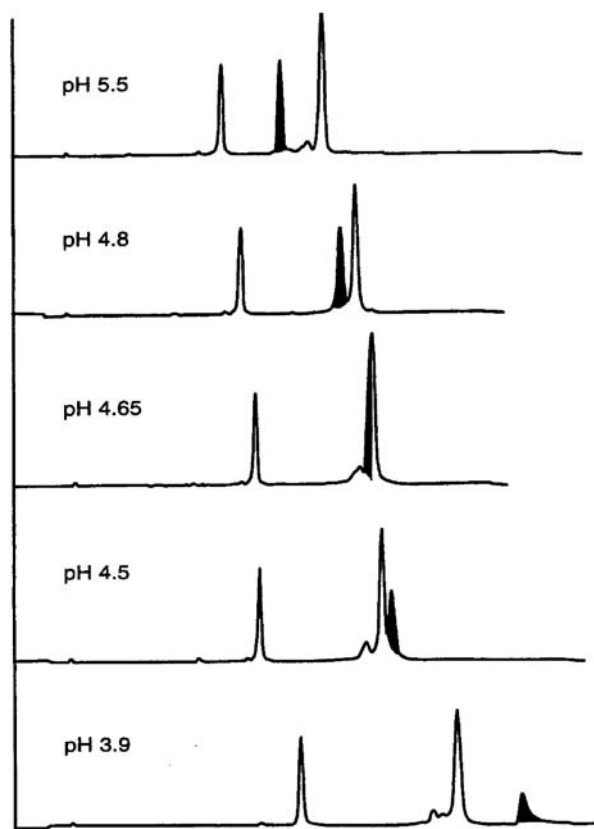
**Figure 9.2** Molecular model of insulin illustrating different properties of the surface. Dark blue areas represent positively charged residues, red areas represent negatively charged residues, green areas represent hydrophobic residues, grey areas represent aromatic residues and light blue areas represent polar residues. The image was made using ICM program (MolSoft LLC, Meutchen, U.S.A.) by entry 9INS in the Protein Databank [14]. By courtesy of Dr. Martin Norin, 1996, Pharmacia & Upjohn.

static and charges may be induced due to dynamic surface interactions, which will affect retention in, e.g. IEC [11]. As many amino acids exposed on the surface of hydrophilic proteins are acidic or basic the pH will have a large influence on the net charge of the protein. At low pH the protein will have a positive net charge (due to the charged amino groups) and at high pH the net charge will be negative (due to the dissociated carboxylic



**Figure 9.3** Variation of surface charge of kallekrein A with pH. Red colour indicates positive charge and blue colour negative charge of the surface. The pH is, from top left; 2, 3, 4, 5 and top right; 6, 8, 10 and 12. The isoelectric point of kallekrein A is 4.6. By courtesy of Dr. Åke Pilotti. Work from GE Healthcare Bio-Sciences AB, reproduced with permission.

acid groups). Thus, at some intermediate pH the net charge of the protein will be zero as defined by that the molecule will not travel in an electric field, this pH is called the isoelectric point. However, this does not mean that the molecule is uncharged. There are patches of both negative and positive charges at the isoelectric point and furthermore, there is a substantial amount of negative charge below and positive charge above the isoelectric point, as illustrated by Figure 9.3. Thus, it may be expected that proteins can be retarded by an anion exchanger below the isoelectric point and cation exchanger above the isoelectric point. This is illustrated in Figure 9.4 where the retention for cytochrome c is affected by pH more than four units away from the isoelectric point [12]. It is interesting to note



**Figure 9.4** Separation of  $\alpha$ -chymotrypsinogen A (isoelectric point 9.0), lysozyme (isoelectric point 11.0) and cytochrome c (isoelectric point 9.4), from left to right in the chromatogram at pH 5.5. The proteins are separated on a cation exchanger, Mono S<sup>TM</sup> HR 10/10 using a 20 column volume gradient from 50 mM buffer to 50 mM buffer in 1 M NaCl, the buffer was automatically prepared by the BufferPrep function of the system used, ÄKTAexplorer<sup>TM</sup>. It is seen that the peak of cytochrome c (shaded) will be more retained than lysozyme at low pH and a reversal of the elution orders is taking place over a very narrow pH interval, i.e. of 0.3 pH units. This indicates that the charge distribution is very different for the molecules. By courtesy of G. Malmquist and N. Stafström. Work from GE Healthcare Bio-Sciences AB, reproduced with permission.

that the effect on lysozyme from a decrease in pH is fairly modest leading to a reversal of elution order between the proteins. The influence of pH on the charge of the protein may be determined by an electrophoretic titration curve where the electrophoretic mobility as a function of pH is plotted [13].

### 9.3.3 Characterization methods of proteins

Proteins normally show absorption at 280 nm due to the content of aromatic amino acids (*cf.* Table 9.1), in addition to the absorption due to the peptide bond. The protein concentration of the effluent may therefore conveniently be traced by an on-line UV detector. Qualitative characterization of proteins include electrophoresis (running in native as well as denaturing conditions is recommended). Digestion of proteins to peptide fragments and separation with reversed phase chromatography provides a fingerprint (peptide map) of the protein. Digests may also be characterized by two-dimensional electrophoresis. The first dimension is run in isoelectric focusing mode using immobiline gels. The fragments are separated due to differences in their isoelectric point. The second dimension is SDS-PAGE (polyacrylamide gel electrophoresis under denaturing conditions, i.e. sodium dodecyl sulphate) where the fragments are separated by size. The individual spots may be directly scanned by a MALDI laser to desorb the peptide for a subsequent determination of the mass by time of flight (TOF) mass spectrometry [6]. LC-MS was used for analysis of glycosylation of monoclonal antibodies [15]. Information about the secondary, tertiary and quaternary structure may be obtained from gel filtration under native and denaturing conditions. Identity and concentration can be obtained by ELISA (enzyme linked immunosorption assay) provided specific antibodies towards the target protein are available.

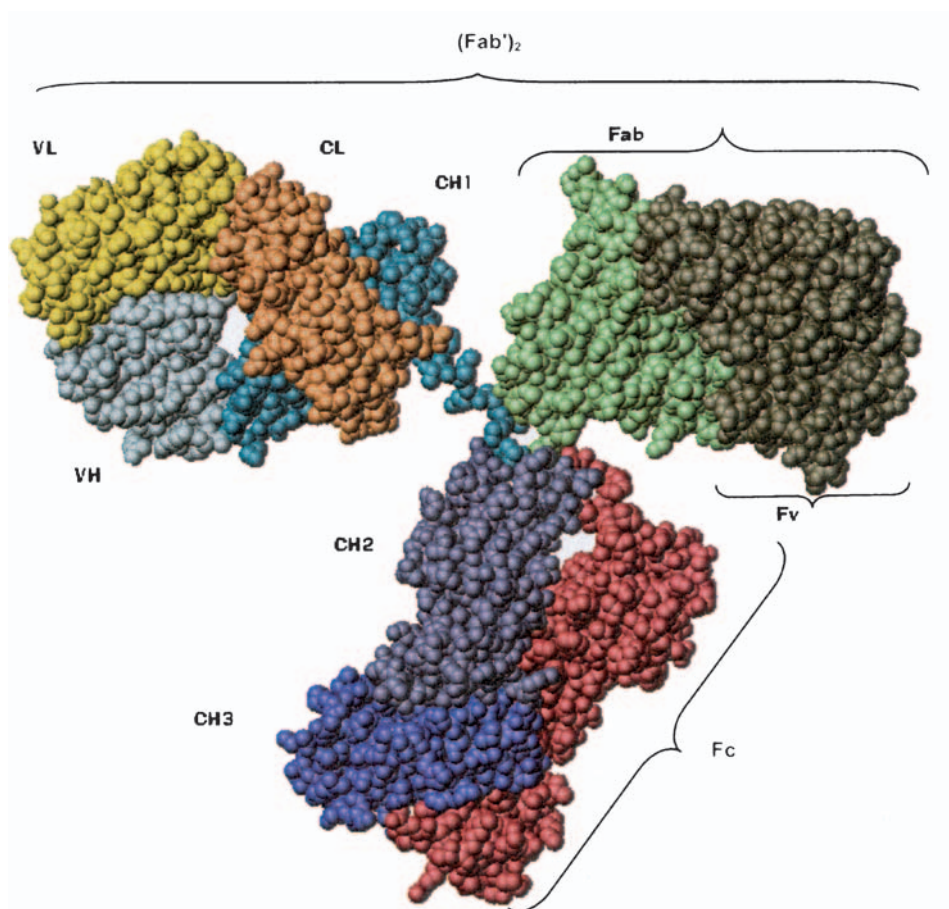
### 9.3.4 Properties of human antibodies and antibody fragments

Human antibodies and antibody fragments are currently the single most-important class of proteins used for development of new biopharmaceuticals (e.g. see Chapters 1 and 2). A short description of the basic properties of antibody and antibody fragments are therefore given here for reference purpose.

The wide use of antibodies stems from the straightforward way of producing antibodies having a specific binding to the target molecule (e.g. a growth factor essential for cancer targeted by Herceptin®, inflammation causing protein targeted by Enbrel® etc., see Chapter 1) and the high affinity achieved for the targets. The potential of antibody treatment has increased considerably with the design of human antibodies replacing the early strategies of employing mouse antibodies or partially humanized antibodies that may have a theoretic risk of undesired immunogenicity reactions. However, today many of the successfully used antibody pharmaceuticals are based on mouse or chimeric mouse antibodies (see Chapter 1). Many of the biopharmaceutical antibodies are monoclonal, i.e. the host cells producing the antibody have one common origin. In this way all antigen-binding sites will be identical. Polyclonal antibodies show a natural variability of the complementary determining regions (CDR's, see below) and these will interact with different parts of the antigen (i.e. the parts that provoked the immunogenic response).



Of the five types of human antibodies, i.e. IgA, IgD, IgE, IgM and IgG, only the last one, IgG is predominantly used for biopharmaceutical purposes. The structure of monomeric antibodies follows the same basic design of two identical units being held together with a disulphide bridge. Each unit is comprised of one heavy chain (H) and one light chain (L) held together with a disulphide bond. This is true for antibodies from most species, one exception being the llama antibody where the unit is comprised of a heavy chain only. Four subclasses of human IgG exists, denoted as IgG1, IgG2, IgG3 and IgG4 with IgG1 being the dominant subclass and the one primarily employed for design of antibody-based biopharmaceuticals. The structure of IgG1 is shown in Figure 9.5. The figure



**Figure 9.5** Space-filled model of the human IgG1 antibody showing the different domains of the antibody molecule. Left-hand part showing the heavy chain in different shades of blue with, from bottom, the constant regions CH<sub>3</sub>, CH<sub>2</sub> and CH<sub>1</sub> and the variable region VH and the light chain in yellow-gold with the constant region, CL and the variable region VL. The light chain may be of two types, denoted kappa and lambda, differing in the amino acid composition of the CL region. Right-hand part showing the Fc-region in red and the Fab-region in green with the Fv part of the Fab-region in dark green. By courtesy of Enrique Carredano. Work from GE Healthcare Bio-Sciences AB, reproduced with permission.

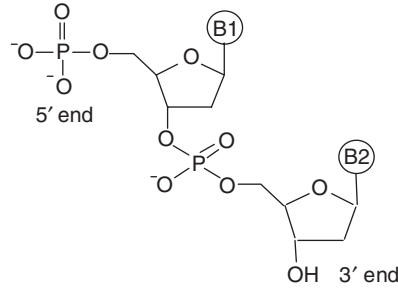
also depicts the various parts of the antibody, i.e. the Fc-region (fragment that crystallizes) and the Fab-region (fragment showing antigen binding). The end part of the Fab-region, the Fv-region (fragment of the variable regions) contains three hypervariable regions, called complementary determining regions, denoted CDR1, CDR2 and CDR3. Since this is the basic functional region of the antibody there is an interest in producing biopharmaceuticals only containing this region, either by exploiting the antibody without the Fc-part, yielding a (Fab')<sub>2</sub> fragment, or only one of these, i.e. the Fab fragment or simply the scFv fragment where the two regions, VH (variable region from heavy chain) and VL (variable region from light chain) are linked together head-to-tail to form a single chain. With this strategy the size of the antibody can be reduced from 150 kg/mole for the entire antibody to 50 kg/mole for the Fab fragment or to 25 kg/mole for the scFv fragment (leading to more favourable pharmacological properties, e.g. administration). This will also require some new purification strategies since the traditional purification of antibodies with protein A is predominantly based upon binding to the Fc-part (i.e. the CH<sub>3</sub>-CH<sub>2</sub> hinge region, in addition to a weak binding in the VHIII region). Protein G will bind to the CH<sub>1</sub>-region in addition to the Fc-part. Protein L is promising since it binds to the VL region of the Fv-part.

## 9.4 NUCLEIC ACIDS

Recombinant nucleic acids, and predominantly deoxyribonucleic acid (DNA), forms the basis for the production of recombinant proteins and peptides. DNA coding for a peptide is frequently chemically synthesized while DNA coding for larger proteins may also be purified from natural sources. The pharmaceutical use of short nucleic acids, oligonucleotides, have received increased interest with the potential of blocking the synthesis of proteins, *in vivo*, with the possibility of binding synthetic single-stranded oligonucleotides to, e.g. messenger RNA (i.e. anti-sense technique). This strategy may be revised with the discovery of the properties of double-stranded short interfering RNA, siRNA, for inhibiting the production of specific proteins. This is called RNA interference (RNAi). Plasmid DNA and mRNA are potential candidates respectively for gene therapy and *in situ* vaccination.

### 9.4.1 Basic structure of DNA and RNA

The building blocks of nucleic acids are the nucleotides. A nucleotide is composed of one purine or pyrimidine base coupled to a 5-carbon sugar (pentose), D-ribose for ribonucleic acid (RNA) and 2-deoxyribose for DNA, to which a phosphate group is attached at the 5' position for DNA and RNA. The nucleotides are then coupled together via a phosphodiester bond between the phosphate group and the 3'-carbon of the sugar moiety of the next nucleotide thus forming a linear polymer, see Figure 9.6. The 3' end of the molecule has a free hydroxyl group attached to the carbon and the free 5' end has a phosphate group (or a hydroxyl group) attached. Nucleotides are negatively charged at neutral pH due to the dissociation of the phosphate group ( $pK_{a1} \sim 4$ ). Differences in properties between nucleic acids are primarily related to the order and nature of the basic groups and the three-dimensional structure of the large DNA and RNA molecules. Properties of the basic



**Figure 9.6** Dinucleotide formed by a condensation reaction between two nucleotides to form a phosphodiester bond. B1 and B2 represent bases as given in Table 9.2.

**Table 9.2**

Purine and pyrimidine bases found in DNA and RNA

Base	Designation	Type	Basic groups <sup>a</sup>	pK <sub>a</sub>
DNA, RNA				
Adenine	A	Purine	–N=	4.1
Guanine	G	Purine	–NH <sub>2</sub> , =CO <sup>–</sup>	3.3, 9.6
Cytosine	C	Pyrimidine	–N=	4.5
DNA				
Thymine	T	Pyrimidine	=CO <sup>–</sup>	9.8
RNA				
Uracil	U	Pyrimidine	=CO <sup>–</sup>	9.5

<sup>a</sup>In addition to the nitrogen used for coupling to the pentose.

*Note:* The pyrimidines are six-member ring structures containing two nitrogens (of which one is coupled to the sugar moiety) and purines are a similar six-member ring structure to which a five-member ring containing two more nitrogens (of which one is coupled to the sugar moiety) is coupled. The acid pK<sub>a</sub> is due to the reaction  $R-NH^+=R \rightarrow R-N=R+H^+$ , or  $R-NH_3^+ \rightarrow R-NH_2+H^+$  and the basic pK<sub>a</sub> is due to the loss of a hydrogen according to  $R-NH-C(=O)-R \rightarrow R-N=C(-O^-)-R+H^+$  where R is the purine or pyrimidine ring.

groups are given in Table 9.2. The sequence of the molecule is given by the designation of the base starting from the 5' end.

### 9.4.2 Surface properties of nucleic acids

As for amino acids, small oligonucleotides will have a linear structure and the entire molecule will be able to interact with a chromatographic medium. Since the phosphate group incorporated into the oligonucleotide is negatively charged at a pH above 4 anion-exchange chromatography is a suitable technique. The different hydrophobicities of the purine and pyrimidine bases can also be used for hydrophobic interaction. The hydrophobic interaction can be further enhanced by suppressing the ionisation of the phosphate groups, i.e. working at low pH or by the formation of ion-pairs between the phosphate group and a cationic agent (e.g. tetrabutylammonium hydrogen sulphate) [16].





**Figure 9.7** Conformation of plasmids (a) supercoiled (sc) plasmid DNA, (b) open circular (oc) plasmid DNA and (c) linear plasmid DNA. Note the compact form of sc pDNA!

An essential property of DNA is the reaction with a molecule of complementary sequence to form a double-stranded alpha helix. This helix is predominantly held together by hydrogen bonds between the specific base pairs, leaving the charged phosphate groups exposed on the surface.

While small molecules, i.e. up to 18 base pairs (bp) behave as globular molecules in gel filtration, molecules of intermediate size behave as rigid rods and large molecules, i.e. having more than 150–200 bp will appear as flexible coils. The free ends of a linear double-stranded DNA molecule may base pair to give a circular DNA. Circular covalently closed DNA that is isolated from mitochondria, virus or bacteria is often supercoiled, i.e. has an extra circular turn and will thus elute as a smaller sized molecule (see Figure 9.7). Plasmid DNA is used as a vehicle for introduction of specific DNA sequences in recombinant protein synthesis. The very large size of plasmids was exploited to purify plasmids in the range 4–150 kbp (kilo base pairs) from protein and RNA by selectively eluting the DNA in the void volume of a SEC column [17]. Plasmids will not be able to enter the pores of conventional resins designed for proteins since plasmids are very large, e.g. the size of a 5.7 kbp plasmid DNA is roughly 800 nm if open circular and 480 nm if supercoiled. However, the size will depend upon, e.g. solvent ionic strength [18]. Due to the large size of DNA only the outer surface of the resin may be available for adsorption with traditional chromatography resins, which leads to low capacity, e.g. a few milligrams per litre for an ion-exchanger.

The phosphodiester bond of oligonucleotides is susceptible to enzymatic hydrolytic cleavage. In chemical synthesis of oligonucleotides oxygen in the phosphate group may be replaced by sulphur to decrease the risk of hydrolytic cleavage by nucleases. This group is called a phosphorothioate (or phosphorodithioate if two oxygens are replaced). A different approach to avoid hydrolytic cleavage is to replace the pentose–phosphate backbone of nucleic acids by a polyamide backbone. This peptide nucleic acid (PNA) to which the purine and pyrimidine bases are attached in a configuration similar to those of DNA or RNA has been shown to be able to form base pairs and is a potential anti-sense agent [19]. During the chemical synthesis of oligonucleotides the bases are protected by a trityl group. This group is hydrophobic which may be used in reversed phase or hydrophobic interaction purification step of the synthesis mixture [20].

### 9.4.3 Characterization methods of nucleic acids

Nucleotides absorb light at 260 nm that provides a convenient way to trace the content of nucleic acids. The ratio of absorption at 260 nm to that at 280 nm may be used to

qualitatively measure the DNA-to-protein ratio (i.e. the ratio would be larger than 1.8 for pure nucleic acid). Nucleotides may be separated by liquid chromatography, e.g. ion-exchange chromatography or reversed phase chromatography [21]. For larger nucleic acids the pore size of the chromatography resin must be large to allow for permeation, unless the separation is carried out on a non-porous particles (of small size to yield high total surface area). DNA fragments of up to 2000 bp have been separated on porous chromatography resins and up to 5000 bp on non-porous packings. Gel electrophoresis, especially using a pulsed electric field, has been used for separation of very large DNA fragments (i.e. up to 12 million bases). Capillary gel electrophoresis is another technique that is used for separation of nucleotides and DNA fragments [21].

The molecular mass of nucleotides may be determined by mass spectrometry. Masses of up to 60 bases can be determined but the technique is rapidly evolving and the limit is continuously increased [6, 22].

The base composition of DNA is traditionally determined by the Sanger method [21]. In this method the DNA is used as a template and a series of fragments of different lengths, each terminated with a labelled nucleotide (e.g. fluorescently tagged A, G, C and T), is synthesized by an enzymatic reaction. Separation of the fragments by electrophoresis yields the length of the fragment (i.e. position of the base) and identification of the tag yields information about the identity of the base. The capability of the method is limited by the electrophoresis separation and DNA sequences up to more than 1000 bp can be resolved and determined with reasonable redundancy (i.e.  $<2$ ). The requirement for fast sequencing of large pieces of DNA as set by the human genome project has led to a rapid development of new technology for DNA sequencing (e.g. sequencing by hybridization, laser-mass spectrometry sequencing, etc.) [23]. Sequences for up to 24-base oligonucleotides can be unambiguously identified by MALDI-TOF MS [22].

## 9.5 VIRUSES

Virus particles form a very inhomogeneous group of bioparticles and some years ago, i.e. year 2000, approximately 40,000 virus isolates have been assigned to any of the 56 families of viruses identified [24]. Of these, approximately 50%, or 23 families represent viruses that infect vertebrates. Virus particles, or fragments of virus particles are used for immunization purposes, either by exploring the properties of the virus itself or as a vehicle for, e.g. a DNA-based immunization strategy. In this case the purity of the biopharmaceutical virus particle suspension must be assured and general purification technologies are adapted to this class of particles. However, in another scenario the purpose may be to eliminate virus particles present as an impurity of a feedstream and then a simple ultrafiltration, exploiting the large size of virus particles, may be a good alternative.

### 9.5.1 Structure of virus particles

Virus particles contain a core of RNA or DNA, which is the essential part of the virus, being transcribed to mRNA in the infected cell leading to translation and in the end production of

**Table 9.3**

Properties of some animal virus particles interesting for vaccine manufacture [24]

Type, member	Nucleic acid	Symmetry	Coat	Size (nm)
Parvo, AAV	DNA	Icosahedral	Naked	18–26
Adeno, Ad-5	DNA	Icosahedral	Naked	70–90
Retro	RNA	Icosahedral	Enveloped	80–130
Orthomyxo, Influenza	RNA	Helical	Enveloped	90–120
Pox	DNA	Complex	Enveloped	170–200 × 300–450

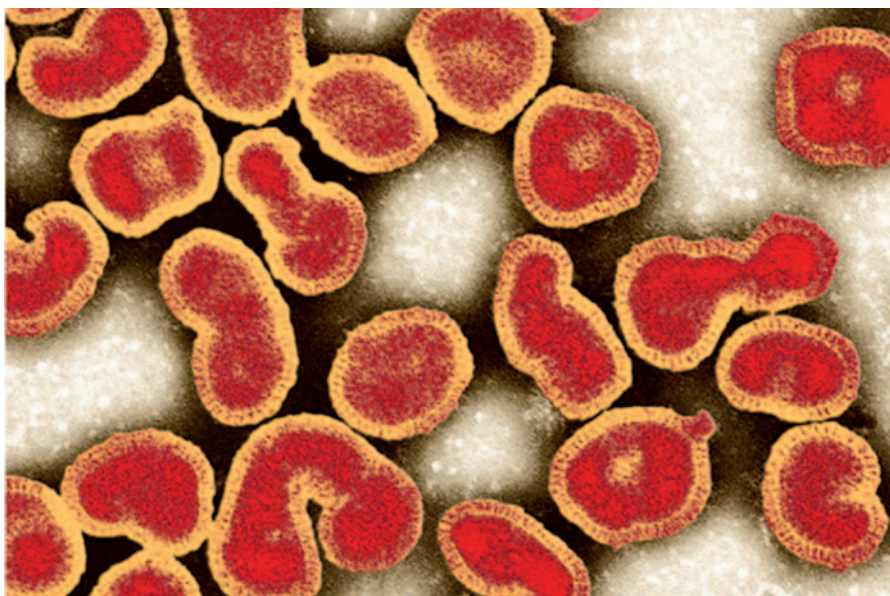
new virus particles with the aid of the cell machinery. The virus nucleic acid is protected by a capsid made from a stable protein shell. Some virus particles have an envelope of a lipid membrane bilayer carrying viral glycoproteins coating the capsid. Virus particles missing this envelope are designated as naked or non-enveloped virus particles. The symmetry of the capsid is in general either helical, leading to a rod-shaped virus particle or icosahedral leading to a more spherical particle. A third type, called complex particles, have a structure that is not yet well known [24]. The size of animal virus particles varies from a diameter of approximately 20 nm, e.g. for Parvovirus, to 450 nm, e.g. for Poxvirus, or more (see Table 9.3).

### 9.5.2 Surface properties of virus particles

The surface properties of virus particles may differ a lot, especially between enveloped and naked virus. The capsid of the naked virus particle is composed of a repetition of a few proteins, typical for each virus particle. Hence, one will expect that properties for proteins, such as isoelectric point, surface charge will be properties that, in addition to size, may be exploited for purification purposes and most purification protocols exploit these properties. The isoelectric point are for many viruses found in the region of pH 5.5–7.3. The lipid bilayer of enveloped virus particles will make them susceptible to detergents, e.g. SDS that is employed for inactivation of viruses. In combination with extreme pH, SDS treatment has proven to be a valuable technique for inactivation of viruses. The surface properties of enveloped viruses vary due to the different glycoproteins embedded in the membrane and exposed on the surface to play an important function in the invasive process of the virus. Properties of an industrially interesting virus, i.e. influenza A virus is schematically illustrated in Figure 9.8.

### 9.5.3 Characterization methods for virus particles

Presence of virus particles have traditionally been noted by their infectivity. This is still a major technique for measurement of the concentration of virus particles in a sample. A dilution series of the sample is inoculated into host cell cultures and infected and damaged cells are visualized as a spot (plaque) by adding a dye that will be adsorbed by the



**Figure 9.8** Transmission electron micrograph of Human Influenza Virus Type A at 191,700 times magnification showing an outer core composed of the lipid-membrane layer and the glycoprotein structures on the surface of the virus whereas the stable protein capsid and the core of RNA is not resolved in this micrograph. The glycoproteins on the surface of Influenza virus are hemagglutinin (HA,  $M_r = 76,000$ ) responsible for the primary infection by binding to sialic-acid-containing cell receptors and neuraminidase (NA,  $M_r = 220,000$ ) important for the progression of infection. The size of the virus particle is roughly 90–120 nm but as seen the size and shape varies considerably [24]. Reproduced by license from Dr. Gopal Murti/Visuals Unlimited/Getty Images.

background uninfected cells. The PFU/ml, i.e. plaque-forming units per millilitre, may thus be calculated. Infectivity demands the use of proper cell culture for the virus in question and it gives only a quantitative measure of infectivity. Sometimes the surface properties of the virus may be used for an assay, e.g. as for influenza virus where the envelope contains a glycoprotein that can be used for a hemagglutination (HA) test. In this test the HA protein binds to sialic acid residues on the surface of red blood cells and forms a network that prevents the blood cells from settling. This assay gives a quantitative measure of virus titre and not of viral infectivity. Another way to measure virus particles is to visualize these by electron microscopy, count the particles and compare with an internal standard of, e.g. latex particles.

Detection of known viruses may of course be made using antibodies raised towards some epitope of the virus particle. Then all common techniques such as direct detection or indirect detection in solution or on solid support with various reporter molecules, e.g. fluorescent, can be employed.

Detection of unknown viruses in a process stream is not an easy task and one will in most cases have to rely upon infectivity tests of animals. The subsequent classification of

an unknown virus will involve sequencing of the viral nucleic acid. Real time quantitative PCR was used for process validation of viral removal in process chromatography [25].

The reader is referred to the excellent book by Flint *et al.* for detailed information about characterisation of virus particles [24].

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# – 10 –

## Optimization of Chromatographic Separations

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### 10.1 INTRODUCTION

A rational design of a purification processes is based upon experience and/or practical implications of chromatography theory. Understanding the relationships governing the separation is, of course, also valuable when troubleshooting the process.

Valuable insights into parameters that are important ones and to what degree these parameters will influence the separation can be gained from varying the values of these parameters and studying the theoretical result obtained. This type of calculations are facilitated by the use of software routines, and some selected applications, which may be found useful for elucidating the effects of various parameters in liquid chromatography, are supplied with this book.

Liquid chromatographic separations are based upon the different degrees of interaction of the dissolved substances with a chromatographic resin. Thus, in order to separate the target substance from impurities by chromatography, conditions that favour interaction with the target substance but not with the impurities or vice versa must be established. This important property of the matrix is loosely referred to as the selectivity (which also is influenced by the solvent and the solute). The solutes are physically separated by passing a solution through the packed bed that will transport solutes confined in the extra-particle space. The concentration of solutes in the extra-particle space will be inversely related to their degree of interaction with the chromatography resin, and desorption is regulated by an eluent. Thus, molecules will be eluted in increasing order of affinity to the chromatographic resin.

While understanding the basic phenomena that regulate separations one may be able to produce a model of the effect of various experimental parameters and use this model to try to *a priori* predict the outcome of a chromatographic separation under a particular set of circumstances. This will yield valuable ideas for how to optimize a separation but it is important to realize that, at best, only a rough approximation of the expected result is achieved and confirmation by experiments is a must.

Obviously, the purpose of the specific purification step, i.e. if the aim is initial capture, intermediate purification or final polishing, will put different requirements for the



optimization process (e.g. the demand for high purity increases further down in the process). Also, step elution involves few parameters and is generally easier to optimize than a gradient elution.

## 10.2 BASIC RELATIONSHIPS

The purpose of process chromatography is to separate, i.e. resolve, one target component from impurities. The resolution is achieved by selectively retarding the target component or the impurities to different extents while keeping the dispersion of solute bands as small as possible. There are a few relationships that are fundamental to all types of chromatography and from which basic parameters regulating the retention and zone broadening of solutes may be described and the resolution calculated. The complete chromatographic process may be described by the material balance of the system.

### 10.2.1 Resolution

The resolution between two solutes is calculated from the difference between their retention volumes,  $V_R$ , as compared to the average of the base widths,  $w_b$ ,

$$R_s = \frac{2(V_{R_2} - V_{R_1})}{w_{b_2} + w_{b_1}} = \frac{k'_2 - k'_1}{2(2 + k'_2 + k'_1)} \sqrt{N} \quad (10.1)$$

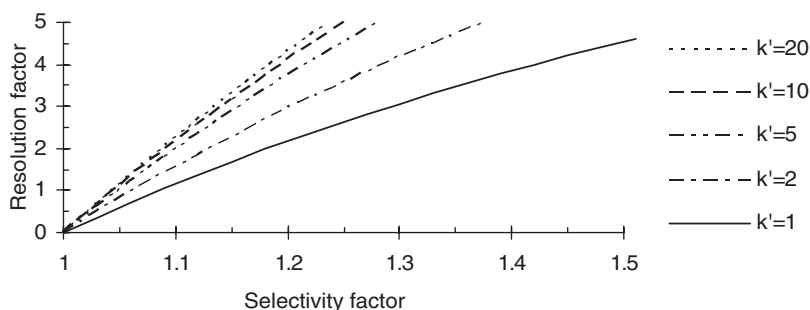
The right-hand side of the equation expresses the resolution in terms of the retention factor,  $k'$  and the plate number of the column,  $N$ , and is valid for isocratic chromatography and under the assumption that the mobile-phase volumes and the plate numbers for the two solutes are identical (*cf.* eqs. (10.4) and (10.9)). By setting  $k'_2 + k'_1 = 2k'$  the equation may be rearranged to the often-used expression

$$R_s = \frac{1}{4} \frac{\alpha - 1}{\alpha} \frac{k'_2}{1 + k'} \sqrt{N} \quad (10.2)$$

which separates the effects from the selectivity factor,  $\alpha$ , the retention factor and the column efficiency in isocratic elution.

A resolution factor of 1.5 yields, in practice, a complete separation of two solutes having Gaussian peak shapes, *cf.* Figure 4.3 (the effect of different resolution factors on the yield and purity may be simulated by the accompanying software routine—see Simulation of separations below).

The graph in Figure 10.1 illustrates that the most important single parameter affecting the resolution by far is the selectivity factor. For instance, the gain in resolution by increasing the plate number 20-fold (e.g. by increasing the column length 20-fold) may be achieved by increasing the selectivity factor from 1.01 to 1.05 (as readily calculated from eq. (10.2)).



**Figure 10.1** Resolution factor as a function of the selectivity factor for different values of the retention factor,  $k'_2$ . Calculated from eq. (10.2) for  $N = 10,000$ .

### 10.2.2 Retention

The retention factor,  $k'$  (the retention factor is sometimes denoted as  $k$  [1]), is given by the amount (or rather, number of moles) of solute in the stationary phase,  $W_S$ , as compared to that in the mobile phase,  $W_M$ ,

$$k' = \frac{W_S}{W_M} \quad (10.3)$$

The relative migration of a solute will be equal to the relative amount found in the mobile phase, and the retention volume of the solute,  $V_R$ , will be related to the retention factor by [2]

$$V_R = V_M + k'V_M \quad (10.4)$$

where  $V_M$  is the mobile-phase volume. The retention factor is related to the distribution coefficient,  $K_D$ , expressing the concentration of solute in the stationary phase,  $C_S$ , over that in the mobile phase,  $C_M$ , by

$$k' = K_D \frac{V_S}{V_M} \quad (10.5)$$

where  $V_S$  is the volume of the stationary phase. It is important to note that the retention factor is proportional to the phase ratio,  $V_S/V_M$ , of the chromatographic resin (e.g. phase ratios may vary with specific surface area of materials). It is also seen from eq. (10.5) that the retention factor (and the retention volume) is constant only when the distribution coefficient is constant. This is not the case for chromatography in non-linear mode, which is common in preparative separations (see below). Thus, varying the sample concentration may result in variations, or even shifts, in retention volumes of solutes due to influences of the isotherm

on the distribution coefficient. In gradient elution the distribution coefficient is gradually changed due to the influence of the mobile-phase composition on dissociation constant (*cf.* eq. (10.29)) and hence the retention factor is not constant.

The definition of mobile-phase volume and stationary-phase volume needs some consideration. The mobile-phase volume is equal to the elution volume of the solute under non-retentive conditions. Thus, while the total-liquid volume (i.e. the extra-particle and intraparticle volume) of the column may be equal to the mobile-phase volume of small solutes this is certainly not true for large solutes, which are excluded from a fraction of the intraparticle volume. It is in most cases not possible to assign a stationary-phase volume to the chromatographic resin (except in size exclusion where the stagnant phase corresponds to the stationary phase). In some cases it may be more appropriate to discuss in terms of surface area [3].

The selectivity factor,  $\alpha$ , for two solutes is affected by the chromatography material at the experimental conditions chosen (i.e. mobile-phase composition, temperature, etc.) and is expressed by the relative retention of the solutes as

$$\alpha = \frac{k'_2}{k'_1} \quad (10.6)$$

While  $k'$  may vary due to the differences in phase ratio of different materials, the separation factor is not affected as long as the difference in phase ratio affects the molecules in a similar way.

### 10.2.3 Zone broadening

The peak width is primarily affected by the zone broadening in the column, and the variance of the zone,  $\sigma^2$ , is proportional to the distance travelled by the zone,  $z$ . The zone broadening per unit length is called the plate height [2] and is denoted as  $H$  (or HETP, height equivalent to a theoretical plate)

$$H = \frac{\sigma^2}{z} \quad (10.7)$$

Conversion from length to volume, and setting  $z = L$ , where  $L$  is the column length, yields the familiar relationship, valid for isocratic elution

$$H = \frac{L}{(V_R/\sigma)^2} \quad (10.8)$$

The number of plates per column,  $N$ , is given by  $L/H$  and thus

$$N = \left( \frac{V_R}{\sigma} \right)^2 \quad (10.9)$$

One description of the variables influencing the plate height of the column in isocratic elution is given by the van Deemter equation [4].

$$H \approx A + \frac{B}{u} + Cu \approx 2\lambda d_p + \frac{2[0.6D_M + D_S((V_R/V_0) - 1)]}{u} + \frac{(V_0/V_R)(1 - (V_0/V_R))d_p^2}{30D_S}u \quad (10.10)$$

where  $\lambda$  is a geometric factor of order unity,  $d_p$  the particle size,  $D_M$  and  $D_S$ , respectively, the diffusion coefficient of the solute in the mobile phase and intraparticle phase, and  $u$  the mobile-phase interstitial velocity.  $A$  is related to eddy dispersion,  $B$  to molecular diffusion and  $C$  to mass-transfer resistance. For small-particle-sized resin or solutes of high diffusivity the effect of eddy dispersion is reduced by molecular diffusion, which leads to an extension of the A-term to incorporate this coupling [5]. However, this effect may be neglected in preparative purifications of biomacromolecules. Eq. (10.10) may be written using so-called reduced parameters, i.e. the reduced plate height,  $h$ , given by

$$h = \frac{H}{d_p} \quad (10.11)$$

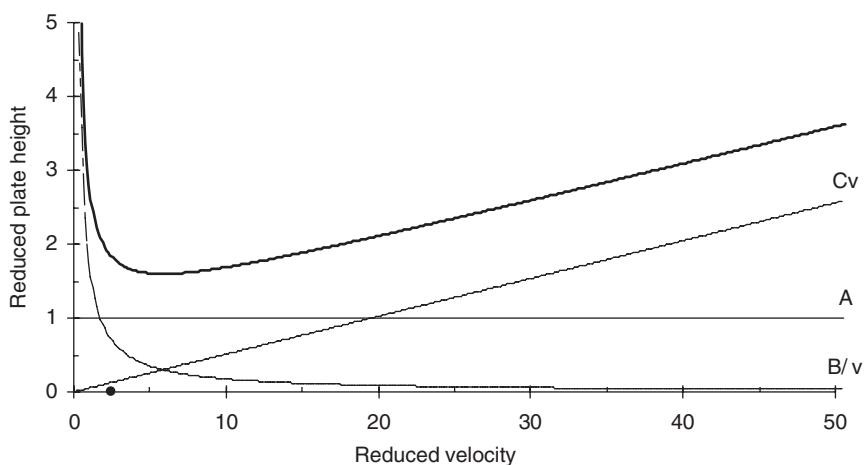
and the reduced velocity,  $v$ , given by

$$v = \frac{ud_p}{D_M} \quad (10.12)$$

giving the following simplified equation

$$h = 2\lambda + \frac{2[0.6 + \gamma_S((V_R/V_0) - 1)]}{v} + \frac{(V_0/V_R)(1 - (V_0/V_R))}{30\gamma_S}v \approx 1 + \frac{1.5}{v} + 0.04v \quad (10.13)$$

where  $\gamma_S$  is a factor to account for the restricted diffusion in the intraparticle space ( $\gamma_S = D_S/D_M$  and is typically 0.05–0.2 for macromolecules) [6]. The right-hand side of the equation comes from the assumption that the relative mobility of the sample zone,  $V_0/V_R = 0.5$  and  $\gamma_S = 0.2$ . Eq. (10.13) may be used for a general, qualitative description of the different contributions to column zone broadening as illustrated in Figure 10.2. It is shown that the B-term has influence only at very low flow rates and for fast diffusing solutes, and that the dominating term at high flow rates and for slow diffusing solutes is the C-term. The restricted diffusion will have a large impact on the slope of the C-term! It may be noticed that  $A$  in some cases may be dependent upon the flow rate (i.e. leading to coupling as mentioned above) and also that convective transport may reduce the C-term at very high flow rates. However, eq. (10.10) has been used successfully in qualitative predictions in preparative-scale isocratic size exclusion



**Figure 10.2** Zone broadening in liquid chromatography as expressed by the reduced van Deemter plot. A-, B- and C-terms as described by eq. (10.13).

chromatography (SEC), ion exchange chromatography (IEC) and reversed-phase chromatography (RPC) [3, 7, 8].

Another equation that has been found useful for calculating the plate height is the empirical Knox equation where the coupling between eddy dispersion and longitudinal diffusion is taken care of in the A-term according to

$$h = A'v^{1/3} + \frac{B'}{v} + C'v \quad (10.14)$$

Bristow [9] noted that  $A' = 1$ ,  $B' = 2$  and  $C' = 0.05$  for well-packed high-performance liquid chromatography (HPLC) columns. This is in good agreement with the van Deemter equation (i.e. the right-hand side of eq. (10.13)). It must be stressed that eqs. (10.10), (10.13) and (10.14) are only valid for isocratic elution; in gradient elution the peak width will generally be smaller due to the self-sharpening effect.

The total plate height of the system will be the sum of different contributions, e.g. from large sample volumes, mixing chambers and other dead volumes and column zone broadening (including transport phenomenon, see below). The effect will vary depending upon chromatography mode employed. Zone broadening in isocratic elution is very sensitive to the quality of column packing, something which is utilized in the control of packed column (see Chapter 12). From Figure 10.2 it is seen that the reduced velocity must be kept constant (e.g. when changing the solute or varying the temperature) if comparable results are to be received (i.e. any solute may be used for test of column packing as long as it does not interact with the chromatography matrix and provided the reduced velocity is constant, e.g. at a value of 5).

### 10.2.4 Mass transfer

The transport of solute through the column is dependent upon the local concentration of solute in the mobile and stationary phases,  $C_M$  and  $C_S$ , respectively, the interstitial velocity of the mobile phase,  $u$ , the dispersion of the zone and molecular diffusion. These factors may be combined to give the following expression for the material balance in the column

$$\frac{\partial C_M}{\partial t} = D_A \frac{\partial^2 C_M}{\partial z^2} - u \frac{\partial C_M}{\partial z} - \frac{V_S}{V_M} \frac{\partial C_S}{\partial t} \quad (10.15)$$

Thus, for an infinitesimal small segment within the column the change of concentration of solute in the mobile phase per unit time is given by the changes in concentration due to dispersion (first term on the right-hand side), and transport of molecules in the mobile phase (second term), and as a result of the adsorption/desorption equilibrium (third term on the right-hand side). Dispersion arises from axial diffusion, eddy dispersion as described by the van Deemter equation. In theory also film diffusion (i.e. diffusion of molecules through the stagnant solvent layer around particles) will contribute to overall dispersion, but this effect is normally small and often ignored. Convective flow transport of molecules through the particles will enhance mass transport and reduce the dispersion caused by long-range intraparticle diffusion. The review of mass transfer in chromatographic separation by Li *et al.* [10] can be recommended for further reading.

Unfortunately there is no analytical solution to eq. (10.15) for gradient elution chromatography and results must be calculated numerically. This together with approximations needed for dispersion of zones and for adsorption/desorption equilibrium at high-sample loading, common in process chromatography, has resulted in different approaches for theoretical simulations of chromatographic purifications. The use of chromatography theory for modelling chromatographic separations is discussed below.

### 10.2.5 Flow resistance of packed beds

Sometimes factors other than chromatographic ones need to be addressed. For instance, even though the separation factor is large enough to allow for a decrease in separation time, the pressure drop over the packed bed at elevated flow rates may exceed the pressure rating of the pump or of the chromatographic resin. In that case a decrease in column length may be a better solution (the resolution is proportional to the square root of the column length, cf. eq. (10.2)).

The pressure drop over a packed bed may be calculated from the Hagen–Poiseuille equation as adapted to packed beds by Blake, Kozeny and Carman, see the review by Allen [11]

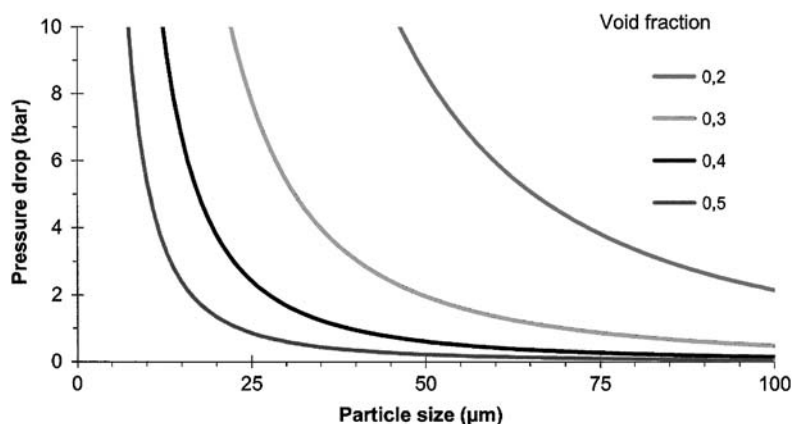
$$\Delta p = u\epsilon \frac{L}{d_p^2} \eta \frac{1 - \epsilon^2}{\epsilon^3} 36k = u\epsilon \frac{L}{d_p^2} \eta \frac{180(1 - \epsilon)^2}{\epsilon^3} \quad (10.16)$$

The first term of eq. (10.16) is a conversion from interstitial liquid velocity,  $u$ , to nominal liquid velocity with the help of the void fraction  $\varepsilon = V_0/V_c$  where  $V_c$  is the geometric bed volume and  $V_0$  is the extra-particle void volume. The pressure drop is given in Pascal,  $\text{Pa} = \text{N/m}^2$ , if the viscosity of the solvent,  $\eta$ , is expressed in  $\text{N}\cdot\text{s/m}^2$ , the velocity,  $u$ , in  $\text{cm/s}$  and the column length,  $L$ , and the particle size,  $d_p$ , in  $\text{cm}$  (see Appendix A for conversion factors). The aspect factor,  $k$ , depends upon the shape of the particles and is close to 5 for spherical beads [12]. The last term of eq. (10.16) is called the flow resistance parameter and is used to compare packing density and permeability of packed beds (see Appendix A for definitions) [1].

It is important to notice the large influence of the void fraction,  $\varepsilon$ , on the flow resistance and the calculated permeability. For instance, a bed of hexagonal close-packed uniform spheres has a void fraction of 0.26 [13], and gives a pressure drop six times that of a bed of randomly packed spheres, having a void fraction of 0.40. It may be noted that the void fraction of silica type of materials is often in the range of 0.42–0.45, that of mono-sized synthetic polymers is around 0.36–0.40 and that of non-rigid polymers is in the range 0.30–0.33. A higher void fraction yields lower pressure drops but a larger contribution to the non-separating volume of the system.

The influence of viscosity on flow resistance needs to be considered when applying viscous samples or adding viscous modifiers to the eluent, and when transferring separation methods to cold room.

Changing the particle size from 100 to 10  $\mu\text{m}$  increases the flow resistance 100 times. This requires the use of high-pressure systems for running chromatography resin of small particle size (e.g. from 5 bar system used for standard chromatography to 100 bar system for HPLC) even though the column lengths for HPLC normally are shorter than those used for standard chromatography. The large influence of void fraction on pressure drop, as illustrated in Figure 10.3, shows that an inhomogeneously packed column will yield larger pressure drop than expected from the measured void fraction; thus, unrealistic pressure drops may be



**Figure 10.3** Pressure drop of packed beds of different void fractions as a function of particle size. Calculated from eq. (10.16) with  $L = 10$  cm, and the nominal velocity,  $u \cdot \varepsilon = 10$   $\text{cm/min}$ .

indicative of column deterioration. Eq. (10.16) is useful for determining whether a packed bed is compressed or not, i.e. if the flow resistance is substantially larger, e.g. 50%, than expected from particle size, length, viscosity, void fraction and the contribution from system factors (i.e. connectors, tubings, frits, etc.). The flow resistance caused by the system may be determined by replacing the packed column with an empty column of the same type.

Flow resistance of a packed bed of semi-rigid chromatography resin will depend not only on the mechanical properties of the material, the friction forces applied to the resin by the fluid, but also on supportive effects, e.g. by the column wall. Therefore, flow properties are generally not linearly scaled when, e.g. the column diameter is increased and the effect may be modelled as discussed in Chapter 12.

### 10.3 PURIFICATION PRINCIPLES

As outlined in Chapter 4 purification may be achieved by selective interaction of the solute with the chromatographic resin or by a non-adsorbing mode, e.g. as in SEC.

Using adsorption for purification provides options for both high selectivity as well as high capacity. The interaction on a molecular level differs between, e.g. size exclusion (affected by steric interactions), ion exchange (being a long-range interaction phenomenon) and reversed phase (which involves surface interactions). This results in different relationships between the retention factor and physical properties of the solute and the chromatography resin.

The separation may be performed by keeping the retention factor,  $k'$ , constant during the elution by keeping the composition of the mobile-phase constant, i.e. isocratic conditions. If the composition of the mobile phase is continuously changed during the separation, e.g. to gradually decrease  $k'$ , we talk about gradient separation. If the change in mobile-phase composition is discontinuous to create abrupt changes in  $k'$ , we talk about step elution. The different modes of elution are illustrated in Figure 10.4.

#### 10.3.1 Gel filtration/size exclusion chromatography, SEC

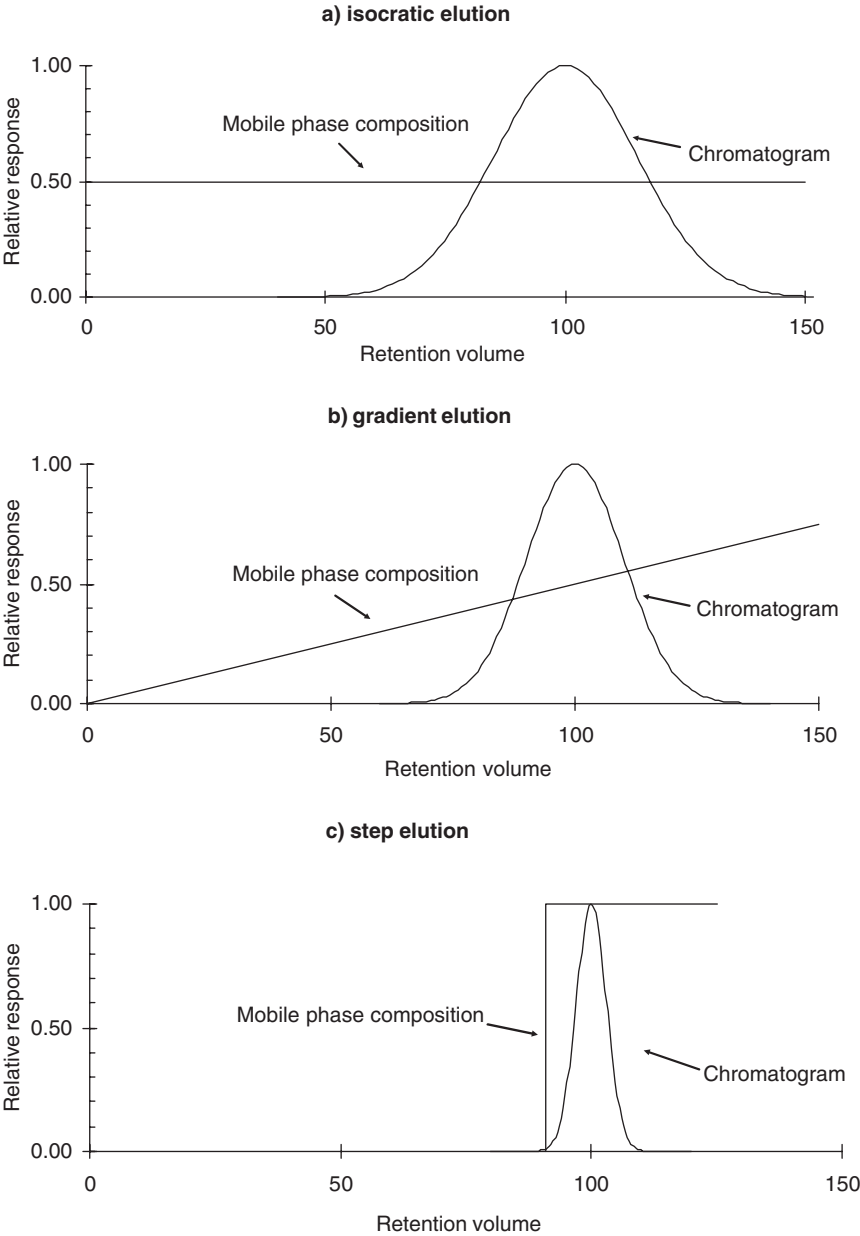
In gel filtration, or size exclusion chromatography, the solutes are separated on the basis of the different fractions of the pore volume that, for sterical reasons, are available for solutes of different size. Thus, the mobile-phase volume of a non-retained solute is equal to the interparticle, or void, volume,  $V_0$ . The stationary-phase volume corresponds conceptually to the intraparticle, or pore, volume,  $V_i$ , of the chromatographic medium. The phase ratio,  $V_S/V_M$  is thus equal to  $V_i/V_0$ , which sometimes is called permeability of the chromatographic particle (not to be confused with the permeability of the packed bed described above).

##### *Retention in SEC*

From eqs. (10.4) and (10.5) and the expression for the phase ratio given above, the following expression for the retention volume,  $V_R$ , in size exclusion is obtained

$$V_R = V_0 + K_D V_i \quad (10.17)$$





**Figure 10.4** Elution modes in liquid chromatography. Chromatogram at low sample loads.

where  $K_D$  is the distribution coefficient. The distribution coefficient is related to the solute and pore dimensions through

$$K_D = \int_R^\infty \left(1 - \frac{R}{r}\right)^a f(r) dr \quad (10.18)$$

where  $R$  is the effective radius of the solute,  $r$  the size exclusion radius of the chromatography resin,  $a$  a factor related to the geometric shape of the pore (i.e.  $a = 2$  for pores of cylindrical shape [14]) and  $f(r)$  the pore size distribution function of the resin. The conclusion from this equation is that the retention time in size exclusion is regulated only by geometric factors and cannot be adjusted by changing the composition of the mobile phase (unless this affects solute or pore dimensions, e.g. as for charged flexible polymers).

The plot of  $K_D$  versus the logarithm of solute radius yields a sigmoid curve (provided solutes of similar shape is used—shape will affect  $r$ , see below) with an approximate linear centre region having a slope of  $dK_D/d \log R$ . This curve is called the selectivity curve. The plot of  $\log R$  versus  $K_D$ , or more common,  $V_R$  yields a calibration curve. A narrow pore volume distribution,  $f(r)$  will yield a steep selectivity curve on expense of separation range covered by the chromatography resin. It is important to note that also a hypothetical support having a single pore size will yield a sigmoid selectivity curve for molecules smaller than the pore size [15]. Thus, the selectivity curve is not identical with the cumulative pore size distribution. However, an apparent pore size distribution can be derived from size exclusion data [16].

The selectivity curve should not be confused with the selectivity factor defined in eq. (10.6). It can be shown that the slope of the selectivity curve is proportional to  $(\alpha - 1)/K_{D1}$ . The selectivity factor,  $\alpha$ , is very high in size exclusion (e.g.  $\alpha$  is 4 for two solutes eluting at the extremes of the separation range,  $K_D = 0.2$  and  $0.8$ , and even higher for solutes eluted at the void volume and total volume, respectively). However, the retention factor is limited to 1 for low porous resins and 2.3 for resins of high pore fraction which sets an upper limit for the resolvability of size exclusion, e.g. a resolution factor,  $R_s$ , of 13 for  $k' = 2.3$ ,  $\alpha = 4$  and  $N = 10,000$ , cf. eq. (10.2).

### *Zone broadening in SEC*

The zone broadening in size exclusion is primarily caused by dispersion of the sample zone of large solutes due to slow mass transfer, i.e. the C-term in eq. (10.10), in addition to eddy dispersion, i.e. the A-term [6, 17]. The zone of the injected sample will also add to the total zone broadening, unless this volume is small (e.g. less than 0.5% of the column volume) [18]. Minimizing extra-column contributions to the zone broadening is important when size exclusion is used for qualification of column performance (see Chapter 12). Neglecting system contributions (e.g. pipes, detector cell volume, etc.) the zone broadening may be approximated by

$$H \approx H_{\text{injection}} + H_{\text{column}} \approx \frac{V_{\text{sample}}^2}{K_{\text{injector}}} \frac{L}{V_R^2} + 2\lambda d_p + \frac{V_0}{V_R} \left(1 - \frac{V_0}{V_R}\right) d_p^2 u \frac{1}{30D_s} \quad (10.19)$$

Eq. (10.19) tells us that the sample volume should be kept low, the injector constant high (see eq. (10.22)) and the linear velocity should be adjusted when the particle size or the solute (i.e.  $D_s$ ) is changed in analytical size exclusion (e.g. for column qualification). In preparative size exclusion, when large sample volumes are applied, the inherent efficiency of small-particle-size chromatography resins is of minor importance, e.g. desalting of large sample volumes may be performed using large particles, which will be advantageous due to low flow resistance (*cf.* eq. (10.16)).

### Resolution in SEC

The resolution of solutes in size exclusion is determined by the size differences between the solute of interest and impurities, the selectivity of the chromatography resin and parameters such as flow rate, particle size and column dimensions. Influence of various parameters may be estimated from the resolution equation, i.e. eq. (10.1), as adopted to size exclusion by using eq. (10.17) and the relationship  $k' = K_D V_i/V_0$

$$R_s = \frac{2(V_{R_2} - V_{R_1})}{w_{b_2} + w_{b_1}} = \frac{1}{4} \log \frac{R_2}{R_1} \frac{dK_D/d \log R}{(V_0/V_i) + K_D} \frac{\sqrt{L}}{\sqrt{H}} \quad (10.20)$$

where  $dK_D/d \log R$  is the slope of the selectivity curve,  $K_D$  the average distribution coefficient,  $L$  the bed height and  $H$  the average plate height of the solutes.

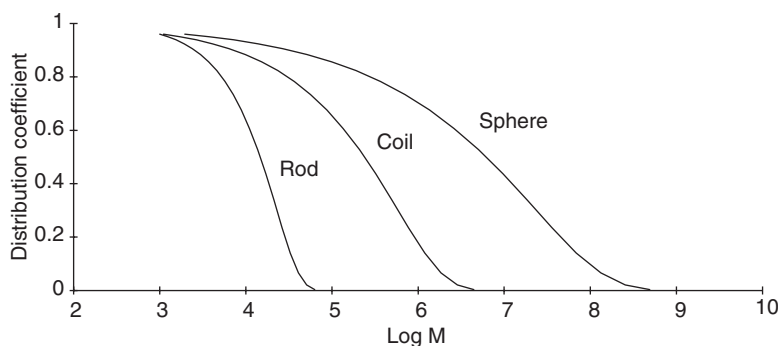
Thus, the resolution increases with increasing slope, pore volume and bed height and with decreasing void volume, distribution coefficient (though there is an optimum value of  $K_D$  as discussed below) and plate height.

### Influence of experimental parameters in SEC

From eqs. (10.17) to (10.20) we can identify that effective size and diffusion coefficient of the solute, mobile-phase flow rate, pore volume and pore size distribution of the chromatography medium, void volume and column length, and sample volume and viscosity are important parameters in size exclusion. The effect of various parameters may be studied by using the modelling software supplied.

### PROPERTIES OF THE SOLUTE

The influence of different molecular shapes on the retention is illustrated in Figure 10.5. The reason for the observed phenomenon is that the relationship between size and molecular mass differs for solutes of various shapes (i.e. the root-mean-square radius is proportional to  $M$ ,  $M^{1/2}$  and  $M^{1/3}$  for rods, flexible coils and spheres, respectively). Thus, it is easier to separate elongated solutes, e.g. DNA of intermediate size, than spheroidal solutes, e.g. globular proteins, having equal difference in molecular mass. However, this parameter is generally of little interest unless the shape of the solute or contaminants may be altered (e.g. by using denaturing resins or detergents) without impairing the recovery of an active product. A high solute diffusivity will give narrow peaks due to the positive effect on the non-equilibrium term (C-term) but may, on the other hand, give broad zones due to longitudinal diffusion (B-term), see eq. (10.10). This must be considered when the temperature



**Figure 10.5** Influence of solute shape on retention in size exclusion.

or viscosity of the eluent is changed or when zone broadening of different solutes is compared (e.g. for column qualification). Thus, zone broadening noted for one solute may not be relevant to the separation situation at hand. An increase in zone broadening due to temperature (e.g. by transferring the purification column to cold room) may be compensated for by decreasing the flow rate in accordance with eqs. (10.10) and (10.20), see Ref. [6].

#### PROPERTIES OF THE MOBILE PHASE

In theory, size exclusion is independent of the composition of the mobile phase (unless this is affecting solute or pore dimensions). In fact, variations in retention time or peak shape with changes in the mobile-phase composition or temperature is indicative of enthalpy effects and mixed-mode interactions. To prevent ionic interactions with the small amount of ionic groups present on most size exclusion materials addition of electrolytes, e.g. 25–150 mM sodium chloride, may be needed. In case of mixed-mode interactions it may be necessary to incorporate the theory of, e.g. ion exchange or hydrophobic interaction chromatography (HIC) for explaining retention behaviour.

The flow rate will influence the diffusional broadening of the sample zone (eq. (10.10)) and the effect may be substantial for large solutes of low diffusion coefficient (e.g. serum proteins) as indicated in Figure 10.2 (i.e. the reduced velocity is inversely proportional to the diffusion coefficient). It may be noted that size exclusion of low-molecular-weight solutes should be carried out at relatively high flow rates to reduce axial diffusion. In theory, using an increasing flow gradient to keep the reduced velocity constant when progressively smaller solutes are separated will be optimal. A decrease in cycle time with a factor of two was reported [6]. However, this may be difficult to apply in large-scale size exclusion. The optimal flow rate, given by a reduced velocity of 5, as seen from Figure 10.2, is proportional to  $D_M/d_p$  and this will be impractically low for macromolecules. Allowing a zone broadening from the C-term to be equal to that from the A-term resulted in the following recommendation for flow rate in size exclusion [6]:

$$F = A_c \varepsilon \times 65 \times K_D \frac{D_M}{d_p} \times 60 \quad (10.21)$$

where  $F$  is the flow rate (ml/min) and  $A_c$  the column cross-sectional area (cm<sup>2</sup>). Incorporating  $K_D$  in this equation will account for different values of  $\gamma_s$  in eq. (10.3) (i.e. setting  $K_D = 0.6$  will yield a recommended reduced velocity of 40 which is in good agreement with Figure 10.2). This recommendation does not hold for desalting where the solute of interest is excluded from the porous phase and very high flow rates may be used without severe zone broadening.

The zone broadening of the solute is affected by solute diffusivity as indicated by eq. (10.19). At high flow rates the plate height may be expected to be inversely proportional to solute diffusivity, provided the sample volume is small. The diffusivity increases proportionally to the temperature and inversely proportionally to the viscosity. The viscosity decreases with temperature. Thus, high viscosity of the eluent is generally avoided. The effect of temperature needs to be considered when performing separations in a cold room. Thus, the transfer of a size exclusion separation from 22 to 3°C resulted in a decrease in resolution by 20%. The resolution was restored by decreasing the flow rate by 47% [7].

#### PROPERTIES OF THE CHROMATOGRAPHY RESIN

The choice of chromatography resin having an optimal pore size distribution is affected by the composition of the sample to be purified. If the target molecule and the contaminants differ substantially in size (e.g. more than a decade in molecular mass) it may be possible to use a chromatography resin that has a pore size that excludes the target molecule but not the contaminants or vice versa. This is the most favourable situation, i.e. creates the highest selectivity factor, and will allow large freedom for the choice of running parameters that will affect productivity, such as sample volume, flow rate, column length, etc., which may generate high zone broadening. Furthermore, by eluting the solute of interest in the void volume the zone broadening of the solute will be minimal and the dilution factor low, e.g. 1.25 times.

If the target molecule only differs slightly in size from the contaminating solutes (e.g. as for oligomeric monoclonal antibodies) the choice of pore dimensions is more critical and a chromatography resin having a narrow pore size distribution (i.e. a high selectivity) and having a pore size from which the target molecule is eluted at roughly half a column volume ( $K_D \approx 0.4$ ) is optimal [6]. The increase in selectivity when going from a conventional size exclusion medium (e.g. Sepharose™) to a medium of maximum selectivity (e.g. Superdex™) may permit a doubling of the resolution or, in theory, reduce the separation time by a factor of four by increasing the flow rate (provided the C-term in eq. (10.10) is dominating) or decreasing the column length by this factor [19].

From eq. (10.20) it may be concluded that the pore volume should be as high as possible. Increasing the pore fraction  $V_i/V_0$  from 1.2 to 2.0 corresponded to a doubling in plate counts from 1400 to 2700 [20]. In this case the column length could have been reduced with a factor of two with retained resolution.

In buffer exchange the pore volume will be the determining factor for the processing rate, i.e. in theory a sample volume equal to the pore volume may be applied. Due to dispersion (i.e. the A-term and system contributions) the applicable sample volume will be slightly less (e.g. 80% of the pore volume).

As seen from eq. (10.20) the void volume should be kept low. This is due to the fact that the void volume does not contribute to the separation but only ‘occupies’ valuable column volume. The void volume is related to the particle structure (size distribution, rigidity and shape) and the packing density (*cf.* eq. (10.16)). Irregular particles yield larger void fractions than spherical resins. Void fraction for different size exclusion resins was found to vary from 0.30 for agarose based to 0.40 for spherical silica [6]. The void volume will influence the flow resistance as shown by Figure 10.3.

The particle size of the chromatography resin influences the zone broadening due to the decrease in diffusion distances with decreased particle radius. Since the C-term is dependent upon the square of particle size the effect is quite large and at large velocities (*i.e.* when the C-term is dominating) the resolution will be inversely proportional to the particle size. However, this is of little relevance in preparative size exclusion when large feed volumes are to be purified and the sample volume will dominate the peak width. Thus, the particle size must be optimized with the sample load in mind (see below).

The resolution is proportional to the square root of the column length. The effective column length may be increased by adding columns in series (*e.g.* as with the stack columns). However, some resolution may be lost due to zone broadening in the connectors between columns. Increasing the bed diameter will increase the pore volume of the system and as outlined above this has a very positive effect on the resolution if the fluid velocity is kept constant (however, the peak width will also increase due to increased retention time and the zone will be more diluted).

#### SAMPLE LOAD

The sample load is a product of the sample concentration and sample volume. In preparative size exclusion the high sample volume will contribute to the total peak width as described in eq. (10.19). The injector-dependent constant,  $K_{\text{injector}}$ , has been found to be close to 5 for ordinary laboratory injectors and approaching 12 for optimal injectors and large sample volumes where the injection profile is a square wave [18]. At very large sample volumes the load will be the limiting factor for peak width and thus resolution.

An optimal sample volume when processing large feeds may be calculated with the help of eq. (10.19). This optimum will balance the detrimental effects of a large sample volume (*i.e.* running few cycles) and the zone broadening running at high flow rates (*i.e.* split the sample into many cycles). A guidance to the optimal sample volume is given by

$$V_{\text{injection,opt}} \approx \left( \frac{V_{\text{feed}} \times K_{\text{injector}} \times V_c V_i d_p^2}{15D_M} \right)^{1/3} \quad (10.22)$$

where  $V_{\text{feed}}$  ml sample is to be processed per hour. The equation was found to support the general rule of processing a volume equal to 2–6% of the column volume for each cycle at cycle times of 5–1 h [7].

The sample concentration that is applicable is restricted by the viscosity of the injected sample plug as compared to the viscosity of the eluent. The general rule is that the relative viscosity of the sample plug should be less than 1.5. This corresponds to a

sample concentration of ca. 70 mg/ml of a globular protein such as serum albumin [6]. High viscosity of the sample will cause a distorted rear zone of the elution band [21]. This may be avoided by using an eluent of matching viscosity (though not generally applicable) or reducing the viscosity effects by special column constructions [22].

### 10.3.2 Ion exchange chromatography, IEC

The interaction in IEC has traditionally been described by a stoichiometric model where the solute will displace a number of counterions from the surface equating the number of interacting sites of the solute [23, 24]. This model has been questioned and a model where general electrostatic interaction theory for charged surfaces is used to explain the retention has been presented [25]. An evaluation of the two concepts using a weakly charged chromatography resins gave results in favour of the electrostatic interaction theory [26]. However, it was suggested that the two models would describe different extremes of IEC and further investigations must be performed before a conclusive statement could be made. Since the stoichiometric displacement model (SDM) currently provides the basis for ion exchange theory it will be used in this section.

The stoichiometric models have been refined to incorporate the steric shielding of ion exchange groups by large solutes. This model is called the steric mass action (SMA) model and was used for modelling IEC in overload mode [27].

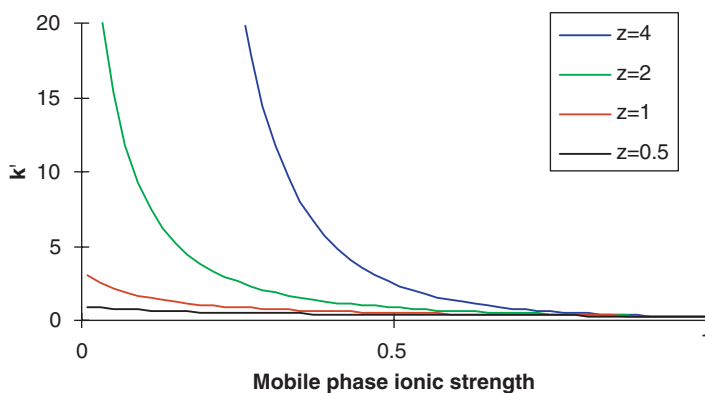
#### *Retention in IEC*

The retention factor in IEC is a function of the concentration of salt in the mobile phase,  $c$ , and the properties of the solute and adsorbent according to [3]

$$k' = k'_0 c^{-z} \Rightarrow \log k' = \log k'_0 - z \log c \quad (10.23)$$

where  $k'_0$  is related to the ion exchange capacity of the medium,  $Q_v$ , ( $k'_0$  is proportional to  $Q_v^z$ ) and  $z$  the interacting, or characteristic, charge of the solute. This equation is given for the stoichiometric model (in the electrostatic model  $\ln k'$  is proportional to  $I^{-1/2}$ , where  $I$  is the ionic strength) [25]. The relationship between the retention volume and mobile-phase concentration is shown in Figure 10.6. The retention varies drastically with  $c$  and  $z$ . It is seen that the retention will be sensitive to the ionic strength only in a limited region, the elution window, and that keeping the ionic strength constant will separate molecules, differing only slightly in  $z$ , far apart. On the other hand, separation of mixtures of solutes varying substantially in  $z$  requires a continuous change in  $c$ , i.e. gradient elution. However, such separations (i.e. simultaneous separations of several components) are only of primary concern in analytical applications. In preparative separations large differences in  $z$  are favourable since this allows step gradient elution. Typical values for the characteristic charge for proteins in IEC are in the range of 3.6–8.2 [24], and 4.8–7.5 [28], although this will vary with pH see Chapter 9.

It may be noticed that eq. (10.23) predicts that all solutes will move along the column bed and that this effect is not negligible unless  $k'$  is large. It is therefore recommended to



**Figure 10.6** Influence of mobile-phase salt concentration and characteristic charge on retention in ion-exchange chromatography (IEC).

apply the sample in a low ionic-strength buffer, however a too low ionic strength may lead to low dynamic capacity (see below). For solutes that are strongly adsorbed, the mobile-phase concentration may need to be increased considerably to desorb the solute. In order to reduce separation times (and excessive dilution of sample zones) the concentration is, in elution chromatography of complex mixtures, varied either continuously (gradient chromatography) or stepwise. For special cases combination of isocratic, step elution and gradient elution may be favourable.

The retention volume is given by eq. (10.4), (i.e.  $V_R = V_M + k'V_M$ ). This equation is valid only for conditions where the retention factor is constant, i.e. under isocratic conditions. In case the concentration of the mobile phase is changed (i.e. as in gradient elution) the retention factor is also continuously changed. The apparent retention factor calculated from the retention volume in gradient elution does not have any physicochemical meaning [3].

If the chromatographic system contains large extra-column dead volumes these will be added to the retention volume (but these should not be incorporated into the calculation of  $V_M$ , see Section 10.7).

### *Zone broadening in IEC*

The zone broadening in isocratic elution will be affected by the same factors as noted for size exclusion. If the adsorption–desorption reaction is not fast this factor will also contribute to zone broadening. However, in gradient elution a sharpening effect from the gradient is obtained (i.e. molecules at the front of the zone sense a lower ionic strength and thus a higher retention factor than molecules at the rear of the zone). This means that a steady state regarding zone broadening will be reached and, furthermore, all sample zones will have the same (narrow) width on the column, provided the elution conditions (e.g. column length and gradient conditions) are sufficient to promote this steady state. The degree of zone sharpening will depend upon the slope of the gradient (higher degree



of sharpening for a steeper gradient) and also on the relationship between  $k'$  and ionic strength for the solute. For traditional resins, peak widths of 50–80% of the ones calculated from eq. (10.10) may be expected [8, 29]. This sharpening effect is, together with the possibility to regulate  $k'$ , the main advantage of gradient elution.

### *Resolution in IEC*

The resolution in isocratic elution ion exchange is given by eq. (10.2). A plot of resolution versus  $k'$  shows that the effect of  $k'$  is low for  $k'$  above 10, see Figure 10.2. Also the largest effect on the resolution by far comes from the differences in  $k'$  between the two solutes, i.e. the selectivity.

Since  $k'$  varies during gradient elution, eq. (10.2) can not be used for calculation of resolution in this elution mode. Yamamoto and co-workers [29] found that the following equation was useful for predicting the influence of experimental parameters on the resolution in linear gradient elution of proteins in ion exchange and HIC.

$$R_s \propto \sqrt{\frac{L}{gV_0H}} \quad (10.24)$$

where  $L$  is the column length,  $g$  the gradient slope (mole/L per litre gradient volume),  $V_0$  the interstitial void volume and  $H$  the plate height. The plate height will be slightly lower than calculated from eq. (10.10) due to the zone-sharpening effect of the gradient provided the sample load is low and that the desorption kinetics is fast. At high flow rates the plate height will be proportional to the C-term. The influence of the column length may be substituted in eq. (10.24) by setting  $L = V_c/A_c$  where  $A_c$  is the cross-sectional area of the column and  $V_c$  is the geometric column volume.

### *Influence of experimental parameters in IEC*

The retention in ion exchange is determined by the charge of the solute and the exchanger, and the ionic strength of the buffer. The particle size, pore dimensions and flow rate will affect the mass transfer rate, and the resolution will be limited by the sample load. The gradient slope is important in gradient elution.

### PROPERTIES OF THE SOLUTE

For amphoteric solutes (i.e. solutes whose charge is pH-dependent) or weak exchangers, the pH of the buffer is of great importance. To ensure complete ionization the pH should differ at least one unit from the  $pK_a$  of the charged groups. For proteins and oligopeptides it may be noticed that the isoelectric point is equivalent to the pH where the net charge of the biomolecule is zero. However, there may exist areas of positive charge above the isoelectric point and areas of negative charge below the isoelectric point that will promote interactions with cation and anion exchangers (effects of pH have been noted as far as four units from the isoelectric point). The pH will affect the characteristic charge and thus have a large influence on the retention factor and resolution (*cf.* Figure 9.3). For basic proteins separated on an anion exchanger the retention will increase with increasing pH. For acidic

proteins separated on a cation exchanger the retention will decrease with an increase in pH. The magnitude of the change will be dictated by the slope of the titration curve (a plot of charge versus pH) for each protein. If the slope is similar (which is often the case) then all proteins will be affected in a similar way by a change in pH and no gain in selectivity will be obtained unless the pH passes the isoelectric point of one protein or if the charge distribution of the proteins is very different (*cf.* Figures 4.9, 4.10 and 9.4).

It must be noted that specific solute properties might cause shift in elution positions when mobile-phase composition or sample load is changed. This may be due to different charge–pH relationships, differences in charge distribution (patches), influence from solute size on  $k'$  or an effect of crossing isotherms (see below).

#### PROPERTIES OF THE MOBILE PHASE

The influence of the type of counterions on selectivity has been discussed (e.g. see Ref. [28]). However, it has been shown that specific effects of the salt used is not to be expected [30]. The governing parameter is the elution strength of the salt and the effect of one type of salt may be obtained by another type of salt by adjusting the concentration. The relative elution strengths of different ions are listed in Table 10.1.

The retention factor is dependent upon the ionic capacity of the chromatography medium raised to the power of  $z$  (eq. (10.23)). However, by keeping  $Q_v/c$  constant also  $k'$  is kept constant [3]. Thus, if the ionic strength needed to desorb a substance is unsuitable an alternative may be to use a chromatography resin having another ionic capacity.

Optimization of the starting conditions (i.e. the ionic strength of the start buffer) and the gradient slope are important for the resolution in gradient elution (i.e. to affect the peak-to-peak distance and keep the dispersion low). In laboratory preparations different shapes of the gradient have been elaborated but this is complicated for large-scale purposes. For the reason of robustness industrial-scale purifications by IEC are preferentially based upon step elution of the target solute. However, since gradient elution generally offers higher resolution of solutes having similar affinities for the adsorbent this elution principle is gaining popularity, especially as equipment for reliable large-scale gradient elution is becoming available (see Chapter 11). Gradient elution will also produce more concentrated zones of eluted product due to the sharpening effect. Furthermore, the influence of characteristic charge on  $k'$  (see Figure 10.6) makes gradient or step elution necessary for elution of large molecules (e.g. proteins), while small solutes (e.g. peptides) may be separated under isocratic conditions [31].

The flow rate is not critical in IEC as long as the contact time (i.e. the time allowed for the sample to equilibrate with the chromatography medium) is sufficient. In most cases,

**Table 10.1**

Elution strength of different ions [30]

Anion-exchange chromatography
Acetate < formate < chloride < bromide < sulphate < citrate
Cation-exchange chromatography
Lithium < sodium < ammonium < potassium < magnesium < calcium

the kinetics are sufficiently fast to allow very high flow rates to be used in gradient or step elution. The dispersion that will take place during elution (i.e. due to size exclusion of the solute) will dilute the zones somewhat but the effect on resolution is compensated for by regulating the selectivity (also, there is a zone-sharpening effect in gradient elution). Therefore, IEC is conducted at high flow rates as compared to size exclusion. However, as evident from eq. (10.24) the resolution will decrease with increasing values of  $H$ , and  $H$  will predominantly be affected by the C-term, i.e. proportional to the flow rate, at high flow rates. On the other hand, keeping the separation time constant means that  $g$  will be inversely proportional to the flow rate and thus the net effect on resolution will be nil.

#### PROPERTIES OF THE CHROMATOGRAPHY RESIN

The selectivity factor is dependent upon the type of charged group and the number of charges but generally not influenced by the matrix as such, unless secondary interaction mechanisms (e.g. hydrophobic interactions or size exclusion) are influencing. Thus the separation pattern on a HPLC type of resins, e.g. Mono Q<sup>TM</sup>, was found to be very similar to that on Q Sepharose Fast Flow (having the same functional group), which facilitated scale up from laboratory conditions [32].

The particle size will influence the plate height the same way as for size exclusion, though the effect may not be so dramatic due to the zone-sharpening effect of gradient elution. The effect of particle size on resolution of proteins in gradient elution was calculated from eq. (10.24), and good correlation to experimental results was found [33]. Smaller particles generally show faster mass transfer due to shorter diffusion paths, which results in a higher dynamic capacity of smaller particles.

The pore size and pore structure of the chromatography resin will have an effect on the accessibility of adsorptive sites for the solutes and the kinetics of the adsorption/desorption process. Thus, for large molecules wide-pore chromatography resins having an open chromatography matrix structure to provide fast access to adsorptive sites and that minimizes the risk of blocking the pathways will be preferential. Results from comparison of chromatography resins of different pore structure indicate that macroporous chromatography matrices are favourable except for very small solutes. Thus, too large pores may yield an unfavourable ratio of surface area to volume resulting in decreased capacity. In most cases the adsorptive site is made more accessible by attaching those to the chromatography matrix surface via a spacer arm, which will have a positive effect on available capacity and adsorption kinetics.

The diffusive mass transport of solutes is the primary limiting factor provided that the adsorption/desorption kinetics is fast (which is often the case in IEC). As noted for catalysts and chromatography resins the mass transport may be enhanced by promoting convective flow through the chromatographic particles [34–36]. This is achieved by increasing the pore dimensions of the particles to be in the same order as that of the void channels between the particles to decrease the flow resistance of the porous bead (*cf.* eq. (10.16)). Applications of large pore size chromatography resins based on synthetic as well as natural polymers for IEC of proteins have been described [37–39]. Superporous resins are characterized by higher resolution at elevated flow rates as compared to traditional

chromatography resins as a result of the increase in mass transfer [39]. The trade-off for speed is the reduced capacity caused by the reduction in chromatographic matrix (i.e. the superpores). It was shown that the productivity of conventional resins may in some situations exceed that of superporous resins due to this loss in capacity [40, 41]. In another comparison, a composite chromatography resin, where transport is reported to take place through a so-called surface diffusion, was found to give high capacity at high flow rates and compared also favourably to the properties of superporous resins [42]. This illustrates that there is a continuous development of process chromatography resins for IEC, and furthermore tools for the correct evaluation of the properties of these resins as compared to traditional alternatives are essential for the practitioner in the field.

#### SAMPLE LOAD

In isocratic elution the sample volume will contribute to the zone broadening as for size exclusion and it was concluded that concentration overload provides maximal throughput [43]. The sample volume is not a critical factor in gradient elution IEC unless the sample solution is of high ionic strength (e.g. has a high salt content). Under unfavourable conditions the solute may be eluted during the sample application, as a result of either isocratic elution (e.g. due to too high salt content) or frontal chromatography (i.e. other components of the sample are more strongly retained). This should be checked by determining the breakthrough capacity of the target solute in the feed solution (see below).

The sample concentration is interplaying with the sample volume and is limited by the amount of sample that may be applied, which in turn is determined by the capacity of the chromatography resin for the solute (and influence from contaminating solutes). Early in the purification process the sample concentration as such is often low and IEC is a very efficient concentration step, in addition to purification from other solutes.

The sample load will influence the resolution since the band will occupy a finite width. Restricting the load to less than 30% of the maximal load will normally be sufficient to avoid overloading and the influence on peak width or retention time will be small [8]. In practice this means that 25% of the column is used for sample loading while 75% of the column is used for the separation (obviously there is a lower limit to the column length for this rule of thumb to be valid).

The effect of overload mode, as a result of volume or concentration overload, is discussed below (see 'Non-linear chromatography' below).

The capacity is, together with the quantitative recovery of solute, the most important feature of the chromatography resin and experimental conditions chosen. For example, some chromatography resins may show very high capacity but low yield and therefore a careful examination of the properties (e.g. breakthrough capacity and material balance calculations) of the chromatography resin under the experimental conditions chosen is important.

The experimental dynamic binding capacity is dependent upon several experimental parameters, e.g. charge and size of target molecule, pore size and charge of the chromatography resin, and ionic strength of the solvent. Thus it was shown that under certain conditions the dynamic binding capacity decreased with decreasing ionic strength, which is contrary to general expectations [44].

### 10.3.3 Reversed-phase chromatography, RPC

The retention mechanism in RPC is still not fully understood [46]. Retention has been explained from a solvophobic model where the solute is forced into the stationary phase due to the strong mutual interaction of the molecules in the mobile phase (thus ‘excluding’ the solute from the mobile phase). The retention, in this model, is related to the solubility parameters of the solute and the components of the mobile phase though the relationship is complicated and only qualitative information is given by applying the theory [3, 46]. Another model that was discussed earlier was based upon liquid–liquid partitioning of the solute between the mobile phase and the stationary phase or the stationary–liquid interface but such effects had not been shown experimentally. However, experimental evidence supporting a partitioning process between small non-polar solutes and the stationary phase has been presented [45, 47]. On the other hand, it was found that polar solutes behaved differently from non-polar solutes [47]. It may also be expected that large solutes will behave differently from small solutes. Therefore, in this scope, implications of RPC theory will be made with reference to the existing theory (e.g. see Ref. [3]).

#### *Retention in RPC*

The retention factor in RPC can be related to the concentration of organic modifier in the mobile phase,  $c$ , according to

$$k' = k'_0 \times 10^{(-mc)} \Rightarrow \log k' = \log k'_0 - mc \quad (10.25)$$

where  $m$  is the ratio of the areas on the resin occupied by one molecule of solute to that occupied by one molecule of the solvent [3]. It should be noted that  $k'_0$  contains solvent and stationary-phase-specific constants and also the phase ratio,  $V_S/V_M$  (which may differ between materials). The retention factor decreases rapidly with increasing concentration of organic modifier and the decrease is attenuated for solutes of large interaction area (i.e.  $m$ ) as seen in Figure 10.7. This effect of the solute size on  $k'$  has been experimentally verified and used to explain the different retention behaviour of proteins and smaller solutes in RPC [31, 48]. The effect is that large solutes elute within a very narrow window of % organic modifier, i.e. a few percent (and the retention has, therefore, erroneously been interpreted as an on-off mechanism) [48]. This also results in that the effect of column length on resolution is rather small for proteins as compared to peptides.

Eq. (10.25) is a simplification where a quadratic term with respect to concentration has been neglected. In some cases the full equation is needed to account for variations of  $k'$  with mobile-phase concentration [3].

#### *Zone broadening in RPC*

Zone broadening in isocratic RPC will be similar to that in size exclusion, provided the adsorption–desorption mechanism is fast. In gradient RPC the sharpening effect, or band-compression effect of the gradient, will result in less zone broadening than in size exclusion. Furthermore, the widths of different peaks will not vary substantially for solutes of

similar size. As a rule of thumb, the peak width will be roughly equal to that of a substance eluted isocratically at a retention factor of 1–2 [3].

### Resolution in RPC

The resolution in RPC is described by eq. (10.1). As for IEC the largest effect on resolution is found for  $k' < 10$  (*cf.* Figure 10.1). The relationship expressed in eq. (10.24) is also applicable to RPC [49]. Snyder and Stadalius found that  $Rs \propto t_G^{0.5} d_p^{-1}$ , where  $t_G$  is the gradient time in RPC. This may be rearranged to eq. (10.24) provided the C-term dominates the plate height.

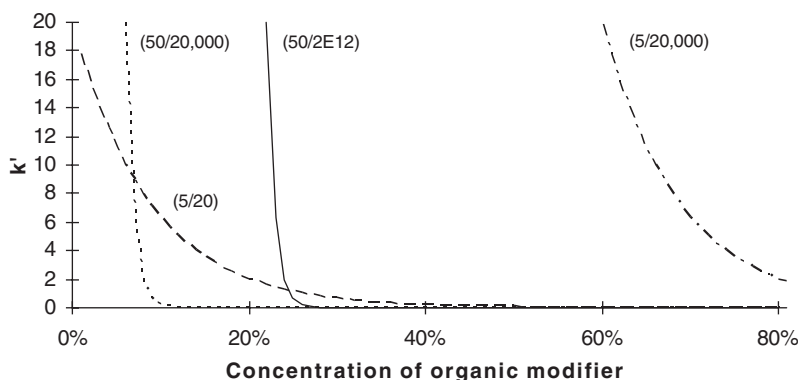
### Influence of experimental parameters in RPC

The parameters that may expect to influence the resolution in RPC are the one affecting the selectivity, i.e. size and the solubility properties of the solute, the type and concentration of the ligand(s) and the type and concentration (gradient) of organic modifier. The zone broadening will be affected by flow rate, particle size, column length, solute size and sorption kinetics. The size and pore structure of the solid phase will influence the kinetics as well as the available capacity.

#### PROPERTIES OF THE SOLUTE

The size of the solute will influence the occupied adsorbent area and it may be expected that the retention will increase with hydrophobic surface area of solute. For instance, an exponential relationship between peptide chain length and retention time has been noted [50]. From the influence of solute size on the retention factor large molecules are eluted within a very narrow range of mobile-phase composition in gradient elution RPC (*cf.* Figure 10.7). It may also be noted that molecules having an ordered structure may undergo conformational changes as a result of solute–sorber interactions. Such conformational changes may be reversible or irreversible, as noted for proteins on reversed-phase resins [51–53].

Relevant solubility parameters of solutes are difficult to extract. However, for molecules such as peptides attempts to relate retention to amino acid hydrophobicity coefficients



**Figure 10.7** Retention in reversed-phase chromatography (RPC) for large and small solutes. Calculated from eq. (10.25) with  $(m/k'_0)$  as stated in figure.

have met with some success [50]. However, this approach is difficult for larger molecules, e.g. proteins, that may expose interior hydrophobic patches as a result of secondary conformational changes induced by solute–surface interactions.

#### PROPERTIES OF THE MOBILE PHASE

The solvent strength of the organic modifier will influence the solvophobic effect and it may be expected that a stronger solvent will more readily accept the lipophilic solute. Thus, the solvents may be arranged according to increasing elution strength as in Table 10.2. A solvent of high hydrophobic strength is selected for solutes of high hydrophobicity. In some cases mixtures of organic modifiers of different properties have been used to increase the resolution; however, this is more suitable for analytical purposes or laboratory-scale purifications. The influence of solvent viscosity on flow resistance (*cf.* eq. (10.16)) may be prohibitive for use of some solvents or solvent mixtures (e.g. the viscosity of water–ethanol mixtures varies greatly with composition).

When choosing the organic modifier, considerations such as handling and disposal of organic solvents in large scale must be addressed. Most authorities put restrictions on the use of large quantities (i.e. 10 L ethanol) of organic solvents, and explosion-proof equipment and environment is required. Also, evaporation or other spontaneous changes of the composition of the mobile phase must be eliminated. Therefore, alternatives to organic solvents for large-scale RPC are currently being investigated.

The concentration of the organic modifier has a profound impact on the retention, as seen from Figure 10.7. In order to keep the separation time within reasonable limits it is, also in RPC, common to use a gradual change of the composition of the mobile phase for elution. A more shallow gradient will result in higher resolution as seen from eq. (10.24); however, on the expense of separation time. If the solutes differ substantially in retention the elution may also be accomplished by step elution, however this requires close control of the mobile-phase composition.

It is common to add a buffering substance to the mobile phase to assure that the solutes are uncharged during the separation (to avoid possible ionic interactions that otherwise may ruin the separation). In one application of RPC the solute is deliberately kept at a pH where it is charged and an organic counterion is added to the mobile phase. The solute and the counterion form an ion pair that is sorbed on the stationary phase. Often trifluoroacetic acid (TFA) is used to regulate the pH for silica-based supports (i.e. to suppress ionization of silanol groups) and the anion will also form an ion pair with cationic proteins. For preparative purposes this technique is of limited interest since it adds an extra separation step (to remove the counterion) and the cost of the ion-pair agent may be prohibitive.

**Table 10.2**

Properties of some organic solvents used in reversed-phase chromatography (RPC) of peptides [9, 50]

Solvent	Hydrophobic strength	Viscosity ( $10^{-3}$ Nsec/m <sup>2</sup> , 20 °C)
2-Propanol	High	2.3
Acetonitrile	Medium	0.4
Methanol	Low	0.6



## PROPERTIES OF THE CHROMATOGRAPHY RESIN

The chromatography resin should preferably be inert towards the components of the mobile phase, otherwise the matrix will swell and shrink with changes in mobile-phase composition which may cause problems with bed stability. Since the major driving force in RPC is the solvophobic effect the surface properties of the chromatography resin will also affect retention. Thus, it has been noticed that the silica backbone influences the retention in RPC [54]. Also the retention behaviour between different types of matrices, i.e. poly(styrene)-divinylbenzene and silica, displays that the backbone has a pronounced effect on the retention characteristics of solutes.

The particle size of the material will influence the diffusion distances of the solutes and thus a small particle size is favourable for a low zone broadening. At the high mobile-phase velocities commonly used in RPC the zone broadening is dictated by the C-term in the van Deemter equation (eq. (10.10)) and the peak width in isocratic elution will increase proportionally to the particle size giving an equal reduction of the resolution factor. For gradient elution the influence of particle size is slightly reduced (due to the sharpening effect) as seen from eq. (10.24).

The pore size and structure of the material will influence the mass transport of solutes as for other types of adsorptive chromatography (e.g. see discussion above for IEC). It has been concluded that RPC of large solutes such as proteins is best carried out with large-pore-size materials, i.e. having nominal pore size exceeding 50 nm [55]. It was shown that chromatography resins of 400 nm pore size yield high resolution of proteins at high flow rates whereas 30 nm pore size was optimal for peptide separations [56]. However, the material was used for fast analysis and the impact of loss in surface area on capacity in preparative purifications was not studied.

The type of ligand attached to the matrix has generally only a small influence on retention of proteins and peptides [56]. However, shorter aliphatic chains of four to eight carbon atoms length (C4, C8) are recommended for the separation of large solutes, i.e. proteins, whereas longer aliphatic chains (C8, C18) are chosen for separation of smaller macromolecules, i.e. peptides. The rationale behind this recommendation is the decreased recovery of proteins noted with more hydrophobic ligands (i.e. longer carbon chains) requiring higher concentration of organic modifier for elution. It may also be noted that the ligand density of reversed-phase resins is much higher than that used for HIC (see below). For instance, C4 was chosen in one industrial-scale purification of recombinant human IGF-1 to maximize product recovery [57].

## SAMPLE LOAD

The sample volume or concentration should not affect the resolution as long as the applied amount of sample (including *all* adsorbing species) is well below the maximum capacity of the sorbent. The general rule used in IEC of loading less than 30% of the maximum capacity for retaining the resolution should be valid also in gradient elution RPC. This means that maximum protein load should be proportional to column length, which also has been noted [54].

Too concentrated samples (e.g. having high viscosity) may be diluted prior to application if the starting conditions are to be chosen so that all material will be adsorbed to the chromatography resin.

The contact time will affect the maximum sample load that may be applied before sample appears at the column outlet. Increasing the mass transport will decrease the residence



time needed for complete adsorption of material in the loading step (i.e. unless equilibrium kinetics becomes a limiting factor).

Loss of active material due to irreversible adsorption, denaturation or conformational alterations must be evaluated in RPC.

### 10.3.4 Hydrophobic interaction chromatography, HIC

Hydrophobic interaction is mediated by the unfavourable energy needed to keep the hydrophobic molecule solvated in the polar solvent. Thus, the molecules are believed to be 'forced' out from the solvent into an interaction with the hydrophobic ligand at high ionic strength, rather than actively 'pulled out' of the mobile phase by the ligand (e.g. as for IEC) [3], hence the description solvophobic effects—i.e. avoiding the solvent. Though the basic principle is rather simple the solvophobic effects are currently not understood in detail and furthermore other effects such as electrostatic interactions will also affect the separation [58]. The role of water structure in biological interactions, which has a decisive influence on HIC, has been reviewed and the conclusion is that one should look at how different conditions affect the interacting surfaces rather than the solvent [59]. Thus, as for RPC the exact retention mechanism is under debate (e.g. see Lenhoff [60]) but currently the solvophobic theory is frequently used for relating experimental observations to theory.

The retention is accomplished by adsorbing the hydrophobic solutes at high ionic strength of the mobile phase (i.e. 'salting out') and desorbing the solutes by reducing the ionic strength.

#### *Retention in HIC*

The retention factor is, in absence of electrostatic effects, given by [58]

$$k' = k'_0 \times 10^{(mc)} \Rightarrow \log k' = \log k'_0 + mc \quad (10.26)$$

where  $m$  in this case is a hydrophobicity parameter (related to the hydrophobic contact area and the molal surface tension increment of the salt),  $c$  the salt concentration and  $k'_0$  a characteristic system constant. The similarity with the expression of the retention factor in RPC is obvious, though the dependence of  $c$  is inverse due to the different influence of  $c$  in the two cases (*cf.* eq. (10.25)). Thus, the retention factor increases rapidly with an increase in mobile-phase ionic strength and the retention factor may also be expected to be influenced by the interaction area of the solute (i.e.  $m$ ).

#### *Zone broadening in HIC*

Application of the sample zone is taking place at high ionic strength, and thus higher viscosity of the mobile phase than in, e.g. size exclusion or ion exchange. This may result in less efficient mass transport and broader sample zones unless compensated for by a decreased flow rate. During the elution, effects similar to those noted for IEC and RPC may be expected (i.e. a size exclusion type of broadening at isocratic elution and a sharpening of the zone at gradient elution).

### *Resolution in HIC*

The largest effect on resolution is, as for other adsorptive techniques, found for  $k' < 10$  (cf. Figure 10.1). Increasing the column length,  $L$ , will have a positive effect on the resolution and so will a decrease of the plate height,  $H$ . The influence of experimental parameters on the resolution as expressed by eq. (10.24) was found to be valid also for linear gradient elution HIC of proteins [29].

### *Influence of experimental parameters in HIC*

The parameters that will affect the resolution include solute, and mobile-phase properties and column length and loadability as for other adsorptive modes. A large influence of the ligand on the retention properties can be noted, in contrast to RPC.

#### PROPERTIES OF THE SOLUTE

Since the separation is based upon the ability of the stationary phase to interact with hydrophobic sites of the molecule it may be expected that charged parts of the molecule would decrease the interaction and thus that the interaction should be largest close to the isoelectric points. However, this is not always the case and retention as a function of pH is different for different proteins (i.e. due to the proximity of titratable and hydrophobic groups, see Chapter 9). Since the effect is arbitrary, pH is not the premier variable to study (e.g. as opposed to the situation in IEC) but must anyway be kept constant.

#### PROPERTIES OF THE MOBILE PHASE

The ionic strength of the feed solution needs to be sufficiently high in order to promote adsorption of the solute, i.e. to prevent elution during the adsorption step. On the other hand, the ionic strength must not cause precipitation of the sample or sample components on the column since this may cause problems with denaturation, high back pressures, etc. [61]. Normally between 1 and 4 M of sodium chloride or 0.75–2 M ammonium sulphate is needed for the adsorption step [61]. The solution is buffered to a suitable pH using a dilute, e.g. 0.01 M, buffer substance. The actual adsorption process is believed to be a multi-step reaction where the initial solvophobic step is followed by a rate-limiting reorientation of the protein on the ligand for maximum interaction. Since the adsorption step is performed in an environment that promotes aggregation of proteins a prolonged residence time may decrease the amount of recovered active material.

The solvophobic effect of the mobile phase is related to the content of cosmotropic salt that has the property of supporting the structure of water, and thus increase the hydrophobic effect, or chaotropic salt which has the property of disrupting the structure of water, and thus decrease the hydrophobic effect. These different properties of electrolytes were noticed well over a century ago and the ability of salt to salt-out proteins formed the basis for the Hofmeister series [62]. This series is given in Table 10.3. The specific influence of salt on the water structure may be quite large and from neutron diffraction studies it was concluded that 4 M of sodium chloride has an effect on water structure corresponding to a pressure of 1.4 bar [63]. The effect of different salts was found to follow the Hofmeister series. The influence of various salts on the retention in HIC has been attributed to their

**Table 10.3**

Hofmeister series of increasing salting-out properties of ions

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Anions
$\text{SCN}^- < \text{I}^- < \text{ClO}_4^- < \text{NO}_3^- < \text{Br}^- < \text{Cl}^- < \text{COO}^- < \text{SO}_4^{2-} < \text{PO}_4^{3-}$
Cations
$\text{Ba}^{2+} < \text{Ca}^{2+} < \text{Mg}^{2+} < \text{Li}^+ < \text{Cs}^+ < \text{Na}^+ < \text{K}^+ < \text{Rb}^+ < \text{NH}_4^+$

---

different molal surface tension [58]. Adsorptive efficacy has been noted to increase linearly with surface tension of the salts [64].

The desorption of hydrophobic solutes is achieved by increasing their solubility in the mobile phase. This is accomplished by reducing the ionic strength of the buffer or decreasing the surface tension by adding ethyleneglycol or isopropanol to the mobile phase. It is also possible to displace the protein with detergents, though the effect is small and the problem of getting rid of them after chromatography has discouraged their use [65].

Since the mechanism is driven by entropy we would expect an increased retention with increased temperature (making the disordered water molecules less prone to form an ordered layer around the hydrophobic molecule). We would also expect a decreased interaction at extremes of pH since the molecules will be highly charged. However, the opposite has been noticed experimentally [66].

The gradient in HIC is, as for other adsorptive modes, an important tool for tuning the resolution. However, gradient time may be more important than for IEC due to the kinetics in HIC may be expected to be slightly slower than for IEC. This is because the interaction in HIC involves direct solute-to-sorbent contact whereas the interaction in IEC is believed to take place over a distance of roughly 7 Å [58].

The flow rate will affect the zone broadening the same way as for size exclusion, i.e. according to the van Deemter equation. However, a zone-sharpening effect will be obtained in gradient elution, as noted for other types of adsorption chromatography. For HIC the viscosity of high salt and the slow kinetics will further increase the zone broadening and thus a larger zone broadening than noticed for IEC is common [58]. The flow rate will also affect the contact time (and thus utilized capacity).

#### PROPERTIES OF THE CHROMATOGRAPHIC RESIN

The influence of the type of ligand on the retention in HIC is large. In general, the retention increases with the length of the alkyl chain and also with increased substitution level. The use of aryl ligands adds the possibility of aromatic interaction to the hydrophobic interaction. This makes the selection of the optimal ligand to a matter of empirical testing though certain guidelines for optimization have been given [67]. From the result of a test of a large number of ligands and salts it may be concluded that selectivity could be obtained by different combinations of hydrophobicity-mediating resins and salts [68]. In principle the combinations, strong ligand–weak salt (e.g. phenyl-sodium chloride), medium ligand–medium salt (octyl-sodium acetate) and weak ligand–strong salt (e.g. butyl-ammonium sulphate) would be expected to yield similar results with respect to selectivity. However, the capacity would differ.

The optimal choice of ligand will be the most hydrophobic one that gives high recovery of active product and with conserved structural integrity. The substitution level on resins for hydrophobic chromatography is roughly 35% of that of reversed-phase resins [69]. It is believed that this low surface coverage will help in preserving the conformational structure of biological macromolecules. Unfolding of proteins in HIC and RPC has been extensively studied by Fernandez *et al.* [70, 71], confirming that chromatographic resins of higher hydrophobicity, or increased salt concentration or large hold times will increase unfolding. Jennissen [72] discussed selection of HIC resins in terms of 'critical hydrophobicity' to find an optimum resin for a specific application.

#### SAMPLE LOAD

The content of competing contaminants in the sample will limit the amount that is applicable in order to get the resolution needed. Thus the content of a more hydrophobic solute would drastically reduce the capacity for the product (and also change the elution position due to sample displacement effects). The utilized capacity of the sorbent is directly proportional to the ionic strength of the initial buffer, up to the point of precipitation (see Figure 4.20).

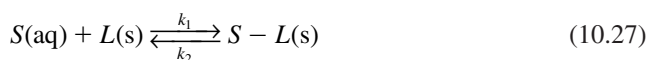
The sample volume will be of no concern as long as conditions for total adsorption of the sample are met. The amount of sample applied will be determined by the maximum capacity of the sorbent and the content of interfering solutes. As for other types of adsorptive chromatography the zone broadening will be affected by high sample loads leading to a broadening of the zone when more than 30% of the maximum capacity is exceeded [8]. If the sample precipitates on the column the time for the adsorption step needs to be minimized which puts restrictions on the sample application time. To reduce the risk of precipitation the technique of on-line sample dilution may be applied [67].

### 10.3.5 Affinity chromatography, AC

The separation principle in AC is the biospecific or group-specific interaction between a ligand and the solute (see Chapter 4 for discussion about different types of affinity ligands). By selecting a ligand that shows high specificity for the target solute a high degree of purification may be achieved in only one step. Due to the high selectivity of AC desorption of the pure solute is in most cases carried out by step elution after washing away impurities. The use of AC for large-scale purification of proteins has been reviewed [73].

#### Retention in AC

The adsorption and desorption process may simply be described by



where  $S$  symbolizes the solute and  $L$  the ligand,  $k_1$  is the forward and  $k_2$  the backward rate constant. The association constant,  $k_A = k_1/k_2$  should exceed  $10^5 \text{ M}^{-1}$  to yield an

efficient sorption of the solute. At lower association constants (i.e.  $k_A < 10^4 \text{ M}^{-1}$ ) the solute is only retarded and may be washed out prior to the desorption step. The dissociation constant,  $k_D$ , should ideally not be smaller than  $10^{-11} \text{ M}$  since this will require very harsh eluting conditions. The two constants are interrelated by  $k_D = 1/k_A$ . Thus, the association constant should be in the range  $10^5$ – $10^{11} \text{ M}^{-1}$ . When calculating association or dissociation constants it must be noticed that association constants determined for free ligand may be several orders of magnitude larger than those of immobilized ligand. An affinity medium with an association constant of  $10^6 \text{ M}^{-1}$  is called a micromolar binder and is generally regarded as a good affinity medium. A nano-molar binder, i.e. having an association constant of  $10^9 \text{ M}^{-1}$ , is suitable for scavenging applications.

### *Zone broadening in AC*

It has been believed that the kinetics of AC is slower than other modes of adsorptive chromatography techniques due to the fact that the interaction requires a favoured orientation of the molecule. However, this has not been experimentally proven and the conclusion is that the molecular orientation kinetics is not the rate-limiting factor for AC resins presently used (the situation may be quite different for other formats, such as affinity membranes).

The contact time needs to be sufficiently long to cope with the slow mass transfer of macromolecules (i.e. as for other adsorptive modes).

Desorption is normally done by a stepwise change of the composition of the mobile phase and the zone broadening can be assumed to be similar to that for size exclusion.

### *Resolution in AC*

Since desorption is carried out in a stepwise manner most of the components being sorbed will be eluted in one peak, unless the eluting conditions are specific. The term resolution as defined in eq. (10.1) has no meaning for stepwise elution (i.e.  $k'$  is momentarily changed from  $\infty$  to 0).

### *Influence of experimental parameters in AC*

The most critical parameter in AC is the selection of ligand and suitable conditions for adsorption and desorption. Since AC is an on-off technique column dimensions, flow rate, etc. are dictated by productivity considerations rather than from purity (i.e. resolution) point of view.

#### PROPERTIES OF THE SOLUTE

Properties of the solute will dictate the selection of ligand. A property that is very specific and also robust (i.e. not affected by normal experimental variations) is sought. Since affinity resins are relatively expensive the content of fouling solutes (e.g. lipids) may be reduced in a prior step.

#### PROPERTIES OF THE MOBILE PHASE

Desorption in AC is achieved by increasing the dissociation constant. This may be performed by varying the pH to change the conformation, or alter the surface charge of the

solute or the charge of the ligand. Another way to desorb the solute is by competitive elution where an agent will compete with the solute or the ligand for the affinity sites. This will introduce a contaminating solute to be removed by a subsequent step, e.g. size exclusion. Increasing the ionic strength, up to 1 M NaCl or the addition of chaotropic salt may sometimes be needed for desorbing the solute.

Desorption step in immobilized metal AC involves reducing the pH, increasing the ionic strength or stripping the metal ion by adding EDTA or another powerful complexing agent.

#### PROPERTIES OF THE CHROMATOGRAPHIC RESIN

A fast mass transport of solutes to the ligand is facilitated by using large-pore-size resins. Convective transport may be beneficial for fast separations in AC, however the trade-off between available capacity and speed must be evaluated. The orientation of the ligand will influence the effective capacity of the affinity resin. A too high concentration of ligand may yield steric hindrance (especially if the ligand is large) and also higher degree of non-specific interaction.

#### SAMPLE LOAD

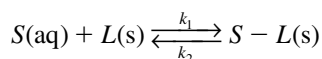
As for other adsorptive modes the capacity increases with increased residence time as shown in Figure 4.22. This illustrates that the mass transfer is currently the rate-limiting step for the adsorption of solutes to these types of chromatography resins. The sample load is only important at weak affinity, where the column will act as in an isocratic elution mode and the rules given for size exclusion may be applied. The maximal applicable sample load is determined by frontal analysis as described below. It may be noted that the degree of utilization of the affinity resin is low for systems having a low association constant, as shown in Figure 4.21 [74].

### 10.3.6 Other modes of chromatography

This chapter has been devoted to the modes of chromatography for which a solid theory has been established, and which allow predictions of separation behaviour. Other modes of chromatography that have been proposed for industrial purifications and whose use is based upon experimental optimizations include hydroxyapatite and multi-mode chromatography resins. The latter involves a purposely combination of different surface interactions such as ionic, hydrophobic and hydrogen bonding which may result in interesting separation properties. However, the separation is not easily predictable from theory.

## 10.4 ADSORPTION

The simplest model for adsorption chromatography assumes that the solute,  $S$ , confined in the aqueous phase (aq) is adsorbed to the ligand,  $L$ , of a solid (s) chromatography surface. The process is as expressed by eq. (10.27), which for convenience is given below



The adsorption process is characterized by an association constant,  $k_A$ , ( $k_A = k_1/k_2$  where  $k_1$  is the forward and  $k_2$  the backward rate constant) and a dissociation constant,  $k_D$ , ( $k_A = 1/k_D$ ). The association constant is given by

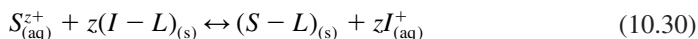
$$k_A = \frac{[S - L^*]}{[S^*][L^*]} = \frac{q^*}{C^*(q_m - q^*)} \quad (10.28)$$

where  $C^*$  is the concentration of solute in the mobile phase, i.e.  $C_M$  and  $q^*$  the concentration of adsorbed solute, i.e.  $C_S$ , at equilibrium and  $q_m$  the maximum (monolayer) capacity of the chromatographic medium for the solute. When  $C_S \ll q_m$  eq. (10.28) may, with the help of the relationship  $K_D = C_S/C_M$ , be used to express the relationship between the distribution coefficient and the association and dissociation constant through

$$k_A = \frac{1}{k_D} = \frac{K_D}{q_m} \quad (10.29)$$

Thus, two solutes having identical retention factors (and sensing the same phase ratio, i.e. having similar  $K_D$ ) may show different maximum capacity due to different association constants. This will lead to a situation where one substance will displace the other if there is a competition for the ligands. Eq. (10.29) elucidates the difference between affinity and retention.

In IEC the solute is displacing one or several,  $n$ , counterions,  $I$ , and the interaction for a cation exchanger may in the simplest case be described by



Eq. (10.30) is based on the simple stoichiometric model for ion-exchange equilibrium [23, 24]. An extension of this model accounting for the ligands sterically shielded by the interacting solutes, the SMA model, was found to fit experimental data for non-linear IEC of proteins [27]. The equation for the association constant is slightly more complicated for the ion-exchange case

$$k_A = \frac{C_S I_M^z}{C_M [z(q_m - C_S)]^z} \quad (10.31)$$

Since the interaction mechanism in ion exchange does not involve surface interaction (e.g. as for reversed phase) the stoichiometric model has been challenged [25]. However, for practical purposes of optimizing ion exchange in preparative purifications the stoichiometric model has been found useful. On the other hand, it is important to realize that an apparent fit of experimental data to a particular model, in most cases, does not provide enough evidence that the model is correct, but merely suggests its applicability for the separation problem at hand. The situation is further complicated by the fact that many models contain lumped parameters or approximations that hinder a strict physicochemical

evaluation of the model. Thus, it is up to the user to validate the applicability of the method for the purpose at hand.

### 10.4.1 Adsorption isotherms

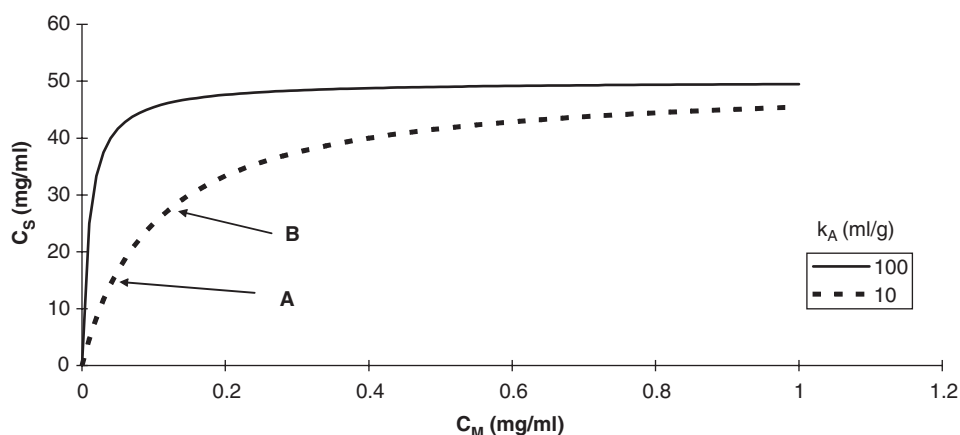
Rearranging eq. (10.28) yields the following relationship for the amount of adsorbed solute

$$C_S = \frac{C_M q_m}{C_M + k_D} = \frac{C_M q_m k_A}{C_M k_A + 1} \quad (10.32)$$

From eq. (10.32) it may be noted that the concentration of adsorbed solute is asymptotically approaching the maximum capacity of the chromatography medium and that this will be reached at lower mobile-phase solute concentration for solutes of higher association constants, see Figure 10.8. The plot of  $C_S$  versus  $C_M$  is called an adsorption isotherm (the temperature is held constant). The relationship given by eq. (10.32) is known as a Langmuir isotherm. This is valid for the simple case of monolayer adsorption of a single solute on a one-to-one relationship with the ligands (i.e.  $z = 1$  in eq. (10.30)) which is not affected by the presence of other solutes. This idealized condition is not generally fulfilled in preparative purifications where several components compete for the adsorptive sites and when secondary interactions (e.g. protein association) may be expected.

The equilibrium adsorption of a multi-component mixture may in the simple case be represented by the multi-component Langmuir isotherm

$$C_{S,i} = \frac{C_{M,i} \times k_{A,i} \times q_{m,i}}{1 + \sum C_{M,j} \times k_{A,j}} \quad (10.33)$$



**Figure 10.8** Hypothetical Langmuir adsorption isotherms for two solutes of different association constants. *A* denotes the upper range for the ‘linear region’ and *B* indicates a non-linear part (i.e. where  $K_D$  is dependent upon  $C_M$ ) of the isotherm.



Other isotherms have been found useful for describing adsorption characteristics such as interactions between adsorbed molecules (e.g. the Fowler isotherm) and adsorption to heterogeneous surfaces (e.g. the Freundlich isotherm). However, even though the Langmuir isotherm is only valid for specific conditions (e.g. monolayer non-competitive adsorption) it has been successfully applied as a first approximation to describe adsorption in preparative chromatography of biomolecules [45, 74–77]. Deviations from the Langmuir isotherm has also been noticed and discussed [78]. A review by Bellot and Condoret [79] addresses the relative merits of different types of isotherms and concludes that the (competitive) Langmuir model of adsorption is one of the most employed in literature.

The simple Langmuir model is not applicable to ion exchange if we want to consider each ligand as a separate binding site (i.e. since one solute may occupy several ligands) and if we want to include the effect of ionic strength on the isotherm. A transformation of the Langmuir isotherm as proposed by Antia and Horvath [80] addresses this situation. The stationary-phase concentration cannot be explicitly derived from eq. (10.31) (as for eq. (10.32)) but an implicit expression is obtained that can be solved for given values of  $k_A$ ,  $z$ ,  $q_m$  and  $C_M$ . A different approach was used by Chase and co-workers [75] who regarded the adsorption site as being the number of ligands a molecule will occupy and disregarded the effect of the ionic strength (i.e. leading to that  $q_m$  will vary with ionic strength). In this way they were able to use the simple Langmuir isotherm for modelling protein adsorption to ion exchangers.

By compensating for the number of ligands,  $n$ , sterically shielded by the solute and replacing  $z \cdot q_m$  with the total ionic capacity  $Q_v$ , the relationship for the SMA model is obtained

$$C_{M,i} = \frac{C_{S,i} \times I_M^{z_i}}{k_A \left[ Q_v - \left( \sum (z_{i,j} + n_{i,j}) C_{S,i} \right)^{z_i} \right]} \quad (10.34)$$

The value of  $C_S$  for different  $C_M$  may be obtained by, for instance, curve fit to experimental data [27]. A comparison of different isotherms for IEC showed that the SMA model (eq. (10.34)) gave better agreement with experimental data than the modified Langmuir isotherm or the basic SDM model (where steric shielding is not accounted for) [27]. However, the SMA model contains a ‘lumped’ parameter, i.e. the steric shielding factor,  $n$ , which together with the characteristic charge,  $z$ , is determined from experimental fit (the other models also contain similar adjustable parameters).

The shape of the Langmuir isotherm is convex as shown in Figure 10.8. This type of isotherm is common in liquid adsorptive chromatography (though other types of isotherms are not uncommon). The Langmuir isotherm displays three distinct regions: one linear region at low-solute concentrations, one non-linear region at high-solute concentrations and one constant region at very high solute concentrations. The distribution coefficient,  $K_D$ , is equal to the slope of the isotherm at any mobile-phase composition. Thus, the retention factor will vary with sample concentration in the non-linear region of the isotherm (cf. eq. (10.3)). To maximize throughput most preparative chromatography is carried out in the non-linear region; this is called non-linear chromatography.

### *Linear chromatography*

Working at concentrations where the solutes are essentially not competing leads to a condition where the amount adsorbed solute is proportional to the concentration of solutes in the mobile phase (region A in Figure 10.8). This condition is called linear chromatography. Working in this region is favourable from resolution point of view (due to less tailing of peaks) and also from a scale-up point of view (constant conditions). As can be seen from Figure 10.8 the linear region is extended to higher  $C_S$  for chromatography resins of high maximum capacities and affinity resins with high association constants. In techniques such as size exclusion no adsorption takes place and the concentration in the stagnant phase is always proportional to the concentration in the mobile phase. One might in SEC expect non-linearity to result from over-crowding at very high concentrations (i.e. due to sterical interference between molecules); however, the limiting factor seems to be the hydrodynamic instability of highly concentrated sample zones which leads to viscous fingering effects.

### *Non-linear chromatography*

In preparative chromatography maximum throughput is sought. This means that most separations are carried out at high mobile-phase concentrations in the non-linear region of the adsorption isotherm (part B in Figure 10.8). From the figure it is seen that as the mobile-phase concentration increases (as for the ascending part of a chromatogram) the quotient  $C_S/C_M$  decreases. This leads to a decrease in the retention factor (*cf.* eq. (10.3)) and will result in an increase in velocity of the solute. The reverse is true as the mobile-phase concentration decreases. The effect is that the ascending part will be steeper than expected and that the descending part will show pronounced tailing, until the concentration is so low that the linear part of the isotherm is reached. The effect on the separation will differ depending on whether the elution is done isocratically or by a gradient. In isocratic elution the zone broadening and elution distance between peaks will vary proportionally to the column length leading to 'proportionate separation pattern' while a 'constant separation pattern' is obtained in gradient elution, provided the conditions are sufficient to promote a 'quasi-steady state' as described below [8].

#### ISOCRATIC ELUTION

The situation described above will result in a peak that will get deformed as the concentration of sample is increased. Furthermore, the leading part of the peak will get displaced to shorter retention times [2]. Thus, for isocratic separation of mixtures, there is an upper limit to the volume that may be applied before the bands start to overlap. On the other hand, increasing the sample volume will result in an increase in zone broadening (e.g. as for size exclusion, *cf.* eq. (10.19)). Knox and Pyper [43] reviewed the situation at overloaded isocratic elution conditions and provided guidelines to maximize the throughput. The influence from the sample volume was found to be more detrimental to resolution than the influence from sample concentration. It was concluded that concentration overload provides higher throughput in isocratic preparative liquid chromatography. Isocratic elution is often applicable to separation of small molecules but generally not to purification of macromolecules (i.e. due to the dependence of retention factor on solute size,

*cf.* eqs. (10.23) and (10.25)). The latter type of substances are predominantly purified by linear gradient or step-elution chromatography.

#### GRADIENT ELUTION

In gradient elution the zones are sharpened due to the compression caused by the different retention factors at the leading and trailing part of the peak (sensing slightly different mobile-phase concentrations of eluting component). This will eventually lead to a 'quasi-steady state' where dispersion effects and compression effects are balancing each other to result in a constant peak width [8]. Prerequisites are a sufficient column length and gradient slope to promote the quasi-steady state. This will result in a 'constant separation pattern'. The zone-sharpening effect of gradient elution allows higher sample loads than applicable for isocratic elution and up to 30% of the maximum capacity may be applied before overload conditions is noticed [8]. The determining factor is total load and no preference for sample volume or sample concentration was noted [29]. However, for protein mixtures, overloading effects (as noted by non-symmetrical peak shapes) may be seen for the component of lowest saturation capacity at total loads of 10% while no significant effect was noted on the peak shapes of the other components, even at a total load exceeding 30% [81].

### 10.5 ELUTION MODES

There are basically three different ways of desorbing bound solute(s) from the chromatography resin, i.e. using the sample as in frontal development, or a competing agent as in elution chromatography, or a displacing agent as in displacement chromatography [2, 82].

#### 10.5.1 Frontal chromatography

In frontal chromatography, or frontal development, the sample is also used for elution. Thus, the sample feed is continuously applied to the column and the sample components will displace each other in order of decreasing affinity for the chromatographic medium (this is called sample self-displacement). The least retained solute will be obtained in a pure form (i.e. depleted of the more strongly retained components) until the other solutes break through. Eventually the column will be saturated with the strongest retained component and the effluent will have the same composition as the feed. Thus, even the strongest retained component may be obtained after washing the column and a desorption step (see sample displacement below).

#### 10.5.2 Elution chromatography

In elution chromatography the solutes are desorbed from the chromatography medium due to the action of a competing agent in the eluent. The competition is a reversible process (as indicated in eq. (10.27)) and the concentration of the competing agent is a key parameter

(*cf.* Figures 10.6 and 10.7). Elution chromatography may be carried out under three different conditions, i.e. constant  $k'$ , a continuous change in  $k'$  or a discontinuous change in  $k'$ . By keeping the composition (e.g. ionic strength) of the eluting buffer constant the capacity factor is also kept constant (*cf.* eq. (10.23)). This is called isocratic elution. Isocratic elution is primarily used for separation of small molecules for which the variation of retention factor with mobile-phase composition is not as large as for larger molecules. In gradient elution the composition of the mobile phase is continuously changed (e.g. increased ionic strength as in IEC or decreased ionic strength as in HIC) with a continuous change in  $k'$  as a result. Gradient elution may be necessary for separation of large molecules for which  $k'$  differs too much for isocratic elution to be feasible. Step elution where the  $k'$  is changed momentarily by discontinuously switching the mobile-phase composition may be used for desorbing components of extreme differences in  $k'$  and is commonly used in manufacturing processes due to the greater robustness as compared to gradient elution.

### 10.5.3 Displacement chromatography

If the affinity of the competing agent for the chromatography resin is much higher than that of the solute then the solute will be effectively displaced by the agent. This will take place even though the concentration of the agent, or displacer, is low, in contrast to elution chromatography [83, 84]. This is basically a non-reversible process under normal conditions. Displacement chromatography has the advantage of being able to concentrate samples. The concentration of a displaced sample zone is a function of the initial concentration of the displacer, and the shapes of the isotherms of the component and the displacer. Thus the concentration of displacer provides a convenient way of regulating the concentration of the eluted component. The technique provides self-sharpening of the solute zones (i.e. the tailing noted in non-linear elution mode is counteracted by the adjacent displacing solute). Displacement chromatography can only be realized under conditions where the isotherms of the different solutes do not cross [85]. Displacement chromatography may be carried out using relatively inexpensive equipment (e.g. as used for step-elution chromatography). However, since sample zones are not spaced by buffer a means of detecting the component of interest is needed. Even though the boundaries between zones may be sharp they may still show some overlap, which will result in a need to sacrifice yield to get the required purity. The potential need to remove contaminating displacer has also hampered the utilization of displacement chromatography for large-scale purifications. However, there is currently strong progress in the development of displacers (e.g. having low molecular mass) and displacement chromatography for preparative purifications using different modes of chromatography [86–88].

### 10.5.4 Sample displacement

In overload mode the maximum capacity of the chromatography resin is utilized. This may be achieved by a combination of frontal chromatography for the sample load, followed by a wash to elute non-bound solutes and desorbing the sample by elution or

displacement chromatography [89–91]. The sample load step has been coined ‘sample displacement’; however, according to the terminology by Karger *et al.* [2], this should rather be called frontal development. The sample molecules are effectively competing for the adsorptive steps leading to a ‘displacement train’ being developed inside the column. The solutes may then be desorbed by using either a strong displacer or conventional elution chromatography.

The advantages of different elution modes for process chromatography were reviewed by Freitag and Horváth [85].

## 10.6 BED CONFIGURATION

Bed configurations developed for analytical chromatography (e.g. layer, as in thin-layer chromatography or paper chromatography, packed columns or capillaries of substantial length) may be used for small-scale preparative work. However, demands of high throughput, or direct application of particulate containing feed, or need for continuous operation makes other configurations such as packed beds, fluidized beds or moving beds more suitable for process chromatography.

### 10.6.1 Packed beds

A linear scaling of separations from ordinary analytical columns has provided suitable solutions also for process chromatography. The demand for high throughput has been met by designing columns of large diameters (e.g. steel columns having 1.2 m diameter are now standard items, see Chapter 11). Improvements in handling these large columns (e.g. automatic packing, emptying of columns, hydraulic adjustable adapters, etc.) facilitate their use. The popularity in using packed beds can be ascribed to the applicability of established chromatography theory and scalability of results obtained at laboratory scale. Also, a packed tube is a convenient format for handling the chromatographic resin and potential toxic substances in the feed. However, there are certain limitations to this configuration, which are addressed by the formats below.

### 10.6.2 Fluidized beds

In a fluidized bed the chromatographic resin is kept suspended while the crude feed is pumped through the bed. It may be visualized as a packed bed, which is allowed to expand due to upward flow of the mobile phase. This permits solutes to be adsorbed while particles, such as cell debris or whole cells, pass unretarded through the expanded bed. The technique has become popular since it combines several steps in the early purification such as initial capture of the target solute, removal of particulates and even initial purification, provided a selective chromatographic resin may be employed [92].

A prerequisite for the bed to be fluidized is that the density of the chromatographic resin is large enough to allow gravitational settling to balance the lifting drag forces from the upwards flowing eluent. This means that there is a flow window where the resin can be

used (i.e. at too low flow there will be no expansion and at too high flow all chromatography resins will be eluted out of the column). Quartz or metal alloys may be incorporated into polymer beads to increase the density of the chromatography resin (to allow for higher flow rates to be used). Two other important parameters are the density range and particle-size distribution of the beads, which, if properly chosen, will create a stable bed of good chromatographic performance, in contrast to traditional fluidized beds where the performance is low due to a large degree of backmixing [92]. The expansion degree, expressed as the bed height of an expanded bed over the bed height of a settled bed, of 3–4 (corresponding roughly to a void fraction of 0.8) has proven to yield good adsorptive properties while providing enough space for particles to pass through the bed [93]. Fouling of the bed, i.e. non-wanted adsorption of cell debris, etc., to the particles may lead to collapse of the stable bed. The risk of fouling will differ between cell culture systems and while expanded bed was found to be suitable for yeast systems difficulties have been reported for CHO systems.

After the adsorption, or capture step, the bed is washed and the target solute desorbed, either directly in an upwards mode or in a downwards mode after the bed has been settled. The latter procedure will reduce volumes of buffer and fractions.

### 10.6.3 Moving beds

In packed-bed or expanded-bed chromatography the bed is fixed while the eluent transports the sample components. In a moving bed the chromatography medium is also moved and used for transporting sample components. The movement may be perpendicular to the eluent as in continuous annular chromatography or in the opposite direction of the eluent (i.e. countercurrent) as in a simulated moving bed (SMB).

In continuous annular chromatography the bed is rotated while the sample is continuously applied. The elution takes the solutes down the column but the radial position from the inlet will increase with the affinity to the chromatographic medium. The solute of interest may be continuously sampled from the bottom of the column. A model for predicting the separation of proteins with continuous annular chromatography has been presented [94].

In a true moving bed (TMB) the sample is added in the middle of the bed and strongly retained solutes will be transported with the bed while less strongly retained solutes will be transported with the eluent. In this way binary mixtures may be separated in a continuous fashion (or complex mixtures separated in two fractions). A TMB is difficult to realize in practice but by using a number of columns (e.g. four) of identical properties a SMB may be created where a fresh column is inserted at the bottom of the column train and the uppermost column is removed, desorbed and regenerated to be inserted at the bottom of the column train at next cycle. The advantages of using a moving bed are the continuous, or semicontinuous operation (i.e. application of sample and collection of product) and the high degree of utilization of capacity of the chromatography resin, which are characteristics for countercurrent adsorption [95]. Disadvantages are that the complex sample components cannot be resolved (i.e. only a binary separation may be achieved by each 'column train'; however, several trains may be linked together to resolve mixtures) and expensive

equipment is needed (e.g. valving system). In a comparison between packed bed and SMB for the purification of *trans*-phytol from *cis*-phytol it was found that the productivity of eight columns packed with 25  $\mu\text{m}$  chromatography resin run in SMB mode was roughly equal to that of an ordinary liquid chromatography system running one column packed with 15  $\mu\text{m}$  chromatography resin [96]. The SMB system was reported to be less sensitive to a decrease in column efficiency compared to traditional column chromatography. A less complex three-column system for continuous production of hIgG by periodic countercurrent protein A chromatography was recently presented by Lacki [97]. The production rate (g/h) was increased by 35% as compared to a traditional fixed bed system.

The system configuration for running a continuous operation may be made simple, e.g. as the system described by Lacki, or more complex, e.g. the system for SMB described by Mazzotti *et al.* [97, 98]. The system by Mazzotti *et al.* had the necessary improvement of incorporating a CIP-step as part of the plasmid purification SMB application.

## 10.7 EXPERIMENTAL DETERMINATION OF BASIC PARAMETERS

In order to evaluate the performance of the chromatographic system or assessing parameters for optimizations of separations using eqs. (10.1)–(10.34) the calculation of retention, zone broadening, resolution, mass transport parameters and solute capacity from experiments is needed. Many of these are only valid for isocratic elution, Yamamoto and Snyder *et al.* have addressed retention parameters in gradient elution [8, 99].

### 10.7.1 Retention

The retention factor,  $k'$ , can be calculated from the retention volume,  $V_R$ , and the mobile-phase volume,  $V_M$ , by rearranging eq. (10.4) to

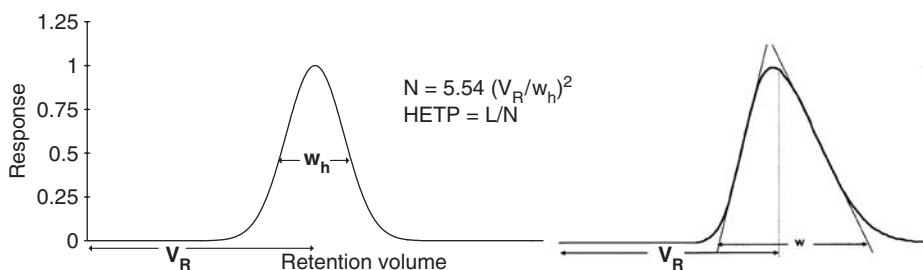
$$k' = \frac{V_R - V_M}{V_M} \quad (10.35)$$

This relationship is valid only for isocratic elution where  $k'$  is constant throughout the run. In gradient elution the retention factor calculated from the retention volume will not have any physicochemical meaning [3]. However, it is useful as a retention parameter to be kept constant during scale-up, provided the gradient volume and the dwell volume are kept constant.

*Retention volume,  $V_R$*

The retention, or elution, volume is given by the volume delivered by the pump(s) from the time when half the sample mass is applied to the column to the time when half the sample mass is eluted from the column. In practice, there are several simplifications made in the calculation of the retention volume. The contribution from the dead volumes in tubings or pipes from the injector to the column and from the column to the detector is often





**Figure 10.9** Calculation of retention and column efficiency from a chromatogram. Left figure illustrates a perfect Gaussian shape, right figure illustrates that calculation of retention volume and peak width from a skewed peak is not straightforward and requires the use of eq. (10.37) and analysis of the statistical moments of the distribution. An estimate of the peak width,  $w_b$ , is given by  $w$ , calculated from the intersection of the tangents with the baseline.

ignored. The contribution from the sample volume to the retention volume in isocratic elution may be ignored if the sample volume is small. The retention volume is often determined from the peak apex, presuming a symmetrical peak shape. In such instances the retention volume is calculated as shown in Figure 10.9.

If, however, the sample volume cannot be ignored the sample application time is set when half the sample volume has been injected. If the data are to be used for scale-up between systems, the influence of the system dead volumes,  $V_{\text{ext}}$ , on the apparent retention factor,  $k'_{\text{app}}$  must be estimated. It is a simple exercise to show that the system dead volumes will influence the measured retention factor according to

$$k'_{\text{app}} = \frac{k'}{1 + (V_{\text{ext}}/V_m)} \quad (10.36)$$

Thus, keeping  $V_{\text{ext}}/V_m$  either small or constant will give an apparent retention factor that is scalable.

If the sample load or other effects causes the eluted peak to be non-symmetrical the retention volume cannot be estimated from the peak apex. In this case the retention volume is set equal to the centre of mass which is obtained from the first statistical moment of the concentration,  $C$ , versus volume,  $V$ , distribution

$$V_R = \frac{\int_{V=0}^{\infty} CV dV}{\int_{V=0}^{\infty} C dV} \cong \frac{\sum_{i=1}^n C_i V_i \Delta V}{\sum_{i=1}^n C_i \Delta V} \quad (10.37)$$

The right-hand side of eq. (10.37) is useful for calculation of retention volumes of non-symmetrical distributions by simple spread sheets or manually (i.e. from the peak height



at different elution volumes). It may be noticed that the denominator in eq. (10.37) is equal to the area of the peak and is the amount of solute eluted.

#### *Mobile-phase volume, $V_M$*

The mobile-phase volume is not a constant but solute dependent. It is the elution volume of the solute under non-retaining conditions (in this case it seems inaccurate to talk about retention volume why elution volume is preferred). In the separation of small molecules by RPC this volume is often set equal to the total liquid volume of the column and coined  $V_0$ . However, this designation is discouraged since  $V_0$  is reserved for the interstitial volume of a packed bed [1]. Symbols and definitions for liquid chromatography are reviewed in Appendix A. Determination of the elution volume under non-retained conditions is performed as described for the retention volume. It is sometimes necessary to vary the conditions to assure that the conditions of non-retention are fulfilled (e.g. running at various ionic strengths). However, the risk of secondary retention mechanisms (e.g. hydrophobic interaction at high ionic strength) must be considered. The difficulty of obtaining correct values of  $V_M$  has been addressed [100, 101]. It is common to arbitrarily set the mobile-phase volume equal to the breakthrough of the solvent front. However, this will not correspond to the thermodynamically correct value of the mobile-phase volume for the solute of interest.

#### *Mobile-phase composition at elution*

The mobile-phase composition at which a solute is eluted in IEC, e.g. elution ionic strength  $I_R$ , may be calculated from the retention volume according to

$$I_R = I_{\text{start}} + (V_R - V_{\text{start}}) \frac{I_{\text{end}} - I_{\text{start}}}{V_{\text{gradient}}} \quad (10.38)$$

where  $I_{\text{start}}$  and  $I_{\text{end}}$  are, respectively, the ionic strength at the start and the end of the gradient,  $V_{\text{gradient}}$  the gradient volume and  $V_{\text{start}}$  the volume pumped through the system from the injection point to the time when the start of the gradient hits the column. It must be noticed that this time will vary due to the dead volumes from the column to the mixer forming the gradient and from the mixer to the pumps delivering the high- and low-ionic-strength buffers. This may be important in scale-up where different system configurations are likely to be used. The last term in eq. (10.38) is called the gradient slope and denoted as  $g$ .

Calculation of the mobile-phase composition for other techniques, e.g. %B for elution in RPC, is analogous to the example with ion exchange given in eq. (10.38).

### **10.7.2 Zone broadening**

#### *Peak width and plate number*

The broadening of the sample zone, either as a function of dispersion caused by mixing chambers outside of the column or fluid transport in the column, or caused by molecular

diffusion, may be determined from the width of an injected pulse (see Figure 10.9). Provided the resulting peak has a Gaussian shape the base width of the peak,  $w_b$ , expressed as four times the standard deviation,  $\sigma$ , may be calculated from

$$w_b = 4\sigma = \sqrt{\frac{2}{\ln 2}} w_h \quad (10.39)$$

where  $w_h$  is the peak width at half peak height. Since  $w_h$  is fairly easy to determine graphically eq. (10.39) is frequently used for calculation of the peak width. Inserting eq. (10.39) into eq. (10.9) yields the following relationship for the number of plates per column,  $N$ , in isocratic elution

$$N = 8 \ln 2 \left( \frac{V_R}{w_h} \right)^2 \approx 5.545 \left( \frac{V_R}{w_h} \right)^2 \quad (10.40)$$

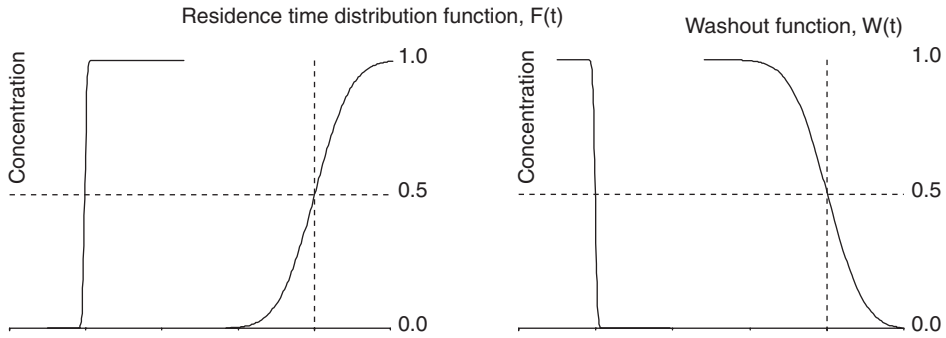
Eq. (10.39) cannot be used if the peak is not Gaussian and the standard deviation of the peak must then be determined from the second moment of the distribution

$$\sigma^2 = \frac{\int_{V=0}^{\infty} C(V - V_R)^2 dV}{\int_{V=0}^{\infty} C dV} = \frac{\int_{V=0}^{\infty} CV^2 dV}{\int_{V=0}^{\infty} C dV} - V_R^2 \cong \frac{\sum_{i=1}^n C_i \cdot V_{Ri}^2 \cdot \Delta V}{\sum_{i=1}^n C_i \Delta V} - V_R^2 \quad (10.41)$$

#### *Residence time distribution, RDT*

For systems of large zone broadening (e.g. fluidized beds) the dispersion becomes so large that measurement of the broadening of an injected pulse may yield large errors and another method is therefore needed. A general approach for calculations of retention and zone broadening in simple flow systems is provided by the residence time theory [102]. The residence time distribution function,  $F(t)$ , is given by the probability that a solute has a residence time less than the time  $t$ . The shape of this function is equal to a positive step function, see Figure 10.10. The washout function is defined as  $W(t) = 1 - F(t)$  and the density function is given by  $dF/dt$ . The mean residence time and the second moment of the distribution are given by equations analogous with eqs. (10.37) and (10.41) with volume transformed to time through  $t = V/F$  where  $F$  is the volumetric flow rate. The statistical moments may also be determined from the washout function by

$$\mu_n = n \int_0^{\infty} t^{n-1} W(t) dt \quad (10.42)$$



**Figure 10.10** Residence–time–distribution function (left curve) and washout function (right curve).

where  $\mu_1$  is the first moment (i.e. retention time) and  $\mu_2$  is the second moment (i.e.  $\sigma^2$  in time units). Time 0 is of course the time when the washout is initiated. Provided the distribution follows that of the normal frequency distribution the normalized washout function may be used to estimate the mean residence time from  $t$  corresponding to  $W(t) = 0.5$  and the standard deviation from  $(t_1 - t_2)/2$  where  $W(t_1) = 0.8413$  and  $W(t_2) = (1 - 0.8413)$ . This is given from the cumulative distribution function, and other values of  $W(t)$  may be chosen and related to the standard deviation of  $t$  from a tabulated data for the standardized cumulative normal distribution. Estimating the standard distribution from several points on the washout function provides the possibility to confirm that the assumption of a normal frequency distribution is correct.

The use of residence time distribution analysis to characterize liquid mixing in packed bed bioreactors has been reviewed [103]. The authors pointed out the problems with getting correct estimates of the parameters from tailing peaks. They recommended that the experimental data should be fit to the function used (i.e. washout function, distribution function or density function) by a least-squares curve fitting technique to avoid erroneously high impact from the tailing part. Also, the quality of the fit to experimental data provides information about the applicability of the residence time distribution analysis to the system under study. It was shown that different methods for measuring and calculating the zone broadening of process columns may yield very different results [104]. The reason for this was not found, and it was suggested that different test procedures would be optimal for characterizing the zone broadening for different types of processes.

#### *Vessel dispersion number*

The zone broadening may be related to different sources, e.g. the terms in the van Deemter equation, as outlined earlier for packed columns. For vessels and non-packed beds, e.g. expanded beds of non-porous beads, the zone broadening is often characterized by a parameter called the vessel dispersion number given by the inverse Peclet number,  $Pe$ , as

$$\frac{1}{Pe} = \frac{D_A}{uL} = \frac{1}{2} \left( \frac{\sigma}{V_R} \right)^2 \quad (10.43)$$

In analogy with dispersion in chromatography it is seen that the axial dispersion coefficient,  $D_A$ , corresponds to the B-term in the van Deemter equation (cf. eq. (10.10)), i.e. eddy dispersion and mass transfer resistance are neglected for these systems. Obviously this is not correct for chromatographic dispersion in vessels containing porous chromatography resins.

### 10.7.3 Resolution

The resolution factor is calculated from the retention volumes and peak widths determined as described above and with the help of eq. (10.1). The effect on resolution factor on recovery at predefined purity is illustrated in Figure 4.4.

### 10.7.4 Mass transfer

Mass transfer coefficients may be estimated from fitting experimental data to different models (e.g. the restricted diffusion in porous chromatography resins may be calculated from a fit of the van Deemter equation to experimental data). However, unless the model used is qualified *a priori* the result can only be regarded as tentative. Modelling of chromatographic purifications is discussed in the next section. The kinetics of mass transfer will have a direct effect on the processing rate, i.e. flow rate that is applicable. The mass transfer may be studied by breakthrough analysis at different flow rates. This is done by applying a step containing the solute (or proper feed mixture, provided the solute of interest may be selectively detected) and observing the response after the column (i.e. very similar to a residence time distribution analysis). Breakthrough analysis is also used for the determination of the maximum capacity of a chromatographic medium and a detailed procedure is given below.

### 10.7.5 Capacity

The maximum capacity of the chromatographic medium for the solute is a very important factor in process chromatography since it will directly influence the productivity. The maximum capacity,  $q_m$ , is needed for the calculation of the adsorption isotherms (cf. eq. (10.32)). In some models (e.g. the SMA model) the total ionic capacity for an ion exchanger is used and thus need to be determined.

#### *Ionic capacity*

The ionic capacity is determined by saturating the ionic groups with a suitable counterion (e.g. one that is easy to assay), washing away the excess counterion, desorbing the counterion and determining the amount. Sometimes the two steps may be performed simultaneously as shown in Table 10.4.

#### *Solute capacity*

The capacity for a biomolecule will always be less than the ionic capacity. This is due to the fact that the biomolecule will occupy a larger surface area than the small counterions.

**Table 10.4**

Test systems for determination of ionic capacity

Type of exchanger	Counterion	Desorbing ion	Assay
Cation exchanger	H <sup>+</sup> (1 M HCl)	K <sup>+</sup> (1 M KNO <sub>3</sub> )	Potentiometric titration of H <sup>+</sup>
Anion exchanger	Cl <sup>-</sup> (1 M NaCl)	NO <sub>3</sub> <sup>-</sup> (1 M KNO <sub>3</sub> )	Ag <sup>+</sup> titration of Cl <sup>-</sup>

The solute capacity may be obtained in batch experiments or with a packed column [74]. The capacity obtained in batch experiments (static capacity) is often larger than that obtained by letting the solution pass through a packed bed (dynamic capacity) due to the shorter contact time in the latter case. It can be noted that this ‘saturation capacity’ may be 6–16 times larger than the loading where effects on the resolution between protein peaks can be observed [105]. Furthermore, the actual capacity for a component in a mixture will often be smaller than that for a pure component due to competition with other adsorbed species of the mixture.

#### DETERMINATION OF BREAKTHROUGH CAPACITY

The breakthrough capacity is defined as the amount of solute taken up by the column at the point when solutes are first detected in the effluent [1]. This is schematically illustrated in Figure 10.11. Since the determination is so important a suggested procedure will be given here.

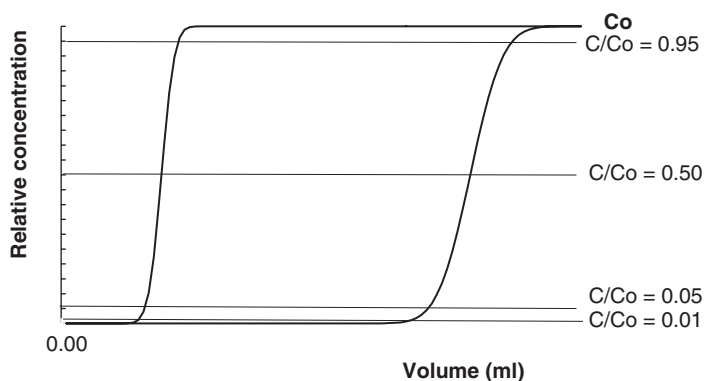
1. Pump a solution containing an appropriate amount of suitable solute through the detector cell to determine the plateau level of the initial concentration,  $C_0$ . Calculate 5% of this level (or any level desired; here 5% is recommended and will be used throughout this description).

*Note:* Make sure the detector response is linear over the whole range. The solute should be dissolved in a solution that promotes adsorption to the chromatography medium.

2. Insert the column in the system and pump the sample solution through the column while continuously tracing the concentration,  $C$ , of the solute in the effluent. Start collecting the effluent in a container at the sample application (see note to step 5).
3. When the concentration of the effluent has reached 5% of the initial concentration, i.e.  $C/C_0 = 0.05$ , then stop applying the sample. The volume collected so far is denoted as  $V_A$  and the concentration of solute is denoted as  $C_A$ . Start pumping a wash solution through the column while collecting the effluent in a new container. Wash the column for at least two column volumes. The volume collected during this step is denoted as  $V_B$  and the concentration of solute is denoted as  $C_B$ .

*Note:* The wash solution should be of the same composition as the solution for dissolving the sample to avoid desorbing bound solute at excessive wash. The concentration of protein may be determined by absorbance at 280 nm using a calibration curve. A proper assignment of the 5% level requires a stable baseline signal; thus, a shift in baseline should be disregarded and a new baseline level be set.

4. Switch container and start desorbing the solute with a suitable desorbing buffer. Follow the step by continuously tracing the concentration of the solute in the effluent.



**Figure 10.11** Determination of breakthrough capacity. Left curve; breakthrough in absence of column (to determine the system dead volume). Right curve; breakthrough of solute (note the lower slope of the curve in this case caused by mass transfer effects).

After the concentration has reached baseline level continue to elute the column for one more column volume. The volume collected during the desorption step is denoted as  $V_D$ , and the concentration is denoted as  $C_D$ .

5. The breakthrough capacity (mg/ml chromatography medium) is calculated from:

$$Q_{B,5\%} = \frac{[V_A C_0 - V_A C_A - V_B C_B]}{V_c} \approx \frac{[V_A C_0 - V_B C_B]}{V_c}$$

where  $V_c$  is the geometric column volume of the chromatography medium bed.

*Note:* In most cases  $C_A$  is negligible as compared to  $C_0$ . In this case there is no need to collect the volume  $V_A$  since it may be determined from the flow rate, provided a high-precision pump is used. It is important that the entire volume from the sample applicator to the column is swept, or dead volumes accounted for, especially for small beds and/or chromatography resins of low capacities.

The recovery of solute is given by:

$$\text{Recovery}(\%) = \frac{100 \times V_D C_D}{[V_A (C_0 - C_A) - V_B C_B]}$$

If the recovery is less than 100% it is of interest to check that remaining solute can be removed from the chromatography medium. This is performed in the same way as step 4 except that a regenerating solution is used instead of the desorbing buffer. The volume (at least two column volumes) is denoted as  $V_F$  and the concentration of solute  $C_F$ . The mass balance should be satisfied, i.e.:

$$V_A C_0 = V_A C_A + V_B C_B + V_D C_D + V_F C_F$$

It is important to realize that the nature and concentration of solute as well as additives (e.g. contaminants) and other factors, e.g. flow-rate, will influence the results obtained, and thus these parameters must be standardized when comparing resins and the conditions chosen must be relevant to the application at hand.

The monolayer capacity may be derived from column experiments provided the isotherm is Langmurian [106].

$$q_m = q\sqrt{2N} \frac{k'}{1+k'} \frac{k'_{\text{low}}}{k'_{\text{low}} - k'} \quad (10.44)$$

where  $q$ ,  $N$  and  $k'$  is measured at high load and  $k'_{\text{low}}$  at low load.

As stated above the frontal analysis procedure used for calculating the breakthrough capacity may be used to study the adsorption dynamics. In this case the application of sample in step 2 is continued until  $C$  is close to  $C_0$  in the effluent. A measure of the dynamics is given from the quotient  $Q_{B,5\%}/Q_{B,95\%}$  and the closer this value is to unity the better the dynamics for the adsorption step. Since the recovery and regeneration of the chromatography medium are not of primary interest in the determination of dynamics the procedure may be simplified by continuously following the step from  $C = 0$  to  $C = C_0$  in two experiments, the first being with the solute dissolved in adsorption buffer and the second with the solute dissolved in the desorption buffer. Provided that the concentrations of the solute in the two solutions are identical and that the flow rate is constant the quotient is given by:

$$\frac{Q_{B,5\%}}{Q_{B,95\%}} = \frac{t_{A,5\%} - t_{D,5\%}}{t_{A,95\%} - t_{D,95\%}}$$

where  $t_{X,Y\%}$  is the time for the solute in the adsorption ( $X = A$ ) or desorption ( $X = D$ ) buffer to reach 5% ( $Y = 5$ ) or 95% ( $Y = 95$ ) of  $C_0$  in the effluent.

#### ADSORPTION ISOTHERMS

To obtain the adsorption isotherms the concentration of solutes in the stationary phase,  $C_s$ , need to be determined for different values of the concentration of solutes in the mobile phase,  $C_m$ . The preferred way is to use the frontal analysis as described above for different  $C_0$  [107].

Another approach is to simulate the chromatogram assuming a shape of the isotherm and compare this with experimental results (see below) [108].

### 10.8 MODELLING OF CHROMATOGRAPHIC PURIFICATIONS

The possibility to design and optimize purifications on the basis of chromatography theory has attained much interest due to the expected savings in time and money from such an approach. However, as outlined above some of the fundamentals of chromatographic theory are still not well understood. It was also concluded that the models proposed are sufficiently complicated to prevent straightforward analytical mathematical solutions. Therefore, approximations have been made to allow for their practical applications, and

these approximations influence the results obtained from such models. To cope with these shortcomings, modelling is frequently supported by experimental data (e.g. to determine the values of lumped parameters), formally restricting the use of the model to the conditions covered by experiments. Furthermore, it must be kept in mind that models are nothing but our attempts to describe processes. These approximations of a real process must be verified by experiments before valid conclusions can be made.

Despite these limitations, modelling of chromatographic purifications provides valuable insights into expected relationships and will help in choosing strategic directions for the design, development and optimization work (e.g. by prioritizing the most favourable alternatives). Modelling can also be used to select critical parameters and their ranges, to be studied in an experimental design for optimization (see Chapter 3). If a model conforms to the purification result obtained on a small scale, this suggests that the system is well behaved. On the other hand, deviations from theoretically expected results are indicative of other types of interaction than anticipated between the solute and chromatography matrix and/or between solutes. This is an important information which needs to be addressed in the optimization of a purification process. In such an evaluation, contributions introduced by the employed model must be disclosed, and therefore a basic understanding of the parameters that govern mass transfer and kinetics is important.

The ultimate goal of modelling a process is to incorporate all individual parts of the process and to optimize these parts not on an individual basis but in concert, in order to give a global optimum of the process. This requires that, e.g. the process economy of the various steps can be calculated and input parameters varied according to requirements from neighbouring steps.

Some simple applications of modelling purifications and calculations of process economy are given to serve as a tutorial.

### 10.8.1 Mass transfer in chromatography

The one-dimensional mass balance in the column may be addressed from the following general expression

$$\frac{\partial C_M}{\partial t} = D_A \frac{\partial^2 C_M}{\partial z^2} - u \frac{\partial C_M}{\partial z} - \frac{V_S}{V_M} \frac{\partial C_S}{\partial t} \quad (10.45)$$

describing the change in concentration of the solute in the mobile phase,  $C_M$ , as a function of time,  $t$ , caused by dispersion along the column axis,  $z$ , for a solute having a total dispersion coefficient  $D_A$ , convective transport as affected by the interstitial velocity of the mobile phase,  $u$ , and accumulation of solutes in the stationary phase, giving the concentration  $C_S$ .  $V_S$  and  $V_M$  represent the stationary- and mobile-phase volumes respectively.

Thus, in order to model an ideal separation using the mass balance equation, we need expressions for the convective transport of molecules in the mobile phase (second term on the right-hand side), the adsorption/desorption equilibrium and kinetics (third term on the right-hand side) and the dispersion (first term on the right-hand side) of the solutes. In the non-ideal case, the mass transfer of a single solute may be affected by self-aggregation,



conformal changes, the concentration and properties of other solutes, etc., which will lead to unexpected results.

### *Mass transfer in the mobile phase*

#### CONVECTIVE MASS TRANSPORT

In the case where there are no retention effects and the dispersion is negligible, the change in solute concentration in the mobile phase is determined solely by convective transport. The negative sign of this term indicates an inverse relationship between velocity and retention time.

#### DISPERSIVE MASS TRANSPORT

The lumped dispersion coefficient,  $D_A$ , is often approximated by  $H u/2$  [10]. By comparison with eq. (10.43) it is realized that this approximation stems from the vessel dispersion number, which may not be applicable to chromatography beds. Sometimes the dispersive mass transport in the mobile phase is lumped together with the interparticle mass transport [109]. Generally, both the A-term and the B-term in the van Deemter equation (eq. (10.10)) should be included in calculating the dispersion coefficient in the mobile phase.

### *Mass transfer in the stationary phase*

The rate of change of the solute concentration in the stationary phase depends upon mass transfer from the mobile phase through the stagnant film layer around the particles (film mass transfer), transport within the bead (diffusion and convection) and adsorption/desorption kinetics.

#### FILM MASS TRANSFER

The film mass transfer parameter,  $K_f$ , is given by [10]

$$K_f = \frac{Sh D_M}{d_p} \quad (10.46)$$

where  $Sh$  is the Sherwood number (see Appendix B for a definition),  $D_M$  the free molecular diffusion coefficient and  $d_p$  the particle size. Since the Sherwood number is normally in the range 2–20, it can be seen that the influence of film mass transfer could, for these cases, be ignored compared with intraparticle pore diffusion, especially since this diffusion will often be small as compared with the free molecular diffusion (see below).

#### PORE DIFFUSION

The diffusional transport within the liquid-filled pores influences the C-term in the van Deemter equation (eq. (10.10)). It could be expected that the solute diffusivity within the particle is 5–20% of the free molecular diffusion coefficient for solutes of the same order of size as the pores [6]. The diffusion of solutes in the pores is for these cases reduced by

the enhanced drag and an expression for the hindered diffusion of spherical solutes in the confined space of pores of cylindrical shape is given by [6, 110–114],

$$D_{S,1} = D_M \left( 1 - 2.104 \left( \frac{R}{r} \right) + 2.089 \left( \frac{R}{r} \right)^3 - 0.948 \left( \frac{R}{r} \right)^5 \right) \quad (10.47)$$

where  $D_{S,1}$  is the local intraparticle pore diffusion coefficient,  $D_M$  the free molecular diffusivity,  $R$  the solute radius and  $r$  the pore radius.

When eq. (10.47) is used to determine zone broadening (i.e. with the help of eq. (10.10)), the effective diffusion distances within the chromatographic particle must be taken into account. This is done by incorporating the tortuosity factor,  $\tau$ , which is the actual pore length (tortuous length) divided by the straight (non-tortuous) length of the pore, into an empirical expression of the pore diffusion coefficient,  $D_s$ , given by  $D_s = D_{S,1}/\tau$  [110]. Unfortunately, the expression for the pore diffusion coefficient does not give results in accordance with experimental data [6, 110]. The reason for this may be deviations from the ideal geometrical shapes assumed (even though slit types of pores give similar results to cylindrical pores) or, perhaps more plausible, the influence of the surface charge of the proteins studied [112]. Thus, it may be concluded that no simple universal equation for the calculation of the pore diffusion coefficient yet exists and empirical-based calculations are therefore regularly used. An empirical relationship of  $D_s \approx D_M K_D/4$  was found to give good agreement with experimental data for  $0.2 < K_D < 0.8$  [6].

Another expression for the effective diffusivity is given by Karger *et al.* [2].

$$D_{\text{eff}} = \frac{K_{\text{sec}} \varepsilon_p D_M}{\tau(K + 1)} \quad (10.48)$$

where  $K_{\text{sec}}$  is the pore fraction available to the solute (i.e.  $K_D$  in the size exclusion mode, cf. eq. (10.18)),  $\varepsilon_p$  the pore fraction of the particle and  $K$  the distribution constant in linear adsorptive chromatography. It must be noted that eq. (10.48) cannot be used for calculating the local pore diffusion coefficient. It is employed to calculate the global flux of solutes into an adsorptive chromatographic particle in linear chromatography (i.e.  $K_{\text{sec}} \varepsilon_p$  accounts for the influence of available volume within the particle on the flux and  $\tau$  accounts for the influence of diffusion distances). It can also be seen that the friction term is missing which limits the use of the equation to small solutes (i.e. for large solutes the expression in eq. (10.47) should replace  $D_M$  in eq. (10.48)).

#### CONVECTIVE MASS TRANSPORT

Transport of solutes within the particle due to convection may take place if the pores are large when compared with the particle size (i.e. to support significant flow through the particle). The size of the interparticle void channels in beds of uniform particles are of the same order as the particle radius [113]. The relative influence of convection as compared to diffusion for intraparticle mass transport is given by the intraparticle Peclet number,  $Pe_i$ ,

expressed by the ratio of the time it takes for a molecule to diffuse from the surface of the particle to the centre to the time for transportation by intraparticle fluid flow,

$$Pe_i = \frac{(r\tau)^2/6D_s}{r\tau/u_i} = \frac{d_p u_i \tau}{12D_s} \quad (10.49)$$

where  $\tau$  is the tortuosity factor,  $u_i$  the effective intraparticle eluent velocity, and  $D_s$  is the intraparticle pore diffusion coefficient [114]. The denominator of the upper term in eq. (10.49) originates from the fact that pore diffusion is a three-dimensional process. The effective intraparticle flow is dictated by the interstitial velocity of the fluid, the pore shape, the intraparticle and interparticle porosities and the pore size relative to the size of the interstitial void channels. The intraparticle Peclet number may be calculated for materials of a defined pore structure, such as porous silica microspheres (PSM) [113]. The intraparticle flow will, for this type of material, be proportional to the square of pore diameter over particle radius (*cf.* eq. (10.16)). From this it may be calculated that convective mass transport will have an influence (i.e.  $Pe_i = 1$ ) when running a protein having  $D_s = 2 \times 10^{-7}$  cm<sup>2</sup>/sec on a 30  $\mu$ m PSM material having uniform pores of 4000 Å diameter, at an interstitial velocity in the column of more than 135 cm/min. Thus, intraparticle convection is not likely to contribute significantly to mass transport in ordinary chromatographic processes. This was also experimentally noted when no convective transport was found for a large pore size chromatography resin at velocities of 5000 cm/h [42].

#### ADSORPTION AND DESORPTION KINETICS

Peak dispersion will result if the kinetics of adsorption and desorption are slow. Slow adsorption kinetics will also influence the 'degree of utilization' as shown in Figure 4.21. The kinetics are described by the forward,  $k_1$ , and backward,  $k_2$ , rate constants of the adsorption process according to [74]

$$\frac{dq}{dt} = k_1 C_M (q_m - q) - k_2 q \quad (10.50)$$

where  $q$  is the amount of solute,  $C_M$  the concentration in the mobile phase and  $q_m$  the maximum adsorptive capacity for the solute. By setting  $dq/dt = 0$ , i.e. the process has reached equilibrium, (eq. (10.28)) and the corresponding expression for the Langmuir isotherm are obtained. It must be noted that contributions to mass transfer other than adsorption and desorption kinetics (e.g. film diffusion and pore diffusion) are sometimes included in the rate constants, making these 'lumped' parameters [74].

Other types of isotherms than the simple Langmuir isotherm may be appropriate as reviewed by Bellot and Condoret and illustrated by Cramer *et al.* [27, 79].

It is generally assumed that the kinetics of the adsorption process is not a rate-limiting factor in chromatographic processes. However, as reviewed by Whitley *et al.* [115] this may not always be anticipated, and non-equilibrium adsorption/desorption kinetics (NAD) may yield unanticipated phenomena. It may also be noted that for other formats,

e.g. adsorptive membranes, the adsorption process has been found to be rate limiting compared with mass transport [116].

When the composition of the mobile phase is changed (i.e. as in gradient or step elution) the retention factor, and thus the distribution coefficient, is also changed. The eluent will promote the breakdown of the adsorbent–adsorbate complex and thus increase the desorption rate constant [117]. From eq. (10.50) it is expected that the adsorption kinetics is dependent upon the sample concentration.

### 10.8.2 Models for mass transfer

Models used for predicting preparative chromatographic separations need to address all contributions to mass transfer that are relevant to the particular separation situation. Solving eq. (10.45) analytically for non-linear adsorption systems is not possible, and several simplifications are introduced in order to arrive at a solution. It is therefore important to realize the limitations of the different approaches used in order to select a model that will be useful for the purification problem at hand.

Another limitation to the application of published models may arise from the use of lumped parameters. Using lumped parameters (e.g. lumped dispersion coefficients) requires fewer experiments to be performed, and is therefore a popular strategy. However, the drawback is that the model is generally restricted to the conditions for which the parameters were determined.

Calculation of mass transfer from the differential eq. (10.45) will depict the separation as a continuous process. This is known as the rate (i.e. the change of solute concentration is studied per unit time) or mass balance model. In another approach the process is conceptually viewed as a large number of discrete stages where the mobile and stationary phases reach equilibrium conditions before the solutes in the mobile phase are transferred to the next stage. This is called the plate model (i.e. the change of solute concentration is studied as a function of stage, or plate, number).

Whereas the rate model is, from a physicochemical point of view, the preferred model, the complex numerical calculations performed may be prohibitive for the general use of this model. Fortunately, the rapid increase in computational power of standard laboratory computers is now changing this situation. The rate model has been extensively used for modelling a wide range of adsorptive chromatographic purifications (e.g. see the review by Velayudhan *et al.* [117]).

The plate model has gained great popularity, mainly owing to its resemblance to other separation processes such as the ‘stirred-tanks-in-series’ model. It has been demonstrated that the plate model is useful for studying and optimizing linear ion-exchange separations [8]. A review showed that the plate model could yield results in accordance with the rate model for linear chromatographic systems [118].

#### *Rate model (mass balance model)*

The rate model has been used for modelling preparative AC for single components [110]. Important factors for the adsorption stage in preparative AC were derived using the assumption that the effects of axial dispersion are negligible [74]. The same approach was

successfully used for modelling protein adsorption to ion exchangers [75]. In another study, the effect of axial dispersion was found to be negligible while the internal mass transfer resistance due to slow intraparticle diffusion was a dominating factor in affinity adsorption of proteins [119].

Cramer and co-workers [27] replaced the Langmuir isotherm by the SMA model, where steric shielding of ionic groups by large solutes is incorporated. They used the rate model, neglected axial dispersion, and found good agreement with experimental data for single solutes in non-linear elution. The model was also useful for modelling multicomponent equilibrium in immobilized metal AC [120].

The mathematical modelling of mass transfer requires the analytical solution of the mass balance equation, after appropriate simplifications, or a numerical approach. Gu [121] described the mathematical modelling of the general rate model and scale-up of preparative purifications. However, this procedure is complex and may not be feasible due to the large number of parameters that need to be determined (as opposed to the lumped parameter approach) [122]. Guiochon *et al.* [123] compared the Craig distribution model (where the process is divided into a number of discrete stages) and the calculation of the mass balance equation using the finite difference method. They found that the two approaches were equivalent. Lightfoot *et al.* [109] compared the dispersion model, where a lumped dispersion coefficient is used, with the extra-particle mass transfer model, where a lumped intraparticle dispersion coefficient is used, and found that they gave almost identical results. However, they noted that this may not be the case when intraparticle flow is contributing to mass transport.

Frey and Grushka [124] presented numerical solutions to the complete mass balance equation using a modified Craig distribution approach. This method seems to be very promising, especially since it can be performed without the need for lumped parameters.

### Plate model

The plate model is based upon the assumption that the column is composed of a number of stages, or plates, in which the equilibrium distribution of solutes between the mobile and the stationary phases is instantaneous and the flow of the mobile phase is continuous without mixing between the plates. The tanks-in-series approach was successfully used by Yamamoto *et al.* [8] to model gradient elution in IEC.

The simplest application of the plate model is the use of the retention factor to calculate the retention volume and to determine the total plate height, e.g. from the van Deemter equation (eq. (10.10)), to give the zone broadening. At low concentrations (i.e. linear chromatography) it is reasonable to assume a Gaussian elution curve (provided the plate count is higher than 100), and the chromatogram may be simulated from the normal curve [2]. Taking into account the effect of sample volume, the elution curve in linear isocratic elution may be obtained from [2, 125]

$$C = \frac{C_0}{2} \left[ \operatorname{erf} \left( \frac{V - V_R}{V_R} \frac{\sqrt{L}}{\sqrt{2H}} \right) - \operatorname{erf} \left( \frac{V - V_{\text{sample}} - V_R}{V_R} \frac{\sqrt{L}}{\sqrt{2H}} \right) \right] \quad (10.51)$$

where  $C$  is the outlet concentration,  $C_0$  the inlet concentration,  $V$  the effluent volume,  $V_R$  the retention volume,  $L$  the column length,  $H$  the plate height,  $V_{\text{sample}}$  the sample volume and  $\text{erf}$  the error function. This approach was applied successfully to the study of the influence of sample volume on the resolution of a protein mixture in small-scale preparative size exclusion [126].

Velayudhan and Ladisch [118] reviewed plate models and described the use of the Craig distribution model for linear chromatography.

They showed that the earlier shortcomings of the discontinuous plate model are due to an incorrect definition of plate number for this model. They also demonstrated that results obtained with this model show excellent agreement with continuous-flow plate theory and also with rate models in linear and non-linear chromatography without the need to resort to lumped parameters [118].

### 10.8.3 Computer modelling of chromatographic purifications

The applications cited above illustrate that computer modelling is necessary to yield a realistic approximation of the resulting chromatogram whereas guidelines for optimization of purifications may be established from general theoretical relationships, e.g. as exemplified in Chapter 4, or from modelling the effect for discrete parameters as treated in this chapter. The prediction of retention parameters with the aid of computers has been reviewed by Baba [127].

## 10.9 SIMULATION OF SEPARATIONS

A computer program for the simulation of peptide separations with the help of size exclusion, cation exchange and RPC has been described [128]. A commercial program for analytical gradient HPLC simulations, named DryLab, is available from LCResources (Walnut Creek, CA, USA) [129].

Commercial software for the modelling of chromatographic purifications is now becoming available, even for large-scale purposes. A simulation of the entire process, including an economical evaluation, can be made with the aid of the Aspen Batch Plus (Aspen Technology, Cambridge, MA, USA) [130]. A fairly recent review of tools for simulation and modelling for industrial bioprocessing is found in Ref. [131].

### 10.9.1 Calculation of process economy

The process economy is closely related to the throughput (i.e. the amount of purified product per unit time) and the productivity (i.e. the throughput per volume chromatography medium). Guidelines for optimizing the productivity may be obtained from general chromatography theory (see Chapter 4) [132]. For more complex situations where many inter-related parameters are studied, computer-aided simulations may be needed to provide accurate information about the effects on productivity [133].

Productivity is but one factor in the calculation of process economy. Solvent costs, loss of crude material (i.e. product recovery) and labour cost are a few examples of other factors that need to be accounted for in the global optimization of the process (see Chapter 8). Software for calculating process economy has been presented for adsorption chromatography [134] and for preparative HPLC [135]. A commercial software, SuperPro Designer (Intelligen Inc., Scotch Plains, NJ, USA) can be used to evaluate large-scale processes to yield an optimal process from a product cost point of view [130].

### 10.9.2 Simulations using the supplied software

The practical implications of chromatography theory have been exemplified in this chapter by some selected illustrations. However, it is virtually impossible to cover all combinations of the different parameters that will be of interest to study for a particular case. We have therefore decided to include a CD containing the most useful equations with this book. The CD is intended to be used as a tutorial aid to facilitate the process of disclosing the relationships governing chromatographic separations. Needless to say, the results provided by the equations on the CD are not more accurate or reliable than the basic equations allow and must therefore not be considered as true data. As with other types of software, results need to be confirmed by experiments.

Applications supplied on CD, see Appendix D for more details.

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## Equipment

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This chapter describes guidelines for selecting pilot and production systems and components, and discusses the advantages and disadvantages of several types of automation approaches. Equipment qualification and validation of automated systems are discussed in Chapter 7.

Disposables are being employed, more and more, in bioprocessing for unit operations ranging from cell culture to final purification steps [1]. Disposables can minimize cross-contamination and cleaning validation. The U.S. FDA's draft guidance on production of Phase 1 clinical trial materials has recommended disposables or dedicated equipment in multiproduct facilities to expedite clinical trials and still maintain compliance with CGMPs that are relevant for this early stage of development [2]. The benefit of rapidly being able to produce clinical grade material should be weighed against costs of reengineering the process should disposables not be available or warranted for the next scale up phase. A decision-support tool for assessing the use of disposable equipment versus stainless steel has been developed [3]. In general, companies will always try to expedite clinical studies as a first priority.

### **11.1 GUIDELINES FOR SELECTING PILOT PLANT AND PRODUCTION CHROMATOGRAPHY EQUIPMENT**

A thorough determination of specifications for pilot plant and production systems can lead to the purchase of the most suitable equipment. Functional, chemical and pressure specifications as well as any impact due to facility area classification, should be clearly examined. Proper hygiene and avoidance of zone spreading should also be considered.

#### **11.1.1 Dimensioning data**

Regardless of the scale and chromatography system, prior to making a purchasing decision, the user should assess the required dimensions. For the liquid delivery system, dimensioning data include column cross-sectional area, linear flow rate, maximum pressure, operating temperature, sample volume and buffer consumption. The system must be able to accommodate feedstream variability—both volume and concentration variabilities. The pumps may have to operate over a wide flow-rate range suitable for sample loading and elution as well

as rapid cleaning-in-place (CIP) and column packing. (Alternatively, different pumps may be used for different operations.)

### 11.1.2 Functional specifications

When choosing equipment for a pilot plant or for production, structure the system by functional units, e.g. for liquid delivery, separation, monitoring, fraction collection and control. For each unit, define in detail what tasks need to be performed as a 'functional specification'. This should lead to a detailed equipment and specifications list. For example, the liquid delivery unit can contain: buffer tank outlet valves and low-level alarms, sample tank drainage alarm, pump with flow-rate control, in-line filters for buffers and sample, air bubble alarm, air trap, over-pressure alarm, filter by-pass, automatic high-pressure filter switch, manual valve override, manual drainage valves, etc.

The functional complexity determines the best automation structure for the system. Automation requirements are very different at different stages (see the section on 'Automation' below). For example, it is not usually necessary to automate alarms on buffer reservoirs for laboratory systems. On the other hand, a production loss of several or more days may be incurred if a 1000 L column runs dry because the buffer tank is empty. As a general rule, functional complexity increases when going from the laboratory scale to production, where reliability of the system is critical.

Defining a good functional specification requires understanding both the overall process and the biochemistry involved. For example, the optimal choice of in-line filters depends on the early recovery procedures (e.g. centrifugation and filtration) and the hydrophobic characteristics of the sample. Proper filters for continuous in-line filtration are determined by long term laboratory scale trials using different filter types. The choice of the proper in-line filter can be an important part of process development, and will have an impact on process performance, time consumption and filter consumption in production. In-line filters affect the life of separation media and, as a result, the economics of the entire process.

Establishment of the functional specifications leads to a piping and instrument (P&I) drawing that includes all instruments and process functions (see Figure 11.1). But before it is possible to choose the appropriate equipment for the defined functions, the chemical and physical specifications of the system must be defined, and hygienic design, zone spreading and documentation requirements should be considered.

### 11.1.3 Chemical specifications

The buffers defined by the purification protocol influence what construction materials are allowed. For example, even common NaCl buffers cause problems with stainless steel at mildly acidic conditions. The sample can also limit the choice of suitable construction materials if the protein of interest is sensitive to contact with certain surfaces. The choice of construction materials may be further restricted by the need for regular cleaning of the system with strong solutions (see also Chapter 6).

The chemical composition of each liquid-handling component should be defined by the supplier. Each component in contact with liquid must be compatible with sample, buffers



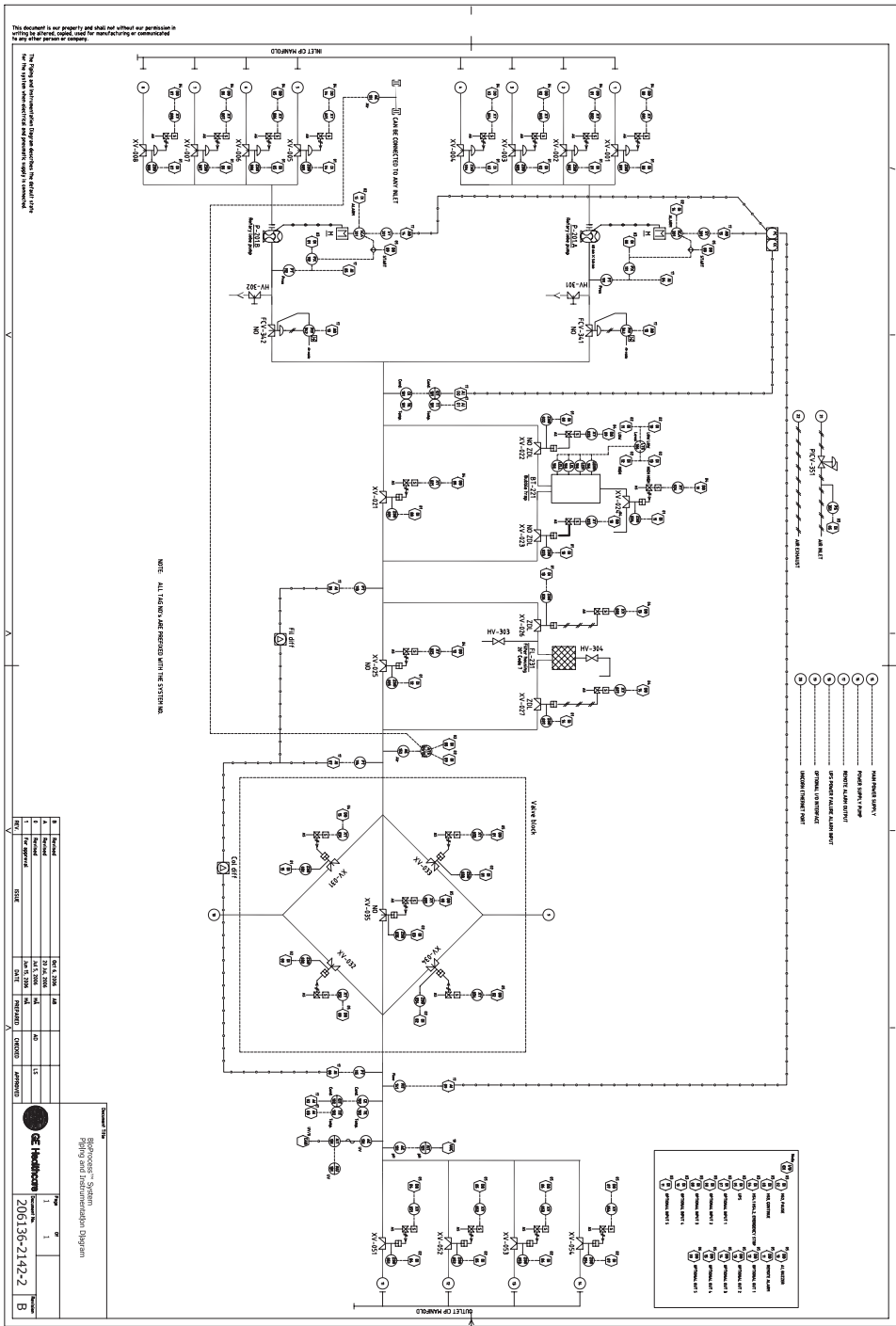


Figure 11.1 A piping and instrument (P&I) drawing of a complex chromatography system.



**Table 11.1**

Materials commonly used in contact with feedstreams in chromatographic processes

Columns	
Tube	Borosilicate glass, stainless steel, PTFE, E-CTFE, polysulphone, TPX, epoxy, high-density polyethylene, polypropylene, Plexiglass
End pieces	Stainless steel, PTFE, E-CTFE, polysulphone, oxirane glass/polycarbonate, polyamide, polypropylene, polyvinyl chloride
O-rings	EPDM, FPM (Viton®), NBR, PTFE, silicone rubber, high-density polyethylene
Nets	Polypropylene, stainless steel, polyethylene, polyester, polyamide, stainless steel, polyethylene, polyvinyl chloride, PTFE
Valves	
Bodies	Stainless steel, polypropylene, polyvinyl chloride, PTFE
Diaphragms	EPDM, silicone, FMP (Viton), PTFE, TPE (Santoprene™)
Filters	PVDF (Kynar®), polysulphone, silicone, cellulose ester, microfibre glass, polyester, polypropylene, urethane
Flow meters	TPX, PVDF (Kynar), sapphire, stainless steel
Conductivity sensors	Polysulphone, stainless steel, PDVF (Kynar)
Ultraviolet cells	Polyvinyl chloride, polypropylene, PTFE, quartz glass, stainless steel

and cleaning agents used in production. Typical wetted surfaces used in column chromatography are shown in Table 11.1. With the exception of stainless steel, equipment used in the production of material for clinical trials and marketed product must be evaluated for extractables. Potential extractables come from alloy metals and fillers in plastics; pigments may leach from rubber—e.g. from column gaskets. Polypropylene, which is used in many manufacturing chromatography systems, has been shown to bind endotoxins [4]. Quality assurance of production materials for biotechnology has been addressed by Miller, who states that the most problematic materials for biotechnology process solution contact are those made from rubber [5]. By performing United States Pharmacopoeia (USP) and European Pharmacopoeia (EP) toxicity testing and qualifying raw material sub-suppliers, today's equipment suppliers can provide firms with sufficient data so that non-stainless steel materials can be used in those situations where they provide beneficial features (e.g. for small systems or multipurpose systems or superior chemical resistance).

Regulators have expressed concern over the long term effects of multiple cleaning cycles on the integrity of plastics. If polymers break down upon repeated exposure to harsh cleaning chemicals, the integrity of the product could be adversely affected. The stability of the plastic and the absence of leachables should be demonstrated.

The chemical resistance chart shown in Figure 11.2 gives guidelines on the chemical resistance of different plastics, elastomers and stainless steel. It is important to note that 'resistance' is not an absolute, but a relative, term. Plastics and elastomers, in particular, may be affected by long term exposure to chemicals. This is not found in standard tests, simply because the duration of the tests is limited. Furthermore, when under pressure, plastics and elastomers may be considerably more affected by chemicals. Extensive tests, therefore, should be performed to verify leakage and resistance of the material to the exact combination of chemicals, pressure and temperature used in the process.

	PTFE	ETFE	CTFE	PFE	PVDF	PEEK	PE	PP	EPDM	Titanium	Platinum	Glass (borosilicate)
Acetic acid (10%)	+	+	+	+	+	+	0	+	+	+	+	+
Acetone	+	+0	+	+	0	+	+	+	+	+	+	+
Acetonitrile	+	+	+	+	02	(+)		(0)		+	+	+
Ammonia (aqueous)	+	+		+						+	+	+
Ammonium sulfate	+	+	+		+			+		+		+
Beer	+	+				+		+			+	+
Chloroform	+	+0		+0	+0	+		0-		+	+	+
Citric acid	+	+	(+)		+	+		+		+	+	+
Cyclohexane	+	+		+	+	+		+0	2	+		
Ethanol	+	+	+	+	+	+		+	+1	+	+	
Ethylene glycol	+	+	+	+	+	+		+	+			
Formic acid	+	+		0-	+	+		+	+2	0	+	+
Glycerol	+	+	+	(+)	+			+	+			
Guanidinium hydrochloride	+	+						+	+			
Hexane	+	+0	+0	+0	+0	+		+0	-2	+		
Hydrochloric acid 10%	+	+	+	+	+	+	(+)	+	+	0	0	+
Hydrochloric acid (concentrated)	+	+	+	+	+	+		+	+	-	0	+
Methanol	+	+	+	+	+	+		+	+1	+	+	+
Methylene chloride	+	+0	(+0)	(+0)	0	+		0-	03	+	+	+
Nitric acid (<25%)	+	+	+	(+)	+	+0	0	+	+	+	+	+
Nitric acid (<50%)	+	+	+	(+)	+	+0	(0)	0	+	+	+	+
Phosphoric acid (20%)	+	+	+	(+)	+	+		+	+	+	+	+
Phosphoric acid (50%)	+	+	+	(+)	+	+		+	+	0	+	+
Potassium carbonate	+				+	+		+	+	+		
Potassium chloride	+				+	+		+	+	+		
Propanol	+	+		(+)	+			+	+			
Sea water	+	+	+	(+)	+	(+)	+	+	+	+	+	+
Sodium acetate	+	+			+	+		+	+	+		
Sodium carbonate	+	+	+		+	+	0	+	+	+		
Sodium chloride	+	+	+		+	+		+	+	+		
Sodium hydroxide (20%)	+	+	+	(+)	0	(+)	0	+	+	+		
Sodium hydroxide (80%)	+	+	(+)	(+)	0	(-)		+	+	-		
Sodium sulfate	+	+	+		+	+		+	+	+		
Sulfuric acid (<50%)	+	+	+	+	+	+	0	+	+	-	+	+
Tetrahydrofurane	+	+0	0	+0	0+			0-	-	+		
Toluene	+	+0	+0	+0	+		+	0-	-2	+		
Trifluoroacetic acid (30%)	+	+0	0	0				(+)		0		
Triton X-100	+	+	+	+	+	+		+	+			
Urea	+		(+)		+	+		+	+			

**Figure 11.2** Chemical resistance chart for selected materials used in process chromatography. Primary system—broad classification: -, not resistant; 0, limited resistance; +, resistant; space, no data available; parentheses, resistance is conditional or the data is from a limited test series. Secondary system—resistance qualification: 0, limited resistance; 1, fair resistance; 2, good resistance; 3, very good resistance.

For production scale chromatography, a general rule of thumb is to utilize only glass, acrylics, fluoroplastics and stainless steel. This gives full freedom in the choice of the separation chemistry and sterilization techniques. (However, fluoroplastics may not be mechanically suitable for sealing components, and glass can only rarely allow for in-line steam sterilization.) Stainless steel components are generally electropolished. Electropolishing leaves a smooth surface that is more corrosion resistant and has been thought to reduce the potential for bacterial growth. However, the latter claim has recently been disputed [6]. Procedures for cleaning stainless steel surfaces have been described by Tuthill [7]. For further information, see Wang and Chien [8] and Cowan and Thomas [9].

### 11.1.4 Pressure specifications

Pressure specifications are estimated from laboratory measurements of the pressure drop over the packed chromatography column and information from equipment suppliers showing the pressure drops over the liquid-handling equipment and the empty column.

Pressure measurements of the assembled system must then be made to determine the specifications. The pressure drop information should be shown as a function of flow rate. The operating system pressure is calculated as the sum of the pressure drop over the packed column and the equipment to be installed between the pump and the fraction-collection system.

With resins smaller than 30–40  $\mu\text{m}$ , the contribution from the extra-column equipment to the overall pressure drop is usually negligible, and, therefore, the system pressure specification is defined largely by the column back pressure.

It is important to ensure adherence to pressure-vessel regulations. These regulations are generally specified by country. National guidelines for pressure vessels are described by Meyer [10]. Since many companies use identical processes in manufacturing sites in more than one nation, early assessment of the local requirements of likely manufacturing sites can save time and money by avoiding the need for redesigning and specifying a system.

### 11.1.5 Hygienic design

Solvent compatibility of equipment and the hygienic design of chromatographic systems have become more obvious with the introduction of resins that withstand strong alkaline solutions suitable for CIP (for further details see Chapter 6).

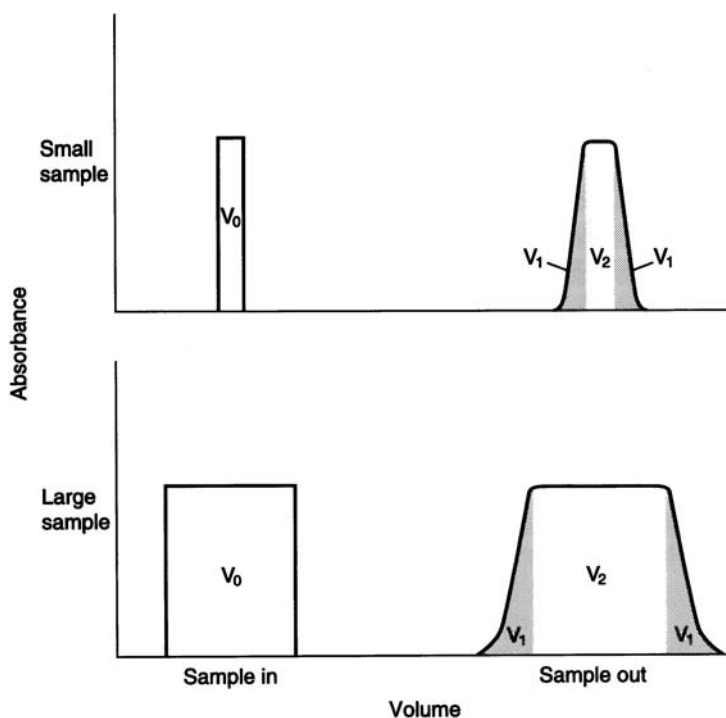
In principle, it is possible to sterilize a chromatographic system in-line. In reality, however, the geometry of the equipment may not allow the sterilizing agent to flush all parts of the system. Equipment should be chosen that does not create stagnant zones. For example, the geometry of a membrane valve allows for free liquid flow over the whole internal surface. Best practices for hygienic design are summarized by the ASME BioProcessing Equipment (ASME BPE) Standard, which is followed by many equipment suppliers. But it is not always possible to find hygienically designed equipment. For example, a small-scale ultraviolet (UV) monitor flow cell with laboratory types of non-sanitary, threaded connections might be needed in the system. It is still possible to achieve good process hygiene, however, by regularly opening the flow cell and cleaning it outside the system. For production systems, flow cells are machined or welded stainless steel or polypropylene with no threaded pieces and tri-clamp connections to the process line.

Good hygiene in process chromatography depends on using hygienically designed equipment where available, 0.2 micron-filtered solutions coming into the system, a method for in-line cleaning and sanitization.

### 11.1.6 Zone spreading

The separation power of a complete chromatographic system is determined by the chemical interactions between separation media and the molecules to be separated. The equipment does not contribute to the actual separation; still, the same separation chemistry can give a different performance when run in different equipment. This performance difference is a result of zone spreading created by back-mixing effects in the liquid-handling system or in the column inlets and outlets.

Zone spreading on different scales can be investigated by running salt pulses through the system without a column. Zone spreading of the liquid-handling equipment is measured as



**Figure 11.3** The effect of sample volume on zone spreading:  $V_0$ , sample volume;  $V_2$ ,  $V_0$  superimposed on 'sample out';  $V_1$ , zone spreading (or peak broadening) on 'sample out'. (Note: absorbance is not to scale)

the dilution factor, which is equal to the volume of the diluted sample leaving the system divided by the volume of the sample entering the system. To obtain a relevant measurement, the incoming sample volume should be the same as the volume of the actual sample to be separated in the system.

Zone spreading in analytical chromatography has been thoroughly investigated [11]. In general, extra-column zone spreading causes fewer problems in large-scale pilot and production chromatography. Dilution from extra-column zone spreading has less effect on larger peak volumes (see Figure 11.3).

In adsorption techniques, equipment installed upstream of the column has minimal dilution effects on the separations performed in the column. This is due to the concentrating effect the actual adsorption has on the sample. Major equipment-related zone spreading is due to dilution in the column outlet, the monitoring system, or the fraction collecting system. To minimize these effects, monitor flow cells, tubing and fractionating valves should be designed to minimize internal volumes.

In size exclusion chromatography (SEC) and other isocratic elution techniques, the dilution effects of the whole equipment system (from the sample tank to the fractionating valves) can affect zone spreading. Therefore, these techniques are generally more affected by the equipment during scale-up, and greater care should be taken in the system design.

**Table 11.2**

Typical technical documentation available with a production chromatography system

Drawing and documentation index	Cabinet layout
Piping and instrumentation diagram	Part list
General system specification	Electrical schematics
Instrument specification	Wiring table
Mechanical specification	Cable diagrams
Spare parts and interchange ability list	I/O list
Inspection and test plan	Software configuration description
Calibration certificates	Software configuration revision document
Inspection reports	Assembly drawings
Configuration of programmable devices	Instrument-specific manuals and cut-sheets
Functional test records	Installation and operational qualification documentation

### 11.1.7 Documentation

Documentation needs for chromatography systems are extensive and should be established prior to purchase of the system. Proper documentation is a requirement for equipment qualification and process validation (see Chapter 7). Table 11.2 shows a listing of typical technical documentation supplied with a chromatography system used for production.

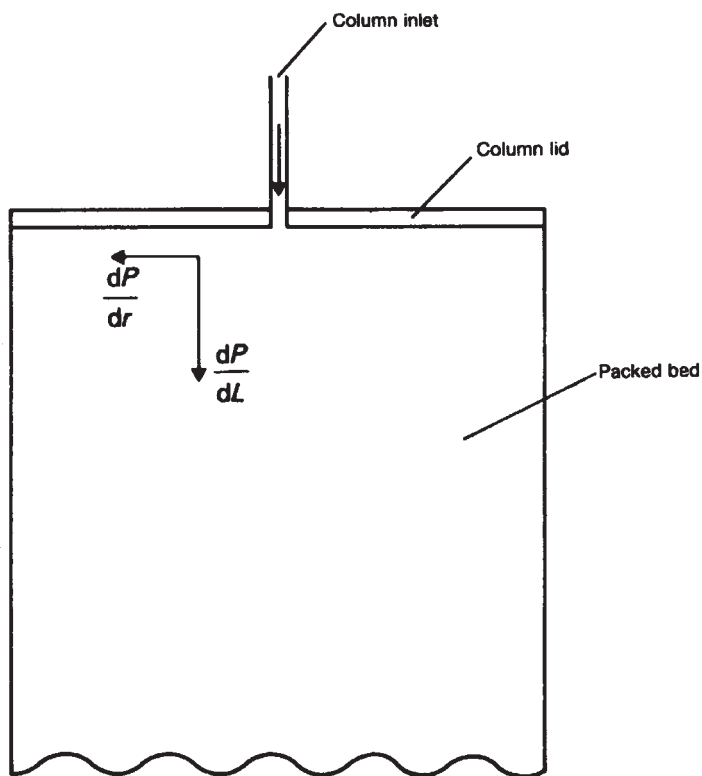
## 11.2 SELECTION OF COMPONENTS

A chromatography system can be quite simple, consisting of only a few components, or very complex. In addition to the automation hardware and software described below, a complete system includes: columns, valves, pumps, monitors and sensors, tubing or piping, and fraction-collection devices. For further reading, see McCabe and Smith [12] and Coulson and Richardson [13].

### 11.2.1 Columns

A chromatography column is designed to create as little zone spreading as possible and to allow for packing the chromatography resins to give the highest efficiency. The column inlets and outlets should allow even sample flow over the chromatographic bed, and the pressure tolerance of the column should be sufficiently high to permit packing of the smallest particle size resins that will be employed. Technically, the construction can vary, but all columns showing an even flow distribution have a radial pressure drop that is negligible in relation to the axial pressure drop in the inlet (Figure 11.4).

An integral part of the column and its flow distribution system is the particle retaining net, also called bed support (not shown in Figure 11.4). At laboratory scale or for HPLC



**Figure 11.4** Radial and axial back pressure in a column distribution system.

columns, frits or woven meshes with high lateral permeability may be used which distribute the incoming liquid within the support structure before it enters the packed bed space.

At pilot and production scale columns with larger diameter to height ratios, and especially when using low-pressure resins however, a separate liquid distribution channel in between the column end cell and the net is required for proper distribution of liquid over the total surface of the packed bed. Large-scale distribution systems typically employ patterns of distinct fluid flow channels alternating with ribs for mechanical support of the net and the packed bed, respectively. Another commercially available type of distribution system uses a coarse woven mesh in between the finer particle retaining net and the end cell. The coarse structure distributes liquid from the central inlet rapidly and uniformly over the full surface area of the net and the packed bed.

At larger column diameters, the distribution channel and its hold-up volume may generate additional zone spreading by differences in residence time between liquid passing the column near the centerline and liquid travelling along the distribution channel towards the column wall prior to entering the packed bed. In order to minimize this source of zone spreading, conically shaped distribution channels with decreasing channel depth towards the end of the channels near the column wall are applied. Thus, a balance can be achieved between a

low radial pressure drop and the need for a sufficiently high liquid velocity throughout the channel to minimize residence time differences.

Other design solutions are columns with multiple inlets, which pre-distribute the liquid before feeding it into end piece and distribution channel, respectively. Equally efficient may be columns with a single inlet employing a means for internal pre-distribution of liquid. For the latter, e.g. an oversized anti-jet device (a disc) transfers the incoming liquid to an intermediate diameter before releasing it into the distribution channel. Today, engineering methods like computational fluid dynamics (CFD) allow for design optimization of a distribution system to match the specific type of chromatography resin with proper control of column efficiency [14].

For columns with larger diameters, a number of constructions are available. Pack-in-place columns utilize unique nozzles that simplify packing of large columns and provide a 'closed-system' environment that minimizes the risk of contamination. The choice of the proper column depends on the chemical and physical specifications and, most important, the separation media. For techniques such as expanded bed adsorption, unique liquid distributor systems at the base of the column are required. For low-pressure chromatography (up to 3 bar), there are now commercially available columns constructed of plastic, glass or stainless steel. These columns range in size from laboratory scale up to diameters of approximately 2 m.

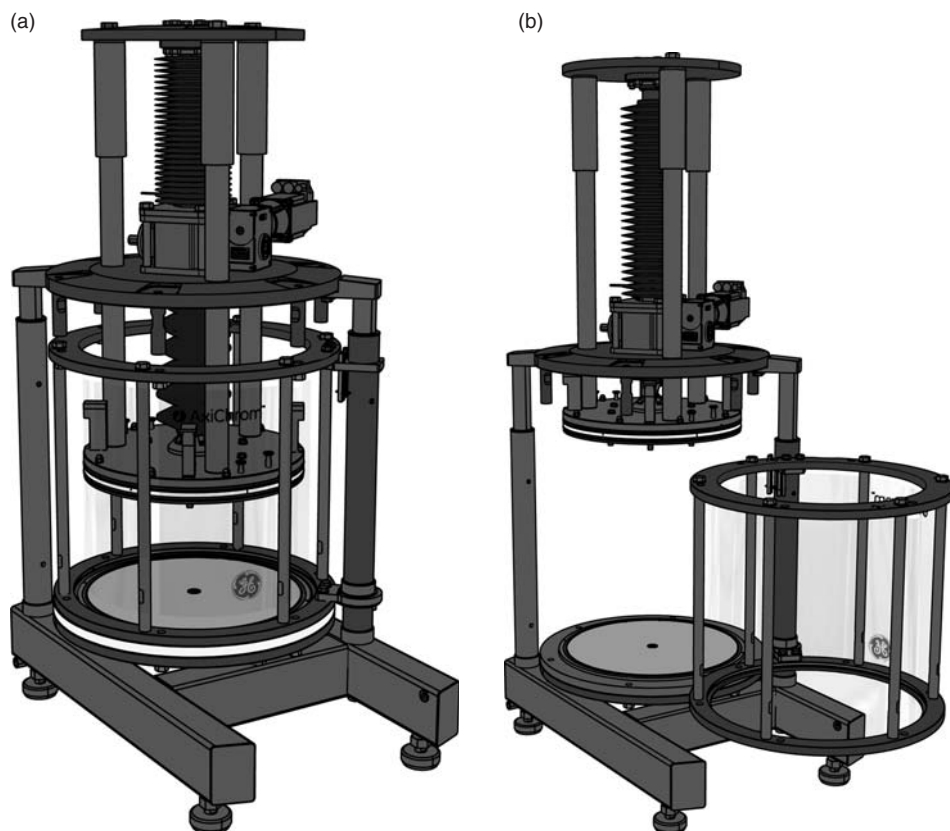
Traditionally, biochemists have preferred columns that allow for visual inspection of the packed media. But it is useful to keep in mind that as the column diameter increases, a smaller portion of the packed media can be seen. Both glass and some plastics allow for visual inspection. Cast polymethylmethacrylate tubes with high chemical and mechanical resistance are often used for manufacturing. These columns are non-toxic, tolerate high pressures and have a relatively high chemical resistance. High quality stainless steel and glass columns allow for high flow rates and meet pharmaceutical industry standards.

As mentioned above, occasionally a normal stainless steel construction might not be compatible with the chemical environment and columns constructed of higher quality stainless steel, e.g. hasteloy that is unaffected even by strongly acidic halide-containing buffers, may be needed. This can be rather costly, and compatibility with wetted surfaces should be considered in process design when selecting solvents and buffers.

For medium pressure chromatography (up to 20–40 bar), columns constructed of glass or stainless steel are available in diameters up to 60–100 mm. Diameters greater than 60–100 mm require stainless steel.

For high pressure process chromatography, the only practical construction material is stainless steel. Unfortunately, as the system pressure increases so does the column cost. At pressures beyond one bar, at process scale, the column is considered a pressure vessel. Pressure-vessel codes differ from one country to another and must be defined before choosing a column for production purposes [15, 16].

During process development, the use of adaptors allows for evaluation of the optimal bed configuration and simplifies the frequent repacking needed when comparing different resins. Hydraulic or motor-driven adaptors can allow for rapid packing (Figure 11.5a). The adaptor is lowered either under constant pressure or with constant velocity, which facilitates fast packing under optimized conditions, followed by locking the adaptor in place. The entire packing operation can be completed in 5 min and may be fully automated. In production, a column with fixed end pieces can be both hygienic and economical, but for both development



**Figure 11.5** AxiChrom™ (a) assembled column, (b) column in maintenance position with swing-out tube.

and manufacturing the adaptor style that allows for bed adjustment seems to be preferred. Large-scale columns with hydraulic or motor-driven adaptors can also simplify the maintenance of the column internals (i.e. o-rings, nets) by eliminating the need for external hoists, which are e.g. required for maintenance of columns with fixed end pieces (Figure 11.5).

### 11.2.2 Valves

Valves are used for directing liquid through the chromatography system, and valve functions are shown in Table 11.3. These functions can be either manual or automatic or both. Valves can be operated either electromagnetically or by compressed air.

For buffer selection and fraction collection, multi-port valves are preferred to minimize dead volumes that provide a location for bacterial growth. Filter and column by-pass are best achieved by manifolded multi-port diaphragm valves. Two-way valves sometimes increase the system hold-up volume, but sanitary three- and four-way diaphragm valves enable the



**Table 11.3**

## Valve functions

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Automatic buffer switch
Fraction collection
Filter by-pass
Air trap by-pass
Air trap, air release
Column by-pass
Pump switch
Forward or reverse flow through the column
Connecting columns in series or in parallel
Selecting on-line monitors
Recycling product
System drainage
CIP routines

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design of efficient systems. The problems previously associated with dead volume and fluid accumulation may be avoided with these multi-port valves that allow the diaphragm of the valve to be almost flush with the interior wall of the pipeline [17].

Depending on the scale of operation, system pressure and chemicals used, diaphragm valves are the valves of choice for hygienic, reliable, production scale chromatography. Miller [5] cautions that ‘a constant flow of low-level toxics can be extracted out of improperly prepared valve diaphragms’. Further information on valve design is presented in a bioprocess-engineering book [18].

### 11.2.3 Pumps

When selecting a pump for process chromatography, hygiene, chemical resistance, pressure/flow rate capabilities, temperature tolerance, shearing, speed control and pulsation should be considered.

#### *Hygiene*

For production scale liquid chromatography, the pump should be easily disassembled for cleaning and permit steam sterilization and CIP. There should be no stagnant zones in the pump where microbial growth may occur.

#### *Chemical resistance*

The materials in the pump must be compatible with cleaning procedures and chemicals used in the process. The number of suitable pumps is rather limited, particularly if steam sterilization is to be used. It is important to know what materials are used in the pump seals. Many chromatographic processes require ethylene propylene diene monomer (EPDM) or fluororubbers because of their chemical resistance.

*Pressure/flow rate*

It is most important that the pump covers the entire flow rate range of the process and that the discharge pressure is adequate over the whole range (Figure 11.6). In addition, the requirements of the pump can be met by suitable placement of tanks. For example, if a buffer tank is located on the floor above the chromatography operation and gravity assists delivery of the fluid, the risk for pump cavitation is less than if the buffer tank is situated on the same level as the system.

*Temperature tolerance*

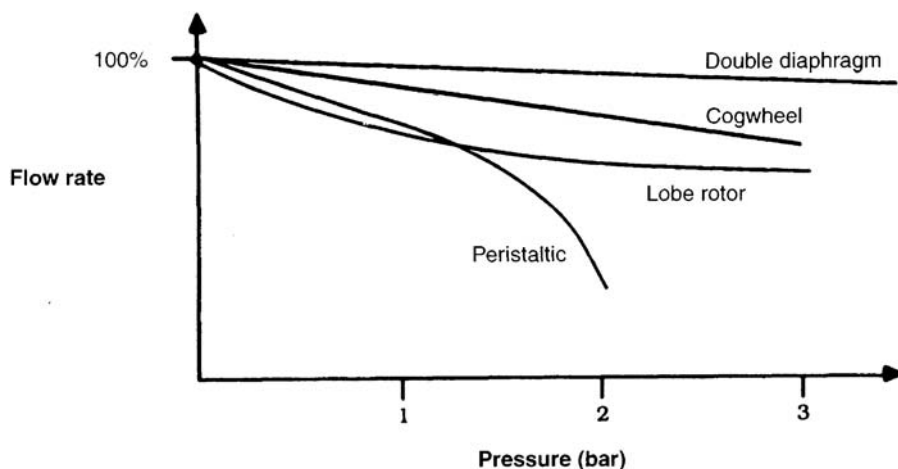
The operating temperature range of different pumps varies. Usually, this does not cause any problems because most chromatographic processes are run at room temperature. However, if steam sterilization or hot water is used for sanitization, considerable care must be taken in selecting the appropriate pump.

*Shearing*

Most proteins are sensitive to shearing. When choosing a pump for protein processing, this is one of the most important aspects to consider. Several commonly used industrial pumps, e.g. centrifugal, gear and screw pumps, cause protein shearing. Pump shearing has been observed to account for up to a 60% loss of protein product.

*Speed control*

Liquid chromatography systems are normally operated over a wide flow rate range. During sample application, viscosity changes may affect flow rates, and the elution flow rate is usually considerably lower than the flow rate during equilibration and cleaning steps. To enable operation over the entire flow rate range, it is necessary to have pumps with automatic speed control.



**Figure 11.6** Pressure/flow rate curves for different types of pumps.

### *Pulsations*

Pump pulsation should be minimized to prevent disturbance of the packed column. If a pulsating pump must be used, care should be taken to minimize pulsation. Air traps positioned after the pump can act as pulsation dampeners, and in-line pulse dampeners can also be used. Pulse dampeners may, however, introduce a hold-up volume and reduce gradient accuracy.

Additional pump features that should be considered include hermetic leak tightness, long-term sterility, lifetime and operator safety. These issues have been discussed by Allee, who describes these and other features for peristaltic, rotary lobe, and rotating disc pumps [19]. Diaphragm, screw, peristaltic, lobe rotor and gear pumps are all used in chromatography. In most cases, the flow rate specification will dictate which pump design will be chosen. Chemical compatibility should also be considered. For gradient systems, two pumps are typically used. In some cases, a single pump is used with two control valves or switch valves to proportion the mixture. Separate sample application pumps are sometimes employed to minimize sample dilution. Each pump type has certain advantages and disadvantages, some of which are discussed by Glaser [17]. For further information on pumps, see Stover [20].

## **11.2.4 Monitors, meters and sensors**

Monitors can be divided into those used for process monitoring and those used to measure system performance and provide safety. Monitors used for process monitoring include those for UV, conductivity and pH. Monitors for system performance and safety include flow meters, pressure, air and temperature sensors. The recorded measurements become part of the batch documentation.

### *UV monitors*

UV monitors measure the absorbance of UV light. Usually the UV absorption of proteins is measured at a wavelength of 280 nm. Multiple wavelength and scanning wavelength monitors may be useful for laboratory and even pilot scale chromatography. In the production chromatography, single wavelength monitors are preferred because of their reliability and simplicity. Furthermore, little of the available scanning equipment is designed for industrial use.

There are functions that are advantageous in both laboratory and process chromatography UV monitoring, i.e. auto-zero base line and event mark. For process chromatography, there are additional features to be considered. These include: flow-through cell, variable path lengths, sanitary connections and cell interior, non-sensitivity to electrical noise, lamp surveillance, explosion protection and adjustable alarm levels.

### *Conductivity monitors*

Conductivity monitors measure ionic strength. Conductivity is the primary input control parameter used in automated chromatography systems to enable the generation of salt gradients or to control buffer dilution or in-line buffer preparation. Conductivity monitors are also useful for monitoring and automating cleaning and equilibration steps. It is important

to keep in mind that both conductivity and pH (see below) measurements are temperature dependent and both can be temperature compensated.

Industrial conductivity monitor requirements are, in many respects, similar to those described above for process scale UV monitors. Since conductivity monitors are used for water purification systems and waste-water treatment units, there are plenty of large-scale flow jackets available. Sanitary design of flow cells and jackets, and minimization of internal volume of flow jackets, should be considered when selecting conductivity monitors.

### *pH monitors*

In many applications, accurate pH control is critical to the success of the separation. Minor fluctuations in the pH might ruin the separation since some proteins elute within 0.1-pH unit of each other. Furthermore, an equally small variation in pH can affect the solubility of a protein and can lead to column blockage. Table 11.4 shows a pH sensitivity investigation on a process for separation of human serum albumin (HSA) from blood plasma. The results indicate that the pH during part of the process may not vary more than 0.05 pH unit to achieve reproducible results. From a technical point of view, this is a very narrow limitation that is not found in today's well-designed biotechnology processes.

Industrial pH monitoring is very difficult, particularly because of the sensitivity of pH electrodes to fouling. The design of a pH-measuring unit, therefore, needs particular attention. Most important, the electrode should be easily removed from its flow jacket for cleaning, calibration or replacement.

### *Flow meters*

The flow rate in a liquid chromatography system is usually controlled using a flow meter signal as input and a controller to adjust the speed of the pump. The flow meter should not be sensitive to viscosity changes, and a sanitary design is preferred. Several types of flow

**Table 11.4**

Possible variations in pH and ionic strength (*I*) in buffers used in the different chromatographic steps for purification of HSA

Step	Correct		Range	
	pH	<i>I</i>	pH	<i>I</i>
Buffer exchange	7.0	0.025	Not critical	0.023–0.027
Anion exchange				
Buffer 1	5.20	0.025	5.10–5.25	0.023–0.027
Buffer 2	4.50	0.025	4.40–4.60	0.025–0.030
Buffer 3	4.00	0.15	Not critical	Not critical
Cation exchange				
Buffer 1	4.50	0.025	4.30–4.70	0.023–0.027
Buffer 2	5.50	0.11	5.45–5.55	0.11–0.12
Buffer 3	8.00	0.40	Not critical	Not critical
Gel filtration		0.05 M NaCl	Not critical	Min. 0.02

meters are available. The most commonly used—mass and electromagnetic flow meters (the latter cannot be used to measure WFI or DI water)—meet both sanitary and accuracy requirements.

#### *Air sensors*

Air sensors are used to protect the chromatography column from air and to enable complete emptying of the sample tank. The column can also be protected by an air trap. Usually an air trap and an air sensor are used in series.

The complete emptying of the sample tank is essential if the sample has a high value. Level sensors can be used to achieve sample tank emptying, but the air sensor has an advantage over level sensors. Since the air sensor is usually placed on the outlet line, it is not in direct contact with the tank contents. Ultrasonic air sensors with sanitary design are recommended and available in both plastic and stainless steel.

### **11.2.5 Tubing or piping**

Buffers and sample are pumped from their respective containers to the chromatography column in tubing or piping. The acceptable pressure drop in a chromatography system is determined by the discharge pressure of the pump in the system and the maximum operating pressure of the respective components in the system, including the tubing. The system should be designed in such a manner that most of the pressure drop is produced by the resin in the column. As a general guideline for low-pressure chromatography, the maximum pressure drop in the tubing should be 10–15% of the available pressure from the pump. This leaves around 50% of the available pressure for the packed bed and the rest for all other components in the system. Tubing is also sized based on the general rule of thumb that the maximum process fluid velocity is approximately 1.5 m/sec.

When selecting tubing or piping for a chromatography system, the following additional aspects need to be considered: dimensions, internal volume, chemical stability, temperature, flexibility and price.

### **11.2.6 Fraction collectors**

In the laboratory, the fraction collector is just as important as the column. At process scale, other considerations affect the choice of equipment for collecting fractions, the most important being the number of fractions collected. Rarely are more than five or six fractions collected in a well-developed production process. During process development, however, more fractions will usually be collected. Multi-port valves or manifolds of two-way valves are the most suitable fraction-collection method for production.

## **11.3. AUTOMATION**

Automation of a process includes control, documentation and evaluation. Automation enables feedback control. Process analytical technologies (PAT) require the use of automation.

PAT is described elsewhere in this book (see Chapter 7). This section describes advantages of automation, different control systems and hardware and software specifications for chromatographic automation systems for development to production stages. Validation of automated systems is addressed in Chapter 7.

### 11.3.1 Advantages of automation

There are many advantages to automating chromatographic processes. These include: decreased labour costs, reduced process development time, decreased capital costs, increased reproducibility, increased reliability, improved work environment, improved overview and improved documentation.

One of the most obvious reasons for automating a process is to reduce labour cost. Automation has the added advantages of freeing competent personnel for less tedious tasks and reducing process development time. Automation increases reproducibility and decreases batch losses due to operator error. Reliability can be increased by adding checkpoints and automatic alarms; the operator knows if the process is working properly. The checkpoints and automatic alarms can be displayed to allow quick access to all parts of the process. Processes that need to be in cold or hygienic rooms can be remotely controlled, which improves the working environment for the operator and reduces the risk of contamination. For a complex process, automation can improve the overview of the process with logical schematic displays, including set and actual levels of control parameters. Methods, process parameters and other process information can be stored automatically for each process run.

### 11.3.2 Control systems

An automation system can be based on different types of control hardware, e.g. dedicated controller, programmable logical controller (PLC), personal computer based system or combinations of these three.

#### *Dedicated control system*

This uses hardware optimized for a specific application. This reduces the capital cost because you only pay for what you need. The controller is easy to use and ready for installation. The built-in software is easy to use, i.e. no programming skills are necessary. The dedicated controller can work as a stand-alone or as a remote unit in a computer system. One disadvantage is that expansion possibilities and modifications can be limited. A dedicated controller has its place both in process development and in production plants.

#### *Programmable logical controller system*

PLC system is a general system that can be used in any type of application. The hardware is often built in a modular style so that the user can change the control capacity by simply adding or replacing modules in the system. The PLC system needs some preparation before the system is ready to use, e.g. assembly of the different modules. Furthermore, extensive programming is necessary. The PLC system can work as a stand-alone or as a remote unit

in a computer system. PLC systems used in production should be designed for process environments.

#### *Personal computer system*

Another general system that can be used in any type of application is the personal computer system. Often the computer communicates with a remote unit, i.e. a dedicated or PLC. Alternatively, input/output (I/O) interfaces are installed in the computer or in an interface box with no intelligence. In control systems consisting of computers combined with remote units, the latter control the process and the computer documents, evaluates and manages one or more systems. In the case of I/O interfaces, the computer itself controls the process. In this case, the computer must have software with multi-tasking and real time capabilities. Today, vendors can provide software that has undergone years of extensive development and is ready for use upon arrival at the manufacturing firm. There are many software programmes available that communicate with laboratory equipment, i.e. computer packages with intelligent front ends.

#### *Distributed control systems and networking*

Centralized distributed control systems (DCSs) are commonly used today in pharmaceutical plants to control processing operations. Personal computer (PC)-based supervisory control and data acquisition (SCADA) packages are also being employed. Networking unit operations and employing standard commercially available control packages are becoming more desirable in the biotechnology industry [21].

### **11.3.3 Hardware and software specifications**

Good automated manufacturing practices (GAMP) describe documentation and building validation into a system during the design and functional specification stages (see also Chapter 7).

Different stages in a project need different types of automation. In the development phase, e.g. a more flexible automation system is needed. In production, reliability is more important.

The hardware specifications for an automation system are basically the same for process development as for production. Any of the control systems above could meet the specifications shown in Table 11.5.

The hardware capacity required varies with the complexity of the process. In process development there is a need for expansion possibilities, while in production the system is often fixed. There are many signals that need to be included in the hardware. These are shown in Table 11.6.

The software specifications for an automation system for process chromatography depend on which stage the process is in, i.e. process development or production. In process development, the same chromatography system can be used for many different processes and the methods can vary to a great extent. Many functions need to be included in the software (Table 11.7).

**Table 11.5**

Automation for process development and production systems: hardware specifications

Compatibility with industrial environments	Voltage fluctuations Static electricity High-electrical noise levels Humidity Dust, dirt Explosion proof, where appropriate
Display of relevant information	Valve status Process step Flow rate Pressure Monitor signals Flow sheet
Number of I/O (digital and analog)	
Documentation	Printer/plotter CD drive USB port for data transfer
Automatic alarm handling	Differential pressure over columns and filters High- and low-pressure levels High- and low-pH levels High- and low-conductivity levels High- and low-flow rates High- and low-tank levels Air detection Valve malfunction Monitor malfunction (especially UV monitor lamp) Documentation of all alarms

**Table 11.6**

Automation for process development and production systems: signal specifications

Outputs
1–3 system pumps, analog control
5–20 on, off <sup>a</sup>
Inputs
1–2 flow meters, analog input
1–3 monitors, analog input
10–40 digital inputs for alarm purposes in production systems
1–2 digital inputs per digital output to be recorded
Control loops
1 proportional integral (PI) control loop per pump

<sup>a</sup>For automatic CIP routines and extensive fraction collection, this number of digital outputs increases dramatically.



**Table 11.7**

Automation for process development systems: software functions	
Flexible number of inputs and outputs	
Fast programming by ‘non-expert’	
Manual control of all outputs during a run	
Automatic documentation and display of:	
Flow rate	
Pressure	
Monitor signals	
Valve status	
Evaluation of results	
Resolution	
Economy	

**Table 11.8**

Automation for production systems: software requirements	
Documentation of cost	
Documentation of yields	
Easy to use for operators	
Trend analysis	
Manual control	
Security, user-defined access to different levels of control	
Automatic documentation and display of:	
Flow rate	
Pressure	
Monitor signals	
Valve status	
Evaluation of results	
Resolution	
Economy	

In production, the automation is used for a well-defined process. The software requirements are shown in Table 11.8. The optimal automation system for process chromatography is a system that can be used in process development, pilot runs and full production. The time required to test and evaluate control of the process will then be minimized.

In summary, selection of chromatography equipment requires careful consideration of the needs for flexibility, documentation, reliability, hygiene, automation and validation. After functional, chemical and pressure specifications are established, components or systems can be selected that meet laboratory, pilot plant and production requirements. Local and national standards, such as those for electrical and explosion-proof requirements, should also be evaluated to ensure compliance. For production systems and even some pilot scale

systems, the user should work with the supplier company to ensure that all design criteria are clearly defined. The user may even wish to inspect large engineered systems at the assembly site. Functional tests (factory acceptance test, FAT) that the supplier may perform include loop tests for conductivity, flow meters, pH, pressure, leakage, UV, valve and air sensors. Other tests include those for alarm and watch functions, gradient performance, pressure/flow, pump and leakage. Calibration should be performed at the time of delivery and the critical function tests will be performed to commission the system, i.e. ensure it is in working order. Training of operators and service engineers on the proper use and maintenance usually take place at the time of the commissioning.

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# – 12 –

## Column Packing

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### 12.1 INTRODUCTION

An efficiently packed column is often critical for the successful purification of biological molecules. The purpose of the separation (i.e. initial capture, intermediate purification or final polishing) as well as the elution mode (i.e. step elution, gradient or isocratic) will influence the requirements for column efficiency. Generally, the smaller the average particle size and the greater the bed height, the more critical the packing becomes in order to fully exploit the inherent benefit from small particles and long beds. Column packing shall not only provide an efficiently packed column but also a stable bed that will not deteriorate over-time.

For aqueous purification of biological molecules, standard chromatography columns range in diameter from approximately 5 mm to 2 m. Column lengths also vary, typically from 5 to 100 cm. Generally, an aspect ratio, i.e. ratio of column diameter to bed height, should not exceed 10 or it may be difficult to form a homogeneous bed. Furthermore, fluid distribution can be an issue with a very large aspect ratio, i.e. excessively short, wide beds. Fluid distribution systems vary from vendor to vendor. The physical properties as well as surface properties of the chromatography resin will also vary between types of resins and this will affect column packing. Clearly, there can be no one method for packing all columns but if the method is not supplied by the vendor there are some general principles that can be applied in the development of a packing method.

### 12.2 THEORY

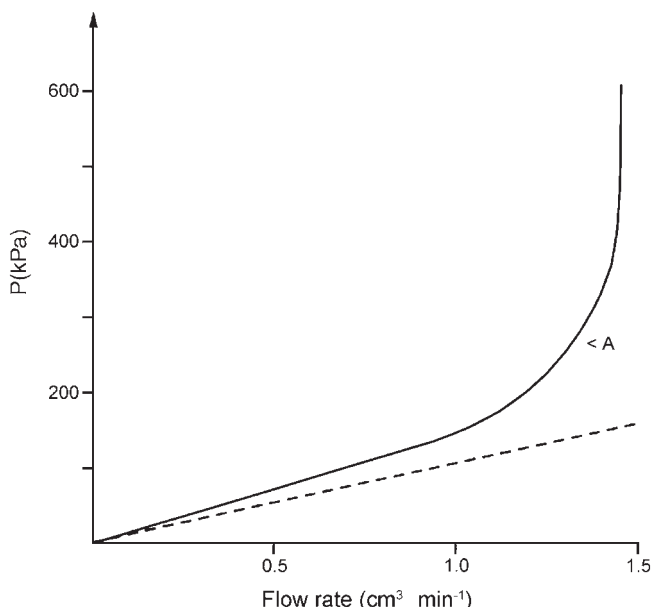
Many factors influence the column packing process and its outcome and theory describing the interplay of these factors has not yet been fully mapped out [1–5]. Therefore experience and know-how is important for development of a suitable packing method. However, there are some fundamental aspects that are valuable in the design of a packing method [6].

The first requirement is to obtain a good dispersion of the resin particles in the slurry buffer used for packing. Additives may have to be added to prevent particle–particle or particle–column-wall interactions. Particle–particle interactions are dependent upon surface roughness, particle shape and surface chemistry and can be studied by measurement of the rheological properties of the slurry.

The actual packing of the homogeneous slurry is often divided into two steps. The objective of the first step is to form a homogeneous consolidated bed. This is achieved by settling the particles at a constant fluid velocity, where particles are packed due to the viscous drag from the fluid. The velocity chosen depends upon the rigidity of the material and shall be low enough to prevent elastic deformation of the particles (see below). The consolidation phase may be simulated using computational fluid models to various levels of detail, where even the tracking of individual particles has been investigated recently for the example of packing small bed sizes [2, 5].

After having formed the consolidated bed, it must be compressed to create a stable bed that will not rearrange or be further compressed if used within the set operation window. The operation window is determined by the deformability of the particles due to the fluid stress and the supportive wall friction (i.e. wall support). This compression to the final bed height can be done either by applying a high flow rate and thereby increasing the viscous drag on the particles and bed, respectively, or by mechanical compression employing a movable adapter, so called axial compression. The deformation of the bed under this compression step has been described by application of the elastic theory based on coefficients such as the Young's modulus, the shear modulus and the Poisson ratio in addition to the wall friction coefficient [1–4]. These may be determined, e.g. by triaxial instrument setup but that is mainly used by vendors and for basic research for characterization of the material [1]. The elastic theory illustrates also the fundamental differences between bed compression by flow and mechanical axial compression: While the axial compression of the bed will result in a uniform compression and a uniform void fraction over the length of the bed, the compression by flow and viscous drag on the particles will result in a gradient of compaction and void fraction, resulting in higher compaction and lower void at the column outlet where the excess liquid is removed. This behaviour is inherent to the fact that axial compression of the bed will result in a compression by both top and bottom end piece, whereas the viscous drag is exerting a force and load accumulating in direction to the column outlet leaving the bed at the column inlet uncompressed.

The viscous drag by the fluid velocity will compact the bed and reduce the inter-particle void fraction, starting from the outlet, leading to a reduction of the permeability and an increase in the pressure drop. A higher pressure drop will in turn cause further compaction, which results in the non-linear pressure–flow behaviour that is characteristic for the compression of a chromatographic bed by flow (compare Figure 12.1). Thus, the characterization of pressure–flow behaviour of the gel is of outmost importance for developing a packing method in case that appropriate data cannot be obtained from the vendor. Both for the purpose of such an experimental characterization, but also for the packing of the column by flow compression, it is essential to have good control of flow rate and pressure drop over both the column and the system in order to achieve the appropriate bed compression without collapsing the bed or even damaging the gel. After packing, a relaxation of stress and void fraction will occur throughout the bed once the top end piece of the column is brought into place and the flow has been stopped. Hence, beds of elastic beads compressed by the flow method were found to give uniform beds as measured by the uniform void fraction of the bed [6]. If the bed has been compressed too hard during the compression step, this relaxation may be disturbed by generation of channels or major displacements in the bed structure, resulting in poor column efficiency.



**Figure 12.1** Pressure drop over a chromatography bed as a function of flow rate (solid line). Deviation from the dotted line, representing the theoretical pressure drop from eq. (10.16), is due to bed consolidation in the linear part and bead compression resulting in decreasing flow channels in the right hand part. The maximum flow for this gel-column-fluid combination is arbitrarily set to 80% of the flow at the inflexion point, A, and is 1.0 ml/min. The working flow rate must be kept substantially lower than this, e.g. 80% and this yields a flow rate of 0.8 ml/min. Experiment performed with Superose™ 6 prep grade (34  $\mu\text{m}$  cross-linked agarose) in a HR 10/30 (10 mm I.D.) column. (Reproduced from L. Hagel and T. Andersson, *J. Chromatogr.* 285 (1984) 299 by permission from Elsevier.)

### 12.3 PREPARATION OF COLUMN AND SYSTEM

In addition to aspect ratio and supported packing techniques (flow packing or axial compression packing), important information for each column includes its pressure rating, materials of construction and chemical resistance (see Chapter 11). To assure that the system will not yield excessive backpressures or that any pressure limit of the resin set by the manufacturer is not compromised a pressure/flow test of both system and column is performed.

Prior to measuring the pressure/flow curve the system (and column) should be checked for leaks. The leak test is performed after assembling the column and ancillary system, which includes pumps, hoses, gauges, valves and fittings. The leak test is typically performed by filling the column with water and pressurizing it up to its pressure rating and then closing the inlet side valve, making sure not to exceed pressure rating. After 1 min, the pressure is recorded and then the measurement is repeated after 30 min. A loss of pressure is indicative of a leak.

The column itself together with tubing and connectors significantly influences pressure. The pressure drop over the empty column is usually higher at laboratory scale columns and columns intended for use with small particles diameters, which is due to small diameter

tubing and fluid distribution devices employing frits of low porosity. Temperature may also influence the pressure/flow performance due to the influence on viscosity (e.g. see eq. (10.16)) and should be consistent with that used for the packed column.

As outlined above, the knowledge of pressure drop over the empty column and the system is essential for proper control of column packing methods that are relying on bed compression by flow. The pressure drop of the system with an empty column should be subtracted from that of the system with a packed column to yield the true pressure drop over the packed bed.

## 12.4 PACKING THE COLUMN

Instructions for packing a resin in columns with specified designs and dimensions are usually available from the supplier. However, users may purchase the resin from one vendor and columns from another. In this case, complete packing instructions are usually not readily available. In the event that packing directions are insufficient or do not give satisfactorily results the following general procedure may be tried.

The steps involved are; choosing packing solution, selecting slurry concentration, defining fluid velocity, pressure or compression factor for the packing step by a pressure/flow test of the consolidated bed and finally packing the column.

### 12.4.1 Packing solution

Most resins are shipped in 20% ethanol, but packing in this solution is usually unacceptable at large scale due to explosion-proof concerns and the cost of solvents and their disposal. In many cases, the ethanol content can be reduced to about 10% by dilution with water. An ethanol concentration of 10% works well in pack-in-place columns and in axial compression columns. Depending on the type of resin and the scale of operation, the best packing buffer may contain salt (e.g. 10–250 mM), or alternatively a hydrophobic solvent such as ethanol (e.g. 10%) to reduce particle–particle interactions. For some ion exchangers salt is added to achieve optimal packing. If corrosion of stainless steel is a problem (e.g. if solutions are left over night) then non-corrosive anions, e.g. sulphate may be useful. Some hydrophobic interaction resins are best packed in a 10–20% ethanol solution.

### 12.4.2 Preparation of the slurry

The slurry concentration should be sufficiently high to allow the appropriate amount of resin to fit into the column, together with any extension of the column tube that may be used for column packing. However the concentration should generally not exceed 70% to prevent particle aggregation and trapping of air bubbles. The most common slurry recommendation is 50%, but it can range between 30 and 70%. The concentration of slurry is determined by letting the resin, dispersed in the packing solution, settle over night and the settled volume of resin is set to 100% slurry. When packing columns with a large ratio of bed height to column diameter (i.e.  $h/d > 1$ ), a vessel of the same dimensions as the column is preferable

for the determination of slurry concentration to account for the impact of wall support. If required, the slurry is diluted with packing solution to the desired concentration. In order to guarantee that the correct bed height is achieved at the correct compression factor (see below), the exact amount of resin has to be transferred to the column. The pressure/flow experiment will give guidance to the relationship between settled volume of slurry and volume of packed bed.

### 12.4.3 Determination of packing parameters

As outlined in the theory section the fluid velocity appropriate for packing a non-rigid resin is depending upon many parameters and therefore the elastic deformation must be tested by running the column at either increased flow (which is recommended) and register the back pressure or by increasing the pressure and register the resulting flow. This is typically performed by adding the slurry (as prepared above) to the column, mounting the adaptor and running a flow gradient while noting the pressure drop. Such an experiment can be seen in Figure 12.1 for the example of a laboratory scale column and the maximum flow applicable to this setup can be estimated to be 1.0 ml/min (i.e. 80% of the flow at the inflexion point of the pressure/flow curve). In case the bed is continuously compressed during this test, e.g. as noted by a continuously non-linear increase in pressure drop also at low flow, the test shall be performed using a stepwise increase of flow letting the bed stabilize, i.e. reaching a constant pressure drop, between each step.

While the pressure drop over the bed and thereby fluid velocity are the controlled parameters for packing methods based on bed compression by fluid flow, it is the degree of volumetric compression that is controlled in axial compression packing methods. The volumetric compression is expressed as compression factor, which is the ratio of the volume occupied by the settled or consolidated bed set to the volume of the packed bed at its final bed height. If the optimal compression factor for a resin is available from the vendor, it is straightforward to determine the required amount of slurry and settled bed volume, respectively. In practice, the compression factor resulting from an optimized flow packing method is usually very close to a compression factor that would be used for axial compression packing. Hence, the characterization of the consolidated bed by pressure–flow curves as described above can also be utilized for developing axial compression packing by measuring the volumetric compaction of the gel during the course of the experiment. The compression factor found at the flow rate optimal for the flow packing method will be suitable for controlling the axial compression packing, 15% compaction is usually a good guideline for elastic resins.

### 12.4.4 Packing methods

Many packing modes and methods exist and columns may be designed to be packed using one or several methods. Combination methods also exist. The methods mainly used are constant flow, constant pressure, suction, pack-in-place and axial compression. In principle, all columns can be packed by the constant flow or pressure methods. If no packing method is supplied by the manufacturer a constant flow or constant pressure method is suggested.



In a constant flow or pressure method, the slurry inside the column is settled and packed using a flow of liquid from the top to the bottom. The best method is a two-step one, where the resin is first settled at a low fluid velocity (e.g. 30% of the maximum flow as determined in 12.4.3) to create a consolidated homogenous bed. The bed is then compressed to the correct compression factor using a higher flow, e.g. 80% of the maximum flow or at the pressure corresponding to this flow. This is the maximum operating flow (or operating pressure) under ideal conditions, but often the actual flow (or pressure) is lower due to e.g. high viscosity of the sample or eluent (e.g. if the column is run at lower temperature).

In suction packing methods, the slurry is packed using a pump on the outlet side of the column. Normally, these columns utilize packing reservoirs to accommodate the total slurry volume. Suction packing is only applicable for soft resins that require a low pressure drop during packing achievable by suction. Pack-in-place methods utilize a nozzle that is inserted into the column, hereby allowing operation in a closed system while pumping the resin into the column. The main advantages of the pack-in-place concept are automation, speed and reproducibility when re-packing (and unpacking) the columns. When utilizing the pack-in-place concept for columns with fixed end pieces, the columns are packed with a high slurry velocity and excess liquid is removed through the outlet while the bed is being formed and compressed. Another concept is the use of a pack-in-place nozzle in an axial compression column. Here, the complete slurry volume can be introduced into the column by use of the movable adapter prior to running bed consolidation and compression by displacement of the adapter in a second step. Compared to the pack-in-place column with fixed end pieces, the latter column concept is more versatile and facilitates beds packed to improved homogeneity and efficiency.

## 12.5 EVALUATING COLUMN PACKING QUALITY

Testing the bed is important to verify that the column is efficiently packed. Periodically repeating the test is recommended to ensure the consistency of the column efficiency and bed integrity. The test conditions are very important since a poorly performed test can lead to rejection of a good bed. Much emphasis must be put on defining the specifications, and correlate these to the required separation performance, e.g. a column for step elution in capture mode will not require the same specification of efficiency as a column for isocratic elution in polishing mode. However, a significant reduction in the measured efficiency, peak symmetry or pressure drop may indicate that the column is deteriorating and should be repacked. As noted elsewhere in this book (see eq. (10.19)), the measured efficiency of the packed bed depends among other parameters on resin particle size, quality of the packed bed, flow rate and sample volume and solvent viscosity. The two most commonly used methods for evaluating column-packing quality are step and pulse method, both are measures of the residence time distribution (see Chapter 10.7.2).

### 12.5.1 The step method

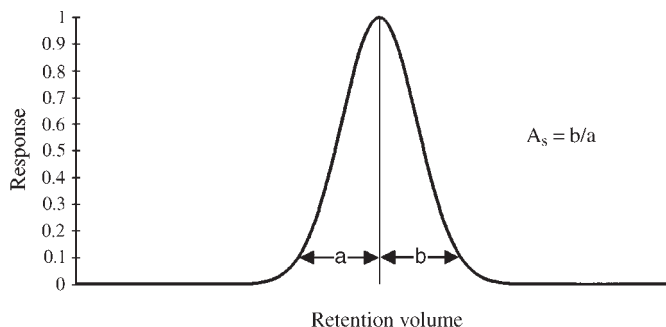
The analysis of the residence time distribution by a step method is practical for manufacturing processes in which buffer salt concentration is changed. In this method, efficiency

is measured by monitoring the transition in conductivity as the buffer is changed from low to high salt or vice versa. The shape of the curve is a step resembling frontal analysis. This analysis can be performed in process and does not require addition of a separate sample to the column. Transition analysis is less sensitive to the external volume of the system; therefore, the results will differ slightly from those of the pulse method. Evaluation of a residence time distribution curve is discussed in Section 7.2 of Chapter 10. In order to apply this evaluation, it shall be verified that there is a linear relationship between the change in concentration of the tracer substance and the measured signal subjected to the evaluation.

### 12.5.2 The pulse method

The most common method for measuring efficiency has been to add a pulse, i.e. a narrow sample zone, of a low molecular weight solute and then calculate the values for zone broadening in terms of  $H$  (or HETP, height equivalent to a theoretical plate) and peak symmetry ( $A_s$ , the asymmetry factor). Calculation of  $H$  is given by Figure 10.9 and the calculation of peak asymmetry factor is given by Figure 12.2.

Traditionally, a pulse of sodium chloride, benzyl alcohol or acetone has been used for tests of zone broadening and peak symmetry. Whereas an ultraviolet (UV) monitor is used to detect acetone and benzyl alcohol, sodium chloride is monitored using a conductivity meter. Alternatively, a sample containing concentrated equilibration buffer may be used to test the packing, e.g. 0.8 M NaCl in a solution of 0.4 M NaCl has been found to be generally applicable. It is important to be aware that, in some cases, salt may interact with the resin and give erroneous absolute values. And acetone or benzyl alcohol may interact with some polymeric packing in such a way that zone broadening occurs. This interaction can be prevented by using acetone in ethanol as the test sample and 100% ethanol as the mobile phase. This is, however, only realistic when a firm is running a process in organic solvents and is prepared to deal with disposal, explosion proof and cost-related issues. In addition, it is imperative that if acetone is used its quality is exceptionally high to avoid contaminating the column with impurities sometimes found in laboratory grade acetone. It may be a good strategy to test a few different substances and eluents to ensure that solute–solvent interactions do not cause false results.



**Figure 12.2** Calculation of peak symmetry, measured at 10% of the peak height. An asymmetry factor,  $A_s$ ,  $< 1$  is called leading and indicates channeling and if it is  $> 1$ , which is more common, the peak is tailing and is typical for dispersion by mixing and solute–resin interactions.

Three separate pulses (i.e. sample injections) are recommended for each packing. The average values for  $H$  and  $A_s$  are more accurate since there is often some variability in sample application volume and speed even with the same system, also considering that the plate height is calculated from the square of the measured peak width. In addition, signs of improving or decreasing bed performance can be seen if results change for each injection. An upward trend may indicate that the bed needs more time to stabilize before the test is started.

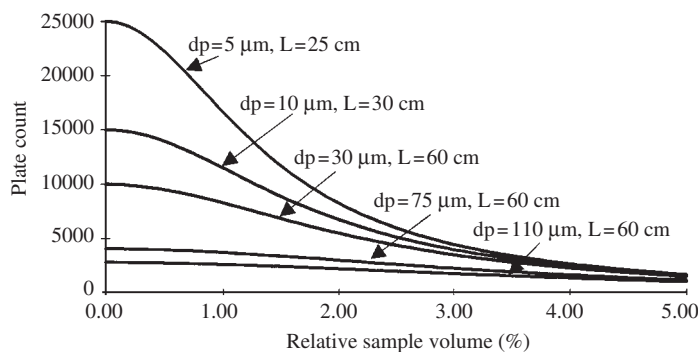
If  $H$  and  $A_s$  are established on a small scale, it is important to keep in mind that lack of wall support at large scale can lead to decreased packing quality that results in columns with lower efficiency. While this may not impact an adsorption technique such as ion exchange, it could result in loss of resolution in a technique such as size exclusion.

In process chromatography, it is important to keep in mind that  $H$  and  $A_s$  are measurements that should be used as tools for comparing the packing quality of different lots of resin and different column sizes and designs and then by calculating the reduced plate height,  $h$  (see eq. (10.11)). Column efficiency, peak symmetry and column pressure drop are useful in assessing the quality of packing between runs, after cleaning, and after storage. A significantly increase in pressure drop may indicate that the column is partially clogged or compressed. The expected pressure drop may be calculated from eq. (10.16). The condition of the column may also be continuously followed by residence time distribution analysis at stepwise buffer changes. Realistic ranges that correlate with column performance (i.e. product purity and recovery) should be specified (see also column qualification in Chapter 7).

It is also important to recognize that column design has a major impact on the quality of column packing, and system design influences the test results (see also Chapter 11). Properly designed column inlet (for liquid distribution) and outlet (for liquid collection) provide uniform flow across the bed surface resulting in uniform packing densities. During testing of the packed column properly designed inlets and outlets minimize band broadening.  $H$  and  $A_s$  measurements reflect not only the quality of the packing in the column but the column design and all components from the point of entry of the test sample to the monitor. Components (i.e. pumps, valves and monitor cells) and tubing diameter and length contribute to zone broadening, which leads to a loss of resolution. For example, if the diameter of the tubing is too large in relation to the column size, zone spreading in the tubing will degrade the output of an otherwise well-packed column. When comparing the packing quality of small-scale to large-scale columns, the influence of extra column components must be taken into consideration.

Extra zone spreading due to large void volume in the bed is seen when a column is not densely packed and/or is not homogenous. If, on the other hand, the bed is packed too densely, the flow may be channeled and this will lead to zone spreading as well.  $H$  cannot distinguish between these very different causes. But determination of peak symmetry can be helpful. An  $A_s$  value less than 1 (leading peak) indicates the bed has been packed too tightly causing channeling in the bed. An  $A_s$  value greater than 1 (tailing peak) may be caused by insufficient packing pressure, air under the distribution net or partial clogging of nets or resin material. On the other hand, tailing may also indicate interaction of the test sample with the resin as discussed above. If tailing is not seen in the original test, but it appears after an extended time and is not alleviated by column cleaning, it may mean that the column needs to be repacked.

After packing a column and measuring  $H$  and/or peak asymmetry, it is recommended that the packed column be allowed to run for a given time (e.g. overnight) at about 80% of the packing flow rate and the measurements of HETP and  $A_s$  performed again. Quite frequently,



**Figure 12.3** The effect of sample volume on measured zone broadening, expressed as column efficiency,  $N$ , calculated from  $N = L/H$  where  $L$  is the length of the bed. Sample volume in % of bed volume.

the column packing stabilizes and the efficiency of the column increases after it has run overnight. This can be repeated a few times to assess the column stability.

The contribution to zone spreading from sample volume is more significant when using resins of small particle sizes. Figure 12.3 illustrates the importance of sample volume when measuring the zone broadening. When a sample volume greater than 1% of the bed volume is used with high efficiency resins, such as those used for size exclusion and reversed phase chromatography, the measured value may not provide accurate information. Under these conditions, column packing could be deteriorating and not observed by determining  $H$ . On the other hand, for those applications in which column efficiency is not critical, the sample volume is less important. For further information on the influence of sample volume on zone spreading, see Hagel [7].

In addition to sample volume, the flow rate used to measure zone spreading must be specified, since increased zone spreading will be seen as a result of non-equilibrium between the mobile and stationary phases due to resistance to mass transfer. This is influenced by the diffusion rate of the solute and the diffusion distance, which is dictated by the size of the beads (see eq. (10.10)). It may also be noted that the diffusion rate is temperature dependent.

Finally, standard operating procedures for measuring  $H$  and/or  $A_s$  as well as column back pressure must be written in such a manner that they are easy to follow and can be repeated. Unless these measurements are made in the same way, the interpretations of the results are often misleading. This could, for example, lead to a decision to repack a column that was in fact performing as it should, wasting time and even causing production delays.

## 12.6 SCALE UP

Since successful scale up depends, in most cases, on maintenance of bed height, accurate measurement of bed height at small scale is important. Unfortunately, bed heights using columns of small I.D. may give an overestimate of the relationship between bed height and gel volume for large-scale columns due to the supportive influence of the column walls

for small-scale columns. In addition, the loss of wall support may lead to increased bed compression at scale-up. A scale-independent technique to achieve packed beds of the same density, irrespective of column diameters up to 1 m was recently described [8].

An effort to enhance large-scale column packing consistency uses elasticity theory to predict limits on mobile phase velocity and pressure drop. This approach enables definition of a predicted window of scaleable column operation as demonstrated for a number of resins [9].

Establishing scaleable parameters at small scale enables manufacturing to implement the process without redeveloping it. A theoretical model for chromatography scale up uses a two-dimensional model to describe compression and pressure/flow properties during steady-state flow. This model has been used to predict large-scale column performance using a limited data set from small scale [10].

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# – Appendix A –

## Symbols and Definitions in Liquid Chromatography

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### A.1 INTRODUCTION

During the development of the different techniques of liquid chromatography for separation and characterization of small and large solutes the designations used has, from an overall view, sometimes led to inconsistencies. Unfortunately, so far, no single suggestion for nomenclature of liquid chromatography has gained total acceptance. However, the proposal by IUPAC referred to in the first edition still seems to provide a good base for a general nomenclature for liquid chromatography. An addition to this nomenclature with reference to nomenclature for non-linear chromatography was made by IUPAC [1, 2]. The proposal by IUPAC has, as far as possible, been implemented in this book and forms the basis for the symbols and definitions given in Table A1. The units given are as suggested by ISO [3]. This may need to be adjusted according to scale (i.e. while a sample volume of  $\mu\text{l}$  is used for the analytical characterization of fractions, L (litres) is a more appropriate unit for process scale feed).

### A.2 SYMBOLS USED IN LIQUID CHROMATOGRAPHY

Table A1

Symbols used in liquid chromatography

Parameter	Symbol	Equation <sup>a</sup>	Unit	References
Relative molecular mass of solute ('molecular weight')	$M_r$			
Diffusion coefficient of solute in free solution	$D$		$\text{m}^2/\text{sec}$	[1]
Diffusion coefficient of solute in mobile phase	$D_M$		$\text{m}^2/\text{sec}$	[1]

(Continued)

**Table A1** (Continued)

Parameter	Symbol	Equation <sup>a</sup>	Unit	References
Diffusion coefficient of solute in stationary phase	$D_S$		m <sup>2</sup> /sec	[1]
Axial dispersion coefficient	$D_A$		m <sup>2</sup> /sec	
Hydrodynamic volume of solute	$V_h$		ml	[4]
Effective radius of solute	$R$		nm	
Hydrodynamic (viscosity) radius of solute	$R_h, R_{vis}$	$(V_h \times 3/4\pi)^{1/3}$	nm	
Stokes radius of solute	$R_{St}$		nm	
Viscosity of solvent	$\eta$		Pa s	[4]
Intrinsic viscosity of solute	$[\eta]$			[5]
Density of solvent	$\rho$		kg/m <sup>3</sup>	
Particle diameter	$d_p$		cm, $\mu$ m	[1, 4]
Pore radius	$r_p$		nm	[1]
Effective pore radius	$r$		nm	
Break-through capacity of adsorptive bed	$Q_B$		mmol/ml g/ml	[1]
Maximum monolayer capacity of bed	$q_m$	10.28, 10.44	mmol/ml	
Ionic capacity of ion-exchange bed	$Q_V$		mmol/ml	[1]
Column inside diameter	$d_c$		m, cm	[1]
Column cross-sectional area	$A_c$	$\pi(d_c/2)^2$	m <sup>2</sup> , cm <sup>2</sup>	[4]
Column length, bed height	$L$		m, cm	[4]
Column inlet pressure	$p_i$		MPa	[1]
Column outlet pressure	$p_o$		MPa	[1]
Pressure drop over a packed bed	$\Delta p$	$p_i - p_o$ , 10.16	MPa	[1]
Void (inter-particle) volume of column	$V_0$		L, ml	[1, 5]
Volume of stationary phase in column	$V_S$		L, ml	[1]
Hold-up volume	$V_M$		L, ml	[1]
Pore (intra-particle) volume	$V_i$		L, ml	[5]
Extra-column volume	$V_{ext}$		L, ml	[1, 5]
Total liquid volume	$V_t$	$V_t = V_0 + V_i + V_{ext}$	L, ml	[1, 5]
Geometric column (bed) volume	$V_c$	$A_c \times L$	L, ml	[6]
Specific permeability	$B_0$	$d_p^2 \varepsilon^3 / (180(1 - \varepsilon)^2)$		[1]
Interstitial fraction, inter-particle porosity	$\varepsilon$	10.16		[1]
Intra-particle porosity	$\varepsilon_p$	10.48		
Tortuosity factor	$\tau$	10.48		
Mobile phase flow-rate	$F$		l/h, ml/sec	[1, 4]
Mobile phase (interstitial) velocity	$u$	$F/(\varepsilon A_c)$	m/h, cm/sec	[1, 4]

(Continued)

**Table A1** (Continued)

Parameter	Symbol	Equation <sup>a</sup>	Unit	References
Mobile phase nominal velocity	$u_n$	$F/A_c$	m/h, cm/sec	[6]
Reduced mobile phase velocity	$v$	10.12		[1]
Gradient slope	$g$			
Retention time	$t_R$		h, s	[1, 4]
Retention (elution) volume	$V_R$	10.4, 10.37	L, ml	[1, 4]
Retention factor	$k'$	10.3, 10.35		[1, 4]
Separation factor	$\alpha$	10.6		[1, 4]
Distribution coefficient	$K_D$	10.5, 10.17		
Association constant	$k_A$	10.28		
Dissociation constant	$k_D$	10.29		
Forward rate constant	$k_1$	10.50		
Backward rate constant	$k_2$	10.50		
Inlet concentration of solute	$C_0$	10.51	mol/L	
Outlet concentration of solute	$C$	10.51	mol/L	
Mobile phase concentration of solute	$C_M$		mol/L	
Stationary phase concentration of solute	$C_S$	10.32	mol/L	
Gel phase distribution coefficient, constant	$K_{av}$	$(V_R - V_0)/(V_c - V_0)$		
Exclusion limit	$V_{h,max}$		nm	[5]
Standard deviation of a Gaussian peak	$\sigma$	10.7, 10.41		[1]
Peak width at base of a Gaussian peak	$w_b$	10.39	L, ml	[1, 4]
Peak width at half peak-height of a Gaussian peak	$w_h$	10.39	L, ml	[1, 5]
Resolution factor	$R_s$	10.1, 10.24		[1, 5]
Number of theoretical plates, plate number	$N$	10.9, 10.40		[1, 5]
Plate height (HETP)	$H$	10.7	cm, $\mu$ m	[1, 5]
Reduced plate height	$h$	10.11		[1, 5]
Peak asymmetry factor	$A_s$			

<sup>a</sup>Figures refers to equations in Chapter 10.

### A.3 DEFINITIONS OF CHROMATOGRAPHIC PARAMETERS AND EQUATIONS

$A_s$ , is calculated from the partial peak widths at 10% of the peak height as  $b/a$  with  $a$  representing the peak width of the ascending part of the peak and  $b$  of the descending part of the peak. Thus, a tailing peak will have  $A_s > 1$  while a gaussian peak will have  $A_s = 1$ .

$\alpha$ , the separation factor expresses the relative retention of two consecutive peaks,  $\alpha > 1$ .



$B_0$ , the specific permeability expresses the resistance of a packed column to the flow of the mobile phase.  $B_0 = d_p^2 \times \varepsilon^3 / (180(1 - \varepsilon)^2)$  and the pressure drop over the packed column is theoretically given by  $\Delta p = u_n L \eta / B_0$ .

$d_p$  represents the particle diameter. Different estimates of the particle size distribution are frequently used to express the “average” particle diameter. Estimates include the first moment of the weight distribution of particle diameters, number-average particle diameter and harmonic mean of the distribution.

$\varepsilon$  represents the interstitial porosity of the packed bed. This is determined from the ratio of the inter-particle volume to the total geometric volume of the bed.

$\varepsilon_p$  represents the porosity of the chromatography bead or particle and is determined from the ratio of the pore volume to the total particle volume.

$g$  represents the gradient slope and is calculated from  $(X_{\text{end}} - X_{\text{start}}) / V_{\text{gradient}}$  where  $X_{\text{end}}$  and  $X_{\text{start}}$  are the mobile phase conditions at the end and the start of the gradient, respectively, and  $V_{\text{gradient}}$  is the gradient volume. For IEC and HIC  $X$  is often ionic strength and  $g$  expressed in M/ml, for RPC  $X$  may be % acetonitrile and  $g$  is %/ml. In chromatofocusing  $X$  will be pH.

$K_D$ , the distribution coefficient (distribution constant) is the concentration of a component in the stationary phase divided by the concentration of the component in the mobile phase.  $0 \leq K_D \leq \infty$  for general chromatography whereas  $0 \leq K_D \leq 1$  for gel filtration (size-exclusion chromatography). Note that the distribution coefficient as suggested by Ref. [1] is denoted as  $K_c$ . However, since this is not generally used the designation  $K_D$  has been employed throughout the book. The relationship  $K_D = (V_R - V_M) / V_S$  is also applicable to gel filtration where, by definition,  $V_M = V_0$  and  $V_S = V_i$  (see eq. (10.17)).

$K_{\text{av}}$  is the gel phase distribution coefficient calculated in gel filtration by setting the stationary phase equal to the bead volume (i.e. also including the matrix volume in the calculation, therefore  $K_{\text{av}} < K_D$ ).

$k'$ , the retention factor (sometimes called the capacity factor which however should be avoided since it may be confused with adsorptive capacity) is the amount of solute in stationary phase divided by the amount of solute in mobile phase. The designation  $k$  is suggested by Ref. [1]. However  $k'$  is used in this book to avoid confusion with  $k$  as a symbol for rate constants.

$M_r$  is the relative molecular mass of the solute and is dimensionless. The designation  $M_w$  is used for weight-average molecular mass of a polymer sample and should therefore be avoided for the traditional use as molecular weight of, e.g. proteins. The designation MW for molecular mass might be regarded as short for molecular weight. We suggest the use of g/mol instead of Daltons as the unit for molecular mass or the use of the dimensionless designation,  $M_r$ .

$N$  is the plate number of the column. It is calculated from an isocratic run by  $N = 5.545 \cdot (V_R / w_h)^2$  assuming a gaussian peak.  $V_R$  and  $w_h$  must have the same unit, for example ml. One may substitute  $V_R$  in the formula with  $t_R$ , but then the unit of  $w_h$  must be time.  $N/L$  is used for calculating the number of theoretical plates per unit column length, often normalized to a column length of 100 cm (i.e. yielding number of plates per meter).

$\Delta p$ , the pressure drop over the packed bed is frequently given in bar, psi (pounds per square inch) or Pa (Pascal). The number may be converted by: 100 kPa (100,000 Pa) = 1 bar = 14.5 psi.

$Q_V$ , the theoretical capacity in mmol of ionogenic group per volume of swollen ion exchanger (H-form of cation exchanger and Cl-form of anion exchanger).

$Q_B$ , is the break-through capacity, the dynamic binding capacity, of a bed at a given level of break-through, obtained experimentally by passing a solution of a particular solute through the column. The amount which has been taken up by the bed when the species is first detected in the effluent or when its effluent reaches some arbitrarily defined fraction of the inlet concentration, i.e.  $x$ , is the break-through capacity of the bed,  $Q_{B,x}$ . It may be expressed in millimoles or milligrams per gram dry resin or per ml bed volume.

$R_s$ , the resolution factor is determined according to  $R_s = 2(V_{R2} - V_{R1})/(w_{b1} + w_{b2})$  provided that  $V_{R2} > V_{R1}$ . For peaks of gaussian shape;  $w_b = 4\sigma$  and  $w_h = \sigma\sqrt{8\ln 2}$  and the resolution factor may thus be calculated from the width at half peak-height according to  $R_s = \sqrt{2\ln 2} \cdot (V_{R2} - V_{R1})/(w_{h1} + w_{h2})$ . Normally, peaks in IEC or RPC show tailing and the relationship is not valid. On the other hand, the equation may still be used to estimate the apparent resolution between overlapping bands for which the base width may not be calculated. However, it should be noted that the number obtained is not  $R_s$  (unless the peaks are gaussian and of roughly the same size) and in order to stress this it is recommended that the calculation procedure used is clearly stated in the text and that another symbol, e.g.  $R_{s(h)}$ , is used to denote that the calculation is made from half peak-height.

$\tau$ , the tortuosity factor is a geometric factor to correct for the actual, tortuous, length of a pore as compared to the straight, non-tortuous, length.

$t_R$ , retention time is the time between the start of the elution and the emergence of the peak maximum. For large sample volumes the correct start point is set at half injected volume (see  $V_R$ ).

$u$  is the linear velocity of eluent in the packed bed or in a pipe. This is expressed in distance per unit time (e.g. cm/min) in contrast to the flow-rate which is measured by the amount of liquid delivered per unit time (e.g. ml/min). Please note that linear flow-rate is a misnomer and should not be used. The velocity may be calculated from flow rate by  $u = F/(\varepsilon A_c)$ . For a pipe  $\varepsilon = 1$ . The calculated linear velocity may be lower than the actual local velocity due to the tortuous flow paths of a packed bed.

$u_n$ , the nominal linear velocity is the linear velocity in a part of the column not containing any packing. Thus this is an artificial measure of velocity. It is used for comparisons between columns of different diameters when the inter-particle porosity is not known,  $u_n = F/A_c$ .

$V_{ext}$  is the external volume and incorporates the volume contributions of all system components external to the column.

$V_i$  is the volume of the liquid (mobile phase) which is stationary in the pores of the gel or solid packing, i.e. the pore volume. In material sciences the designation  $V_p$  is used for pore volume. The ratio  $V_i/V_0$  is expressing the maximum separating volume over the non-separating volume in gel filtration and is referred to as permeability (not to be confused with the specific permeability,  $B_0$ ).

$V_h$ , the hydrodynamic volume of solute is a property that is proportional to  $[\eta]M_r$ .

$V_{h,max}$ , is the hydrodynamic volume of the largest solute that is able to permeate into a porous chromatography resin, i.e. defining the exclusion limit of the resin.

$V_M$  is the mobile phase volume of the system that is sensed by the solute under non-retentive conditions. It includes any volumes contributed by the sample injector, the

detector and connectors [1]. For small solutes this is equal to the total liquid volume,  $V_t$ , but for large molecules, e.g. being excluded from the matrix, this is equal to the void volume,  $V_0$ .

$V_0$ , the void volume is the volume of the mobile phase in the interstices between the gel beads or the solid particles contained in the column. This is calculated from the peak apex of a totally excluded substance (chosen to avoid secondary exclusion effects). The true void volume is obtained by subtracting the external volume.

$V_R$ , the retention volume is the time between the start of the elution and the emergence of the peak maximum. In case the sample volume is constant it may simply be regarded as part of the external volume. In case the sample volume is varied the correct start point is set at half injected volume. In SEC it is more frequent to talk about elution volume,  $V_e$ .

$V_S$  is the volume of stationary phase in column, i.e. the volume of the stationary liquid phase. For gel filtration  $V_S$  is equal to the pore volume,  $V_i$ .  $V_S/V_M$  is called the phase ratio and is sometimes denoted  $\phi$ .

$V_t$  is the total volume of the liquid phase in the system. In RPC and IEC this is often denoted set equal to  $V_M$  under the assumption of that the solutes are very small (see  $V_M$ ).

$w_b$  is the peak-width at base, i.e. the segment of the baseline intercepted by the tangents drawn through the inflection points of the chromatogram.

$w_h$  is the peak-width at half height, i.e. the length of the line, drawn parallel to the baseline, that is intercepted by the peak at 50% of the peak height. Please note that the use of  $w_{1/2}$  for this estimate is discouraged (see Ref. [1]).

$\eta$  is the viscosity of the mobile phase or the sample at ambient temperature.

$[\eta]$  is the intrinsic viscosity, equivalent to the reduced specific viscosity at infinite dilution.

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# – Appendix B –

## Dimensionless Numbers

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### B.1 INTRODUCTION

In order to arrive at results that are generally valid, i.e. not limited to a specific measuring situation, dimensionless numbers are frequently used in chemical engineering. Some dimensionless numbers that are used for liquid chromatography are explained in this section.

*Bi*, Biot number expresses the ratio of time for film mass transfer to the time it takes for a molecule to diffuse through the chromatographic particle (*cf.* eq. (10.46)). It is given by

$$Bi = \frac{K_f r}{D_s}$$

where  $K_f$  is the film mass-transfer parameter,  $r$  the particle radius and  $D_s$  the effective intra-particle diffusion. Thus if  $Bi \ll 1$  then it may be expected that film mass transfer will be rate limiting as compared to pore diffusion and vice versa.

*Pe*, Peclet number is used in some different context. One is to estimate the overall extent of axial dispersion for a tanks-in-series model. The Peclet number is in this case calculated by

$$Pe = \frac{uL}{D_A}$$

where  $u$  is the velocity of the mobile phase,  $L$  the length of the vessel and  $D_A$  the axial dispersion coefficient (*cf.* eq. (10.43)). Large values of  $Pe$  indicate small dispersion (e.g. plug flow in a vessel) and small values are caused by large dispersion (e.g. mixed flow in a vessel). The assumption of that back mixing follows a Fick's-law-type of equation may be an oversimplification [1]. The Bodenstein number, *Bo*, is identical to the Peclet number for a closed reactor.

The local axial dispersion of packed beds has also been characterized by the Peclet number. In this case the Peclet number is defined by

$$Pe = \frac{ud_p}{D_A}$$

where the characteristic length now is the particle diameter,  $d_p$ . This definition of Peclet number yields the relative mass transport of solutes in the mobile phase caused by convection, i.e. the velocity of the mobile phase, as compared to diffusion. It may be noticed that the Peclet number is very similar to the reduced velocity (see eq. (10.12)), i.e. assuming that the dispersion is only caused by the solute diffusivity in the mobile phase (i.e.  $D_A = D_M$ ) yields  $v = Pe$ .

Peclet number has also been used to express the ratio of time for mass transport of solutes through porous media by molecular diffusion as compared to convective flow. The characteristic length is the length of the pore, given by  $d_p\tau$ , where  $\tau$  is the tortuosity, the dispersion is set equal to the pore diffusivity,  $D_s$ , taking place in three dimensions and the intra-particle flow is  $u_i$  (see eq. (10.49))

$$Pe_i = \frac{u_i d_p \tau}{12 D_s}$$

For values of  $Pe_i \gg 1$  mass transport by convection is dominating over diffusion. However, calculation of  $Pe_i$  is not trivial since accurate data for intra-particle flow, and restricted diffusion generally are not easily obtainable.

$Re$ , the Reynolds number expresses the ratio of inertial force and viscous force. At  $Re \ll 100$  the viscous force dominates and the flow through a packed bed is laminar or viscous. At high Reynolds number the flow is turbulent which disrupts the laminar layers and results in a plug flow profile [2]. Radial transport of molecules is dominated by molecular diffusion at low  $Re$  and by turbulent mixing at large  $Re$ . The Reynolds number is given by

$$Re = \frac{\rho u d_p}{\eta}$$

where  $\rho$  is the density and  $\eta$  the viscosity of the mobile phase.

$Sc$ , the Schmidt number is given by [2]

$$Sc = \frac{\eta}{\rho D_M}$$

The Schmidt number is equal to  $Pe/Re$  and thus express the ratio of convective to diffusive mass transport related to the ratio of the inertial to the viscous force of the liquid.

$Sh$ , the Sherwood number is similar to the Biot number but expresses the mass transport in the mobile phase due to active mass transport (e.g. caused by flow) relative to molecular diffusion. It is defined by

$$Sh = \frac{K_f d_p}{D_M}$$

The film mass transfer parameter,  $K_f$ , can be calculated from the Sherwood number by the relationship [3]

$$Sh = 2 + 1.45\sqrt{\varepsilon Re Sc}^{1/3}$$

where  $\varepsilon$  is the bed voidage.

$\Theta$ , the Thiele modulus of reaction expresses the reaction rate as compared to the intra-particle diffusivity and is calculated from [4]

$$\Theta^2 = \frac{d_p^2 k q_m}{4C_s}$$

where  $k$  is the forward reaction rate constant and  $q_m$  the solute capacity of the adsorbent.

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# – Appendix C –

## Activities for Biopharmaceutical Production from Genetically Engineered Mammalian Cells

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### C.1 INTRODUCTION

One of the more confusing issues is what to do and when to do it. In this chart, we provide an outline of the critical activities and considerations from toxicology studies to license application.

### C.2 ACTIVITIES CHART FROM TOXICOLOGY TO LICENSE APPLICATION

Activity	For Tox studies	For IND and during Phase 1 and 2	For Phase 3 and validation during P3	After validation/ pre-BLA
Assays			All validated <sup>a</sup>	All validated <sup>a</sup>
Release	Meaningful	Qualified/validated		
Safety	Qualified/validated	Validated		
Stability	Meaningful	Qualified/validated		
Bioburden	Controlled	Control at CGMP level	Control at CGMP level; establish reasonable limits	Control at CGMP level; employ action limits or stays within predeter- mined specs
Calibrate	Instruments	Instruments	Instruments	Maintain calibration schedule

(Continued)



Activity	For Tox studies	For IND and during Phase 1 and 2	For Phase 3 and validation during P3	After validation/ pre-BLA
Characterization	Cell banks: minimal testing may be sufficient: sterility, mycoplasma, <i>in vitro</i> or if appropriate MAP/HAP; product and impurities: assays are meaningful	Raw materials; fairly good understanding of product and impurities profiles	Characterization/ qualification of process (robustness studies); increased understanding of product and impurities; review cell bank characterization	Characterization may continue along with efforts to develop process improvements for future implementation. Control charts may dictate where further characterization is necessary
Clean	Everything <sup>b</sup>	Everything <sup>b</sup>	Everything, validate assays for cleaning validation, validate cleaning	Maintain validated cleaning; routine monitoring; revalidation dictated by changes or increased knowledge; complete review (at least yearly)
Comparability		To Tox material	To Tox and Phase 1 and 2	To clinical material, especially Phase 3
Compliance <sup>c</sup>	GLP	GMP (graded)	GMP	GMP
Contaminants <sup>d</sup>	Employ unit operations predicted to minimize contaminants	Better control. Test harvests for adventitious agents with at least <i>in vitro</i> test, mycoplasma and bioburden	Better control. Test according to ICH Q5A once process is finalized	Maintain control
Define	Product, process, raw materials	Product, process, raw materials	Product, process, raw materials	Stay within validated acceptance criteria for defined product, process, and raw materials
Document	Everything	Use SOPs; batch records; written QC responsibilities	Validation protocols and validation master plan approved by QA	Everything: formalized and QA approved; maintained
Equipment	Calibrate, qualify (must be reliable)	Calibrate; qualification exercise similar to abbreviated IQ/OQ/PQ for critical equipment (note: Phase 1 equipment not likely to be used in subsequent stages). Define wetted materials	Re-calibrate and re-IQ/OQ/PQ when changes made	Fully qualified, maintained; routine calibration according to established SOPs
Extractables	Avoid (potential to cause tox failures)	Evaluate potential prior to entering clinical studies <sup>e</sup> ; define limits for those that are potentially harmful	Use wetted materials within defined limits; perform clearance studies if relevant	Maintain vendor audits and awareness of vendor changes that could impact extractables

(Continued)

Activity	For Tox studies	For IND and during Phase 1 and 2	For Phase 3 and validation during P3	After validation/ pre-BLA
Formulation	Define preliminary	Put on stability for at least length of clinical study. Continue formulation development	Refine w/dose requirements and further understanding of stability  Start final conformance lot stability (end of Phase 3)	Maintain
Impurities	Major: defined	Much better definition	Increased understanding; application of validated assays; detect and monitor most at 0.1% of product; all over 1%; perform risk assessment to determine necessary level of detection <sup>f</sup>	Defined and specified limits
Process	Sufficient for capture of API, employ unit operations predicted to minimize contaminants	Defined but improving; need not be final process scheme, deviations investigated for root cause; change control	Still improving until finalized after characterization/robustness studies; major changes after start of Phase 3 are a bad idea. Use, change control; deviations investigated	Defined and maintained. Change control. Deviations investigated
Product	Defined	Increase understanding	Further increased understanding	Defined and meeting specifications
Production quantity	Sufficient for tox and formulation studies; retention samples for comparability	Sufficient for early clinical studies and further stability and process-improvement studies; comparability demonstration; retention samples	Sufficient for pivotal clinical studies, robustness studies, stability, process-improvement studies; comparability demonstration; retention samples	Sufficient for marketing; retention samples
Qualify		Equipment abbreviated IQ/OQ/PQ (scale too small for later development); raw materials; cleaning	Characterization and compendial assays	
Raw materials	Defined	Define quality attributes and specifications; identity tests; start stability evaluation for some raw materials	Meet all specifications; start validation of assays; audit vendors of critical raw materials	Audit vendors; re-audit periodically

(Continued)

Activity	For Tox studies	For IND and during Phase 1 and 2	For Phase 3 and validation during P3	After validation/ pre-BLA
Specifications	Preliminary working specs	Working specs	Established specs after characterization/ robustness studies	Defined and specifications met
Stability studies	Start preliminary stability studies	Study changed material/new formulations on stability; ensure stability for entire clinical study (concurrent with clinical program)	Start forced degradation of finalized formulation if warranted; ensure stability during clinical trials	Maintain conditions that ensure stability
Training		Critical for manufacture of clinical materials, esp. GMP training	Ongoing	Ongoing
Validate	Bioburden assays; endotoxin assay	Toxic impurities removal, specific impurity removal (e.g. Protein A for MAb production). Start viral clearance (usually 2 virus prior to Phase 1)	Assays as needed due to changes (e.g. sterility, <i>in vitro</i> adventitious agents); viral clearance if potential to be impacted by changes; finalized process validation; once finalized process exists, perform viral clearance studies for license application, where relevant	Maintain validated state

<sup>a</sup>Except characterization assays, which should be meaningful and qualified.  
<sup>b</sup>Consider disposables for tox and early clinical studies to minimize cleaning validation.  
<sup>c</sup>Risk assessment will dictate level of compliance required for clinical studies.  
<sup>d</sup>Contaminants defined as those agents not expected to be part of process (e.g. adventitious virus).  
<sup>e</sup>Vendor data may be sufficient.  
<sup>f</sup>These numbers are only estimates.

# – Appendix D –

## Simulations Using the Supplied Software

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### D.1 INTRODUCTION

Many of the fundamental equations describing theoretical relationships of chromatography were outlined in Chapter 10. Insight into how different parameters may affect the end result can be achieved through simulations and therefore a number of selected applications are supplied on a CD, which is delivered together with this book. The CD is intended to be used as a tutorial aid to facilitate the process of disclosing the relationships governing chromatographic separations. Needless to say, the results provided by the equations on the CD are not more accurate or reliable than the basic equations allow and must therefore not be considered as true data. As with other types of software, results need to be confirmed by experiments.

Applications supplied on CD

File name	Equation	Description
Pressure drop	(10.16)	Pressure drop over a packed bed
Sample volume	(10.19)	Influence of sample volume on zone broadening
Resolution in SEC	(10.20)	Resolution in size exclusion
Resolution in IEC RPC HIC	(10.1)	Resolution in adsorption chromatography
Isocratic separation	(10.51)	Simple simulation of a linear isocratic separation
Yield and purity		Plots the yield and purity as function of resolution
Langmuir isotherm	(10.32)	Adsorption isotherm, Langmuir type

### D.2 HOW TO USE THE SOFTWARE?

In order to run the applications you must have a PC-based version of Microsoft® Excel.

- (1) Start Microsoft Excel.
- (2) Insert disk.

- (3) For running the applications; open the specific file (file name as given in the table above).

Each application is divided into *Information* and *Calculation*.

*Information* gives basic information of what the application contains, how to run the application, the equations used for the calculation and copyrights.

*Calculation* is where new data are inserted, the calculation performed and a printed report obtained.

Start by entering the values for the variables you want to study in Calculation. The maximum and minimum value for the calculation as well as the fixed value used for calculations of other variables is entered for each variable. Note that this fixed value can be chosen to be outside of the range selected for maximum and minimum value. Only data in the green fields in the box is to be changed (to prevent loss of information the document is protected).

Results are immediately given by the graphs illustrating the results as a function of the different input values. A one-side printout of the results is obtained by pressing the print symbol. The size of the printing area may need to be adjusted by changing the scale of the print format to fit paper size used.

Information regarding the CD and how to use it are also found in the file README.TXT on the CD.

### D.3 PRESSURE DROP

This application calculates the flow resistance of a packed bed and prints a report of the results.

#### D.3.1 Calculations

The pressure drop is calculated from eq. (10.16)

$$\Delta p = u_n \frac{L}{d_p^2} \eta \frac{180(1 - \varepsilon)^2}{\varepsilon^3}$$

where  $u_n$  nominal is the velocity of the mobile phase,  $\varepsilon$  the void fraction,  $V_0/V_c$ ,  $L$  the length of the packed bed,  $d_p$  the particle size (spherical particle shape is assumed) and  $\eta$  the solvent viscosity (0.001 Nsec/m<sup>2</sup> for H<sub>2</sub>O at 20 °C).

A graph illustrating the flow resistance parameter,  $180(1 - \varepsilon)^2/\varepsilon^3$ , as a function of  $\varepsilon$  is also given. This is useful for comparing permeability of packed beds of different packing density. The large change of flow resistance for a small change in void fraction at low void fractions may be noted!

The pressure drop over system components is not included in the equation above. System effects may be substantial at high velocities and may be checked by running the eluent through an empty column.

## D.4 SAMPLE VOLUME

This application calculates the influence of sample volume on the peak width, plate number and indirectly the resolution, in isocratic elution and prints a report of the results. Results are immediately given by the graphs illustrating the influence of sample volume as a function of the different input values.

### D.4.1 Calculations

The peak width is calculated from eqs. (10.19), (10.10), and (10.8) from the total plate height,  $H$

$$H = H_{\text{injector}} + H_{\text{column}}$$

$$= V_{\text{sample}}^2 \frac{L}{K_{\text{injector}} V_R^2} + 2\lambda d_p + 2 \frac{(0.6D_M + D_S(V_R/V_0 - 1))}{u} + \frac{V_0}{V_R} \left(1 - \frac{V_0}{V_R}\right) d_p^2 \frac{u}{30D_S}$$

where  $V_{\text{sample}}$  is the applied sample volume,  $L$  the column length,  $K_{\text{injector}}$  an injector-dependent constant,  $\lambda$  a geometric packing-dependent factor,  $d_p$  the particle diameter,  $D_M$  the diffusivity of the solute,  $D_S$  the restricted pore diffusion coefficient,  $V_R$  the retention volume,  $V_0$  the inter-particle void volume and  $u$  the mobile phase velocity.

The input parameters that are needed for the calculation are the mobile phase volume  $V_M$  (here  $V_M = V_t$ ), either the retention volume  $V_R$  or the retention factor  $k'$  (if  $k'$  is not given it is calculated from  $V_R$ ,  $V_M$  and  $V_i/V_0$ ), the diffusion coefficient  $D_M$ , or the relative molecular mass  $M_r$ , if the solute is a globular protein (i.e. if  $D_M$  is not given it is calculated from the molecular mass by the approximate relationship  $D_M = 260M_r^{-1/3}$ , which is applicable to globular proteins), and the injector constant (this is close to 5 for laboratory injectors, for large sample volumes the constant has a higher value, e.g. close to 12). The variable parameters are the sample volume, the particle size, the velocity of the mobile phase, the phase ratio of pore volume over interstitial volume,  $V_i/V_0$  and the column length. The fixed value of the sample volume is given as % of the bed volume.

The plate height is converted to peak width with the aid of eq. (10.8).

The results elucidate the influence of sample volume on the total peak width as compared to the contribution from only the column (i.e. at infinitely low sample volume). Another useful plot is the effect on plate number. Here this is given as  $N/N_{\text{max}}$  which shows the plate number measured at a certain sample volume as compared to the plate number at an infinitely low sample volume. The degree of 'loss' in plate number depends upon the particle size, pore volume of the chromatography medium, the column length and the velocity of the mobile phase. The influence on column length is calculated assuming a constant bed volume, i.e. the column plate number increases with column length and thus the relative influence from a certain sample volume also increases. The effect from velocity will follow the van Deemter plot and will thus be affected by, e.g. the diffusion coefficient of the solute (see eq. (10.10)).

The effect on plate number may be converted to resolution between the peaks with aid of eq. (10.1) stating that the resolution factor is proportional to the square root of the plate number (e.g. a reduction in  $N/N_{\max}$  from 1.0 to 0.9 gives a reduction in resolution factor by 5%).

## D.5 RESOLUTION IN SEC

This application calculates the influence of different variables on the resolution in gel filtration (size exclusion chromatography) and prints a report of the results.

Data for exploring the influence of parameters may be taken from the sheet named *Typical data*. This sheet provides a calculation of the distribution coefficient from retention volume according to  $K_D = (V_R - V_0)/(V_i - V_0)$ , see eq. (10.17). The radius of globular proteins may be calculated from the molecular mass and the slope of the selectivity curve can be estimated from two solutes.

### D.5.1 Calculations

The resolution is calculated from eq. (10.20)

$$\text{Resolution} = \left( \frac{1}{4} \right) \log \left( \frac{R_2}{R_1} \right) \left( \frac{b}{(V_0/V_i + K_D)} \right) \left( \frac{\sqrt{L}}{\sqrt{H}} \right)$$

where  $R$  is the radius of the molecule,  $b$  the slope of the selectivity curve ( $dK_D/d \log R$ ),  $V_i$  the intra-particle pore volume and  $V_0$  the inter-particle void volume,  $K_D$  is the average distribution coefficient (here this is set equal to the value of 'Distribution coefficient'),  $L$  the bed height and  $H$  the average plate height. The plate height is calculated for 'Solute2' from the van Deemter equation (eq. (10.10)) assuming that the relationship between size and diffusivity for proteins is applicable.

## D.6 RESOLUTION IN IEC, RPC AND HIC

This application calculates the resolution between two solutes in isocratic elution chromatography and prints a report of the results. Results are immediately given by the graphs illustrating the resolution as a function of the different variables.

### D.6.1 Calculations

The resolution factor is calculated from eq. (10.1)

$$R_s = \frac{k'_2 - k'_1}{2(2 + k'_2 + k'_1)} N^{1/2}$$

where  $k'$  is the retention factor, and  $N$  the plate number of the column. The retention factor for solute 1 is calculated from the selectivity factor,  $\alpha$ , with aid of the relationship  $\alpha = k'_2/k'_1$ .

## D.7 ISOCRATIC SEPARATION

This application simulates a separation of a mixture of up to five components in linear isocratic mode.

### D.7.1 Entry of parameters

Start by entering the values for the components in 'Fixed parameters for the solutes'. Entry fields for data are green while result fields are bright yellow. You may enter the retention volume,  $V_R$ , or the  $k'$  of the solutes ( $k'$  is used in the calculation to allow for variations in bed volume and is therefore calculated from the retention volume and mobile phase volume,  $V_M$  or  $V_i$ , if not explicitly given. Thus  $V_M$  must be stated together with  $V_R$ . An approximation of  $V_M$  is given from the column data in the second entry table). You may enter the diffusion coefficient,  $D_M$ , or the relative molecular mass for globular proteins,  $M_r$  (thus, if you have other solutes than globular proteins you must enter the diffusion coefficient and not use the inbuilt conversion between molecular mass and diffusivity).

The second entry table contains the running parameters that may be interesting to study. The particle size, the superficial velocity and column length may be altered to see effects on the column zone broadening. The sample volume can be changed to study contribution from extra-column effects (the sample volume relative to the bed volume is calculated by the program). The bed volume need to be adjusted if the column length is changed and the column diameter is to remain constant. The pore fraction  $V_i/V_0$  illustrates effects from chromatographic media of different intra-particle porosity. Finally, lambda may be used to simulate the influence from how good the column is packed (lambda = 0.6 corresponds to an excellently packed column,  $\lambda = 1.6$  may be obtained for an acceptable column while  $\lambda = 4.6$  indicates that the column is not acceptable).

In order to get a plot of highest-possible resolution the range for the calculation need to be entered in the third entry table.

Results are immediately given by the graph illustrating the simulated chromatogram. The resolution between successive solutes is also given. A printout of the results are obtained by pressing the print symbol.

### D.7.2 Calculations

The zone broadening is calculated from eq. (10.10) and expressed by the plate height,  $H$

$$H = 2\lambda d_p + \frac{2(0.6D_M + D_S(V_R/V_0 - 1))}{u} + \frac{V_0}{V_R} \left(1 - \frac{V_0}{V_R}\right) d_p^2 \frac{u}{30D_S}$$



where  $\lambda$ , lambda, is a geometric packing-dependent factor,  $d_p$  the particle size,  $D_M$  the diffusivity of the solute,  $D_s$  the restricted pore diffusion coefficient,  $V_R$  the retention volume,  $V_0$  the inter-particle void volume and  $u$  the mobile phase velocity.

The elution curve is then simply calculated with aid of eq. (10.51).

For globular proteins the diffusion coefficient is calculated from the relative molecular mass,  $M_r$ , by the approximate relationship  $D_M = 260M_r^{-1/3}$ .

## D.8 YIELD AND PURITY

This application calculates the influence of the resolution factor on the product yield as a function of required purity of the product at different feed purity and prints a report of the results. Results are immediately given by the graph illustrating the relationship between the yield at various requirements for purity. Yield for optional levels of purity is interpolated from the data and given in a table.

### D.8.1 Calculations

In this application it is assumed that the elution peaks have Gaussian shapes and that the peak widths are the same for the two peaks (i.e. since they are eluting quite close to each other). For sake of simplicity it is assumed that the impurity is eluted in the second peak.

The elution peaks are simulated from

$$C = C_0 \exp \left( -\frac{1}{2} \left[ \frac{(V_R - V)4}{w_b} \right]^2 \right)$$

where  $V_R$  is the retention volume of the target,  $V$  the volume of effluent and  $w_b$  the peak width.

The yield and purity of the first peak as a function of volume of effluent is calculated from the simulated peaks. It can be noticed that if the concentration ratio of the two peaks is 1/10 the minimum purity that is achieved is 91%, i.e. 10/11 (equal to the feed).

To judge the relevancy of chosen parameters the plate number for the major peak and the retention volume for the impurity is calculated.

The resolution factor for different experimental conditions may be calculated from the applications Resolution in IEC, HIC and RPC or Resolution in SEC (see above).

## D.9 LANGMUIR ISOTHERM

This application calculates the concentration of solute adsorbed as a monolayer as a function of experimental parameters and prints a report of the results.

### D.9.1 Calculations

The calculation is based on the Langmuirian adsorption isotherm given by eq. (10.32)

$$C_S = C_M q_m \frac{k_A}{C_M k_A + 1}$$

where  $C_S$  is the concentration of adsorbed solute,  $C_M$  the concentration of solute in the mobile phase,  $q_m$  the maximum capacity of the chromatographic resin for the solute and  $k_A$  the association constant for the solute.

The results consists of a hypothetical Langmuiran adsorption isotherm, a graph of  $C_S$  as a function of the association constant (to illustrate the influence of  $k_A$  on the sorption efficiency at different  $C_M$ ) and a plot of  $C_S$  as a function of bed capacity (to show the excess capacity needed to achieve a target adsorptive capacity as a function of  $k_A$  and  $C_M$ ) in addition to two graphs illustrating the variation of the distribution function.

## D.10 ABOUT THIS SOFTWARE

This is a non-commercial software supplied free of charge with the book entitled 'Handbook of Process Chromatography, Development, Manufacturing, Validation and Economics, 2nd Edition, L. Hagel, G. Jagschies, and G. Sofer, Elsevier 2007'. The software is a tutorial tool and results are interpreted and used at user's own risk. Suggestions for improvements are welcome and can be sent to the author.

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