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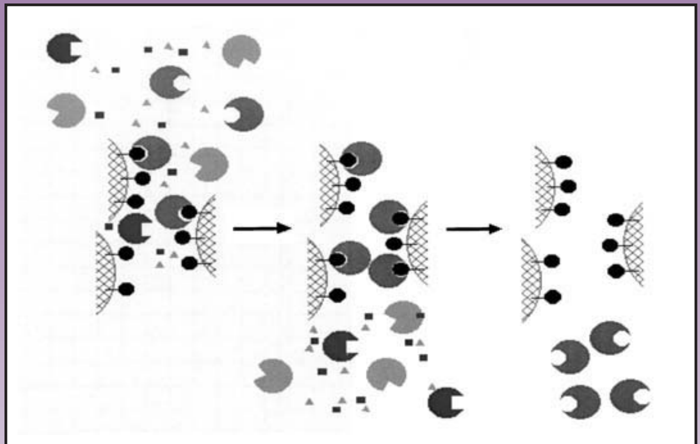
Downstream Processing of Proteins

Methods and Protocols

Edited by

Mohamed A. Desai



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Downstream Processing of Proteins

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Edited by

Mohamed A. Desai

Medeva Pharma Ltd., Liverpool, UK

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Preface

Considerable effort and time is allocated to introducing cell culture and fermentation technology to undergraduate students in academia, generally through a range of courses in industrial biotechnology and related disciplines. Similarly, a large number of textbooks are available to describe the applications of these technologies in industry. However, there has been a general lack of appreciation of the significant developments in downstream processing and isolation technology, the need for which is largely driven by the stringent regulatory requirements for purity and quality of injectable biopharmaceuticals. This is particularly reflected by the general absence of coverage of this subject in many biotechnology and related courses in educational institutions.

For a considerable while I have felt that there is increasing need for an introductory text to various aspects of downstream processing, particularly with respect to the needs of the biopharmaceutical and biotechnology industry. Although there are numerous texts that cover various aspects of protein purification techniques in isolation, there is a need for a work that covers the broad range of isolation technology in an industrial setting. It is anticipated that *Downstream Processing of Proteins: Methods and Protocols* will play a small part in filling this gap and thus prove a useful contribution to the field. It is also designed to encourage educational strategists to broaden the coverage of these topics in industrial biotechnology courses by including accounts of this important and rapidly developing element of the industrial process. The hope is that this will result in graduates having a reasonable understanding of downstream processing principles and techniques, and thus be better prepared to fulfill the ever-increasing demand for competent isolation scientists in industries.

This is, of course, achieved with the help of the dedicated contributing authors of *Downstream Processing of Proteins: Methods and Protocols*, without whose willingness to contribute and patience it would not have been possible. I would also like to thank the Humana Press and Prof. John Walker (the series editor) for their encouragement and prompt feedback. My thanks are also due to the Medeva Pharma Development management for providing me with the time and opportunity to fulfill this task, and without whose support it would have been impossible. Finally, I wish to thank my wife, children, and family members for allowing me to persevere with my editing activities in perhaps what should have been their time.

Mohamed A. Desai, PhD

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1

Downstream Processing in the Biotechnology Industry

An Overview

Manohar Kalyanpur

1. Introduction

Crude medicinal preparations that were aqueous or alcoholic extracts of plant materials were known for centuries to practitioners of the indigenous methods of medicine. The pain-killing salicylates and antimalarial compounds extracted from the bark of certain trees are notable examples of older medicines. Similarly, animal organs such as the pancreas, placenta, and the urine of pregnant females have been a source of hormones for therapeutic use.

Until recently, human albumin was manufactured from pools of human placenta collected from Third World countries. But the high risk of virus contamination from unidentified donors of placenta and the impracticality of identifying the donors, forced the discontinuation of this process. Today, plasma from unpaid donors is the major source of albumin and the risk of transmission of viruses calls for extensive purification including the use of dedicated virus removal and inactivation steps to render the product safe for human use.

Although crude preparations from plant or animal sources are still used as medicinals in some parts of the world, modern medicines in most countries are extremely pure. The high level of drug safety and purity are demanded by the regulatory authorities in such countries as the United States, Europe, and Japan. Fortunately, the biopharmaceutical industries are able to meet the stringent demands because they have access to a variety of excellent purification techniques.

The science of biotechnology covers the exploitation of microorganisms and cell cultures, which form the major source of high value compounds. More

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recently, geneticists have succeeded in breeding transgenic sheep and goats, and methods have been developed to get these animals to express the desired products in their milk. The industry today manufactures on a large scale compounds that would otherwise have been difficult, if not impossible, to produce in significant quantities for treating many diseases. Whether produced from plants, animal tissue, microorganisms, or from cell culture, the desired products are present in rather complex process streams and need extensive purification. A great majority of these products are proteins, which makes this task even more difficult. If these were nonprotein molecules, such as antibiotics for example, one could use simpler solvent extraction methods to isolate the compounds from the solutions in which they are present.

Thus, in the biotechnology industry, there is quite a challenge to the biochemists and chemical engineers in the downstream processing departments of the companies. They employ diverse purification methods in the research laboratory at the bench scale and these are eventually scaled up to the production floor. The methods are used in complementary fashion to develop cost-effective methods in quick time and enable the companies to bring the products to market ahead of their competitors.

This chapter attempts to give the reader an overview of the techniques available for downstream purification of biotechnology products. Readers are advised to refer to specific chapters in later sections of this volume where these techniques are described in detail.

2. Manufacturing Processes in the Industry

As stated before, the industry manufactures products from a number of sources and their downstream processing varies not only from product to product, but also varies depending on the source of the product. Each process, therefore, needs to be finely tailored depending on the properties of the product and the process stream from which it is recovered and purified.

2.1. Products of Recombinant Bacterial Fermentation

The first step in these processes is the separation of the biomass from its surrounding broth. The protein of interest is expressed within the cell as a soluble protein, but it is quite often present in the form of an insoluble refractive mass called the "inclusion bodies." The recovery of the biomass is sometimes performed by preparative centrifugation, but the preferred method today is by means of tangential-flow filtration systems using microporous membranes of appropriate pore diameters. The different filter manufacturers like Millipore Corporation (Bedford, MA), Pall Corp. (Port Washington, NY) and other companies offer membranes with 0.22, 0.45, and 0.65 µm pore sizes, and the scientists developing the process select the membranes best suited to their needs of

biomass concentration. The particulates from the process fluids can get into the membrane pores and cause a significant drop in filtration rates. The phenomenon can be controlled by fine tuning the process conditions to obtain the optimum feed and permeate flow rates and transmembrane pressures.

The composition of the fermentation broths can have significant effects on the filtration rates. One component that has such an impact is the antifoam used to control foaming during fermentation. These hydrophobic chemicals are quickly absorbed to the surface of the membrane and cause a drop in flux. Under certain process conditions such as temperature for example, some antifoams come out of solution to form insoluble micelles, which can easily adsorb to the membrane surface. Therefore, an appropriate antifoam is selected for the fermentation process bearing in mind the downstream processing steps.

During cell harvesting, simultaneous cell washing (also referred to as diafiltration) can be performed by adding a suitable solution to the cell concentrate, which also helps to maintain the desired pH or ionic strength of the cell suspension to avoid cell lysis.

Diafiltration also helps to wash away the soluble impurities from the process stream. This step is usually started when the cell concentration reaches a specific point where rapid flux decay is observed.

2.2. Cell Lysis and Clarification of the Lysate

The recovered bacterial cell mass is next lysed by mechanical cell disruption under high pressure. This step releases the desired product from inside the cells for further processing. The lysate, which consists of both soluble and insoluble components, notably the cell debris, is then clarified by a tangential flow filtration step with an appropriate membrane device. Here, once again, the choice of the right microporous membrane is critical. The smaller pore diameters, such as the 0.22 μm, perform better. The larger pores can get plugged by the cell debris or other particulate contaminants. However, ultrafiltration membranes with even smaller pore diameters most often perform better than the microporous membranes because the debris cannot get lodged in the pores. One can, therefore, avoid flow decay. However, the fluxes through the ultrafiltration membranes are, in general, lower than those with the microporous membranes. If the desired protein is in the soluble fraction of the lysate, it passes the membrane in this step to end up in the permeate and it is then sent to the next purification step.

If the product is present as inclusion bodies, it is present in the retentate of the above step and has to be first solubilized by the addition of an agent such as guanidine or urea. The solubilized protein is then separated from the particulates by ultrafiltration. The selected membrane should permit the passage of the solubilized protein while retaining the debris and particulates in the retentate.

Aggregrates of proteins and colloidal material are also retained, and care must be taken to make sure that the desired protein is recovered in good yields in the permeate. Washing the retentate with a suitable buffer helps to improve the protein recovery in the permeate. The product is then sent for further purification.

2.3. Harvesting Mammalian Cell Cultures

The desired products in these fermentation processes are in the extracellular fraction. If the cells are lysed during the cell concentration, the intracellular proteins can spill out of the cells and contaminate the extracellular product. The extremely fragile mammalian cells, therefore, need careful handling. An elevated transmembrane pressure and high filtration rates can damage the cells. An excellent membrane-based tangential-flow filtration system was developed by Millipore in the early 1980s. The system contains a microporous membrane, usually with pore diameters of 0.45 μm, a feed pump much like in the conventional TFF systems, but a permeate pump replaces the usual valve used for restricting the permeate flow. The second pump helps to accurately control the permeate flux and to maintain a low transmembrane pressure. Under high transmembrane pressures, the fragile cells can be pushed into the membrane pores and get damaged. With these systems, a high product recovery can be achieved without cell lysis. Diafiltration helps to further improve product recovery.

2.4. Concentration of Viruses

The first step in the manufacture of viral vaccines and antigens is the concentration of the viruses or portions of the viral coat. This is best done by the use of ultrafiltration with the membrane typically having a nominal molecular weight limit of 100 kDa or higher. During concentration, diafiltration is employed to wash away into the permeate all lower molecular weight contaminants. This helps to accomplish simultaneous purification of the retained viruses. For the concentration of viral antigens, ultrafiltration membranes with a lower cutoff in the range 10–30 kDa are often used. In any case, for each process the membrane giving the best results is carefully selected. Ultrafiltration is employed in the commercial manufacture of several vaccines, e.g., influenza ***(1)***, Epstein Barr ***(2)***, and measles ***(3)***.

3. Further Purification of Biotechnology Products

Once the desired protein is obtained in a particulate free process stream, the task of product purification continues until the product is obtained at the desired level of purity. Simultaneously, efforts continue to maximize the product yield in each step. The techniques employed fall into two major groups: membrane-based methods such as ultrafiltration and nanofiltration (reverse osmosis), and a variety of chromatographic procedures.

3.1. Ultrafiltration

The method is commonly employed to concentrate proteins of different molecular weights whereas smaller molecules such as salts, sugars, and sometimes smaller peptides are removed. A very wide range of molecular weight cutoffs is available from several suppliers. Prior knowledge of the molecular weight of the desired protein helps to choose the membrane with the best cutoff for the application. Again, diafiltration is used, if necessary, to wash off the smaller molecular-weight impurities. In short, the ultrafiltration step helps to enrich the retentate in the higher molecular-weight products while enriching the permeate in the smaller molecules from the complex process stream. The permeate streams are further concentrated, if necessary, by nanofiltration.

3.2. Nanofiltration or Reverse Osmosis

The reverse osmosis membranes can retain low molecular-weight compounds such as salts, sugars, and small peptides. These membranes were originally developed for desalination of sea water to make potable water. But these membranes have found a small niche of applications in the biopharmaceutical industry. They are quite often used for the concentration of antibiotics, peptides, and other molecules with molecular weight between 100 and 3000 kDa. The newer membranes in this class also permit the passage of salts and allow the products to be desalted at the same time. In the biotechnology industry the application is limited to the concentration of small peptides.

3.3. Chromatography Methods

Chromatography plays a very important role in the purification of therapeutic compounds. These are highly versatile and selective techniques that serve well in the downstream processing of biotechnology products. As mentioned in the Introduction, therapeutic proteins in this industry are recovered from complex sources and have to be very pure and efficacious to be approved for use as medicinal products. The use of chromatographic techniques helps to purify the biotechnology proteins to a state of very high purity, devoid of undesirable side effects. For most biotechnology products, purification costs are certainly a major proportion of the total production costs ***(4)***. Each chromatographic technique has its advantages and disadvantages and one single chromatography step is seldom capable of giving a product of the desired quality in terms of homogeneity and purity. The different types of chromatography procedures that are used in downstream processing are briefly described as follows.

3.3.1. Ion Exchange Chromatography

Ion exchange chromatography is a relatively inexpensive technique and is quite widely used in the purification of biologicals. The development chemist

chooses between the weak ion exchangers such as DEAE (diaminoethyl) and CM (carboxymethyl) or the strong ion exchangers such as quaternary ammonium. The strong ion-exchange resins show a higher capacity over a wide range of pH values.

3.3.2. Hydrophobic Chromatography

This technique separates molecules by taking advantage of the differences in their polarity ***(5,6)***. Alkyl or aromatic groups are immobilized as ligands on silica, glass or cellulose matrices. The biologicals in an aqueous stream adsorb to specific ligands on columns from which they can be eluted and thus separated from components that do not bind to the ligands.

3.3.3. Affinity Chromatography

This is a highly selective technique that separates molecules based on their unique biological or chemical interaction with ligands ***(7,8)***. These separations are based on the interaction of the proteins that are undergoing purification with peptides, saccharides, and other proteins that serve as ligands. A list of several commercially available ligands and their applications is available ***(9)***.

3.3.4. Size Exclusion Chromatography

This technique, which is also referred to as gel chromatography, is employed for exchanging solvents or buffers in which the product is dissolved. The highly cross-linked gels such as dextrans and polyacrylamide gels give rapid results. The more porous gels such as certain sephadexes and sepharoses work well when used for protein purification at the research scale, but suffer from problems of limited loading capacity when used on a large scale at the production level. Also, the flow rates with the softer gels are slow. However, for solvent exchange, the Sephadex 25 (Armersham-Pharmacìa, Uppsala, Sweden) has a high loading capacity of about 25% of column volume. The columns operate at high flow rates.

4. Final Purification of Biotechnology Products

4.1. Virus Removal and Inactivation

The biotechnology products of high therapeutic value are derived from human plasma and other fluids, animal tissue, milk of transgenic animals, and cell culture processes. The products carry the inherent risk of transmitting to the human recipients viral contaminants coming from the starting material of their process of manufacture. There is also some risk of the contaminant viruses coming from certain downstream processing steps, e.g., the use of proteolytic enzymes of animal origin such as porcine trypsin or pepsin and the monoclonal

antibodies used in affinity chromatography. Finally, there always exists the risk of adventitious viruses entering the process stream because of the failure of GMP (good manufacturing practice). The regulatory authorities, notably the Committee for Proprietary Medicinal Products (CPMP) in Europe and the United States Federal Drug Administration have issued clear guidelines for removal or inactivation of viruses from biotherapeutic proteins. In 1994 , the FDA's Center for Biologics Evaluation and Research (CBER) issued a directive asking industries to validate the removal or inactivation of the viruses from all biologicals. Plasma-derived proteins like the albumin, immunoglobulin, and the different blood clotting factors can carry viruses like hepatitis A, B, and C and the human immunodeficiency virus (HIV). The CPMP has been particularly strong in recommending measures to remove or inactivate the viruses from the blood derivatives. The biotechnology products manufactured by cell culturing can be contaminated with viruses such as the murine leukemia virus coming form the particular cell line. Products derived from animal tissue can also transmit viruses from these source materials to the recipients of the drugs.

The authorities have recommended that the purification steps should achieve an overall virus reduction of at least 12 logs. The blood products industry has been using for some time different methods for virus inactivation. These are heat inactivation (pasteurization), pH inactivation, solvent/detergent treatment, UV and gamma ray irradiation, and the addition of certain chemical inactivating agents like β-propiolactone. Claims have been made by some companies that chromatographic procedures can also reduce the virus content of biologicals.

However, all of these methods have some limitations. For example, the solvent/detergent treatment can inactivate the lipid-coated viruses, but the method is innocuous against the non-lipid coated viruses and leaves polio virus and other viruses of this type viable. The physical inactivation by heat can cause denaturation of the biologicals. With chemical inactivation, one has the task of removing the added chemical completely from the product, not to mention the fact that the chemicals are not without fault. The chromatography methods give a rather low level of virus reduction and are not considered robust enough to work well under all conditions of process.

Membrane filtration has recently emerged as an effective method of virus removal from biologicals. The method is well accepted as is evident from the published literature ***(10–13)***. An important point to remember is that no single method from among those listed above can alone give the complete removal of viral contaminants. However, when membrane filtration is used with the other methods in a complementary fashion, one can achieve the overall virus reduction to satisfy regulatory requirements. The subject of virus contamination in

biologicals and the merits and drawbacks of the different methods employed for their removal and inactivation is the topic of a recent review ***(14)***.

4.2. Removal of Endotoxins

Endotoxins are agents that cause fever when injected intravenously to animals or humans. They are lipopolysaccharides present in the cell wall of gram-negative bacteria and are released into the surrounding liquid when they are killed. They are present as aggregrates in solutions and their size depends on the composition of the surrounding liquids. Endotoxins can be effectively removed from process streams by ultrafiltration using membranes with a low nominal molecular weight limit, typically 10 kDa or lower. The protein being purified goes across the membrane whereas the larger endotoxin molecules stay behind in the retentate. The method is, therefore, applicable to low molecular-weight products with molecular weights lower than 10 kDa that will easily pass the membrane during ultrafiltration. The method is not suitable for depyrogenating larger proteins. But there are ways to restrict the introduction of pyrogens into these products by following the procedures listed below.

1. Sterile filtration or heat sterilization of all liquids including buffers, salt solutions, and so on, used in the downstream process to remove the contaminant bioburden.
2. Steam sterilization or chemical sanitization of all production equipment including chromatography columns, filtration equipment, and membrane filters.
3. Maintaining good manufacturing practices in all production areas to avoid the risk of bacterial contamination, post-use cleaning all equipment to bring it to an endotoxin-free condition, and maintaining the equipment in that condition between successive production runs.

The industry is able to keep the endotoxin content of products below the maximum permissible level by rigorously following the aforementioned procedures.

4.3. Sterile Filtration

The therapeutic agents manufactured by the biotechnology industry are mostly injectables and fall in the general classification of parenteral drugs. These need to be dispensed in a sterile form. Because these proteins are thermolabile, there is no question of sterilizing them by the method of terminal heating. These products can be sterilized by filtration through 0.22 μm-membrane cartridge filters in the normal flow or dead-ended mode. This is an accepted practice in the pharmaceutical industry, but the process needs to be validated following the guidelines for parenteral drugs issued by the USFDA ***(15)***.

5. Conclusions

The products of the biotechnology industry are derived from diverse sources and their downstream recovery is a combination of numerous purification

methods. A well-developed process at the laboratory bench scale needs to be carefully scaled up to the production level. The production must proceed following the good manufacturing practices and, at the same time, paying attention to keeping both production and purification steps within the conditions specified in the validation reports of the company. Earlier batches are used to make products for different phases of clinical trials for the drug. By paying attention to all details, the manufacturing personnel help the company to obtain the regulatory approval required to market the drug ahead of its rivals. In today's highly competitive environment, the first company that gets the marketing approval for a drug stands to benefit immensely from its sales and takes a significant share of the market for that particular drug. This shows just how critical downstream processing is in this industry and the responsibility that rests on the shoulders of a company's R & D and production personnel.

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2

Microbial Cell Disruption by High-Pressure Homogenization

Anton P. J. Middelberg

1. Introduction

The disruption of a cell's wall is often a primary step in product isolation, particularly when hosts such as *Escherichia coli* and *Saccharomyces cerevisiae*, which generally do not excrete product, are employed. Of the available methods, high-pressure homogenization is dominant at moderate or large process volumes.

The high-pressure homogenizer is essentially a positive-displacement pump that forces cell suspension through a valve, before impacting the stream at high velocity on an impact ring. Operating pressures range up to 1500 bar. Several valve designs are available, but cell-disruption applications (as opposed to fat-globule dispersion) generally utilize a tapered cell-disruption design (*see* **Fig. 1**). The mechanism of disruption is still a matter of some debate ***(1,2)*** and of little concern in the current context. Disruption performance is, however, optimized by maintaining small valve gaps and hence high-velocity jets, with short impact-ring diameters. As complete disruption is rarely achieved with a single homogenizer pass, multiple passes are often employed.

This chapter describes some practical issues surrounding microbial cell disruption, and highlights issues not discussed extensively in the general scientific literature. It will, therefore, be of most use to those inexperienced with homogenization, or those with a practical focus. For more detailed information, the reader is referred to reviews, which provide pointers to the literature and information on other methods of cell disruption ***(2–4)***.

The structure addresses four major themes. Equipment layout is discussed in some detail. A simple method for cell disruption is then provided, and issues that affect performance are discussed in the Notes section. Some time is also

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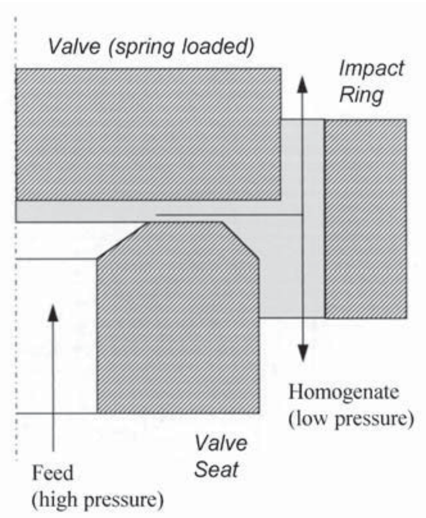

Fig. 1. Cross-section of a typical high-pressure homogenizer valve for use in cell-disruption applications.

spent discussing the analysis of both disruption and debris size. The latter is a particularly difficult problem, and often not of concern in laboratory settings. At process scale, however, a reasonable quantification of debris size is critical for optimal process design and operation.

2. Materials

2.1. Solutions and Reagents

Buffer may be required to dilute the cell suspension prior to homogenization. Buffer choice depends on product stability. Redox reagents may be required in the buffer to prevent the oxidation of certain products. Similarly, improved yields of soluble protease-sensitive proteins can be obtained by the addition of appropriate inhibitors. For stable products such as inclusion bodies, the use of a simple buffer such as 50 m*M* phosphate pH 7.4 is often acceptable. Where the fermentation broth is not concentrated prior to homogenization, simple dilution with water may prove adequate. For analysis of disruption, reagents for soluble protein quantitation are required. The Bradford dye-binding assay ***(5)*** is widely employed. This is now available as a commercial kit (Bio-Rad Laboratories, Hercules, CA).

2.2. Equipment and Layout

It should be stated at the outset that there is no universally optimal homogenizer system design. The final design depends very much on the scale of

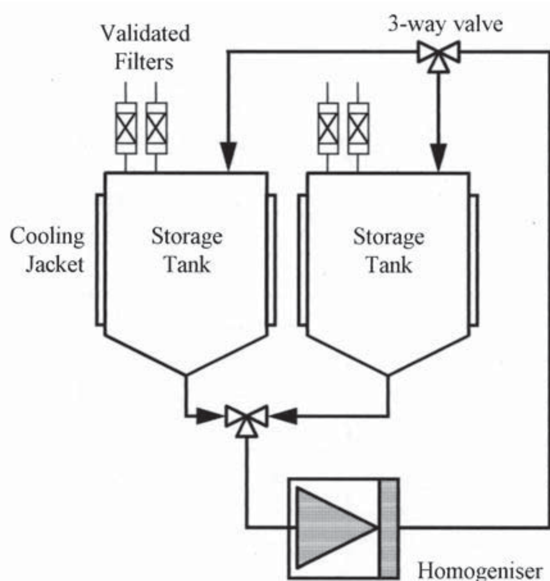

Fig. 2. Example of a high-pressure homogenization system.

application, the need for process cleanability with or without equipment disassembly, containment requirements, and process validation considerations (*see* **Notes 1** and **2**).

Several manufacturers such as APV-Rannie (Copenhagen, Denmark), Niro-Soavi (Parma, Italy), and APV-Gaulin (Wilmington, MA) offer competing homogenizer designs. Features include double-seal arrangements to prevent accidental release to cooling water, steam-sterilization of the high-pressure side, and simplified high-pressure delivery systems to facilitate cleaning. For laboratory-scale work, small homogenizers such as the APV-Gaulin 15 MR are well suited to processing typical fermentation volumes (e.g., 2–10 L) rapidly and efficiently. These often require disassembly after use for thorough cleaning of the valve assembly (*see* **Note 2**).

Figure 2 shows a typical layout for a cell-disruption system based on high-pressure homogenization. Two storage tanks are employed as improved disruption efficiency is achieved by operating a discrete-pass strategy, where the homogenizer feed is drawn from one tank, whereas the homogenate is passed to the other. The location of the three-way valves enables feed to be drawn from, and fed to, either tank. The feed and homogenate tanks, therefore, alternate for multiple-pass strategies (the normal operational mode). As homogenization generates considerable heating of the suspension (typically 2.5°C per 10 MPa of operating pressure), the tanks are jacketed and cooled at 5°C. Additional heat-transfer capacity can be obtained by including internal cooling

coils or an in-line heat exchanger, but with considerably more difficulty in final cleaning. For most laboratory or pilot-scale applications, the external jacket should suffice. Suspension of the tank contents is important, particularly where storage before subsequent processing is required. Tanks fitted with stirrers are ideal but expensive, and cleanability is again an issue. In laboratory settings, effective suspension can be obtained using a recirculation loop on each tank. This can be conveniently implemented using, for example, a double-headed peristaltic pump to simplify cleanability.

2.3. Cell Disruption, Analysis of Disruption, and Analysis of Debris Size

The procedures described here are defined as simply as possible, to rely on standard equipment available in most biological laboratories. Specifically, cell-disruption analysis will require access to a microscope (preferably with phase-contrast optics) and a spectrophotometer. The spectrophotometer is used in conjunction with a dye-binding assay such as the Bradford assay ***(5)*** to estimate the released protein concentration, and hence the extent of cell disruption. This assay is now available as a commercial kit (Bio-Rad).

3. Method

3.1. Cell Disruption

1. With reference to **Fig. 2**, load the cells to be disrupted into one tank. The cell suspension can be the fermentation broth without pretreatment, or may be preconcentrated and resuspended (e.g., by filtration or centrifugation) if removal of the fermentation media or volume-reduction is required.
2. Adjust the cell broth to an appropriate concentration by dilution with a suitable buffer. Cell concentration can vary considerably as disruption efficiency is essentially independent of this parameter although analysis is complicated at higher concentrations (*see* **Notes 3** and **4**). Dilution may be unnecessary if the fermentation broth is not concentrated prior to disruption. Buffer choice is dictated by the stability of the product being released, as homogenization efficiency is relatively insensitive unless specific pretreatments such as EDTA-containing buffers are employed (*see* **Notes 5–7**).
3. Take a small sample of the feed cells for microscopy (*see* **Subheading 3.2.**) and for protein estimation. Sediment the cells and determine the supernatant protein concentration using the protein estimation kit (full instructions are provided in the kit). Alternatively determine the concentration of the specific product of interest. For concentrated feed suspensions, correct the protein concentration for volume fraction (*see* **Note 4**). This value is the feed protein concentration and is a measure of the initial cell disruption (e.g., by upstream units or pretreatments).
4. Switch the three-way valves to feed material from the tank containing the material to be homogenized, with homogenizer discharge set to the other tank.

5. Connect the cooling-water supply to the homogenizer and ensure it is switched on. Connect and switch on other utilities as required for the specific homogenizer design (e.g., steam).
6. Commence homogenization with the operating pressure set to zero. Watch the pressure rise on the instrument gauge to ensure a flow path is available, especially if the homogenizer is not fitted with a high-pressure cutout.
7. Cautiously adjust the operating pressure to the desired value, watching for system problems (e.g., seal leaks, etc.).
8. Allow disruption to proceed while monitoring the system. Ensure an adequate supply of feed by monitoring the tank level.
9. When the feed supply runs low, release the homogenizer pressure back to zero and shut off the system (a system of tank-level detectors coupled to an alarm or a homogenizer shutoff system is advisable).
10. Determine the extent of cell disruption (*see* **Subheading 3.2.**).
11. Allow the homogenate to cool to the desired temperature, and then repeat the above procedure as necessary until the desired performance criterion is met (adequate cell disruption, maximum product release, or acceptable debris size).
12. Thoroughly clean and disinfect the system, using installed clean-in-place systems and adequate flushing. Dismantle and clean, if necessary, after chemical sterilization.

3.2. Analysis of Disruption

1. Analysis of disruption is desirable as soon as possible after cell disruption. Viable cells will remain in the broth after disruption, and these may multiply using substrate available from the disrupted cells (sample fixation using, e.g., 0.2% formaldehyde can inhibit this growth without compromising disruption estimation).
2. Observe the homogenate sample using a phase-contrast or bright-field microscope. Compare with the feed sample to qualitatively estimate the extent of cell disruption. Phase-contrast optics facilitate cell-debris observation, also providing qualitative information on debris size and its impact on subsequent processing.
3. Sediment a sample of the homogenate and determine the protein concentration in the supernatant using the protein estimation kit again. Alternatively, measure the specific product of interest (*see* **Notes 8** and **9**). For concentrated feed suspensions, correct the protein concentration for volume fraction (*see* **Note 4**).
4. Compare the supernatant protein concentration (C_n), with that for the previous homogenizer pass (C_{n-1}), and decide whether further homogenizer passes are required. When protein concentration reaches a plateau or begins to decline, then homogenization should be terminated. Note that a decrease in protein concentration indicates product loss through inactivation ***(6)***. The final plateau value estimates the maximum protein concentration achievable (C_{max}). This may be used to estimate the fractional release of protein after each homogenizer pass (C_n/C_{max}). This ratio, expressed as a percentage, is the simplest measure of cell disruption, although in the strictest sense disruption can only be quantitated using a direct method (*see* **Notes 8** and **9**).

3.3. Analysis of Debris Size

1. Obtain a qualitative assessment of debris size using the phase-contrast microscope. Latex standards of defined size may be incorporated into the sample if calibration is required.
2. Decide whether a quantitative assessment of debris is required (e.g., for process optimization). Several methods are available, but all are either tedious or prone to error (*see* **Note 10**). Cumulative sedimentation analysis (CSA) is a recently developed method that overcomes the limitations of other methods and requires only equipment available in a standard laboratory (e.g., centrifuge with swing-out rotor, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), scanning densitometry) (*see* **Note 11**). It involves centrifuging the homogenate sample in a swing-out rotor of known dimensions for various times, thus sedimenting different fractions of the particulate cell debris. The supernatant and concentrate samples are then analyzed by SDS-PAGE with quantitation of outer-membrane proteins by scanning densitometry. A comparison with the initial homogenate provides an estimate of the fraction of debris sedimented at each centrifugation time. This can then be used to construct a cumulative size distribution using standard mathematical techniques.

4. Notes

4.1. Equipment and Layout

1. As indicated in the Introduction, a homogenizer is essentially a high-pressure positive-displacement pump that forces the cell suspension through a homogenizer valve. In designing the equipment layout, it is important to ensure that an unconstricted flow path is provided while the homogenizer operates. The three-way valves in **Fig. 2** should not be capable of positive shutoff. Furthermore, the storage tanks must be sealed to prevent aerosol release, usually by validated absolute filters (e.g., 0.22 μm). It is important that the filters be designed and selected to minimize blocking potential (e.g., hydrophilic filters mounted on an adaptor (e.g., elbow), with an integrated condenser for rigorous applications). Pressure-relief systems or connection to a validated air removal system at regulated pressure can also be employed to prevent tank overpressure. It is also important that the feed to the homogenizer be maintained without interruption. Manually monitoring tank levels to ensure feed does not exhaust is tedious; level alarms on the tanks are strongly recommended for moderate-scale laboratory operation. At higher automation levels, these can be tied to a cut-out system for the homogenizer. A pressurized feed system can also enhance delivery to the homogenizer, but is generally not required as the core of the homogenizer consists of a positive-displacement pump with no net-positive-suction-head requirement (provided the tanks are above the intake plane of the homogenizer).
2. Cleanability in these systems is a major concern. Spray balls or heads on the tanks are highly recommended, as is chemical disinfection by recirculating appropriate cleaning solutions through the homogenizer. Given the extreme pres-

sure that these systems operate under, regular maintenance is most definitely required. It is strongly recommended that the manufacturer's suggested maintenance schedules be followed, and that a sufficient stock of spare parts be maintained if operational downtime at critical junctures is undesirable.

4.2. Cell Disruption

3. Kleinig et al. ***(7)*** examined the effect of cell concentration for *E. coli* in a Gaulin 15 MR high-pressure homogenizer. In the range of 5–150 g/L wet weight, a small decrease in homogenization efficiency was observed at higher concentrations. **Eq. 1** described the effect of wet cell concentration, X (g/L), on disruption efficiency

$$\ln (1/1 - D) = (0.0149 - 2.75 \times 10^{-5} X) N^{0.71} P^{1.165} \quad (1)$$

where D is the fractional release of protein (the protein release at a specific point divided by the maximum release attainable), N is the number of discrete homogenizer passes, and P is the homogenizer operational pressure in MPa. It was concluded that the decrease in homogenization efficiency at high concentrations was not sufficient to warrant dilution of the suspension before homogenization. The decrease in homogenization efficiency could be easily compensated by additional homogenizer passes. This approach proves more cost effective than diluting the broth and homogenizing the larger volume. However, viscosity increases significantly at the higher concentrations, and it therefore appears that the maximum homogenization concentration is limited by practical constraints related to high viscosity.
4. Protein analysis of highly concentrated samples is prone to error because of the excluded-volume of the cell mass. As disruption proceeds, the volume-fraction of packed material can change significantly. This in turn affects the supernatant volume in a given sample, and hence the protein concentration (when comparing samples throughout the disruption procedure, and calculating D in the above equation). A dilution method of correcting for this increase in aqueous volume fraction has been developed ***(8)***. For samples containing partially denatured protein, dilution during protein estimation can lead to variable results. A method using Kjeldahl nitrogen analysis is available that overcomes this problem ***(9)***, but is considered to be less accurate than the dilution procedure because of several assumptions in the analysis.
5. Homogenization efficiency can be improved, with consequent reduction in the need for homogenization, using chemical pretreatments. Strategies for weakening the cell wall focus largely on enzymatic attack of the strength-conferring elements. For example, treatment of *Bacillus cereus* with the lytic enzyme *cellosyl* prior to homogenization increased disruption efficiency to 98% from 40% after a single homogenizer pass at 70 MPa ***(10)***. For *E. coli*, pretreatment with a combination of ethylenediamine tetra-acetic acid (EDTA) and lysozyme has been used to marginally increase the efficiency of mechanical disruption ***(11)***. Yeast, such as *S. cerevisiae* and *Candida utilis*, may be effectively weakened by pretreatment with zymolyase preparation, available commercially from

Seikagaku America (Rockville, MD) ***(12,13)***. In general, however, the cost of these enzyme preparations can be quite high and recovery and recycle is difficult and costly to implement. Significantly enhanced disruption is required to justify this cost, and results of pretreatment will be very organism- and condition-specific. Often, a simpler and more practical strategy is simply to increase the number of homogenizer passes.

6. It is often desirable to inactivate the broth prior to release from the fermenter for downstream processing. An attractive method for cell inactivation is thermal treatment, as chemical addition to the broth is unnecessary. However, thermal deactivation can significantly reduce the efficiency of cell disruption during homogenization ***(14)***. Results are very procedure-specific, reflecting changes in cell wall composition and cell size. Collis et al. ***(14)*** were able to show that by charging stationary-phase cells with glucose prior to thermal deactivation, an increase in disruption efficiency was actually obtained. Furthermore, product release can actually be enhanced through thermolysis at higher temperatures, provided the product is thermally stable. For example, incubation of *E. coli* at 90°C is reported to release cytoplasmic contents within 10 min. The effects are clearly dependent on the state of the microorganism, and the regime of heat treatment (specifically the temperature and the rate of deactivation).
7. Some products may be degraded during homogenization. For example, Augenstein et al. ***(6)*** have clearly demonstrated product degradation when homogenizing *B. brevis* for the release of a shear-sensitive enzyme. Perhaps the greatest problem arises because of heat generation, which can usually be mitigated by precooling the feed to 5°C (and rapidly cooling the homogenate). The literature also suggests that protein denaturation is intimately tied to the existence of interfaces. Consequently, degassing before homogenization may provide benefits.

4.3. Analysis of Disruption

8. Methods for quantifying disruption may be broadly classed as direct or indirect ***(2)***. Direct methods measure the number or volume-fraction of cells destroyed during the homogenization process. Indirect methods infer the volume or number fraction of cells by measuring, for example, the release of total protein during homogenization. In the procedure described earlier, microscopy provides a direct qualitative measure of disruption, whereas the measurement of total protein release provides an indirect quantitation of the volume fraction of cells destroyed. In this case, the indirect method allows definition of a fractional protein release. Several other methods for quantifying disruption are also available ***(2)***. Microscopy can be conducted in a quantitative manner by cell counting. This can be automated using a hemocytometer with methylene-blue dye exclusion ***(16)*** or an Elzone particle-size analyzer (Coulter Electronics, Fullerton, CA) ***(10)***. The Elzone method provides difficult quantitation, however, because of overlap with the debris resulting from cell disruption. Centrifugal disk photosedimentation (CDS) also provides a rapid and direct measure of cell disruption for *E. coli* ***(17)***.

9. The most appropriate method for monitoring cell disruption in a practical sense is to follow the release of the specific product of interest. If the product is an enzyme, then monitoring the release of specific activity using a standard test will be most appropriate. If the product is nonenzymatic, then immunofluorescent methods offer a rapid and relatively simple means of monitoring the rate of product release. Under this approach, maximizing the fraction of cells destroyed is of secondary importance to maximizing the release of product. It is particularly appropriate if the product degrades during homogenization, as the point of maximum product recovery is unlikely to occur at complete cell disruption.

4.4. Analysis of Debris Size

10. Several techniques are available to analyze debris size, but each has limitations. Consequently, the only method provided above is a qualitative assessment of debris size by light microscopy. Methods previously employed to characterize debris include photon correlation spectroscopy (PCS), CDS, electrical sensing zone measurement (ESZ), and CSA. PCS is an inherently low-resolution technique, so sample preparation including the removal of undisrupted cells is required. This may be achieved by filtration ***(18)***. Mild centrifugation has also been used to separate debris from inclusion bodies before sizing ***(19)***. However, fractionation will selectively remove larger debris and distort the measured distribution toward lower sizes. For example, Jin ***(20)*** has shown that up to 47% of the cell debris is removed from the supernatant (the "debris" fraction) using Olbrich's ***(19)*** fractionation scheme. ESZ has the disadvantage of low sensitivity at smaller debris sizes. Sensitivity can be improved by reducing orifice size, but at the risk of continual blocking. It is typically unsuitable for analyzing *E. coli* debris. It has been used with some success for yeast debris sizing ***(21)***, although sensitivity is lost below 1 μm (where a significant amount of debris should be detected). CDS also suffers from low sensitivity below approximately 0.2 μm ***(17)***, where a considerable fraction of *E. coli* debris lies after homogenization. Resolution is limited by baseline problems and uncertainties associated with light extinction as particles approach the wavelength of light ***(17)***.
11. CSA, developed by Wong et al. ***(22)*** for sizing *E. coli* debris in the presence or absence of inclusion bodies, suffers none of the limitations of PCS, CDS, and ESZ. Its key limitation is that full determination of debris-size distributions is labor intensive. For downstream operations, such as the centrifugal fractionation of inclusion bodies and cell debris, however, information on debris size is important for optimal results. In such cases, CSA is the method of choice as it provides a Stokes sedimentation diameter for direct use in the relevant centrifuge performance equations (*see* **Chapter 5**).

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3

Protein Concentration and Buffer Exchange Using Ultrafiltration

Andrew L. Zydney and Ralf Kuriyel

1. Introduction

Ultrafiltration (UF) is a pressure-driven membrane process used throughout downstream processing for: (1) protein concentration, (2) buffer exchange and desalting, (3) removal of small contaminants, (4) protein purification, and (5) virus clearance. This chapter will consider the first three applications—other chapters in this volume discuss the final two processes. Separation in UF is primarily owing to differences in solute size, with the larger species retained by the membrane whereas the solvent and smaller components pass into the filtrate through the membrane pores. Electrostatic (and other long-range) interactions can also affect the rate of solute transport, e.g., charged solutes are strongly excluded from the membrane pores during operation at low salt concentrations ***(1)***.

UF membranes are cast from a wide range of polymers in both flat sheet and hollow fiber form. These membranes have an asymmetric structure with a very thin skin layer (approximately 0.5 µm thick), which provides the membrane its selectivity, and a more macroporous substructure which provides the required mechanical and structural integrity. UF membranes have mean pore size ranging from 10–500 Å. However, most manufacturers rate their membranes by the nominal molecular weight cutoff, which is defined as the molecular weight of a solute with a particular retention coefficient *(R)*:

$$R = 1 - C_p/C_F \qquad (1)$$

where C_p and C_F are the solute concentrations in the permeate solution and feed stream, respectively. Data are typically obtained with different model proteins or with polydisperse dextrans ***(2)***. Unfortunately, the procedures for

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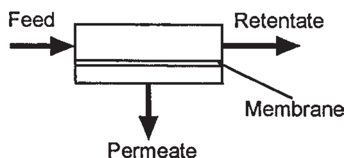

Fig. 1. Tangential flow filtration.

assigning molecular weight cutoffs, including the choice of solutes, the specific buffer and flow conditions, and the chosen retention value (e.g., $R = 0.9$) vary widely throughout the industry, making it difficult to use these classifications for actual process development.

Although small-scale UF processes can be performed using dead-end filtration, almost all large-scale UF is performed using tangential flow filtration (TFF) in which the feed solution flows parallel to the membrane surface. A fraction of the feed is driven through the membrane by the imposed transmembrane pressure drop to form the filtrate or permeate, with the remaining solution collected as the retentate (*see* **Fig. 1**). The tangential flow sweeps the surface of the membrane, reducing membrane fouling and increasing the filtrate flux (defined as the volumetric filtrate flow rate per unit membrane area). Typical filtrate flux in UF range from 25–250 L m^{-2} h^{-1} (often written as LMH). Typical transmembrane pressures (TMP) in UF are 0.2–4 bar.

During UF, the retained biomolecules accumulate on the upstream surface of the membrane forming a concentration polarization layer ***(3)***. This layer reduces the effective pressure driving force and can provide an additional resistance to flow, both of which decrease the filtrate flux. In addition, the increase in solute concentration at the membrane surface increases the rate of solute transmission through the membrane. At high TMP, the solute concentration at the membrane reaches a critical value, at which point the flux (J) becomes essentially independent of the transmembrane pressure. This critical concentration (C_w) may be related to the protein solubility or it may arise from osmotic pressure effects ***(3,4)***. A simple stagnant film model can be used to estimate the flux under these conditions ***(3)***:

$$J = k \ln(C_w/C_b) \quad (2)$$

where C_w and C_b are the protein concentrations at the membrane surface and in the bulk solution, respectively. The mass transfer coefficient (k) characterizes the rate of back transport of solutes from the membrane surface. It is a function of device hydrodynamics (e.g., shear rate), solution properties (viscosity and difffusion coefficient), and module geometry ***(4)***. The flux in the pressure-

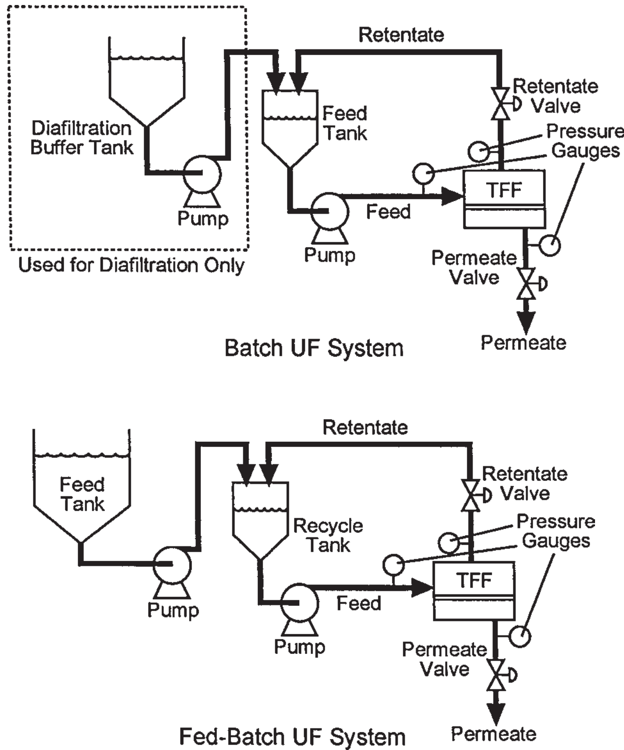

Fig. 2. Ultrafiltration systems.

independent regime can be increased by increasing *k* (typically by increasing the tangential flow velocity) or by decreasing the bulk concentration of the retained species.

Ultrafiltration is generally performed in batch mode as shown in the top panel of **Fig. 2**. The entire volume of feed is contained within a recycle tank. Protein concentration occurs by removal of filtrate through the membrane. Batch operations use a minimum of hardware, provide simple manual or automatic control, and provide the highest filtrate flux. However, it can be difficult to obtain very high concentration factors (large volume reduction) using batch operation, and it can also be difficult to maintain adequate mixing throughout the process. The fed batch configuration utilizes an additional tank to feed into the recycle tank (bottom panel of **Fig. 2**). Fed batch processes can provide greater concentration factors than batch systems, and they also provide better mixing and increased flexibility for use in multiple processes. However, the fed batch configuration requires greater process time. In addition, the number of passes through pumps and valves is much larger than in batch operation, and

this can lead to increased protein denaturation and aggregation. Buffer exchange and desalting are accomplished using diafiltration (DF) in which the low molecular-weight components are washed away from the protein by simultaneously adding fresh buffer (or solvent) to the feed during UF. Diafiltration is generally performed in batch mode, with the DF buffer added at a rate so as to maintain a constant retentate volume (top panel in **Fig. 2**).

2. Materials

UF systems consist of a tank containing the feed solution, pumps, membrane module, valves, and associated instrumentation. These systems can be purchased fully assembled from a number of different manufacturers (e.g., Millipore, Pall, A/G Technology, Koch, Osmonics, Sartorius), or they can be assembled on-site from component parts.

2.1. Membrane

A wide range of polymeric UF membranes are available with different surface chemistries and morphologies. Cellulosic (including both regenerated cellulose and cellulose acetate) and polysulfone (including polyethersulfone) are most commonly used in bioprocessing. Ceramic membranes (alumina or silica) are also available, but tend to be considerably more expensive. Many membranes are modified during or after casting with proprietary chemical treatments, thus the actual surface chemistry and surface charge may be considerably different than that of the base material.

Membrane selection should start with the choice of a high-quality vendor because robustness and reliability are of paramount importance in bioprocessing operations. The most important engineering parameters to consider are product retention, process flux, and chemical compatibility. Product retention and process flux really need to be determined with the actual feed stream using small-scale devices. However, a general rule of thumb is that the nominal molecular weight cutoff of the membrane should be at least 2–3 times smaller than the molecular weight of the protein that is to be retained to insure high product retention ($R > 0.99$ is a typical target). The adsorptive properties of a given membrane play an important role in determining process flux, but are often less important with regard to actual product losses because of the relatively low binding constants (1–10 mg/m^2) and small membrane surface areas (on the order of 0.01 m2/g protein) required for bioprocessing. Cellulosic membranes bind the least amount of protein, but are more susceptible to damage by extremes of temperature and pH than either polysulfone or polyethersulfone.

Chemical compatibility needs to be considered for both the feed solution and for the regeneration or cleaning cycle. Long-term stability of the mem-

branes in bacteriostatic storage solutions should be evaluated by studying product retention and process flux after appropriate storage times.

2.2. Modules

A variety of modules have been developed to achieve the desired mass transport characteristics and, at the same time maintaining high membrane-packing densities. Hollow fiber and flat-sheet cassettes are used most extensively for bioprocessing. Hollow fiber cartridges use an array of narrow bore, self-supporting, fibers potted at the ends in an epoxy or polyurethane resin and housed within a cylindrical cartridge of plastic or steel. Flat-sheet cassettes use a sandwich arrangement of a permeate screen, membrane, and retentat screen. The screens define the flow paths above and below the membrane. The screens generally have a mesh-like structure to promote mixing and increase mass transport. Spiral wound modules also provide very effective mass transfer. However, these devices are susceptible to particulate fouling, and the dead space between the outer edge of the element and the cylindrical housing can be difficult to clean and sterilize. A variety of enhanced mass transfer modules have been developed over the past few years which exploit flow instabilities or turbulence to increase transport. The most successful of these use rotating cylindrical membranes or rapidly spinning disks. These systems provide very high filtrate flux, but they have lower packing densities, are difficult to scale-up, and have uncertain long-term reliability because of the moving parts. Coiled hollow fiber devices can provide enhanced transport without the need for moving parts through the generation of Dean vortices. Additional information on the economics, fluid mechanics, and mass transfer characteristics of these modules are available in the literature ***(4)***.

2.3. Tanks

Tanks are typically cylindrical, but have a conical bottom to provide adequate mixing, even at the very low retentate volumes achieved in processes with large concentration factors. The retentate returns to the tank through a line in the top, which is extended down to the bottom via a dip tube to minimize foam formation. A second inlet dip tube is used for the diafiltration buffer and/or returning the filtrate during cleaning. A motor-driven impeller is used to achieve adequate mixing. Baffles can be placed in the conical bottom to prevent vortex formation. A spray ball can be used for thorough cleaning. A separate drain line is typically provided. Cooling jackets are installed around the outside of the tank when needed. These jackets can also be used to maintain high temperatures during cleaning and sanitization. Vessels operated above 15 psi, which are greater than 6 in in diameter, must be pressure rated. Tanks are typically constructed of 316L stainless steel to reduce corrosion. Mechanical polishing and chemical

treatment are sometimes used to improve performance. Tanks are typically instrumented to provide level, temperature, and conductivity measurements. All connections should be sanitary and tightly sealed to maintain sterility and environmental safety.

2.4. Pumps

Pumps supply both the tangential flow and required TMP. Although centrifugal pumps are inexpensive and deliver a smooth, nonpulsating flow, they are rarely used in bioprocessing becaise of potential particle formation by abrasion. Sliding-vane and gear pumps are also avoided because they can shed particles. The rotary lobe pump with two counterrotating impellers is used most frequently. Fluid is drawn around the outside of the impellers and squeezed out at the discharge port. The tight tolerances required for the impeller spacing make rotary lobe pumps fairly expensive. However, their sanitary design and minimal particle generation make them very attractive for bioprocessing. Progressing-cavity pumps, in which a helical metal rotor rotates inside an elastomeric double helical stator, have recently become more popular. Neither rotary lobe pumps nor progressing cavity pumps are available at laboratory scale. Instead, peristaltic roller pumps are used in which the fluid is forced through the pump by progressive squeezing of flexible tubing (e.g., silicone, Tygon, or Viton). Diaphragm pumps rely on the back-and-forth motion of a flexible diaphragm within a housing. High pressures can be generated, but the flow is highly pulsatile.

The proper pump size and motor for a particular application can be selected using Pump Capacity Charts supplied by manufacturers. These charts show the flow rate that can be delivered by the pump (at different motor speeds) as a function of the back pressure at the pump outlet. The required power can also be read from a chart or calculated using correlations provided by the manufacturer.

2.5. Valves

Valves are used to isolate solutions and control flow rates and pressures. Diaphragm valves are used most frequently. They are cavity-free, self-drainable, and isolated from the solution by a bonnet. The valve body is typically 316L stainless steel. The diaphragm is made of TFE, EPDM, or medical-grade silicone. Ball valves and butterfly valves can also be used. Valve size should be selected based on the required flow/pressure. The valve coefficient supplied by the manufacturer (C_v) gives the volumetric water flow rate (in gal/min) that would pass through the valve under a pressure differential of 1 psig at 60°F.

2.6. Instrumentation

TFF systems are typically designed to provide on-line monitoring of flow rates, pressures, temperature, and liquid levels. Flow rates can be measured

with rotameters, magnetic meters, or turbine flow meters. Flow meters should be calibrated using the actual process fluids. Temperatures are measured using resistance thermocouples. Standard pressure gauges or transmitters are used. Liquid levels are typically measured using displacement floats.

2.7. Solutions

Chemical solutions are required for cleaning, sanitization, depyrogenation, storage, rinsing, and conditioning. Cleaning is typically done using sodium hydroxide (0.1–0.5 *N*), sodium hypochlorite (300–500 ppm), nitric acid (0.1 *N*), phosphoric acid (0.1 *N*), citric acid (0.1 *N*), urea (7 *M*), Tween 80 (0.1%), Tergazyme (0.2%), or Henkel P3-53 (0.5%). The most common cleaning agents for protein foulants are sodium hydroxide and hypochlorite. Sanitization is performed using sodium hypochlorite (20–50 ppm), peracetic acid (100–200 ppm), sodium hydroxide (0.1–0.5 *N*), or formaldehyde (1–2%). Depyrogenation can be accomplished with sodium hydroxide (0.1–0.5 *N*), sodium hypochlorite (600 ppm), hydrochloric acid (0.1 *N*), or phosphoric acid (0.1 *N*). Typical storage solutions are sodium hydroxide (0.1 *N*), formaldehyde (1–2%), sodium azide (0.05%), Roccal™, and sodium bisulfite (1%). The choice of cleaning and storage chemicals remains largely an art, although guidelines are available in the literature ***(4)*** and from most membrane manufacturers. UF systems are typically rinsed and flushed with purified water, and are then conditioned with an appropriate buffered salt solution prior to filling with the feed solution.

3. Methods

3.1. System Assembly

Sanitary components should be used for assembly of all TFF systems. Triclover fittings are typically used for all connections. Precleaning and sanitization can be done to remove microorganisms and pyrogens following the procedures in **Subheading 3.8.** The system should then be flushed with purified water (typically processed with a reverse osmosis system) to remove any remaining solution. Use approx 10 L of purified water/m^2 of membrane area to flush the retentate side, with both permeate and retentate lines directed to drain. Reconnect the retentate line to the feed tank and then flush the permeate side using 30 L/m^2.

3.2. Water Permeability Measurement

The water permeability is typically used as a quality control and to verify the effectiveness of the cleaning cycle. The water permeability is measured using total recycle of the retentate and permeate.

1. Recirculate water through the system for approx 3–5 min at standard operating pressure and flow rate.

2. Record the filtrate flow rate, water solution temperature, and the feed, retentate, and permeate pressures.
3. Calculate the permeability by dividing the filtrate flow rate by the mean transmembrane pressure drop and the membrane area. Values should be normalized to 25°C using a viscosity correction factor (typically supplied by the manufacturer).
4. Check the calculated value against the manufacturer's specifications and any prior membrane lots or process runs.

3.3. Integrity Test

Membrane integrity is usually tested prior to each use with a wetted membrane air flow test.

1. Flush the membrane and module with water, and drain the retentate flow path.
2. Connect a regulated gas source (typically air or nitrogen) to the inlet feed port of the module, and adjust the gas pressure to approximately 5 psi.
3. Close the retentate exit valve and increase the gas pressure to the level recommended by the manufacturer (typically 10 psi). Allow the system to stabilize (at which point the water flow rate should stop).
4. Measure the gas flow rate through the membrane using an inverted graduated cylinder or flow meter.
5. Check the measured flow rate against the manufacturer's specification. Unacceptably high gas flow rates indicate the presence of large defects and typically requires membrane replacement.

3.4. Buffer Conditioning

Some proteins can denature if added directly to purified water. Thus, the UF system should be drained and refilled with appropriate buffer prior to use. Recirculate the buffer through the system for 3–5 min, filtering about 20 mL/m^2 of membrane. Drain the entire system and refill with the feed solution.

3.5. Ultrafiltration/Diafiltration

TFF systems are used for concentration and/or diafiltration. Frequently these operations are combined to a single UF/DF/UF process. The selection of the optimal diafiltration conditions is discussed in **Note 4**. The UF system should be started with the permeate valve closed and the retentate line returned to the feed tank.

1. Start the feed pump and slowly increase to the desired flow rate. Recirculate the flow for 5 min to equilibrate the system.
2. Slowly open the permeate valve while adjusting the retentate valve to give a transmembrane pressure of about 5 psi.
3. Slowly ramp the transmembrane pressure to the desired level.
4. Direct the permeate line to the permeate tank, and continue operation until the desired concentration factor is obtained (*see* **Note 3**). Use the same start-up pro-

cedure for diafiltration, with the diafiltration buffer flow rate initiated when the permeate valve is opened. Continue operation until the desired number of diavolumes is obtained (*see* **Note 2**). For process development and scale-up refer to **Notes 5** and **6**.

3.6. Product Recovery

Residual product in the retentate lines and module can be recovered by pumping air or nitrogen through the system (the use of nitrogen minimizes protein oxidation/denaturation). Fresh buffer is then placed in the feed tank at a volume slightly larger than the hold-up volume of the system. Recirculate buffer through the system at low pump speed and low TMP with the filtrate and retentate line connected back to the feed tank. Pump air/nitrogen through the system to recover the remaining product.

3.7. System Flush

The UF system should be flushed with buffer prior to cleaning to prevent denaturation or precipitation of residual protein. Recirculate the buffer at low pump speeds and low TMP for several minutes with the filtrate and retentate lines recycled back to the feed tank. Pump out buffer solution.

3.8. Cleaning and Sanitization

Cleaning cycles are typically performed at temperatures of 40–60°C to improve sanitization and removal of foulants. Fill the feed tank with the selected cleaning solution. Pump approximately one-third of the cleaning solution through the module with the retentate and permeate lines directed to drain. Then redirect the retentate and permeate lines to the feed tank, and recirculate the remaining cleaning solution through the system for 30–60 min at high tangential flow rate. Drain the entire system and rinse with purified water.

3.9. Storage

Pump appropriate storage solution through the membrane module. Drain the system. Disassemble and store the membrane module in a holding tank filled with storage solution.

4. Notes

1. Fouling can adversely affect membrane operation by reducing flux and increasing solute retention ***(4)***. Fouling can be minimized by: (1) using a membrane with low protein-binding capacity; (2) operating at lower TMP and/or higher tangential flow rate; (3) preventing air interfaces (e.g., bubbles and foaming); (4) minimizing cavitation in the pump by maintaining a positive head on the pump inlet; (5) and using a module with better mass-transfer characteristics. Ramping the transmembrane pressure more slowly during start-up can also reduce fouling.

Some UF users impose a nitrogen overlay on the feed tank to minimize protein oxidation and pump cavitation, and thus reduce fouling. The extent of fouling can be characterized by measuring the water permeability of the used membrane. This is done after flushing the module with buffer but before cleaning. This information can be used to select conditions minimize fouling.

2. The concentration of contaminants or salts in the feed solution (C_F) decreases during diafiltration as:

$$C_F = C_{FI}e^{-N}(1–R) \quad (3)$$

where C_{F0} is the initial contaminant concentration and N is the number of diafiltration volumes, equal to the total permeate volume divided by the (constant) retentate volume. 99.9% removal of a contaminant with $R = 0$ is thus attained after 6.9 diavolumes. It is important to note that components can have nonzero retention even if they are much smaller than the molecular weight cutoff of the membrane. Contaminants can be retained by Donnan exclusion effects with charged membranes or by association with retained species. Detergents in the feed or diafiltration buffer can form micelles, which may entrain small contaminants. Deviations from **Eq. 3** can also be caused by poor mixing in the feed tank. The yield of retained product during diafiltration ($Y = C_F/C_{FI}$) can also be calculated from **Eq. 3** with R the retention coefficient of the product.

3. The ratio of the initial to the final retentate volume in a concentration process is called the volumetric concentration factor X. The product yield for a given concentration factor is:

$$Y = X^{R–1} \quad (4)$$

Eq. 4 can also be used to calculate the contaminant concentration after a concentration step as $C_F/C_{F0} = YX$.

4. In processes requiring both concentration and diafiltration, the total process time will depend upon the point at which the diafiltration is performed. The minimum process time is attained by first concentrating the product to a bulk concentration of ***(5)***:

$$C_b = C_w/e \quad (5)$$

where C_w is the protein concentration at the membrane surface. Cw can be estimated by extrapolation of a plot of J vs C_b to zero flux using **Eq. 2**. More sophisticated methods for determining Cw are discussed in ***(4)***. A subsequent UF concentration is then used to reach the desired product concentration or volume reduction.

5. UF process development involves the selection of membrane area, recirculation flow rate, and transmembrane pressure drop. Tangential flow rate and transmembrane pressure are generally selected based on experiments in which the flux is measured as a function of transmembrane pressure at several flow rates. The transmembrane pressure is generally chosen at the knee of the pressure excursion curve. The filtrate flux typically increases with increasing tangential (recircula-

tion) flow rate, but this requires larger pumps and associated components. It is also important to measure solute retention as a function of tangential flow rate and TMP. In general, product retention decreases with increasing filtrate flux due to the greater degree of concentration polarization (**Eq. 2**). Even small sieving coefficients ($S = 1–R$) can cause significant product loss when a large number of diavolumes are required (**Eq. 3**). The choice of membrane surface area involves a direct trade-off with process time. Most UF processes in the biotechnology industry are designed to operate within 3 hr for ease of scheduling and to minimize protein denaturation and degradation.

6. Scale-up in UF requires the use of the same membrane material, pore size, module configuration, tangential flow velocity, and channel height. The membrane area is then scaled proportional to the filtrate volume. The most effective approach to scale-up is linear scaling, in which the pressure, fluid flow rate, and concentration profile along the length of the filtration module are all kept constant when changing scale of operation *(6)*. Linear scaling can only be achieved by keeping the channel length constant since the retentate velocity, concentration, and pressure all vary with position in the feed channel because of fluid removal and frictional pressure losses. Thus, the membrane area should be increased by increasing the number of hollow fibers or parallel channels. Linear scaling of enhanced mass transfer modules can be difficult to achieve over a wide range of membrane area owing to practical limitations on system geometry.
7. Most UF systems are operated at constant transmembrane pressure. However, in certain applications it is not possible to maintain constant TMP without operating at very low filtrate flux or with unacceptable fouling. Under these conditions it may be advantageous to operate at constant filtrate flux. This can be achieved using a pump on the filtrate line or by regulating the retentate pressure control valve. An alternative control strategy is to maintain constant wall concentration of the retained species during UF/DF *(7)*. Process control would involve a PID loop that measures the flux and controls the TMP or filtrate pump to yield constant C_w as evaluated from **Eq. 2**. Constant wall-concentration control has been shown to give better product yield and quality, more consistent operation, and smaller membrane area than either constant TMP or constant flux operation *(7)*.
8. Repeated circulation of protein solutions through the pumps and valves in the recycle line can lead to unacceptable product denaturation and degradation. The proper selection of pumps and valves is critical. Batch processes have fewer pump passes than fed-batch systems. It may also be possible to employ single-pass operation with the retentate line taken directly to a product holding tank *(4)*.
9. Buffer conditions can also be optimized for UF. In general, higher flux and less fouling is obtained by operating at pH away from the protein isoelectric point at low to moderate salt concentrations or by adding specific stabilizing agents for a given protein.
10. Feed temperature also effects UF/DF processes. Higher temperatures can be used for heat stable proteins to reduce solution viscosity and increase filtrate flux (primarily owing to the increase in solute diffusivity). Low temperatures (as low as

4°C) may reduce fouling for highly heat labile proteins. Heat exchangers can be added to UF systems to maintain the desired temperature control.

11. Some UF systems are sterilized prior to use. Sterilization is typically performed after cleaning, but prior to integrity testing. Saturated steam is more effective in killing bacteria and spores than dry heat. Steam sterilization is performed at 121°C and 15 psig for a minimum of 15 min. Steam is introduced from the top of the feed tank, with condensate removed through valves or steam traps at the bottom. Some UF devices allow steam to be introduced from both the feed and permeate ports.
12. Care should be taken not to exceed the manufacturer's specifications for back pressure limits. Excessive back pressure can cause membrane delamination and failure.
13. UF systems used in biotechnology must be validated to demonstrate process consistency within predetermined specifications and quality attributes ***(8)***. Validation typically requires demonstration of: (1) compatibility of wetted components with all process and storage solutions; (2) adequacy of integrity tests; (3) effectiveness of sterilization and sanitization procedures; (4) and maintenance of protein retention and contaminant removal over the entire lifetime of the system.

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4

High-Performance Tangential Flow Filtration for Protein Separations

Andrew L. Zydney and Ralf Kuriyel

1. Introduction

High-performance tangential flow filtration (HPTFF) is an emerging technology that uses semipermeable membranes for the separation of proteins without limit to their relative size ***(1)***. HPTFF can be used throughout the purification process to remove specific impurities (e.g., proteins, DNA, or endotoxins), clear viruses, and/or eliminate protein oligomers or degradation products. In addition, HPTFF can effect simultaneous purification, concentration, and buffer exchange, providing the unique capability of combining several different separation steps into a single scalable unit operation.

As originally described ***(2)***, HPTFF obtained high selectivity by careful control of filtrate flux and device fluid mechanics to minimize fouling and exploit the effects of concentration polarization (discussed subsequently). Effective separations in HPTFF are obtained by operating in the pressure-dependent, rather than the pressure-independent, regime. In addition, cocurrent flow on the filtrate side of the membrane could be used to maintain the optimal transmembrane pressure, and thus the maximum selectivity, throughout the module ***(2)***. It was subsequently recognized that significant improvements in performance could be obtained by controlling buffer pH and ionic strength to maximize differences in the effective volume of the different species ***(1,3)***. The effective volume of a charged protein (as determined by size exclusion chromatography) accounts for the presence of a diffuse electrical double layer surrounding the protein ***(4)***. Increasing the protein charge, or reducing the solution ionic strength, increases the effective volume thus reducing protein transmission through the membrane.

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HPTFF can thus effect separations by exploiting differences in both size and charge, with the magnitude of these contributions determined by the properties of the proteins as well as the choice of buffer conditions. Optimal performance is typically attained by operating close to the isoelectric point (p*I*) of the lower molecular weight protein and at relatively low salt concentrations (around 10 m*M* ionic strength) to maximize electrostatic interactions ***(3)***. Even lower salt concentrations can be used, although there can be problems caused by pH shifts because of insufficient buffering capacity. Direct charge effects can be further exploited by using a membrane that has an electrical charge opposite to that of the more highly retained protein ***(5)***. Note that it may be possible to exploit electrostatic interactions even for solutes with identical pI because of the different charge-pH profiles for the different species and the combined effects of protein charge and size on protein transmission through the membrane.

The feed flow in HPTFF is parallel to the membrane surface, with a fraction of the flow driven through the membrane by the applied transmembrane pressure drop to form the filtrate solution. This tangential flow "sweeps" the membrane surface, reducing the extent of fouling and increasing the filtrate flux (the volumetric filtrate flow rate per unit membrane area) compared to that obtained in dead-end systems. Typical flux in HPTFF range from 15–200 L $m^{-2}h^{-1}$ (often written as LMH). During HPTFF, the retained biomolecules accumulate at the upstream surface of the membrane. This effect can be described by a simple stagnant film model ***(6)***:

$$C_w = C_b \exp(J/k) \tag{1}$$

where C_w and C_b are the solute concentrations at the membrane surface and in the bulk solution, respectively, and J is the filtrate flux. The mass transfer coefficient *(k)* characterizes the rate of back transport of solutes from the membrane surface. It is a function of the module geometry, the fluid flow, and the solution properties (e.g., solution viscosity and solute diffusivity). Concentration polarization has often been cited as an inherent limitation in using membrane systems for high resolution separations. However, proper choice of filtrate flux and mass transfer coefficient can be used to increase C_w, which increases the protein concentration in the permeate, resulting in significant improvements in overall system performance.

Protein separations in HPTFF are accomplished using a diafiltration mode in which the impurity (or product) is washed out of the retentate by simultaneously adding fresh buffer to the feed reservoir as filtrate is removed through the membrane (*see* **Fig. 1**). This maintains an appropriate protein concentration in the retentate, minimizing membrane fouling and reducing protein aggregation/denaturation. Diafiltration is typically performed at constant retentate volume by controlling the rate of buffer addition to match the filtrate

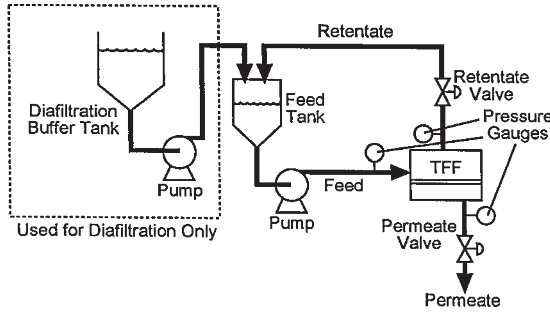

Fig. 1. HPTFF in the diafiltration mode.

flow rate. Differential diafiltration can also be used, with the diafiltration and permeate flow rates adjusted to give the optimal concentration or dilution of the feed during HPTFF.

The optimization of an HPTFF process involves trade-offs between product yield *(Y)*, purification factor *(P)*, number of diafiltration volumes *(N)*, membrane area *(A)*, and process time *(t)*. The performance of an HPTFF system can be conveniently characterized in terms of two dimensionless numbers **(7)**, the selectivity:

$$\psi = S_2/S_1 \tag{2}$$

and a throughput parameter:

$$N\,\Delta S = (JAt/V)\,(S_2 - S_1) \tag{3}$$

where S_1 and S_2 are the sieving coefficients ($S_i = C_{\text{filtrate},i}/C_{\text{retentate},i}$) for the less and more retained proteins, respectively. The number of diafiltration volumes *(N)* is equal to the ratio of the total permeate volume *(JAt)* to the feed volume *(V)* where J is the filtrate flux (assumed to be constant). Membrane area and process time should be chosen to satisfy economic and manufacturing criteria. A typical value based on existing membrane processes is $At/V = 1$ m^2 L^{-1} h^{-1}, in which case $N\Delta S = J\Delta S$ with J in units of L m^{-2} h^{-1}. van Reis and Saksena ***(7)*** developed equations for the yield (final product mass divided by initial mass) and purification factor (yield of desired product divided by yield of impurity) for a product in either the retentate *(R)* or filtrate *(F)*:

$$P_R = Y_R^{1-\psi} = \exp(N\,\Delta S) \tag{4}$$

$$P_F = Y_F / [1 - (1 - Y_F)^{1/\psi}] = Y_F / [1 + (Y_F - 1)\exp(N\Delta S)] \tag{5}$$

For a retentate product, the process starts at $P_R = 1$ and $Y_R = 1$ (all of the product and impurity are in the retentate) and proceeds along a line of constant selectivity (assuming S_1 and S_2 remain constant), with the purification factor increasing and the yield decreasing as the impurities get washed through the membrane ***(7)***. Thus, an HPTFF process with $\psi = 200$ and $J\Delta S = 50$ L m^{-2} h^{-1} would approximately generate a retentate product with $P_R = 1000$ and $Y_R = 96.5\%$ using only 17 diafiltration volumes (assuming $S_1 = 0.40$ and $S_2 = 0.002$).

In contrast, a process for a filtrate product would begin with $Y_F = 0$ and $P_F = \psi$ (for the first drop of product in the filtrate), with the yield increasing and the purification factor decreasing throughout the diafiltration as the product collects in the permeate tank. Different experimental conditions (e.g., buffer chemistry, membrane chemistry and pore size, filtrate flux, and so on) can be compared by evaluating their impact on ψ and $J\,\Delta S$, with the best combination chosen based on overall process objectives.

2. Materials

HPTFF systems consist of a tank containing the feed solution, a tank for the filtrate (in the case where the product is collected in the filtrate stream), pumps, module, valves, and associated instrumentation. The selection of tanks, valves, and pumps was discussed in Chapter 3 and will not be repeated here.

2.1. Membrane

The criteria governing the choice of membrane in HPTFF are similar to those discussed for UF/DF (*see* Chapter 3 in this volume). Membrane selection should start with the choice of a high-quality vendor because robustness and reliability are of paramount importance in any bioprocessing application. The selectivity and $J\Delta S$ are determined by the membrane pore-size distribution and surface chemistry. In order to obtain the high selectivity needed for HPTFF processes, membranes must be completely free of large defects and have reasonably narrow pore-size distributions. Most manufacturer's rate membranes using a nominal molecular weight cutoff, which is defined as the molecular weight of a solute with a given retention (or sieving) coefficient. Unfortunately, the process of assigning molecular weight cutoffs is poorly standardized, making it difficult to use these classifications for actual process development. However, a rough rule of thumb is that the nominal molecular weight cutoff of the membrane should be around the molecular weight of the more retained species.

Membrane chemistry and charge affect the extent of fouling and they control the magnitude of the electrostatic interactions. The membrane chemistry should be selected to maximize electrostatic exclusion of the more highly retained species, i.e., the membrane surface charge should generally be of the same sign as the charge of the more highly retained component *(8)*. Cellulosic and polysulfone (including polyethersulfone) are the most commonly used membrane polymers, although these can be surface-modified to produce more desirable fouling and electrostatic characteristics. It should be noted that membrane fouling can, under some circumstances, enhance the overall separation by reducing the breadth of the pore-size distribution and/or altering the surface charge.

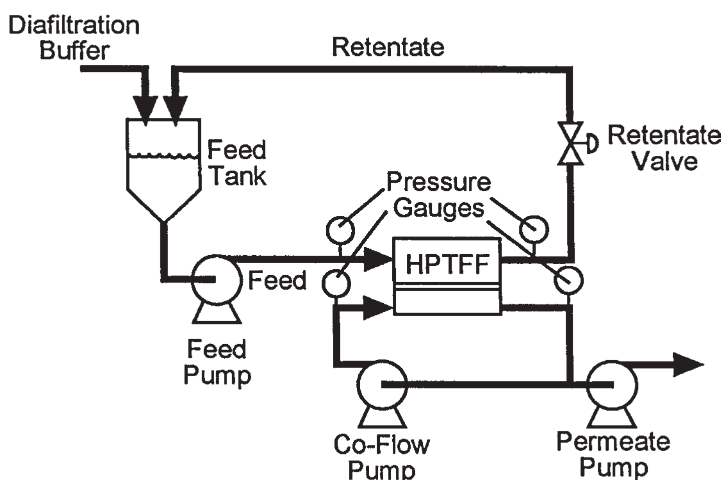

Fig. 2. HPTFF with permeate coflow.

Chemical compatibility of the membranes needs to be considered with respect to the feed solution, cleaning cycle, and storage chemicals. Long-term stability of the membranes in bacteriostatic storage solutions should be evaluated by studying product retention and process flux after appropriate storage times.

2.2. Module

Flat sheet cassettes are used most extensively for HPTFF. These cassettes employ a sandwich arrangement of a permeate screen, membrane, and retentate screen. The screens define the flow paths above and below the membrane. The screens generally have a meshlike structure to promote mixing and increase mass transport.

In order to achieve optimal selectivity and $J\Delta S$ along the entire length of the module, the transmembrane pressure drop must be essentially uniform over the full filtration region. This can be accomplished by placing a recirculation pump on the filtrate line to generate a pressure drop in the filtrate channel that balances the pressure drop in the feed channel *(1)*. A schematic of this "coflow" operation is shown in **Fig. 2**. Alternatively, an "open" channel configuration, e.g., a flat sheet cassette with a suspended screen, can be used to minimize the pressure drop because of feed flow through the device. Optimization of module geometry, feed-flow rate, and filtrate flux generally requires a series of experiments to determine the best combination of selectivity and $J\Delta S$ for the given process. The actual choice of conditions is facilitated using a process optimization diagram *(7)* to evaluate the yield and purification factor corresponding to each set of operating conditions.

2.3. Instrumentation

HPTFF systems should be designed to provide on-line monitoring of flow rates, pressures, temperature, and liquid levels. Flow rates can be measured with rotameters, magnetic meters, or turbine flow meters. Flow meters should be calibrated with the actual process fluids. The accurate measurement of pressure at the inlet and outlet of both feed and permeate sides is important to implement the proper coflow operation. Pressure can be measured by gauges or transducers. Temperatures are typically measured using resistance temperature devices. Standard pressure gauges or transmitters are used. Liquid levels are typically measured using displacement floats in the tanks. In addition, on-line conductivity and UV absorbance should be measured to track buffer composition and total protein concentration during HPTFF.

2.4. Solutions

Chemical solutions are required for cleaning, sanitization, depyrogenation, storage, rinsing, and conditioning. Cleaning is typically done using sodium hydroxide (0.1–0.5 *N*), sodium hypochlorite (300–500 ppm), nitric acid (0.1 *N*), phosphoric acid (0.1 *N*), citric acid (0.1 *N*), urea (7 *M*), Tween 80 (0.1 %), Tergazyme (0.2%), or Henkel P3-53 (0.5%). The most common cleaning agents for protein foulants are sodium hydroxide and hypochlorite. Sanitization is performed using sodium hypochlorite (20–50 ppm), peracetic acid (100–200 ppm), sodium hydroxide (0.1–0.5 *N*), or formaldehyde (1–2%). Depyrogenation can be accomplished with sodium hydroxide (0.1–0.5 *N*), sodium hypochlorite (600 ppm), hydrochloric acid (0.1 *N*), or phosphoric acid (0.1 *N*). Typical storage solutions are sodium hydroxide (0.1 *N*), formaldehyde (1–2%), sodium azide (0.05%), and sodium bisulfite (1%). The choice of cleaning and storage chemicals remains largely an art, although guidelines are available in the literature ***(9)*** and from most membrane manufacturers. HPTFF systems should be rinsed and flushed with purified water, and then conditioned with the appropriate buffered salt solution prior to filling with the feed solution.

3. Methods

3.1. System Assembly

Sanitary components should be used for assembly of all HPTFF systems. Triclover fittings are typically used for all connections. Precleaning and sanitization can be done to remove microorganisms and pyrogens following the procedures in **Subheading 3.8.** The system should then be flushed with purified water (typically processed with a reverse osmosis system) to remove any remaining solution. Use approximately 10 L of purified water/m^2 of membrane

area to flush the retentate side, with both permeate and retentate lines directed to drain. Reconnect the retentate line to the feed tank and then flush the permeate side using 30 L/m^2.

3.2. Water Permeability Measurement

The water permeability is typically used as a quality control and to verify the effectiveness of the cleaning cycle. The water permeability is measured using total recycle of the retentate and permeate.

1. Recirculate water through the system for approx 3–5 min at standard operating pressure and flow rate.
2. Record the filtrate flow rate, water solution temperature, and the feed, retentate, and permeate pressures.
3. Calculate the permeability by dividing the filtrate flow rate by the mean transmembrane pressure drop and the membrane area. Values should be normalized to 25°C using a viscosity correction factor (typically supplied by the manufacturer).
4. Check the calculated value against the manufacturer's specifications and any prior membrane lots or process runs. For the use of water permeability as a measure of the extent of fouling refer to **Note 1**.

3.3. Integrity Test

Membrane integrity is usually tested prior to each use with a wetted membrane air flow test.

1. Flush the membrane and module with water, and drain the retentate flow path.
2. Connect a regulated gas source (typically air or nitrogen) to the inlet feed port of the module, and adjust the gas pressure to approximately 5 psi.
3. Close the retentate exit valve and increase the gas pressure to the level recommended by the manufacturer (typically 10 psi). Allow the system to stabilize (at which point the water-flow rate should stop).
4. Measure the gas-flow rate through the membrane using an inverted graduated cylinder or flow meter.
5. Check the measured flow rate against the manufacturer's specification. Unacceptably high gas-flow rates indicate the presence of large defects and typically requires membrane replacement. HPTFF systems can also be examined using a CorrTest to obtain more detailed information on the membrane pore-size characteristics as discussed in **Note 2**.

3.4. Buffer Conditioning

Some proteins can denature if added directly to purified water. Thus, the HPTFF system should be drained and refilled with appropriate buffer prior to use. Recirculate the buffer through the system for 3–5 min, filtering about 20 L/m^2 of membrane. Drain the entire system and refill with the feed solution.

3.5. HPTFF Operation

The HPTFF system should be started with the *permeate pump off* and the retentate line returned to the feed tank.

1. Start the feed pump and slowly increase the flow rate.
2. As the feed flow increases, turn the permeate coflow pump on and slowly increase the coflow rate to obtain the same pressure drop on the feed and permeate sides. Increase the feed-flow rate until it reaches the desired level while continually adjusting the coflow rate to balance the pressure drops in the feed and permeate sides. Recirculate the flow for approximately 5 min to equilibrate the system.
3. Slowly turn on the permeate pump and gradually increase the filtrate flux to the desired level.
4. Direct the permeate line to the permeate tank, and continue operation until the desired purification factor (or number of diavolumes) is obtained. For a discussion of scaling-up HPTFF Systems refer to **Note 10**.

3.6. Product Recovery

Residual product in the retentate lines and module can be recovered by pumping air or nitrogen through the system (the use of nitrogen minimizes protein oxidation/ denaturation). Fresh buffer is then placed in the feed tank at a volume slightly larger than the hold-up volume of the system. Recirculate buffer through the system at low pump speed and low TMP with the filtrate and retentate line connected back to the feed tank. Pump air/nitrogen through the system to recover the remaining product.

3.7. System Flush

The HPTFF system should be flushed with buffer prior to cleaning to prevent denaturation or precipitation of residual protein. Recirculate the buffer at low pump speeds and low TMP for several minutes with the filtrate and retentate lines recycled back to the feed tank. Pump out buffer solution.

3.8. Cleaning and Sanitization

Cleaning cycles are typically performed at temperatures of 40–60°C to improve sanitization and removal of foulants. Fill the feed tank with the selected cleaning solution. Pump approximately one third of the cleaning solution through the module with the retentate and permeate lines directed to drain. Then redirect the retentate and permeate lines to the feed tank, and recirculate the remaining cleaning solution through the system for 30–60 min at high-tangential flow rate. Drain the entire system and rinse with purified water.

3.9. Storage

Pump appropriate storage solution through the membrane module. Drain the system. Disassemble and store the membrane module in a holding tank filled with storage solution.

4. Notes

1. Fouling can adversely affect membrane operation by reducing flux and increasing solute retention ***(9)***. Fouling can be minimized by: (1) using a membrane with low protein-binding capacity; (2) operating at lower TMP and/or higher tangential flow rate; (3) preventing air interfaces (e.g., bubbles and foaming); (4) minimizing cavitation in the pump by maintaining a positive head on the pump inlet; and (5) using a module with better mass-transfer characteristics. Ramping the transmembrane pressure more slowly during start-up can also reduce fouling ***(1)***. The proper selection of pumps and valves is critical because repeated circulation of protein solutions through the pumps and valves in the recycle line can lead to unacceptable product denaturation and degradation. The extent of fouling can be characterized by measuring the water permeability of the used membrane. This is done after flushing the module with buffer, but before cleaning. This information can be used to select conditions which minimize fouling.
2. The CorrTest ***(10)*** provides a sensitive method for characterizing the pore-size distribution of HPTFF membranes. This porosimetry technique is based on the displacement of one fluid from the membrane pores by a second (highly immiscible) fluid (e.g., two phase systems of alcohol—water or ammonium sulfate, polyethylene glycol, and water). Mix the liquid components and allow them to phase separate. Recirculate one of the liquid phases through the module and membrane. Drain the feed and permeate channels. Introduce the second fluid phase into the feed channel, with the retentate line recycled back to the feed tank. Increase the transmembrane pressure drop to the desired level and measure the filtrate flow rate. Flush the device, refill with buffer, and then measure the buffer-filtrate flow rate at the same transmembrane pressure and feed rate. The CorrTest value (CTV) is defined as the logarithm (base 10) of the ratio of the buffer flow rate to the CorrTest fluid flow rate. Any changes in CTV are indicative of changes in the membrane pore-size distribution and will likely have an adverse effect on system performance.
3. Membrane consistency is critically important in HPTFF because the purification factor and yield can both be very sensitive to changes in membrane properties. Membrane samples at the upper and lower limits of the nominal molecular weight cut-off range should be obtained from the manufacturer and tested as part of the HPTFF process development. Acceptable performance over the full range of membrane properties is needed to ensure reproducibility and consistency in the actual commercial process.
4. HPTFF devices are operated in the pressure-dependent regime. The transition point can be evaluated from a plot of the filtrate flux as a function of transmembrane pressure drop at a fixed feed flow rate ***(2)***. The optimal TMP may be well below the transition point in some systems. Selection of optimal conditions is done by comparing the projected yield and purification factor at different TMP, feed flow rates, and buffer conditions.
5. Buffer selection is critically important in optimizing HPTFF systems. Care must be taken to insure sufficient buffering capacity because the high protein con-

centration at the membrane surface (the critical region) can cause pH gradients in the bulk solution. In addition, specific interactions between the buffer components and the product (or impurity) can occur. Ion-binding interactions have been shown to alter protein charge, effective hydrodynamic volume, and protein-sieving coefficients ***(11)***. Specific stabilizing agents can be used to reduce denaturation and improve system performance.

6. Initial experiments should be done at pH slightly above and below the isoelectric points of the product and impurity to maximize the difference in effective hydrodynamic volumes. Note that HPTFF systems can be designed to operate with "reverse" selectivity, i.e., with greater transmission of the larger molecular weight component, by operating near the isoelectric pH of that component (and far from the pI of the other component).
7. A cascade configuration can be used in HPTFF when the product is in the retentate (*see* **Fig. 3**). The permeate from the HPTFF unit is sent to a holding tank, which is then used as the feed for a separate UF system. The permeate from the UF device is recycled back to the original (product) retentate tank, allowing large numbers of diavolumes to be obtained with minimal buffer consumption. The system is started using fresh diafiltration buffer with the permeate from the HPTFF system allowed to accumulate to the needed volume in the holding tank. The UF system is then started (*see* Chapter 3), with the recirculation flow rate (tangential velocity) and transmembrane pressure drop chosen to provide the required flow rate of recycled buffer back to the HPTFF feed tank.
8. The bulk-protein concentrations can also be adjusted to optimize the performance of HPTFF. Increasing the bulk-protein concentration (by performing an initial UF concentration) will significantly reduce buffer consumption and may thus allow greater overall purification. Reducing the bulk concentration allows operation at higher filtrate flux. The optimal conditions must be determined experimentally by performing small scale runs at different bulk concentrations, with the results extrapolated to process performance by calculating the yield and purification factor using **Eqs. 4** and **5**.
9. HPTFF processes can also be designed with a gradient in solution pH (or ionic strength) to allow the removal of multiple impurities in a single operation. For example, a series of small impurities with different isoelectric points could be very effectively removed from a product with a high p*I* by performing the diafiltration at several different pH (with each impurity removed primarily when the pH is near its isoelectric point). Thus, an initial diafiltration would be performed for an appropriate number of diavolumes at a pH near the p*I* of one impurity, the pH of the diafiltration buffer would then be shifted to that near the p*I* of a second impurity, and the diafiltration would be continued to remove the second impurity. Alternatively, a shift in pH could be used to allow an initially retained product to be collected in the permeate. In this case, a small impurity could be removed during the initial diafiltration, with a shift in pH or ionic strength allowing the product to pass into the permeate while a large impurity (e.g., a viral contaminant) is removed during the final stage of the diafiltration.

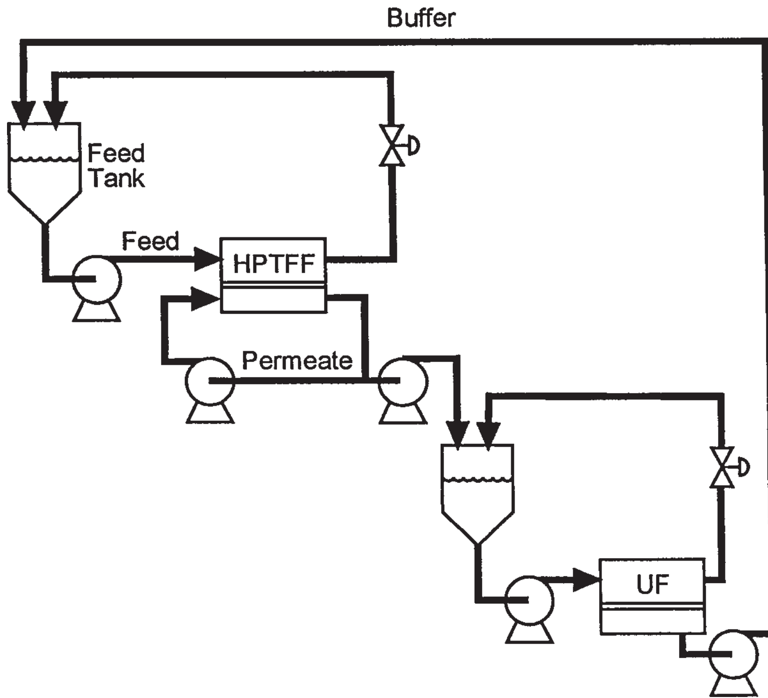

Fig. 3. The use of cascades in HPTFF.

10. Scale-up in HPTFF requires the use of the same membrane material, pore size, module configuration, tangential flow velocity, and channel height. The membrane area is then scaled proportional to the filtrate volume. The most effective approach to scale-up is linear scaling, in which the pressure, fluid flow rate, and concentration profile along the length of the filtration model are all kept constant when changing scale of operation *(12)*. Linear scaling can only be achieved by keeping the channel length constant because the retentate velocity, concentration, and pressure all vary with position in the feed channel because of fluid removal and frictional pressure losses. Thus, the membrane area should be increased by increasing the number of parallel channels.
11. Care should be taken not to exceed the manufacturer's specifications for back pressure limits. Excessive back-pressure can cause membrane delamination and failure.
12. HPTFF systems must be validated to demonstrate process consistency within predetermined specifications and quality attributes *(13)*. Validation typically requires demonstration of: (1) compatibility of wetted components with all process and storage solutions; (2) adequacy of integrity tests; (3) effectiveness of sterilization and sanitization procedures; (4) and maintenance of protein retention and contaminant removal over the entire lifetime of the system.

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5

Large-Scale Recovery of Protein Inclusion Bodies by Continuous Centrifugation

Anton P. J. Middelberg

1. Introduction

Inclusion bodies (IBs) are micron-sized solid protein particles that form within the cytoplasm of certain host cells such as *Escherichia coli* following overexpression of a protein. IBs are comprised primarily of the recombinant protein of interest. Some contaminants including nonproduct protein, nucleic acids, and cell-envelope contaminants can also be incorporated into the granules. However, it is believed that the majority of contaminants actually adhere to the IB surface following release from the cytoplasm during processing ***(1)***. This indicates that IB formation in vivo is a rather specific process that offers certain advantages for downstream processing. Specifically, the protein of interest already exists in a relatively pure state as a small granule that can be recovered by physical separation from nonassociated contaminants. Of course, if a suitably efficient protein refolding strategy is not available ***(2)***, then any gains achieved through inclusion body formation may be easily lost.

Strategies for the large-scale processing of proteins formed as IBs within *E. coli* are highly conservative, and based largely on laboratory procedures. IBs are initially released from the cell by mechanical disruption (*see* Chapter 2 in this volume), providing a mixture of solid cellular debris (cell-wall particles, and so on), soluble host contaminants, and the IBs. Continuous centrifugation is then employed to separate the denser IBs from contaminants. Continuous centrifugation differs from laboratory centrifugation, as the machines can be operated to provide a degree of separation of the solid IBs and the particulate cell debris. Following collection, and usually washing, the IBs are dissolved in strong denaturant prior to protein refolding and recovery by high-resolution

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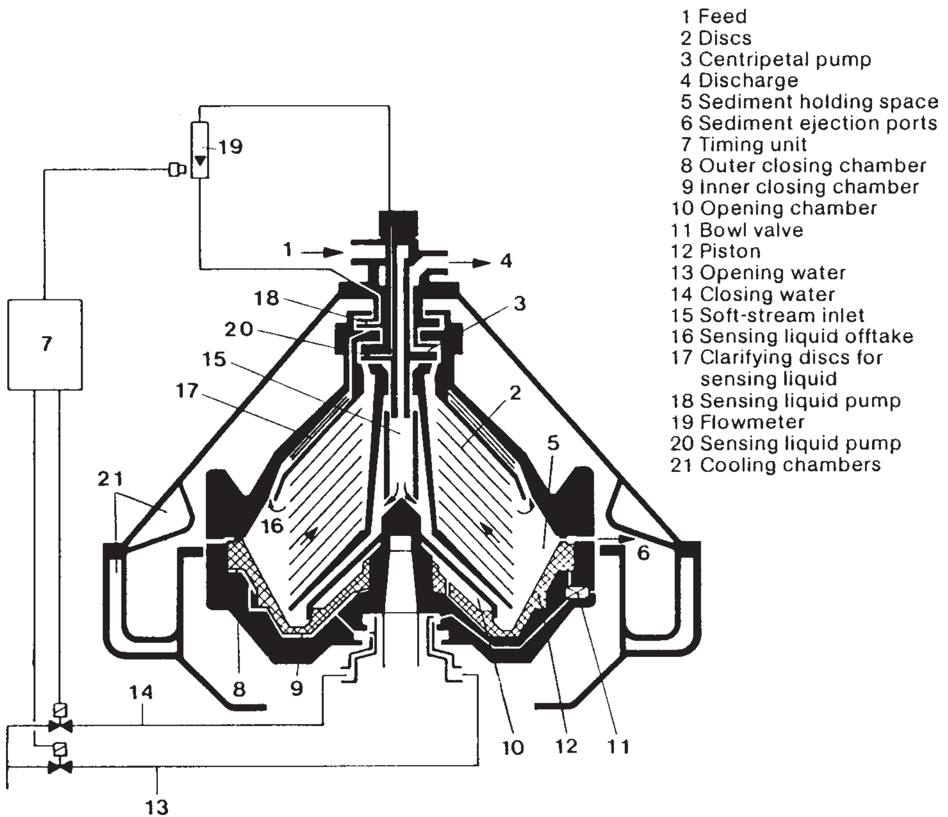

Fig. 1. Cross section of a typical disk-stack centrifuge for inclusion-body collection. Reproduced with the kind permission of Westfalia Separator AG, Oelde, Germany, from the booklet "Centrifugal clarifiers and decanters for biotechnology."

methods such as chromatography. Further description of typical processes is provided elsewhere *(3,4)*.

IB recovery depends strongly on the centrifuge design and operational parameters. The most commonly employed centrifuge for large-scale IB collection is the disk-stack design (**Fig. 1**). Material is fed through the center of the disk stack to the outer periphery, where it is then forced between the disks which are rotating at a high speed. Solids are collected on the disks and flung to the outer periphery by centrifugal force. A sludge of particles (IBs and some debris) is collected in the bowl periphery, where it can be discharged (*see* **Note 1**). Clarified supernatant flows between the disks and exits at the machine centre, carrying any uncollected particles. Essentially continuous separation of sludge and supernatant is therefore achieved, without the need to disassemble

the centrifuge for solids removal. Machines ranging from laboratory size (e.g., 200 mL/min of feed suspension) through to full-scale production are available from the two main manufacturers, namely Westfalia Separator AG (Oelde, Germany) and Alfa Laval Separation AB (Tumba, Sweden).

Centrifuge performance is most suitably described using the fractional collection efficiency, $f(d)$, which provides the fraction of particles of size d collected by the centrifuge. The collection efficiency at a fixed flow rate and with a fixed feed suspension is established by monitoring the feed and supernatant streams:

$$f(d) = 1 - C_L(d)/C_o(d) \quad (1)$$

where C_o is the concentration of particles of size d entering the centrifuge and C_L is the concentration of particles of size d in the exiting supernatant. A curve of $f(d)$ vs d is termed the centrifuge grade efficiency. A useful functional form to describe grade efficiency is given by **Eq. 2**:

$$f(d) = 1 - \exp\left[-k\,(d/d_c)^n\right] \quad (2)$$

where k and n are parameters determined by regression to experimental data and d is the particle size in meters. The critical diameter d_c is given by **Eq. 3**:

$$d_c = \sqrt{18\mu Q/\Delta\rho g\Sigma} \quad (3)$$

and clearly depends on the feed viscosity (μ, Pa.s), the centrifuge flow rate (Q, m^3/s), the density difference between the particles and the suspending liquid ($\Delta\rho$, kg/m^3), gravitational acceleration (9.81 m/s^2), and the machine-specific parameter Σ. Σ is an effective settling area for the centrifuge (m^2):

$$\Sigma = (2\pi/3g)\,\omega^2 n \cot\theta(\rho_1^3 - r_2^3) \quad (4)$$

and depends on the number of flow channels between centrifuge disks (n), the angular speed (ω, rad/s), the inclined angle between the axis and the disk surface (θ, degrees), and the inner (r_1, m) and outer (r_2, m) radii of the disks. Empirical equations for Σ that account for flow nonidealities are also available. For example, the empirical KQ correction substitutes $\omega^{1.5}$ and $r^{2.75}$ into the above equation in place of ω^2 and r^3.

The extent of fractionation of IBs from particulate debris is strongly affected by the settling velocity distribution of each species. There must be minimal overlap between the IB and debris settling distributions for fractionation to be achieved. IB size can vary up to approximately 1.5 µm, and is highly dependent on a variety of factors including plasmid design, host-cell characteristics, and fermentation conditions. The debris-size distribution is controlled primarily by the severity of mechanical disruption (*see* Chapter 2 in this volume), as well as homogenizer design characteristics, operational pressure, and host-cell

characteristics. Debris particle-size distributions may be described using a Boltzmann-type equation:

$$1 - F(d) = 1 \{[1 + \exp (d - d_{50})/w]\} \tag{5}$$

where $F(d)$ is the cumulative undersize mass fraction, d is particle size, d_{50} is the median particle size, and w is a parameter related to the width of the distribution ***(5)***. For *E. coli*, Wong et al. ***(6)*** have shown that, at an operating pressure of 55 MPa in an APV-Gaulin 15 *M* homogenizer (APV-Gaulin, Wilmington, MA), the parameters in **Eq. 5** are correlated with the number of homogenizer passes N:

$$\ln (1/d_{50}) = k_1 N^{0.29} \tag{6}$$

$$\ln (1/w) = k_2 N^{0.1} \tag{7}$$

where k_1 ranged from 0.48–0.66 pass$^{-0.29}$ and k_2 ranged from 1.62–1.92 pass$^{-0.1}$, depending on the properties of the feed cells. Clearly, additional homogenizer passes beyond those required for complete cell disruption reduces the debris size. IB size is generally unaffected. The homogenization conditions can thus be manipulated to facilitate centrifugal fractionation.

Examination of the above equations and comments suggests a method for the centrifugal collection of IBs. The necessary parameters for the above equations and the properties of the feed stream (size distributions and viscosity) must first be established. Critical diameter d_c is proportional to the square root of centrifuge flow rate, so by **Eqs. 2** and **3** an increase in centrifuge flow rate will reduce the fractional collection of particles of a given size $f(d)$. An initial prediction of the required flow rate to achieve a given collection of IBs is, therefore, possible. The actual centrifugation trials use this as a starting point, with adjustment following analysis of the centrifuge performance. Each of these stages will now be addressed.

2. Materials

2.1. Solutions and Reagents

Buffer is required to dilute the homogenate prior to centrifugation, and to resuspend the inclusion body paste prior to recentrifugation. Dilution buffer is very dependent on the nature of the product. Simple phosphate buffer (composition (g/L) $ZnCl_2$, 0.068; KH_2PO_4, 1.57; Na_2HPO_4, 2.62; NaCl, 1.27) has been employed with success ***(7)***. In some cases, problems with IB dissolution and protein renaturation may occur because of the oxidation of surface-exposed cysteine residues. In such cases, the addition of an appropriate reducing agent (e.g., 20 m*M* dithiothreitol or β-mercaptoethanol) to the buffer is recommended. For quantative analysis by centrifugal disk photosedimentation (**Sub-**

heading 3.3.), 20% v/v ethanol-water and 95% ethanol are required. Other reagents (e.g., for sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) are standard to the method employed.

2.2. Predicting Recovery

Prediction of recovery requires a knowledge of the suspension viscosity (e.g., using a cone-and-plate viscometer) and an estimate of the density difference between the particles and suspending fluid (e.g., by density-gradient centrifugation). When a centrifugal disk photosedimentor is employed to establish the IB grade-efficiency curve, then explicit knowledge of the density difference may not be necessary (*see* **Note 2**). The parameters in **Eq. 2**, namely k and n, are also required and may be known by previous characterization of the grade efficiency curve for the particular centrifuge in question, for example using **Eq. 1**. The settling area Σ must also be calculated using **Eq. 4** or empirical forms of the same equation, or may be obtained from the manufacturer. Calculations using the above equations may be easily conducted on a PC-based spreadsheet.

2.3. Centrifugation

A disk-stack centrifuge suitable for inclusion body collection is required. Suitable machines at pilot or process scale are the Westfalia SC35 (Westfalia Separator AG) and the Alfa Laval BTPX-205 (Alfa Laval Separation AB). In this instance, it is assumed that the centrifuge is equipped with an on-line sensor that automatically detects filling of the bowl sludge space and initiates discharge of the solids in an intermittent fashion. The form of on-line detection varies between manufacturers, but is often based on supernatant turbidity measurement or the loss of flow through an interdisk channel when solids reach the disk periphery. Necessary utility and instrumentation lines, and control modules, vary significantly between both manufacturers and applications, and must often be tailored to individual needs. Consequently, issues concerning the selection of a specific machine will not be discussed in detail.

Qualitative analysis of centrifuge performance is easily achieved using a bright-field microscope, preferably equipped with phase-contrast optics to assist in cell-debris observation. Quantitative analysis is best performed using a centrifugal disk photosedimentor (CDS), also known as an analytical disk centrifuge (Applied Imaging, Gateshead, UK or Brookhaven Instruments, Holtsville, NY). The procedure described in **Subheading 3.3.** assumes a DCF4 centrifugal disk photosedimentor from Applied Imaging. CDS provides information on the size distribution of IBs in a sample (e.g., feed and supernatant sample). Quantitative analysis of the concentrate requires standard SDS-PAGE capacity to resolve the product protein from the cell-debris protein bands

located between 31 and 45 kDa, preferably coupled with scanning densitometry to quantitate the relative proportions of product and contaminant proteins ***(6)***.

3. Methods

3.1. Predicting Recovery and Fractionation

1. Determine the size distribution of the IBs in the homogenate using the CDS (*see* **Subheading 3.3.**). Discretize the distribution, on a normalized mass-frequency basis, into appropriate bin sizes (each bin centered on a diameter d_i) in a spreadsheet. The mass in each bin size is, therefore, $m_i \delta d_i$ where δd_i is the size of bin *i*.
2. Measure the IB density using density gradient centrifugation (*see* **Note 2**), and the viscosity of the suspension using a viscometer (e.g., cone-and-plate).
3. Estimate a centrifuge feed rate, and hence, calculate the critical diameter d_c from **Eq. 3**. Set this as a dynamic calculation, dependent on the estimated centrifuge feed rate.
4. Using predetermined constants *k* and *n* describing the centrifuge grade efficiency for IBs, calculate the fractional collection efficiency for each bin size $f(d_i)$ using **Eq. 2**. Wong et al. ***(8)*** found $k = 0.15$ and $n = 2.5$ for a small solid-bowl Veronesi disk-stack centrifuge. Grade-efficiency curves determined using polyvinylacetate particles are also available for larger machines, but explicit parameter values are not provided ***(9)***. Values are highly machine explicit, so determination of the individual machine grade efficiency is optimal.
5. Estimate the total recovery, at the estimated flow rate *Q*, as $\sum_i f(d_i) m_i \delta d_i$. Iterate by adjusting the flow rate *Q* to give the target overall IB collection (e.g., 95%). This is the estimated centrifuge feed rate for the first centrifuge pass.
6. Fix the flow rate to the determined value. Repeat the above with the measured cell debris-size distribution (e.g., measured using CSA as discussed in Chapter 2 in this volume). Use constants k and n appropriate for cell debris. Wong et al. ***(8)*** found $k = 0.13$ and $N = 2.1$ for a small Veronesi solid-bowl centrifuge. Note that debris density will be extremely difficult, if not impossible, to measure accurately. An estimated value is appropriate for this calculation. Wong et al. *(6)* used $\Delta\rho = 1085$ kg/m^3 for *E. coli* cell debris (*see* **Note 2** regarding the need for explicit density estimates). In this case, the calculation provides an overall estimate of the fractional collection of cellular debris (*see* **Note 2**). This should typically be lower than the IB recovery, unless the IBs are very small (in which case consider filtration!) or the debris is very large (indicating the need for further homogenizer passes).
7. Estimate the IB recovery and the cell-debris removal for additional centrifuge passes. Additional centrifuge passes provide enhanced removal of cellular debris, and soluble contaminants not fully removed during the first centrifugation (*see* **Note 3**). This calculation is completed iteratively. The size distribution of the feed material for centrifuge pass $j + 1$ is simply the particle-size distribution of the product from the previous pass (pass *j*). Note that the viscosity will be reduced after the first centrifuge pass because of the removal of DNA, and so on. Subtle

changes in grade efficiency resulting from concentration changes and changes in the properties of the feed stream may also affect the predictions.

3.2. Centrifugation

1. Prepare the centrifuge feed suspension. Large centrifuges can produce significant heating of the product. It is suggested that the feed be precooled to 5°C before centrifugation. The suspension should also be relatively dilute for optimal separation (although dilution can impose a substantial economic penalty if significant reductions in viscosity are not achieved). Dilutions to dry weights below 50 g/L DCW using an appropriate buffer (*see* **Subheading 2.1.**) are commonly employed.
2. Start the centrifuge, carefully following all manufacturer's instructions. These centrifuges, being continuous-flow machines, must usually be started with solution flowing through them. This can be water. When the machine is operating satisfactorily at speed, ensure that the sludge discharge triggers and mechanisms are working correctly, and that the bowl is sealed (for intermittent discharge machines). Also be careful to ensure that all containment procedures are strictly adhered to, as aerosol formation during discharge is of considerable concern.
3. When satisfied that the machine is functioning correctly, start feeding the IB suspension at the calculated feed rate. Note that the predictive ability of the above equations may be quite poor unless accurate grade-efficiency and size data are available. In this case, take a conservative approach if the aim is to minimize product loss, by commencing feeding at a lower feed rate (e.g., 75% of the calculated feed rate).
4. After allowing sufficient time for the suspension to displace the initial water present (typically 3–4 bowl volumes), sample the feed and supernatant streams. Estimate the loss of IBs to the supernatant using the microscope (qualitative, ca. 2–3 min procedure). This simply involves preparing microscope slides of both the feed and supernatant streams, and estimating the relative IB concentration.
5. Assess the level of IB loss. If it is judged to be too high, then reduce the centrifuge feed rate to improve the collection. When assured that product loss is acceptable, analyze the IB loss using the centrifugal disk photosedimentor (quantitative, ca 15–20 min) as described in **Subheading 3.3**. Adjust the centrifuge feed rate as required.
6. Following completion, switch back to feeding water. Dilute the IB-rich sludge using an appropriate buffer (volume at this stage will typically be 50% of the starting volume).
7. Repeat the centrifugation procedure as required, until adequate purity is achieved (*see* **Notes 3–5**).
8. When finished, thoroughly clean the machine and shut it down following the manufacturer's procedures.
9. Use the collected data to refine information on the centrifuge grade efficiency curves, thus improving subsequent estimates of feed rate and performance.
10. Estimate the purity of the separated IBs using SDS-PAGE after each centrifuge pass. Scanning densitometry can be employed to quantitate the relative propor-

tions of product and contaminant proteins. Sedimentable cell-wall proteins are located between 31 and 45 kDa ***(6)***. Comparison with SDS-PAGE analysis of the uncentrifuged feed sample enables the extent of cell-debris removal to be estimated. This can be compared with the estimated removal in the preceding section, and the grade-efficiency curve refined as necessary.

3.3. Quantitation of IB Recovery by CDS

1. Start the Applied DCF4 disk centrifuge at a rotational speed of 8000 rpm, with gain set to maximum for highest sensitivity.
2. Inject 20 mL of water of phosphate buffer into the disk cavity.
3. Inject 1.5 mL of 20% v/v ethanol-water into the disk cavity. This will form a distinct phase on the top of the buffer.
4. Form a density gradient of the disk cavity by mixing the ethanol-water into the buffer using an appropriate machine "boost" strategy. Various strategies are available ***(10)***.
5. Prepare the centrifuge supernatant sample to be analyzed by thoroughly mixing 4 parts of sample with 1 part of 95% ethanol.
6. Inject the sample suspension into the disk cavity and record the instrument output (adsorbance at a given radius in the disk cavity) as a function of time. Convert this output to absorbance *(A)* vs settling diameter *d*, giving an approximate size distribution where the ordinate is proportional to the amount of material at a given size (*see* **Note 2**).
7. Repeat the above **Steps 2–6** for the centrifuge feed sample.
8. Create a curve of $A_L(d)/A_o(d)$ where L is the supernatant sample and o is the feed sample. The centrifuge collection efficiency as a function of diameter d may be calculated by replacing $C_L(d)/C_o(d)$ with $A_L(d)/A_o(d)$ in **Eq. 1**.
9. Appropriate extinction coefficients may be applied to correct the ordinate to a mass rather than absorbance basis. The resulting curves may then be integrated and ratioed to give the overall inclusion body collection efficiency.

4. Notes

4.1. Centrifuge Discharge Mechanisms

1. Centrifuge manufacturers provide a variety of discharge designs for disk-stack centrifuges. Selection is often made on the basis of personal preference. In choosing a design, it is worthwhile considering whether the discharge system is prone to clogging at high slurry concentrations. In this respect, split-bowl discharge systems offer advantages over nozzle-based systems. Machines capable of partial discharge are also preferable, as a high slurry concentration is achieved with minimal discharge of the aqueous phase containing soluble contaminants and/or wash chemicals.

4.2. Is an Explicit Measure of Density Required?

2. In the above procedure, density is required explicitly to predict IB recovery and debris fractionation. However, measurement of density is laborious and prone to

error. Fortunately, explicit density estimation is not required if grade efficiency curves and size distributions are determined using CDS for inclusion bodies and CSA for debris particles. For inclusion bodies, CDS gives a plot of absorbance *A(t)* vs sedimentation time *t*. Absorbance is related to the concentration of particles in the sample, whereas time is related to particle size and density according to **EQ. 8**:

$$d^2\Delta\rho = 18\ \mu \ln (r_d/r_o)/t\omega^2 \tag{8}$$

where ω is the CDS rotational speed (rad/s), μ is spin-fluid viscosity (Pa.s), and r_d and r_o are the detector and start radii of the instrument (m) ***(10)***. The plot of *A(t)* may, therefore, be converted into a plot of $A(d^2\Delta\rho)$. An experimental grade-efficiency curve may then be defined by analogy with **Eq. 1**:

$$f(d^2\Delta\rho) = 1 - A_L(d^2\Delta\rho)/A_o(d^2\Delta\rho) \tag{9}$$

The experimental data may be fitted to a modified form of **Eq. 2**:

$$f(d^2\Delta\rho) = 1 - \exp\{-k[d^2\Delta\rho/(18\ \mu Q/g\Sigma)]^{n/2}\} \tag{10}$$

Determination of *k* and *n* may thus be conducted without reference to absolute density, simply by working in the domain of settling velocity (i.e., $d^2\Delta\rho$). Similar arguments apply for cell-debris distributions determined by CSA ***(6)***. CSA generates $d^2\Delta\rho$ data by sedimentation, that is then converted into *d* data for presentation reasons, using an assumed density. By truncating the analysis at the $d^2\Delta\rho$ level and avoiding assumptions regarding density, calculations may be conducted as described above for IBs. It should be noted that in both of the above cases an explicit density is not required as size analysis was done by sedimentation methods, thus providing an estimate of settling velocity. As the centrifuge operates by sedimentation, the results from size analysis are directly applicable. When other sizing methods such as Electrical Zone Sensing and Photon Correlation Spectroscopy are employed (*see* Chapter 2 in this volume), then an explicit density is required for **Eq. 2** as this information is not inherent in the analysis. Sizes determined by these methods are also prone to error when employed in sedimentation equations because of subtle shape and surface-roughness effects that are not detected by the analytical sizing method.

4.3. Achieving Adequate Purity

3. Contaminants may be broadly classed as soluble contaminants in the centrifuge feed (i.e., in the homogenate), particulate debris that cosediments with the IBs during centrifugation, contaminants adhering to the IB surface, and those incorporated into the IB proper. Some soluble contaminant invariably remains after a single centrifuge pass, as the centrifuge discharges a sludge or slurry of solid IBs and debris suspended in the original liquor (with contaminants), but at higher solids concentration. Resuspension of this slurry in buffer and recentrifugation readily removes nonassociated soluble contaminants, effectively by dilution.
4. Fractionation of the IBs from particulate cell debris is dependent on the settling-velocity distributions. Fractionation is optimized by having heavy, large IBs and

small cellular debris, with minimal overlap of the distributions. Poor fractionation is achieved when the IBs and debris have similar sizes, with considerable overlap of the size distributions. **Eq. 6** predicts that the mean size of the debris particles decreases as the number of homogenizer passes *N* increases. Additional homogenization beyond that required for complete cell disruption can, therefore, have a positive benefit on the removal of cell debris during centrifugation. Note that residual particulate debris will often have an adverse affect on downstream units such as packed columns. The product may also be sensitive to outer-membrane proteases associated with cellular debris that can significantly reduce protein yields during dissolution and refolding ***(7,11)***.

5. Contaminants adhering to the surface of the IBs, if detrimental to downstream operations, can be removed by selective washing. In this approach, the IBs are first collected to wash away soluble contaminants. The slurry is resuspended in buffer containing an appropriate wash chemical, and allowed to react. The IBs are then collected and washed with buffer by repeated centrifugation until adequate removal of the chemical is achieved. A variety of wash chemicals are available, as reviewed at small scale by Fischer et al. ***(2)*** The detergent Triton X-100 (0.1%–4%) and low concentrations of denaturant such as 2 *M* urea are common choices. The use of a chemical wash also has the added benefit of solubilizing particulate debris to some extent, thus facilitating debris fractionation for small IBs. Note that the use of wash chemicals is common practice at small scale, but can substantially increase process cost at large scale. Overall economic benefit must be clearly indicated. As centrifugation is a physical separation method, it is not able to remove contaminants incorporated into the IB (except by partial dissolution of the structure, using chaotropes or detergents). Such contaminants must generally be removed downstream.

4.4. On-Line Estimation of Inclusion Body Loss

6. A key problem with the procedure above is that real-time monitoring of IB loss to the supernatant is not achieved. Microscopic observation by an operator is required. To overcome this deficiency, an on-line control method based on supernatant turbidity has been developed ***(12)***. The ratio of absorbance at 600 nm to that at 400 nm was shown to be a good correlator of the amount of IB material in the centrifuge supernatant. This empirical approach will require definition of the exact correlation for each specific feed material, and sensitivity to subtle changes in feed properties such as IB size (e.g., during fermentation) were not defined. Nevertheless, the simplicity of the method makes it appealing for on-line control of centrifuge feed rate.

4.5. Grade Efficiency and Machine Scale-Down

7. An accurate grade efficiency curve (i.e., parameters k and n) is required for estimation of fractional collection. Performance is very machine-specific, even for a given machine type, owing to subtle variations in parameters such as interdisk spacing. It is, therefore, desirable to characterize the specific machine being

employed. Limited material is often available for such characterization, so scale-down procedures are valuable. A detailed experimental study of the scale-down of a Westfalia BSB-7 centrifuge has been completed using polyvinylacetate emulsion *(9)*. By removing active disks from the centrifuge, it was shown that the centrifuge could be scaled down to 10% of its nominal separation area without significantly altering the grade-efficiency curve at high collection efficiencies. Accurate prediction of the parameters in **Eq. 2** is, therefore, possible at full scale using this approach, provided that the aim is to collect the majority of IBs (as is the norm).

4.6. Empirical Equations

8. In some cases insufficient information may be available to estimate centrifuge feed rate using the above relationships. In such cases empirical relationships provide a possible first estimate of centrifuge performance. The recovery of porcine somatotropin IBs in a Westfalia SB-7 centrifuge ($\Sigma = 7272$ m^2) has been described using the following equation:

$$f = (-0.00516\, Q/60 + 0.0636)\, \nu_g/\mu + (0.4857 - 0.1116\, Q/60) \quad (11)$$

where μ is the homogenate viscosity in cP, Q is the centrifuge feed rate in *L/h*, and ν_g is the Stokes settling velocity of the IBs (nm/s):

$$\nu_g = d^2 \Delta\rho g / 18\, \mu \quad (12)$$

Converting to standard SI units and normalizing the centrifuge feed rate gives:

$$f = (-2.25 \times 10^6\, Q/\Sigma + 0.0636)\, (\nu_g \times 10^6/\mu) + (0.486 - 48.7 \times 10^6\, Q/\Sigma) \quad (13)$$

The above correlations were determined for porcine somatotropin IBs of median diameter 0.41 µm and density 1260 kg/m^3, with limited data ($2.3 \times 10^{-9} < Q/\Sigma$ (m/s) $< 6.9 \times 10^{-9}$, $\mu = 2.85$ cP, $5 < \nu_g$ (nm/s) < 15). For $\mu = 2.85$ Pa.s, $d = 0.41$ µm, and $\rho = 1260$ kg/m^3, **Eq. 13** becomes:

$$f = 1.0 - 67.5 \times 10^6\, Q/\Sigma \quad (14)$$

This gives a simple relationship between fractional collection efficiency and normalized centrifuge feed rate for the specific IBs used in the study. It is instructive to compare equation (14) with a study on the collection of prochymosin IBs in a Westfalia CSA-8 centrifuge ($\Sigma = 10{,}600$ m^2). The prochymosin IBs had a median diameter of 0.94 mm and a density of approximately 1160 kg/m^3. Viscosity was not stated. The data for overall inclusion body recovery may be adequately described by the following relationship:

$$f = 1.0 - 12.1 \times 10^6\, (Q/\Sigma) \quad (15)$$

Eq. 14 and **15** clearly have different gradients. This will mainly be because of differences in homogenate viscosity and inclusion body size, as effects of differing centrifuge size are incorporated simplistically by normalizing the centrifuge flow rate Q by effective settling area Σ. This demonstrates the importance of accurate information on centrifuge performance and feed properties.

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6

Application of Density Gradient Ultracentrifugation Using Zonal Rotors in the Large-Scale Purification of Biomolecules

Mohamed A. Desai and Sandra P. Merino

1. Introduction

Density gradient ultracentrifugation has been widely used to fractionate animal, plant, and bacterial cells, viral particles, lysosomes, membranes, and macromolecules in a range of processes. Its application has been of particular significance in the commercial preparation of viruses for vaccine and immunotherapy products in both batch and continuous-flow zonal modes. These methods have been traditionally used to purify influenza vaccines ***(1)***, but more recently have been used in devising new vaccine purifications such as Hepatitis B ***(2)*** and Rabies ***(3)***.

Traditional preparation of vaccines, e.g., influenza vaccine has improved 10-fold by using ultracentrifugation techniques which increase the efficiency of immunization by allowing higher dosage forms to be implemented. These methods have given superior purifications than other conventional techniques, often with one-step purification being sufficient to obtain the desired purity.

Although ultracentrifugation has been largely superceded by significant advances in chromatography techniques for a typical commercial purification process, however, density gradient ultracentrifugation still enables sufficient and rapid purification of macromolecules for initial protein characterization studies without the requirement of a lengthy process of development and optimization of a chromatography technique. Furthermore, density gradient ultracentrifugation still remains a preferred cost-effective route for the commercial separation of large particulate viruses and vaccines.

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1.1. Density Gradient Ultracentrifugation

1.1.1. Separation Principle

In order to devise a separation, some physical characteristics of the target protein need to be determined either from analytical experimentation or, more usually, determined empirically.

The knowledge of sedimentation coefficient (S_{20},w), size, and buoyant density of the target protein can be very valuable in defining the separation parameters and, hence, reduce a number of trial-and-error experiments. Otherwise, these can be estimated from preliminary separations performed subsequently.

Three basic strategies are available for the operation of density gradient ultracentrifugation: (1) to pellet the target protein to the rotor wall; (2) sediment onto a dense liquid; or (3) banding in a gradient. Pelleting is only suitable for extremely robust particles or cells whereas sedimenting enables recovery of the target protein with minimal losses resulting from denaturation. Banding in a gradient enables removal of impurities owing to high resolution, however, it requires a lengthy development and optimization to define conditions of operation.

Separation using size differential is usually described as rate-zonal centrifugation as the target protein moves from the top of the gradient downwards and bands differentially based on size. Separation based on differences in densities between proteins is termed isopycnic centrifugation. Often a gradient purification would employ both of these techniques together to give a separation, e.g., large heavy particles are normally easy to resolve from the usually smaller impurities. The separation of particulate macromolecules at small scale can be performed in a discontinuous gradient mode. However, scale-up of this would involve the use of continuous-flow mode for large volume processing.

This chapter will focus on the application of density gradient ultracentrifugation in the separation of a particulate protein using the Ti-15 Zonal rotor (Beckman Coulter Inc., Fullerton, CA) in a discontinuous gradient mode.

1.1.2. Type of Rotor Used in Zonal Ultracentrifugation

Batch-type zonal rotors are generally made of titanium with a core of 4 septa made from Noryl®. Beckman Ti-15 provides a total capacity of 1675 mL, which may be too large for laboratory preparations, but is a suitable scale for pilot operations. **Figure 1A** shows the typical rotor format and **Fig. 1B** details the parts of the seal assembly used for loading the rotor.

The rotor is loaded through ports at the top via a removable seal assembly. A pump is used to introduce gradient material and samples to the rotor. Dense material is loaded in the same direction to unload postcentrifugation. Dynamic loading and unloading allows sample removal while enabling sharp product peaks to be achieved.

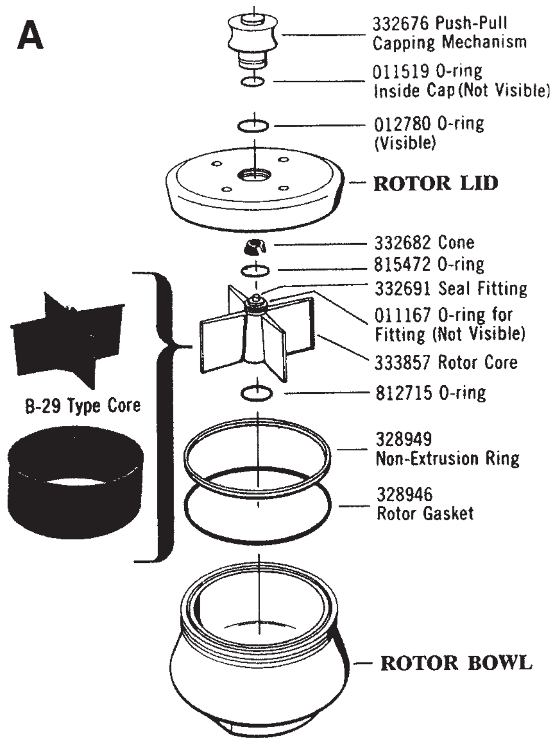


Fig. 1A. Typical rotor format for a batch type Ti-15 zonal rotor (Beckman).

1.2. Gradient Types

Density gradient ultracentrifugation as a process step can be used for concentration as well as purification of the target product. Initially, a dilute fermenter stream is concentrated (e.g., by ultrafiltration) and sample loaded to the rotor (at 50–80% v/v) with a dense cushion of gradient material (CsCl, sucrose, and the like) underneath to sediment the product. The main purpose of this is to remove impurity proteins of lower density.

A second gradient would involve the loading of selected product fractions from the first gradient (after increasing the density of the sample) at the bottom of the gradient and cause the target protein to float upward removed from the denser impurities. Depending on the protein load to this "flotation" gradient, it is possible to increase the purity of the product significantly.

1.3. Gradient Materials

The choice of gradient material depends on the product and impurity stabilities and densities. Commonly used ionic matrices are alkali metals, e.g., cae-

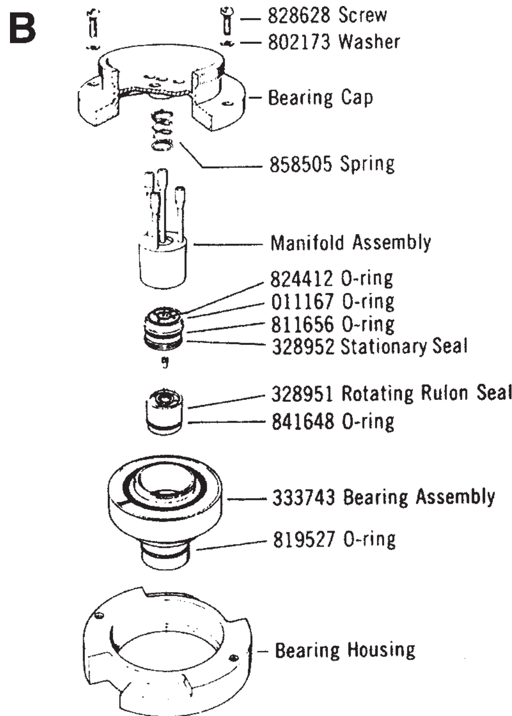


Fig. 1B. Representative parts of a Seal Assembly for use with the Ti-15 Zonal Rotor (Beckman).

sium chloride, potassium tartrate ***(4)***, and potassium bromide ***(5)***, which create high densities with low viscosity, although they are all corrosive.

CsCl is frequently used and can achieve high density (up to 1.9 g/cm^3), but can denature certain proteins. CsCl has the further drawback of cost and corrosive nature in that it is unsuitable for use with aluminium rotors and can corrode the steel of the seal assembly used with the Ti-15 Zonal rotor (Beckman).

Potassium bromide can reach high densities, but only at elevated temperatures, e.g., 25°C, which may be incompatible with stability of proteins.

Sucrose is more widely used as it is a cheaper gradient material and covers sufficient density range for most operations (up to 1.3 g/cm^3) and the viscosity allows formation of step gradient used for banding product in a narrow and sharp peak. The high osmotic potential of sucrose gradients, though, can be damaging to some proteins and should be tested before use. The viscosity also means that it is preferable to heat the higher densities, e.g., 37°C when unloading (as the displacement layer may be as high as 46% w/v) to prevent back pressure problems.

1.4. Discontinuous Gradients

Loading the gradient as discontinuous steps or as linear gradients enables the use of preformed gradient without having extended run times needed to form the gradient. The reduced run time of the separation may be useful for sensitive samples or small particulate proteins, which need longer run time to sediment sufficiently to resolve impurities. Loading discontinuous gradients may give a steeper gradient, which provides a better separation than a linear gradient. For batch-zonal operations on a routine basis, the loading of discontinuous step gradients is a simple technique to operate with a high degree of reproducibility.

1.5. Determination of Run Parameters

1.5.1. Theoretical Run Time

Theoretical run time can be determined for a rotor using the following equation:

$$t = k / S_{20},w \quad (1)$$

here, t is time (h), k is rotor efficiency, and S_{20},w is the sedimentation coefficient. This determination will indicate the minimum run time for a rotor at a specific K factor (speed dependant) required to ensure theoretical completion of product banding.

1.5.2. Rotor Efficiency

The efficiency of rotor performance is expressed as K factor and gives an estimate of the time required to band a product of known sedimentation coefficient at a set rotor speed. The efficiency *(K)* is usually supplied with the rotor handbook, but can also be determined by using the following equation:

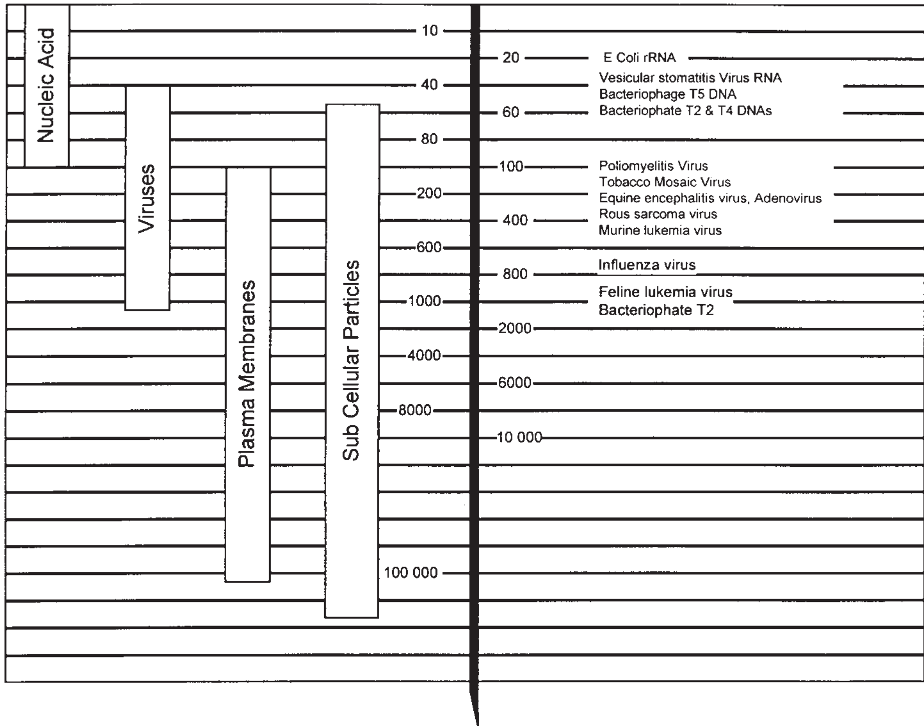
$$K = \ln \frac{(r_{max})}{r_{min}} \cdot \frac{2.53 \times w^2 \times 10^{11}}{Q^2} \quad (2)$$

where w is equal to 0.10472 × revolutions per minute (rpm), r_{max} is the maximum radial distance from the center of rotation (cm), and r_{min} is the minimum radial distance from the center of rotation (cm).

1.5.3. Sedimentation of Sample

The sedimentation coefficient (S_{20},w) of numerous particulate proteins and macromolecules are known and have been described in the literature for selected particles (*see* **Table 1**). Particulate proteins will tend to fall in the range of small viruses 40S–1500S. For a protein of interest, it is useful to obtain this information to calculate the separation time. However, it is not essential to determine S_{20},w and, therefore, this should not stop the determination of operating conditions empirically.

Table 1
Sedimentation Coefficients (in svedbergs) for Select Particulate Protein



1.5.4. Rotor Transfer

If rotor efficiency (*k* factor) and the run time of a tube rotor is known from a successful separation, then if the *k* of the zonal rotor is also known, the run time of the zonal rotor can be determined without the need to calculate S_{20},w as below:

$$t_1 = k_1 \times t_2/k_2 \tag{3}$$

where k_2 is the efficiency of rotor A; t_2 is the run time of rotor A; K_1 is the efficiency of rotor B; and t_1; is the run time of rotor B.

1.5.5. Derating of Rotors for Density and Recalculation of *K* Factor

With the batch-type zonal rotors, which are wide and squat, there is a need to down rate the maximum run speed if density materials are to be used that exceeds 1.2 g/cm^3 at the edge of the rotor. If a higher density is used at top speed, there is potential for the gradient materials crystalizing and damaging the rotor-bowl surface. Therefore, the run speed is decreased according the formula shown as follows:

$$\text{rpm} = \text{max speed} \times \sqrt{}\,(1.2\,/\rho_{max}) \tag{4}$$

where rpm is revolutions per minute and ρ_{max} is the maximum density of solution being used (g/cm^3).

This reduced speed decreases the *K* factor of the rotor accordingly, which can be recalculated below:

$$K_{new} = K\,(Q_{max}/Q_{new})^2 \quad (5)$$

Q_{max} is the rotor maximum speed (rpm) and Q_{new} is the new rotor speed (rpm).

2. Materials

The materials and reagents outlined here are those related to the application involving a Ti-15 zonal rotor (Beckman) for the purification of a particulate protein in a discontinuous gradient mode.

Feed stock solution containing the product from cell-culture supernatant or fermentation broth. This should preferably be concentrated 10–100-fold prior to loading (*see* **Note 1**). Intracellular proteins may show a tendency to associate with impurities and, therefore, influence the gradients. This is minimized by pretreatment of the sample with detergents prior to centrifugation (*see* **Note 2**).

2.1. Physiological Buffers

1. 10–50 m*M* PBS pH 7.0–7.7.
2. 10–50 m*M*-Tris HCl pH 8.0.

2.2. Density Gradient Material

1. CsCl stock 1.6 g/cm^3.

These are used to create the discontinuous layers of the gradient, e.g., CsCl at 1.1, 1.2, 1.3, and 1.4 g/cm^3 densities.

2.3. Equipment

1. Zonal rotor Ti-15 (Beckman) with a seal assembly.
2. Ultracentrifuge Model L8 type fitted with zonal rotor band.
3. Peristaltic pump to deliver 20–50 mL/min.
4. Tubing rig (3 mm ID) to deliver sample to the rotor via a bubble trap and to collect fractions as set up in **Fig. 2a** for loading and **Fig. 2b** for unloading.
5. Abbe 60 Refractometer (Bellingham Stanley, Kent, UK) or density meter (DMA 38 Parr Instrument Co., Moline, IL).
6. Spectrophotometer to measure absorbance at 280 nm.
7. Bench-top centrifuge (low speed) for clarification.

3. Methods

3.1. Determination of Run Parameters

Some key operational parameters will need to be established prior to starting the ultracentrifuge run. These include the following:

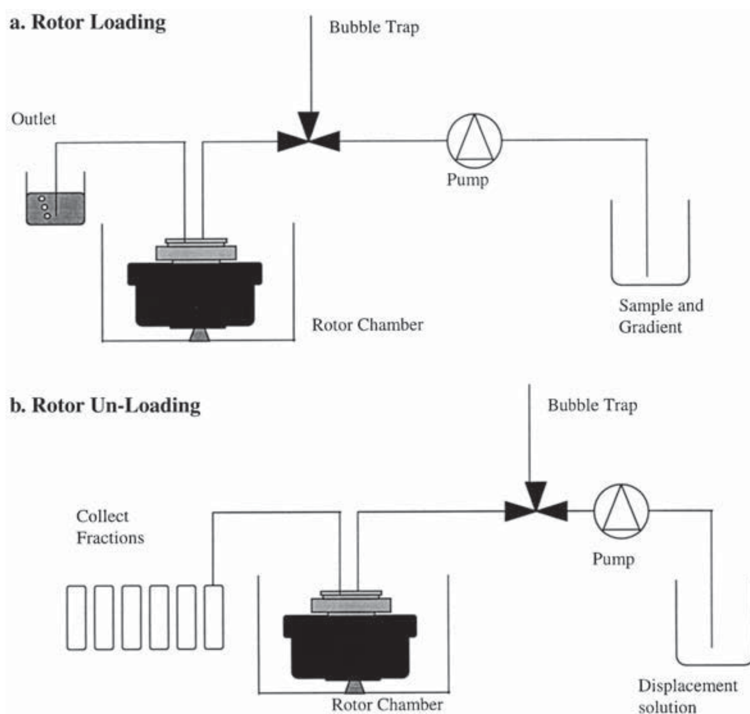


Fig. 2. Tubing set up for loading **(a)** and unloading **(b)** of a Ti-15 zonal rotor (Beckman) during purification of particulate proteins.

3.1.1. Run temperature

The operating temperature will depend on the stability of product over the run time and is typically 20°C for shorter runs, but may require operation at 4–10°C for longer runs (*see* **Note 3**).

3.1.2. Theoretical Run Time

The run time can be determined from the sedimentation coefficient of the protein and the known efficiency of the rotor. If this is not known, the most convenient times may be 20–24 h run for a smaller protein and 4–8 h run for a large protein (*see* **Note 4**).

3.1.3. g*-Force*

The operating *g*-force can be determined to give the required run time depending on the target protein. Lower *g*-force will lead to longer run time and, for convenience, the maximum the centrifuge *g*-force should be used (after downrating for the use of higher density material).

For the Ti-15 rotor, the *K* value at top speed (102,000*g*) is 481. For example, a 50S protein would take 9 h, 37 min to band. If a gradient is set up with a maximum density of 1.4 g/cm^3 then the top speed available would be 74,752*g* and the K_{new} for 74,752*g* would be 656, which for a 50S protein would increase the run time to 13 h, 7 min. Therefore, a convenient run time and operating conditions for a Ti-15 rotor would be overnight for 18 h at 72,585*g* and at 10°C.

3.1.4. Fraction Collection

This is most conveniently set at the collection of 50–100 mL fractions when the Ti-15 zonal rotor is used generating 17–34 fractions from a full rotor volume to determine the position of the product in the separation profile (*see* **Note 5**).

3.2. Preparation and Loading the Zonal Rotor

3.2.1. Rotor Assembly

Operation of the centrifuge and assembly of rotor and seal components should be according to the manufacturer's instructions (shown in **Figs. 1a** and **1b**), which detail all the precautions involved to ensure good performance of the ultracentrifuge ***(6)***. Complete a test run on the assembled rotor, e.g., run a dry cycle then run up to 9957*g* for 10 min. Damage to the seal and subsequent leakage can be prevented by taking the precautions indicated in the instructions (*see* **Note 6**).

3.2.2. Loading of Rotor

The density gradient material (CsCl) should be loaded to the rotor when it is spinning at 398*g* via the tubing assembly and rotating seal (*see* **Figs. 1** and **2**). The inlet is placed in the density gradient material and solutions are loaded to the rotor-edge port using a peristaltic pump. The outlet is placed in a pot of buffer so air displaced is seen bubbling out (**Fig. 2a**).

The peristaltic pump is set to deliver gradient materials at between 30–50 mL/min. Loading starts with the lightest density first, followed by increasing density layers, ending with a heavy cushion of material as the last material loaded. Two examples of gradient separations using CsCl are provided in **Table 2**:

3.2.2.1. Product Concentration

Sample is loaded as the majority of the rotor contents at 50–80% v/v with a cushion of high density CsCl 1.4–1.6 g/cm^3 underneath, which enables the product to sediment on to the cushion where a narrow band of product is formed.

Table 2
Two Examples of Zonal Gradient Separations Commonly Achieved by the Ultracentrifugation Method

Product concentration		Product purification	
Density material	Percentage of rotor volume (v/v)	Density material	Percentage of rotor volume (v/v)
1.0 g/cm^3	2%	1.0 g/cm^3	2%
Sample	50–80%	1.1 g/cm^3	10%
1.4–1.6 g/cm3	20–50%	1.2–1.3 g/cm^3	15%
		1.3–1.5 g/cm^3 sample	50%
		1.6 g/cm^3	10%

3.2.2.2. Product Purification

Fractions from the concentration gradient where the protein of interest has banded (buoyant dense point) are pooled and loaded onto the product purification gradient after increasing the density of the product pool, e.g., at 1.3–1.4 g/cm^3. This means that from a denser point the protein of interest (and any other proteins that are of lower density) will move up the gradient to the top of the rotor. Larger proteins will move faster than smaller molecules.

Loading is complete when buffer is seen emerging from the center port of the seal assembly. At this point, the pump is switched off, tubing to the seal assembly is clamped, and the seal removed from the rotor top (*see* **Note 7**). When the rotor is capped then the centrifuge lid can be closed and the rotor enters the high-speed part of the run.

3.2.3. Unloading the Rotor

The centrifuge will automatically end the run according to the time set and decelerate to 398*g* where the chamber door can be opened. At this point, the seal assembly is attached and the unloading can commence. Unloading is by displacement with high-density material and it is essential to prime the lines with the high-density material before attaching the seal assembly to prevent air from entering the rotor. This displacement material should be 5–10% (w/v) denser than the last layer loaded to the rotor. Typically, 20–40 fractions are collected per rotor and stored at 4°C until required for analysis.

The seal assembly system and the zonal rotor should then be disassembled and cleaned thoroughly, including the centrifuge with Beckman 555 detergent, deionized water followed by 70% IMS before air drying in preparation for next run (*see* **Notes 8–10**).

3.3. Safety

With the use of biologically hazardous proteins or viruses, it is recommended to use the safety shield, which reduces aerosols (generated during centrifugation) from entering the lab air, also when a low-temperature run is to be undertaken the shield allows continuous cooling of the chamber without ice buildup as moisture is drawn from the lab air.

3.4. Analysis of Fractions

3.4.1. RI and Density

Gradient slope can be determined from refractive index (RI) measurements, which can be converted to densities from standard tables ***(7)***, alternatively, direct density measurements using DMA38 density meter (Parr Scientific). Comparing this data to the product peak, the buoyant dense point of the protein of interest can be determined and this information can be used to further modify the gradient to improve the purification (*see* **Note 4**).

3.4.2. Purity and identity by SDS-PAGE and Western Blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of fractions collected can be used, to determine the purity of the profile fractions using standard published procedures ***(8)***. Where a concentration gradient has been used there may be significant levels of impurity that make identification of product bands impossible. In this case, it may be useful to use Western Blot analysis of product fractions to compare product to impurity bands. The SDS-PAGE and Western Blot procedures can be followed from generic molecular biology/biochemistry text books.

Western Blot may also be useful to identify the impurity present where antibodies for the host cell line and/or media impurities are available, or where major impurities are known and can be identified from commercial antibodies, e.g., *E. coli* impurities.

3.4.3. Product Analysis

If an antibody is available against the product, a dot blot can be employed to identify the position of the product peak within the gradient profile. This can be used to give a semiquantitative analysis of the gradient performance and separation of the product.

If an enzyme-linked immunosorbent assay (ELISA) assay is available, information regarding the percentage recovery from the gradient can be determined. Generally, a product recovery of 70–90% in peak fractions would be expected from an optimized gradient separation. Lower recoveries may mean that the gradient needs to be altered to obtain a better separation.

3.5. Optimizing Separation Performance

Separation performance can be analyzed by calculating the product recovery and the clearance of impurities (in terms of total protein) to give a fold purification and a specific activity (units/mg protein) of the product pre- and postcentrifugation.

The recovery and purity will depend on the criteria used to select fractions to take to the next step. If a threshold is used where all fractions with a percentage of the peak activity are pooled, then a wide shallow peak will cause a high carry over of impurities to the next step. This can be improved by narrowing the selected fractions, but this will reduce yield.

An improvement in the gradient can be made to create a narrower zone of product by making the gradient steeper so that the product bands in fewer fractions. Alternatively, the run time can be extended if it is thought that the protein of interest has not reached its buoyant dense point. This may be the case if the gradient has been determined empirically and not through calculation from the sedimentation coefficient of the protein of interest.

For a purification gradient where the protein of interest is floated in the gradient then creating a steeper gradient will decrease the resolution of any impurity peak from a product peak. In this case, a shallower gradient will resolve these two peaks, but may cause a wider product peak unless the run time is extended to allow narrower banding of the product. Alternatively, the sample load can be decreased, which will improve resolution, but will require more runs to process the same amount of sample.

Typically, the protein of interest may not reach the desired purity using solely the centrifugation methods and, therefore, further purification steps may be needed before or after the centrifugation step(s) in an industrial process to achieve the desired purity. If large volumes are to be processed in a production process, then continuous-flow ultracentrifugation should be considered for a given product.

4. Notes

1. The target protein in feed-stock solution can be concentrated 10–100 fold using a tangential flow filtration (TFF) system, such as the Pellicon system (Millipore) or centramate (Pall) consisting of ultrafiltration membrane(s) of a suitable molecular weight cutoff.
2. Proteins extracted from cell debris may bind or associate with impurities possibly forming aggregates and sedimenting to the rotor wall and be lost from the purification. To reduce this potential loss of product, additives may be introduced to the sample and/or the gradient, e.g., 1 *M* urea or DTT during gradient separation, which are subsequently removed in later stages of the process.
3. Most of the bench to pilot scale ultracentrifuges will have a temperature-controlled rotor chamber to enable gradient runs to be performed at lower temperatures for the more labile and potentially unstable products.

4. If sufficient information about the product is not available to calculate parameters such as theoretical run times, *g*-forces, and gradient densities for the separation of a particular protein, this can be rapidly determined on a small scale swing out rotor (SW 28; Beckman) to provide estimates of these parameters.
5. The final fraction sizes can be determined after a series of optimization experiments to achieve resolution of product peak from impurities. A threshold for cutting the product peak can subsequently be established.
6. Preparation of the Rulon seal surface by polishing with crocus cloth (*see* Beckman manual) is key to the good performance of the seal assembly. Any scratches, dents, or unevenness will cause cross leakage or leaks to the rotor bowl.
7. The seal surface can be damaged usually when the tubing is removed from the ports after loading. This can cause substantial leakage problems. Removal of tubing should be done by easing the tubing off the ports using a spatula and not by pulling the tubing. It is also preferable to have a spare seal assembly.
8. Leakage from the seal assembly will cause product and gradient materials to be sprayed into the rotor chamber and affect the routine operation of the zonal rotor and the centrifuge. Spillage of the corrosive CsCl should be removed immediately and thorough cleaning of the seal assembly undertaken after every run to reduce wear on the bearing tracks to minimize excessive vibration of the seal and subsequent leakage.
9. For routine use of the zonal rotor, it is advisable to change the O-rings of the assembly system and the rotor bowl on a regular basis to prevent malfunction and subsequent gradient failures.
10. Solution (particularly CsCl) spillage in the rotor bowl and chamber will be drawn by the vacuum pump and cause deficient vacuum pool. This will affect the speed of the centrifuge and temperature control. Ensure rapid cleaning of any spillage and subsequent drying of the rotor bowl/chamber during use.

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7

Application of Chromatography in the Downstream Processing of Biomolecules

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1. Introduction

The ability to separate and purify biomolecules of interest from a range of complex and diverse biological media such as cell-culture supernatants, fermentation broths, and crude plant and animal extracts has been the cornerstone of biomolecular separation technology. The stringent regulatory requirements for specified purity levels in therapeutic products has been a challenge to the downstream processing practitioners in the biopharmaceutical industry. The increased knowledge, understanding and the development of analytical techniques for the identification of potential biologically hazardous elements and/or components within a biological system, particularly those associated with recombinant technology sources, have rightly imposed an ever more cautious approach to the use of therapeutics in humans.

This in turn has encouraged the purification scientists to design and develop better purification schemes for such products. A downstream process (or a purification scheme) must not only achieve the required purity and recovery levels of the desired product in a safe, reliable, and reproducible manner, but also do so in an economically viable and cost-effective manner.

The exponential development of sophisticated chromatography techniques and associated media/resins to address the separation and purification of virtually any bioproduct is a testimony to the effort and progress in this field.

The common strategy in a chromatographic separation process of biological macromolecules involve initial purification (or primary separation), intermediate purification, and polishing stages. Primary separation usually encompass steps such as cell disruption, clarification, and concentration depending

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on the nature and origin of the target product. Traditionally, this stage of the process has not involved the use of chromatographic separation. However, the emergence of expanded bed adsorption technology and the potential of fluidized bed adsorption is becoming more popular and is being evaluated in a range of products and processes.

Intermediate purification in a process is normally performed on clarified and/or concentrated product containing feed from the primary separation. This stage normally results in the removal of significant levels of impurities such as media components, DNA, viruses, and endotoxins. Commonly used techniques here are ion-exchange, hydrophobic interaction, and, in some cases, affinity resins. Selectivity and capacity of the chromatography media are the key prerequisites for the successful intermediate purification.

The purification process is finally completed by the polishing stage, which removes trace impurities and contaminants resulting in an active and safe product suitable for formulation. Size exclusion chromatography or gel filtration is commonly used at this stage, however, ion-exchange, hydrophobic interaction, and reversed-phase chromatography are also being used in some processes. Chromatography resins, which demonstrate very high resolution and high recoveries are generally suitable for this stage of the process.

The key element to the success of a chromatographic separation is the nature, strength, and specificity of the interactions between the covalently bound ligands on a solid phase (resin) and the target molecule in the feedstream. These interactions generally fall into several broad classes or modes of chromatography. Each mode represents a unique binding mechanism resulting from the characteristics of both the resin and the product.

This chapter will focus on the development, optimization, and scale-up of the three most commonly used chromatography modes: ion-exchange; hydrophobic interaction; and size exclusion (gel filtration). The application of these modes in the separation of biological macromolecules from complex mammalian cell culture and/or microbial fermentation broths will also be discussed.

1.1. Ion-Exchange Chromatography

The adsorption technique of ion-exchange chromatography is widely used in the purification and separation of biomolecules such as proteins, polypeptides, nucleic acids, polynucleic acids, and antibodies. The method of ion exchange is widely used in industrial purification processes as it offers binding and elution conditions that are relatively mild allowing the retention of biological activity, presenting high resolving power with high capacity, it can therefore, be applied to virtually any charged molecule that is soluble within an aqueous system. Ion-exchange chromatography separates on the basis of ionic interaction between molecules of different charge. There are two methods of separation available based upon either pH or ionic strength.

Table 1
Functional Groups Available on Ion Exchangers

Anion exchangers type	Functional group
Diethylaminoethyl (DEAE)/weak	$-O-CH_2-CH_2-N+H(CH_2CH_3)_2$
Quarternary aminoethyl (QAE)/strong	$-O-CH_2-CH_2-N+H(C_2H5)_2 CH_2-CHOH-CH_3$
Quarternary ammonium (Q)/strong	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-N^+(CH_3)_3$
Cation exchangers type	Functional group
Carboxymethyl (CM)/weak	$-O-CH_2-COO^-$
Sulphopropyl (SP)/strong	$-O-CH_2-CHOH-CH_2-O-CH_2-CH_2-CH_2SO_3^-$
Methyl sulphonate (S)/strong	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2SO_3^-$

1.1.1. Ion Exchange Matrix

The property and character of the ion exchanger are determined by the presence of the charged ligand group. The solid chromatography matrix "supports" electrically charged groups for example carboxyl or quarternary ammonium groups. To each of these "charged" groups is associated a counter-ion of opposite charge. The functional group can be either positively (anion exchange) or negatively charged (cation exchange). Associated to the charged groups are counter ions of opposite charge, which can be exchanged by ions having the same charge though greater affinity for the matrix.

Cation exchangers are used to separate negatively charged molecules whereas anion exchangers separate positively charged molecules. There are a number of different functional groups available for use on ion-exchange matrices as shown in **Table 1**.

The functional groups are classified into two different groups being either "strong" or "weak" exchangers. The definition of strong/weak refers to the effect of pH on the group and not to the strength of binding. The charge on weak exchangers is influenced by changes in pH, i.e., they are titratable with ionization only occurring above the dissociation constant. The most common weak anion exchanger, diethylaminoethyl (DEAE), loses its charge above pH 9.0 and the weak cation exchanger containing carboxymethyl groups (CM) loses charge at pH values less than 4.0. Strong ion exchangers are completely ionized over a wide pH range, therefore, maintaining their charge irrespective of pH.

Therefore, if the working conditions exceed the ranges of the weak exchanger, strong ion exchangers should be used. The properties of strong ion exchangers mean that experiments, in particular using pH elutions, can be more easily controlled.

Table 2
The Prediction of Protein Behavior in Ion-Exchange Chromatography Based on p*I* of a Protein

	Cation exchanger	Anion exchanger	Protein net charge
Above p*I*	Does not bind	Binds	Negative
Below p*I*	Binds	Does not bind	Positive

The selection of the ion-exchange matrix, whether anion or cation, strong or weak, is influenced by the nature of the molecule to be separated, effect of pH upon its charge characteristics, stability, and solubility.

1.1.2. Charge Characteristics of Biomolecules

Proteins are made up of amino acids containing ionizable side chains. Therefore, the pH of the mobile phase will affect the charge characteristic of the protein. Proteins are termed amphoteric as they carry both positive and negative charges. Generally, the molecules will be more positively charged at lower pH levels and negatively charged at higher pH values. The isoelectric point (p*I*), as determined by isoelectric focusing electrophoresis of the protein, is the pH at which the protein has "net" zero charge, i.e., where the number of negative charges equals the number of positive charges.

At neutral pH, molecules having a high p*I* (basic molecules) will be positively charged and those with a low p*I* (acidic molecules) will be negatively charged. The isoelectric point can be used to predict the behavior of a sample on an ion exchanger as shown in **Table 2**. However, the behavior is actually dependent upon the distribution of accessible charges on the surface of the molecule. Therefore, although a molecule could have a zero net charge at a certain pH, it can still show some binding characteristics because of the distribution of the charge.

1.2. Hydrophobic Interaction Chromatography

The mass, structure, and function of a protein molecule are determined by its genomic sequence and exhibited through its constituent amino acids ***(1)***. One of the major properties expressed by selected amino acids, and thus protein molecules, is that of hydrophobicity. Although elementary biochemistry suggests that such hydrophobic amino acids would spontaneously try to move away from the aqueous bulk upon protein formation, often hydrophobic residues are found in clusters on the surface of the macromolecule. It is these domains that are exploited during hydrophobic interaction chromatography (HIC) to facilitate the preferential adsorption and subsequent elution of target protein molecules.

Conventional hydrophobic interaction chromatography has found widespread use in biotechnology, both at research and commercial scale. Products such as recombinant human epidermal growth factor (h-EGF) produced from *Saccharomyces cerevesiae* and monoclonal IgG, anti-IgE produced by a murine hybridoma culture grown in a hollow fiber bioreactor ***(2)*** have successfully exploited HIC as an integral unit operation in their purification.

1.2.1. Mechanism of Hydrophobic Interaction Chromatography

On the surface of the protein molecule, the hydrophobic residues are shielded by water molecules, which prevent interaction between the nonpolar amino acids and the bulk aqueous environment. If a neutral salt, such as ammonium sulphate, is added to the aqueous solution, the surface tension of the liquid phase will increase and the water molecules shielding the protein surface will move away from the interface and into the bulk environment. The nonpolar residues on the surface of the molecule can therefore, express their hydrophobic properties.

A protein can be encouraged to adsorb onto a hydrophobic adsorbent by increasing the neutral salt concentration in the aqueous environment. The chromatographic adsorbent is washed in high salt buffer to remove any unbound, or loosely bound, proteinaceous material from the matrix. As the concentration of the neutral salt is lowered, the surface tension of the liquid phase decreases accordingly resulting in the eventual dissociation of the product from the matrix. After each cycle, bound substances are washed out from the column and regenerated with sanitizing agent such as NaOH or 70% ethanol followed by distilled water. **Figure 1** shows an example of a typical hydrophobic-interaction chromatography profile depicting hydrophobic adsorption of the target product with subsequent elution by a reduction in neutral salt concentration.

The concentration of ligands immobilized on to an HIC matrix, i.e., the degree of substitution generally affects the intensity of interaction between the protein of interest and the matrix. Higher substitution generally results in an increase in the binding capacity of the matrix. However, the binding capacity will reach a plateau above which the degree of substitution has no influence as indicated by **Fig. 2**.

1.3. Size-Exclusion Chromatography

Size-exclusion chromatography (SEC), commonly referred to as gel permeation or gel filtration, separates proteins according to their effective molecular size. It is a popular laboratory method normally used to purify small samples of proteins on size differences, for determining the molecular weight estimation of native and other forms of the protein of interest, molecular weight distribution of polymers, determination of equilibrium constants, and desalting

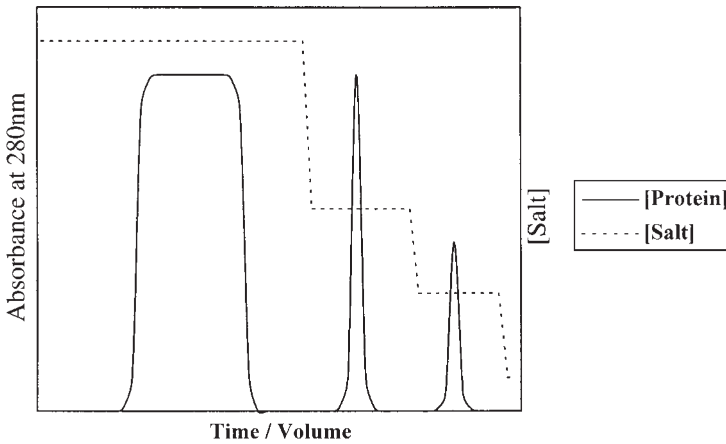

Fig. 1. A typical separation profile obtained during purification of biomolecules using hydrophobic-interaction chromatography.

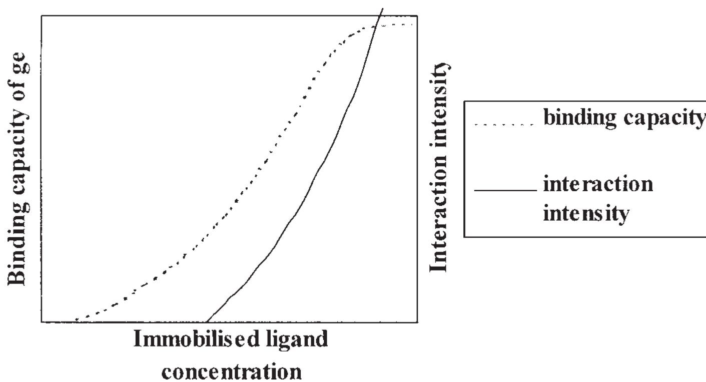

Fig. 2 . Graphical description of the effect of increasing ligand concentration upon the binding capacity of the HIC matrix and the strength of interaction between the matrix and the protein.

(3). SEC is a relatively low resolution-separation technique capable of processing only small sample volumes (1–5% of column volume) and, therefore, would require very large columns at production-scale operation.

However, it is an excellent alternative to diafiltration or buffer exchange and removing salts and low molecular-weight impurities. The sample volume of 10–30% of the column volume can be processed in this manner. Its use is, therefore, confined to late-stage purification steps normally used as "polishing steps" in a production process where the protein of interest is available at gen-

erally high concentrations prior to formulation. Generally, SEC can be used to separate proteins in the range 5–1000 kDa.

1.3.1. Principle of SEC

SEC method depends on the ability of a molecule to penetrate the porous solvated particles of the stationary phase (gel matrix). The smaller the molecular size, the more volume it will have available for distribution and more frequently it will enter through the pores of the column matrix. These pores are usually of a defined diameter and will exclude molecules that are larger than the pores and, therefore, stay in the void volume of the gel. The elution is thus in the descending order of molecular size, the larger proteins having the least volume available, elute first, and the smallest proteins last. When a sample is applied to a packed column, the largest molecules will be fully excluded from the pores and eluted with the mobile phase (eluting buffer) in the retention volume. Small molecules will diffuse into the pores and will be eluted in total bed volume. Intermediate-size molecules will be eluted in between as demonstrated by the schematic representation of SEC in **Fig. 3**.

The principle feature of SEC is its inability to interact with the sample, enabling high retention of biomolecular activity while separating the impurities. Resolution in SEC generally depends on matrix particle size, pore size, flow rate, column length, diameter of product, and the sample volume.

2. Materials

2.1. Ion Exchange Chromatography

1. Anion exchange matrices: Mono Q (Amersham-Pharmacia Biotech, Sweden); DEAE Cellulose (DE 52) (Whatman, Kent, UK); POROS HQ (Perkin-Elmer Biosystems, Norwalk, CT).
2. Cation exchange matrices: Mono S (Amersham-Pharmacia Biotech, Uppsala, Sweden); QA Cellulose QA 52 (Whatman); POROS HS (Perkin-Elmer Biosystems).
3. Liquid chromatography columns either C-series or XK-series in a range of sizes depending on the scale of operation (Amersham-Pharmacia Biotech).
4. Feed-stock solution containing the target protein dialyzed against the appropriate loading buffer to be used (*see* **Note 1**).
5. Liquid-chromatography system comprising of pump(s), gradient mixer, ultraviolet (UV) monitor, chart recorder, fraction collector, conductivity meter, and a pH monitor, e.g., an FPLC system (Amersham-Pharmacia Biotech) or similar.
6. UV spectrophotometer for absorbance measurements at 280 nm.
7. Equilibration/loading buffers: 20 m*M* Tris-HCl + 150 m*M* NaCl pH 8.0; phosphate buffered saline pH 7.4 (PBS); 20 m*M* Bis-Tris pH 6.0.
8. Elution buffers: 20 m*M* Tris-HCl + 1 *M* NaCl pH 8.0; phosphate buffered saline (PBS) + 1 *M* NaCl pH 7.4; 20 m*M* Bis-Tris + 1 *M* NaCl pH 6.0.
9. Regeneration buffers: 0.5 *M* NaOH.

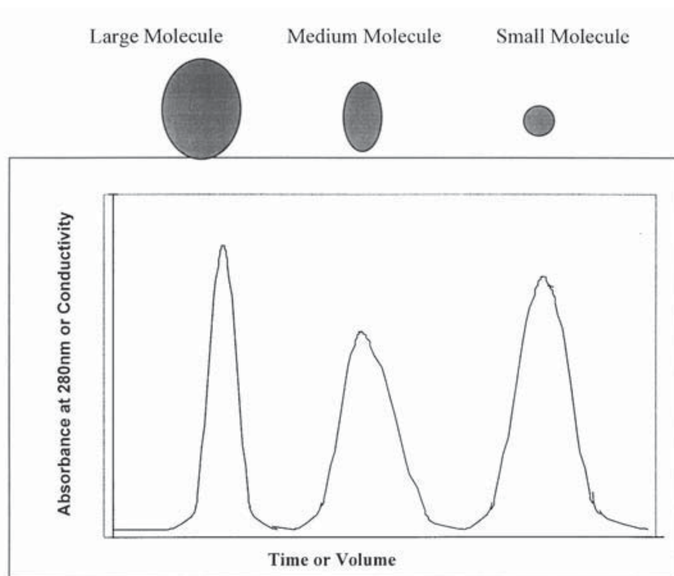

Fig. 3. A Typical size-exclusion chromatography profile.

2.2. Hydrophobic-Interaction Chromatography

1. HIC matrices: Ready-packed HIC Media Test Kit (Amersham-Pharmacia Biotech); Toyopearl HIC Kit (TosoHaas); Hydrophobic Media Kit (Sigma Chemical Co., St. Louis, MO) Alternatively, bulk HIC media is available from the suppliers for in-house packing of columns for operation at various scales. **Table 3** demonstrates the physical and chemical comparison of the ligand chemistry on the commercially available HIC media.
2. Columns: Liquid chromatography columns either C-series or XK-series (Amersham-Pharmacia Biotech.) if packing own columns of various sizes depending on scale of operation. Otherwise, the HIC kits mentioned under matrices are prepacked ready-to-use columns.
3. Equilibration/loading buffers: 20 m*M* sodium phosphate pH 7.0 and 1–3 *M* sodium chloride; 20 m*M* sodium phosphate pH 7.0 and 1–3 *M* ammonium sulphate; 10 m*M* Tris-HCl pH 8.0 and 1–3 *M* ammonium sulphate.
4. Elution buffers: 20 m*M* sodium phosphate pH 7.0; 10 m*M* Tris-HCl pH 8.0.
5. Regeneration buffer: 0.5–1.0 *M* NaOH, distilled water, 70% ethanol.
6. Sample: Feed-stock solution from mammalian cell culture or fermentation broth containing the target protein (*see* **Note 1** and sample preparation and loading in **Subheading 3.2.**).
7. Liquid-chromatography system comprising of pumps, gradient mixer, UV monitor, chart recorder, fraction collector, conductivity meter, and a pH monitor, e.g., an FPLC system from Amersham-Pharmacia Biotech.

Table 3
A Physical and Chemical Comparison of the Ligand Chemistry on the Commercially Available Hydrophobic Matrices

Solid phase adsorbent	Supplier	Nominal particle size	Base matrix
Phenyl Sepharose high performance	Amersham Pharmacia	34 µm	Agarose
Phenyl Sepharose 6 fast flow (low sub)	Amersham Pharmacia	90 µm	Agarose
Macro-Prep Methyl HIC support	Bio-Rad	50 µm	Methacrylate
Butyl 650-S	TosoHaas	35 µm	Methacrylate
Fractogel® EMD Propyl 650S	Merck	30 µm	Methacrylate
Hexyl agarose	Affinity Chromatography Ltd.	110 µm	Agarose
Octyl cellufine	Millipore	90 µm	Cellulose bead
Poros PE (phenyl ether)	Perkin Elmer Biosystems	20 µm	Cross-linked poly(styrene-divinylbenzene)

8. A UV spectrophotometer for absorbance measurements at 280 nm.
9. A product-specific assay for quantitative recovery of product (*see* **Note 12**).

2.3. Size-Exclusion Chromatography

1. Matrices: Range of Sephacryl, Superdex, Superose, Sephadex, and Sepharose SEC media (Amersham-Pharmacia Biotech), having correct fractionation range for separation of target protein; a range of TSK-gel media (TosoHaas); and Fractogel-EMD BioSEC range (Merck).
2. Columns: Liquid chromatography columns either of C-series or XK-series (Amersham-Pharmacia Biotech) for packing own columns of appropriate dimensions depending on scale of operation (*see* **Note 26**).
3. Buffers (*see* **Note 25**). Packing and equilibration buffers: Deionized water (Milli-Q or similar [Millipore]); 20 m*M* phosphate buffered saline pH 7.2 (PBS); 1 *M* NaCl and 0.1–0.5 *M* NaOH. Elution buffers: 20 m*M* phosphate-buffered saline pH 7.2 (PBS); 20 m*M* sodium bicarbonate pH 8.0 + 150 m*M* NaCl; 20 m*M* Tris-HCl pH 8.0 + 150 m*M* NaCl. These can be used with organic solvents or detergents for optimized separation such as 1 *M* Urea or 1–2% SDS. Regeneration buffers: 1 *M* NaCl with or without organic solvents or detergents. Alternatively, 0.1–0.5 *M* NaOH solution.

4. Sample: Feed-stock solution from mammalian cell culture or fermentation brothor more commonly, process intermediate containing the target protein (*see* **Note 1**).
5. Liquid chromatography system comprising of pumps, gradient mixer, UV monitor, chart recorder, fraction collector, conductivity meter, and a pH monitor, e.g., an FPLC system from Amersham-Pharmacia Biotech.
6. Helium or nitrogen line for degassing buffers or 0.2 μm filters.
7. UV spectrophotometer for manual absorbance measurements at 280 nm.
8. A product-specific assay for quantitative recovery of product (*see* **Note 12**).

3. Methods

3.1. Ion-Exchange Chromatography

There are five key stages to the operation of ion-exchange chromatography:

1. Determination of starting point.
2. Matrix preparation and column packing.
3. Sample preparation and loading.
4. Sample elution and matrix regeneration.
5. Analysis.

3.1.1. Determining the Starting Point

Establish the p*I* of the molecule to be separated from lists of p*I* for proteins in the literature ***(4,5)*** and the pH range over which it is stable. If the molecule is stable below its p*I*, then use a cation exchanger and if stable above the p*I* then use an anion exchanger (*see* **Table 2**). Both cation and anion exchangers can be used if the molecule is stable over a wide pH range (*see* **Note 2**). The choice of whether a strong or a weak exchanger is used is dependent on the pH conditions of the operation as weak exchangers lose the charge above their dissociation constants.

3.1.2. Matrix Preparation and Column Packing

Ion-exchange matrices are often supplied as either prepacked columns (Mono Q, Mono S) or as preswollen matrix (DEAE Sephacel: Amersham-Pharmacia Biotech). However, some are supplied as a dry powder (e.g., Sephadex ion exchangers: Amersham-Pharmacia Biotech.) requiring swelling.

3.1.2.1. Dry Matrix

Matrices supplied as a dry powder should be preswollen using the start buffer of choice. Swelling can take between 2–48 h dependent upon the matrix. Swelling, for example, of the Pharmacia matrix Sephadex will take 24–48 h at room temperature or 2 h in a boiling water bath (*see* **Note 3**). Prepare a slurry with starting buffer and pack column according to manufacturers instructions and check the packing efficiency of the column (*see* **Note 4**). Once packed, equilibrate the matrix with 5–10 column volumes of the loading buffer.

3.1.2.2. Preswollen

Decant off the supernatant and replace it with loading buffer to generate a 75% slurry. Pour carefully into column using a glass rod as a guide to prevent trapping of air bubbles. Pack at flow rate and pressure as recommended by the supplier. Check the packing efficiency of the column (*see* **Note 4**). Equilibrate column with loading buffer with 5 to 10 column volumes (*see* **Note 5**).

3.1.2.3. Prepacked Columns

Equilibrate the column thoroughly with the loading buffer, e.g., 20 *mM* Tris-HCl pH 8.0 (minimum 5–10 column volumes) to remove the storage buffer, usually 20% ethanol, before the application of the sample.

3.1.3. Sample Preparation and Loading

1. To ensure that the sample binds to the selected matrix, preparation of the sample is required. The sample should be dialyzed against the loading buffer of choice (see Note 6). Check the pH and conductivity of the sample to show comparability to that of the start buffer. On completion of dialysis either filter or centrifuge the sample to remove any particulate matter.
2. The sample is loaded in the appropriate conditions of pH and ionic strength usually at a lower flow rate of approx 15–30 cm/h to cause the binding of the target molecule to the matrix. Under these conditions the target protein has a greater affinity for the matrix ligands displacing the associated counterions. The unbound impurities will breakthrough and result in an increase in absorbance measurements.
3. When the product has been loaded, continue washing the column with approximately 5 column volumes (see Note 7) of the loading buffer to elute any proteins that have no affinity for the matrix. These proteins will pass straight through the column and absorbance (280 nm) peak will be observed. Collect this material into fractions for analysis.

3.1.4. Sample Elution and Matrix Regeneration

The aim of elution is to displace the target molecule from the matrix with high resolution, separated distinctly from the other components considered as impurities. There are three common methods of elution during ion-exchange chromatography:

1. Isocratic (*see* **Note 8**).
2. Linear gradient.
3. Step gradient.

The linear and step gradients are the most widely used elution techniques. Linear gradients are most frequently used in the development and optimization of an ion-exchange method where the pH or ionic strength is increased con-

tinuously and gradually over time (approximately 5–10 column volumes). Step gradients are most commonly used in manufacturing processes where the use of pH or ionic strength in elution has been optimized for large-scale operations. Such an elution method usually require up to 2–3 column volumes to quantitatively recover the product.

These two methods are the most common, however, a third method "Displacement chromatography" ***(6,7)*** can be used where the target molecule is displaced with another protein, however, this method results in the requirement of removing the displacement molecule, which would be considered to be an impurity. Protocol here describes the step gradient elution.

3.1.4.1. The elution is effected by either a change in pH or by increasing the ionic strength using a buffer containing a higher salt concentration (e.g., Tris-HCl pH 8.0 and 1 *M* NaCl). Elution by increased ionic strength (the most commonly used form of elution) causes a reduction in the affinity of the molecule to the matrix in relation to the ions present within the buffer (*see* **Note 9**). The linear elution through change in ionic strength is the simplest to control (*see* **Note 10**).

3.1.4.2. As the protein is eluted from the column a 280 nm absorbing peak will be observed, which is collected into fractions either manually or using a fraction collector, e.g., Frac 100 as supplied by Amersham-Pharmacia Biotech.

3.1.4.3. The ion-exchange column is regenerated to remove the strongly adsorbed protein returning the matrix to its original counterion condition. This is achieved by either a high ionic strength buffer (e.g., 20 m*M* Tris-HCl pH 8.0 + 1 *M* NaCl) or by an alkali (0.5 *M* NaOH) solution causing hydrolysis of protein followed by a high ionic strength buffer to restore the counterion. The ion-exchange column can be reused for another cycle of operation or stored for future use (*see* **Note 11**).

A typical ion-exchange purification profile showing sample loading, column washing, elution using a linear gradient, followed by regeneration is shown in **Fig. 4**.

3.1.5. Analysis of Fractions

To determine the point of product elution, fractions are analyzed for protein content by absorbance measurement at 280 nm. The fractions where product is expected to elute should be measured by an appropriate product specific analytical technique, e.g., ELISA, SDS-PAGE, and/or Western Blot (*see* Chapter 9 of this volume for further details on analysis).

3.2. Hydrophobic-Interaction Chromatography

The HIC operation and evaluation normally consists of four key stages.

1. Matrix preparation and Column packing.
2. Sample preparation and loading.

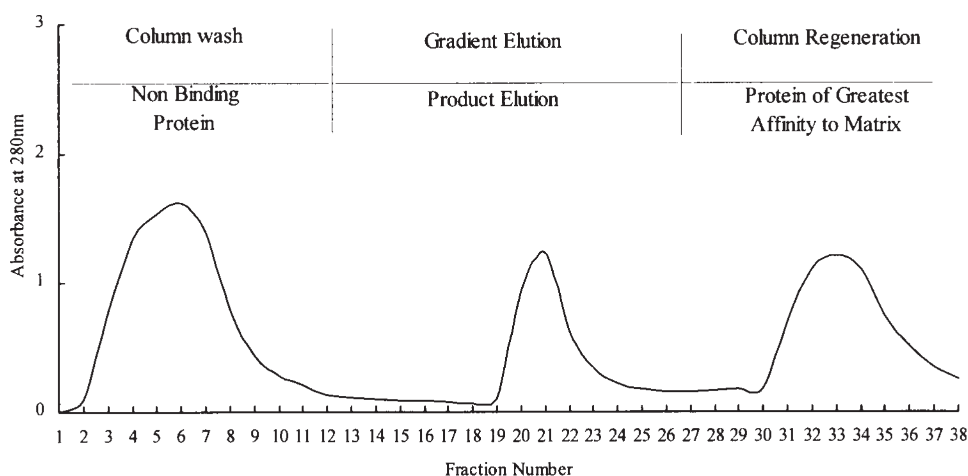

Fig. 4. A typical ion exchange profile during purification of proteins.

3. Sample elution and matrix regeneration.
4. Analysis.

3.2.1. Matrix Preparation and Column Packing

The following protocol should be followed for the initial screening experiments using the commercially available prepacked HIC test kits consisting of a range of HIC media to be evaluated. Wherever possible, the supplier's instructions should be followed for the initial evaluation of the HIC test kits ***(9)***. Alternatively, in-house HIC columns of various sizes can be packed using the following protocol.

1. Prior to packing an in-house column, ensure that all the materials to be used during the operation are allowed to attain the temperature at which the chromatographic operation will be performed.
2. When temperature is equilibrated, prepare an adsorbent slurry to an approximate concentration of 75% v/v using a low-strength buffer (elution buffer) or 20% ethanol and allow to degas overnight (*see* **Note 13**).
3. Having ensured that any air pockets in dead volumes in the column, plungers, and associated tubing have been removed, a small residual volume of packing buffer should be left at the base of the column.
4. The adsorbent slurry should then be poured into the column in a single motion to eliminate the entrapment of air (*see* **Note 14**).
5. When the desired volume of 75% v/v adsorbent slurry has been poured into the column, (and a packing reservoir if required) the unfilled volume should be filled with low-salt elution buffer, at which point the upper plunger should be inserted into the column and the column integrated with the chromatography platform.

6. When a constant bed height is attained, a further three-column volume of low-salt elution buffer should be passed through the bed. On completion of this operation the bed height should be marked on the column and the adaptor should be lowered to approx 3 mm below this mark.
7. After washing out the elution buffer from the chromatography column at a flow rate of 70% of the packing flow rate, the adsorbent should then be equilibrated in high-salt equilibration buffer whose salt concentration is the same as, or even slightly higher than, that in the sample under investigation.

3.2.2. Sample Preparation and Loading

Sample preparation prior to the initiation of HIC is limited to ensuring that its chemical composition is suited to the promotion of hydrophobic interactions between target proteins and adsorbent matrix. Following protocol should be used to prepare and load sample during HIC operation.

1. The elevated concentration of neutral salt that is a prerequisite for HIC should be attained by the addition of a stock solution of a neutral salt at high concentration (*see* **Note 15**) from those listed under equilibration/loading buffers in **Subheading 2.2.**
2. Any "salting-in" compounds (e.g., urea, potassium thiocyanate) present will severely affect the potential for HIC and must, therefore, be removed prior to adding a neutral salt and initiating adsorption (*see* **Note 16**).
3. The pH of the sample solution may need to be altered in order to minimize the overall net charge on the target protein, which could potentially interfere with the interaction between adsorbent and macromolecule (*see* **Note 17**).
4. The temperature at which the HIC experiment is carried out is also of importance, therefore, the temperature at which comparative experiments are carried out should be kept constant (*see* **Note 18**).
5. Load the sample (1–10 mL depending on the size of the column), in the initial experiments at low flow rates (0.1–1.0 mL/min) to allow increased residence time for maximal binding.
6. Monitor the breakthrough by absorbance measurement at 280 nm. Collect fractions of the breakthrough (*see* **Note 19**).
7. When the adsorbent has attained its dynamic binding capacity for the applied sample, any further addition of the sample will result in a breakthrough.
8. Wash the column with 2–3 column volumes of loading buffer to remove unbound or loosely bound material on the matrix until the absorbance at 280 nm has dropped to the baseline level.

3.2.3. Sample Elution and Matrix Regeneration

Product recovery or elution in HIC can be carried out by either a linear or a step reduction in the salt concentration. Elution using a linear gradient will facilitate protein separation, but will result in product dilution resulting from peak broadening. Such an effect can be eliminated by the use of step elution. A

step elution is normally used when the elution behavior of the target protein is understood in order to avoid the coelution of any contaminating proteins (*see* **Note 20**). The protocol outlined below can be followed for step elution during HIC operation.

1. The elution is effected by a decrease in the concentration of salt usually in a stepwise mode, which is the simplest to control in large-scale operation.
2. As the protein is eluted from the column, a 280-nm absorbing peak will be observed, which is collected into fractions either manually or using a fraction collector, e.g., Frac 100 as supplied by Amersham-Pharmacia Biotech.
3. Various proportions of organic solvents such as 40% ethylene glycol or 30% isopropanol can be added to the elution buffer to decrease the polarity or surface tension of the eluent (*see* **Note 21**).
4. Neutral detergents (usually 1%) can also be added to the elution buffer for specific elutions (*see* **Note 22**).
5. The HIC column is regenerated to remove the strongly adsorbed protein returning the matrix to its original condition. This is achieved usually by washing the column with distilled water (*see* **Note 23**).
6. The HIC column can be reused for another cycle of operation or stored for future use (*see* **Note 24**).

3.2.4. Analysis of Fractions

To determine the point of product elution, fractions are analyzed for protein content by absorbance measurement at 280 nm. The fractions where product is expected to elute should be measured by an appropriate product specific analytical technique, e.g., ELISA, SDS-PAGE, and/or Western Blot (*see* Chapter 9 of this volume for further details on analysis).

3.3. Size-Exclusion Chromatography

The SEC operation and evaluation normally consists of four key stages.

1. Matrix preparation and column packing.
2. Sample preparation, loading, and elution.
3. Column regeneration.
4. Analysis of fractions.

3.3.1. Matrix preparation and Column Packing

Select the SEC matrix of appropriate fractionation range supplied by the manufacturer that is suitable for the separation and resolution of the target protein (*see* **Note 27**). In-house SEC columns of various sizes and dimensions, depending on scale of operation, can be packed using the following protocol.

1. Prior to packing an in-house column, ensure that all the materials to be used during the SEC evaluation are allowed to attain the temperature at which the chromatographic operation will be performed.

2. When temperature equilibrated, prepare the appropriate volume of SEC media slurry using deionized water and allow to settle after gentle stirring.
3. Decant the water and resuspend the media in more deionized water and decant to remove any fines from the media. Finally, add more deionized water and degas to an approximate concentration of 50–70% v/v slurry. (*see* **Note 28**).
4. The adsorbent slurry should then be poured into the column in a single motion to eliminate the entrapment of air (*see* **Note 14**). Follow manufacturer's instructions for packing flow rates and pressures.
5. When the desired constant bed height is attained, connect the column outlet to the conductivity and/or UV monitor of the chromatography system.
6. Pass a further 3-column volume of deionized water through the column. On completion of this operation, the bed height should be marked on the column and the adaptor should be lowered to approximately 3 mm below this mark.
7. Condition and equilibrate the media in high salt (1 *M* NaCl) or 0.1–0.5 *M* NaOH regeneration solutions (1–2 column volumes) at twice the flow rates to be used in the study before reequilibration with the appropriate elution buffer (*see* **Note 5**). Linear flow rates of 10–20 cm/h are generally used for equilibration of SEC columns.
8. Evaluate the packing integrity of the column by measuring the HETP and A_s (*see* **Note 4** and **Fig. 5**), of a tracer compound such as 1 M NaCl (conductivity) or 1% (v/v) acetone (absorbance at 280 nm). The limits of HETP and As for the SEC media should be available in the literature ***(1)***.

3.3.2. Sample Preparation, Loading and Elution

Minimum sample preparation or pretreatment is required prior to the initiation of SEC and the following protocol should be followed for loading and elution.

1. Ensure that the viscosity (or density) of the sample is slightly higher than that of the elution buffer (*see* **Note 29**).
2. Filter the sample using a 0.5 or 0.2 μm filter and determine the protein concentration by a protein assay or absorbance at 280 nm prior to loading (*see* **Note 30**).
3. Load the sample onto the column at low flow rates (5–10 cm/h) using the sample loop or an external pump. Volume of sample will influence the column size and performance of the separation (*see* **Note 31**). Use small volume of sample for preliminary evaluation of the separation.
4. Elute the sample with appropriate elution buffer at a flow rate of 5–10 cm/h.
5. Collect the fractions (1–10 mL) depending on the size of the column as soon as the product has been loaded.
6. Store the fractions at 4°C until ready for analysis.

3.3.3. Column Regeneration

When the SEC run is complete, follow the following protocol for column regeneration for the next cycle of operation.

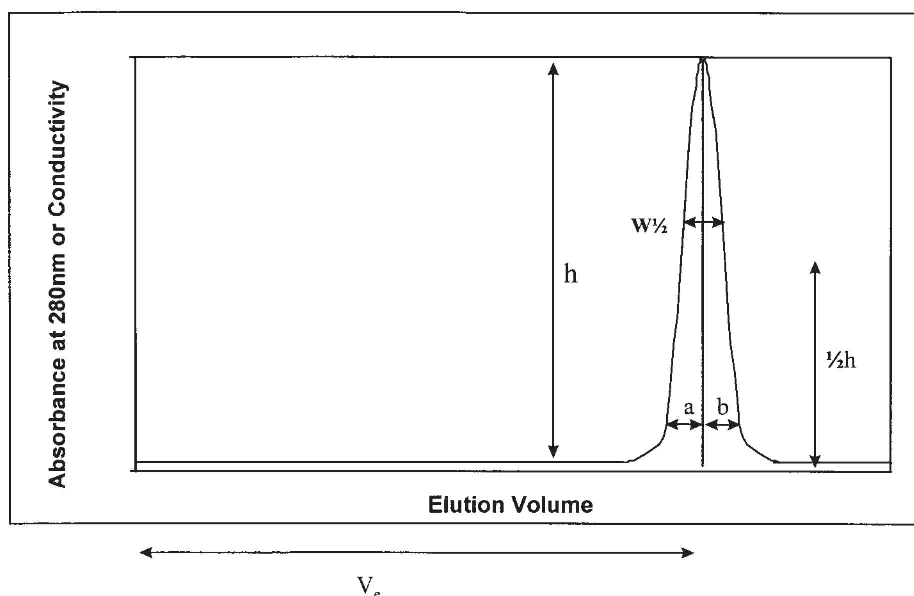

Fig. 5. Evaluation of column packing integrity—measurement of HETP and asymmetry factors after packing a liquid chromatography column.

1. Elute the column with high salt such as 1 M NaCl to remove bound proteins.
2. Sanitize the column with 0.1–0.5 M NaOH (*see* **Note 23**) at half the flow rate used in separation.

3.3.4. Analysis of Fractions

To determine the point of product elution, fractions are analyzed for protein content by absorbance measurement at 280 nm. The fractions where product is expected to elute should also be measured by an appropriate product specific analytical technique, e.g., ELISA, SDS-PAGE, and/or Western Blot (*see* Chapter 9 of this volume for further details on analysis). Mass balance based on total protein or product-specific assay will determine the efficiency of the SEC column under the conditions.

4. Notes

1. This could be a cell-culture supernatant from a mammalian cell-culture system or fermentation broth from a microbial system for extracellular products. Alternatively, it could be lysed cell components suspended and dialyzed in loading buffer in the case of intracellular products.
2. If the p*I* of the target molecule is not known, then a small screening experiment could be undertaken with strong anion or cation exchangers in test tubes over a

range of pHs to establish the binding characteristics of the target product and, hence, estimate the p*I*.

3. Heating the matrix during swelling serves to deaerate the matrix. Alternatively, the swollen matrix could be degassed by vacuum suction or using an inert gas such as helium or nitrogen.
4. Packing efficiency can be determined by the following procedure.
   a. Load 2% column volume of a test solution, e.g., 1% (v/v) acetone or 1 *M* NaCl.
   b. Elute at a flow rate to prevent peak broadening, e.g., at 15–30 cm/h.
   c. Record absorbance at 280 nm of the acetone elution or conductivity for 1 *M* NaCl from point of injection.
   d. Calculate height equivalent to theoretical plates (HETP) and the asymmetry (As) according to the measurements in the literature ***(8)***. Also refer to **Fig. 5** for evaluation of these parameters.
5. If weak buffers are to be used during the procedure, e.g., 10–20 *mM* Tris-HCl pH 8.0 then equilibrate the column using a concentrated buffer (3-5 column volumes), e.g., 0.5 *M* Tris-HCl pH 8.0 initially, followed by 3–5 column volumes of the loading buffer. Equilibration of the matrix is complete when the pH and conductivity of the column outlet are equivalent to that of the loading buffer.
6. To prepare the sample for loading to ensure correct binding, first dialyze against the loading buffer using either a dialysis bag or an ultrafiltration cell with a low-molecular-weight cutoff (e.g., 10 kDa; Millipore).
7. The fraction collection (normally 1–5 mL depending on column size), should be initiated at the same time as the loading of sample begins. After loading of the sample, wash the column through with 3–5 column volumes of loading buffer or until the absorbance baseline on the chart recorder returns to zero or levels off.
8. Isocratic elution is where the ionic strength remains unchanged and the unwanted substances (impurities) are adsorbed to the matrix allowing the purified product to flow through. This type of elution does not require gradient apparatus, however, it is rarely used in the conventional ion-exchange systems.
9. The best conditions for resolution of the product is when the product has the lowest affinity for the matrix of all the adsorbing proteins. Therefore, at the time of elution, the product is the first to elute requiring only a slight increase in the ionic strength or change in pH.
10. Linear pH gradients are difficult to perform because as the pH changes, the ionic strength also changes; therefore, ionic strength cannot be controlled. Linear salt gradients are controllable and, hence, reproducible.
11. The column can be reused after regeneration for another cycle of operation. Alternatively, the column could be equilibrated with 1–2 column volumes of loading buffer containing 0.02% sodium azide and stored at 4°C until required.
12. The product specific assay would be required for quantitative analysis of the product eluted peak. Such assays are usually biospecific for the product such as ELISA.
13. If ethanol is used as the preferred slurry agent, a minimum of 12 column volumes of buffer should be used to wash the column after packing to ensure that the ethanol has been removed prior to the initiation of any adsorption investigations.

Trace amounts of ethanol will adversely affect hydrophobic interaction between protein and adsorbent, and could potentially affect experimental results.

14. If possible, this action should be carried out with the aid of a glass rod to further reduce the likelihood of gas entrapment.
15. Although this will result in unwanted dilution of the sample solution, it will help avoid potential protein precipitation resulting from localized areas of high salt concentrations if the salt was to be added in solid form. A common starting point is the elevation of the neutral salt concentration to 1 *M*. Of course, such an elevated salt concentration should not be used if preliminary experiments have shown that the target product is likely to be adversely affected by this concentration.
16. This can be achieved by microdialysis of the sample against a suitable buffer initially if the volumes are small. For larger volumes, diafiltration using an ultrafiltration system of a defined molecular weight can be used.
17. Bringing the pH of the solution closer to the p*I* of the protein will help reduce electrostatic interference between the matrix and protein. It is important to remember that the solubility of a protein will be reduced as the p*I* is approached, and, therefore, this may lead to unwanted protein precipitation.
18. This technical philosophy also applies to any scale-up experiments that will be carried out as a result of previous bench-scale investigations. An alteration in the temperature could dramatically affect the apparent efficacy of an adsorption operation.
19. The elution profile will be monitored by the UV meter set at 280 nm on the liquid chromatography system. However, the fractions (1–5 mL) should be collected and absorbance measured manually as well using a spectrophotometer.
20. By implementing an elution strategy based upon empirical knowledge of the hydrophobicity of the proteins, it is possible to separate coadsorbed proteins while at the same time maximizing product concentration by keeping dilution resulting from peak broadening to a minimum. In an initial experiment, a linear gradient up to 10 column volumes should be used to allow the precise elution conditions for the target product to be determined. When this value is known further steps can be taken to optimize the elution protocol.
21. The addition of organic solvents to the elution buffers should only be done when it has been established that the product of interest is stable upon exposure to such solvents. Sometimes the linear increase in concentrations of organic solvents with decrease in salt concentration during gradient elution can lead to increased resolution of bound proteins.
22. Some detergents can bind too strongly and are difficult to wash out completely with organic solvents (e.g., ethanol). This might lead to a decrease in the capacity of the HIC matrix during subsequent applications.
23. NaOH (0.5–1.0 *M*) is a very efficient cleaning agent used for solubilizing irreversibly precipitated protein and lipid material. This can be effectively combined in HIC with solvent or detergent based cleaning agents.
24. The column can be reused after regeneration for another cycle of operation. Alternatively, the column could be equilibrated with 1–2 column volumes of loading buffer containing 0.02% sodium azide and stored at 4°C until required.

25. All buffers should be either degassed using vacuum or use of inert gases (helium or nitrogen). However, the buffers can also be filtered using a 0.2-μm filter prior to use. This will also help degas the buffers.
26. Generally, SEC are long and thin to effect better resolution of proteins as well as adequate buffer exchange or desalting. However, there is limit to the length of a column for large-scale processing because of high pressure drops and, therefore, short and wide columns (of reasonable length) are recommended for process scale SEC.
27. Appropriate selection of SEC media can be made from the supplier's media charts if the molecular weights of the target protein and key impurities to be resolved are known. Wherever possible, manufacturer's instructions should be followed for use of the selected SEC media.
28. Degassing of solutions can generally be done either through the use of vacuum or using an inert gas such as helium or nitrogen. Use of vacuum may be more appropriate for degassing polymeric media such as those in SEC.
29. Normally, the sample should be denser than the eluent, this can be made by adding a small amount of glucose, sodium chloride, or a suitable inert material. Generally, the sample will be denser because of the presence of protein at reasonable concentrations.
30. Determination of protein content and/or product concentration by a specific assay will enable the calculation of product recoveries as well as determine loss of product resulting from interaction with the media from mass balance.
31. Larger columns will be required for processing larger volumes. The actual sample volume that can be applied for a specific separation can be determined experimentally. For maximum resolution of difficult fractionations, a sample volume of 1–5% of the column volume is recommended. However, in desalting and buffer-exchange operations, volumes up to 30% of the total column volume can be used to minimize dilution of the product.

5. General Scale-up Comments

The scale-up comments in this section are common and applicable to each of the chromatography modes discussed in this chapter. They should help in the optimization and scale-up of the chromatography techniques discussed here.

The important considerations given to the matrix of choice for a large-scale chromatography process are normally their rigidity, minimal nonspecific interaction, minimal and quantifiable ligand leakage, reproducibility of performance, stability on exposure to sanitization and cleaning agents, availability of validation information, and assurance of long-term availability from the supplier of the matrix.

The operational efficiency of fixed-bed chromatography processes in terms of resolution and peak separation is usually determined by quality of packing. This is expressed by the measurement of height equivalent to the theoretical plate (HETP) and peak asymmetry (A_s). These parameters are measured by

injecting a tracer compound such as NaCl or acetone after packing, and monitored by a conductivity or a UV meter, respectively. The determination and achievement of such values within defined acceptance criteria for specific chromatography operation is important to the success of large-scale unit operations.

The size, type, and dimensions of column selected for large-scale operation of a chromatography process will depend on the mode of operation as well as the capacity of the matrices. However, it is important to keep the bed height to a minimum, but increase the diameter of the column for any scale-up considerations in order to minimize the pressure drop generally caused by longer columns. Selection of columns should also be of the type generally approved by the regulatory authorities with validation information available from the suppliers.

A suitable scale-down model should be developed as part of process development for performing the validation studies such as cleaning and sanitation validation, reproducibility and consistency of usage, evaluation and validation of storage stability, validation of operational parameters, and so on, at this scale.

Wherever possible, an optimized stepwise elution procedure should be developed for large-scale operation of a chromatography step for avoiding technical issues in relation to gradient elutions. Use of biocompatible eluting agents and those consisting of physiological buffer systems should always be considered, particularly, for chromatography steps in the final stages of a purification process.

Logistics of buffer preparation, supply, filtration, and their use should be considered carefully to maximize the sanitary operation of the chromatography unit operations in a process.

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8

Affinity Ligand Technology

Optimal Protein Separations for Downstream Processing

Ken Jones and Dev Baines

1. Introduction

Historical approach of downstream processing of proteins is to develop a sequential column-based procedures where a series of purification steps are used one after the other until the desired purity is achieved. The primary separation steps provide a crude material, which is first applied to "capture" the bulk of the target protein, while removing the major contaminants. Subsequent steps are designed to progress through various additional steps to achieve the desired purity while retaining biological activity. However, each additional step in the overall process results in product loss and incurs processing costs. Even at the excellent average step yield of 90% after 10 steps, the overall process yields are below 40% (**Fig. 1**). On the other hand, affinity chromatography offers a means to separate and purify any given protein in one step directly from crude solution ***(1)***. However, where very low cost proteins ($0.1–0.2/1000) are required in multitonne quantities, for example, egg white for food processing purposes, simple precipitation by heating may suffice. At the other extreme are very high-purity protein pharmaceuticals. Thus, worldwide demand for Factor VII, a blood protein used to treat haemophiliacs, is less than 500,000 per year, but commanding a price of $300,000. This extraordinary 3×10^6-fold cost differential conveys a clear message; a common strategy to develop downstream separation processes is not possible. Furthermore, developing a separation strategy needs more than technical facts. Also, data relevant to economics have to be studied if an optimum separation is to be obtained.

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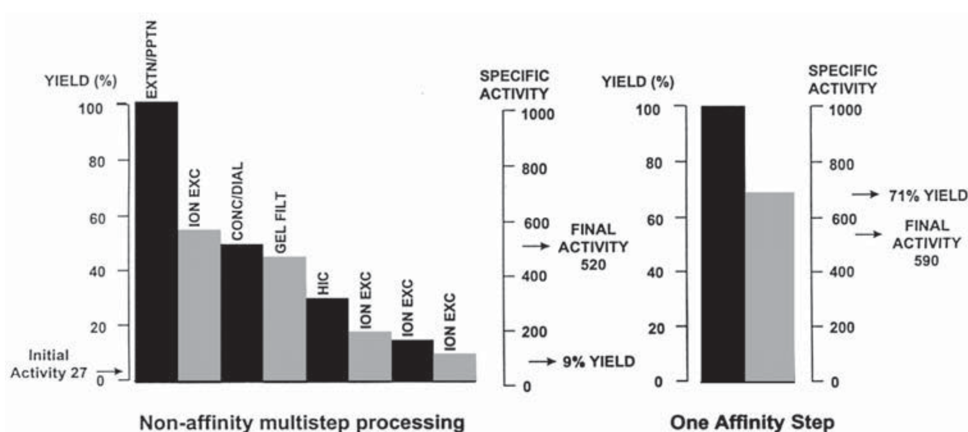

Fig. 1. Comparison of multistep and affinity bioprocessing of a protein.

These include the scale of potential demand, the price users are willing to pay, the degree of purity required, involvement of regulatory authorities, and many other factors. The description of different types of affinity chromatography applications have been described in detail in the companion volume to this series *(2)*. Description in this chapter is therefore, limited to considerations required for large-scale downstream processing by affinity chromatography to manufacture of biopharmaceuticals.

In principle, all downstream processes should have the lowest possible costs. This primary objective is, however, often overlooked during the research and development phase. Consequently, decisions taken by early researchers concerning a separation strategy are very important. The inherent lability of many proteins would indicate affinity chromatography as method of choice for downstream processing of these molecules. Additional advantages of affinity methods include their unique ability to concentrate dilute starting materials, ability to stabilize the target molecules, and simple scale-up procedures. The commercial acceptance of affinity chromatography has been hampered by several problems; the perceived high cost of traditional affinity media, the difficulty in making such media operationally stable in multicycle pyrogen-free environment, regulatory issues, and the resistance to change syndrome.

Recent developments in designed ligand affinity chromatography *(3)*, application of combinatorial chemistry for ligand design *(4)* and use of peptide display libraries *(5)* for peptide ligand discovery is likely to greatly increase the use of affinity chromatography in downstream processing. These techniques of ligand discovery are specialized and readers should consult technology suppliers for further details.

Table 1
Support Matrices for Affinity Chromatography

Support matrix	Operational pH range
Agarose	2–14
Cellulose	1–14
Dextran	2–14
Silica	<8
Glass	<8
Polyacrylamides	3–10
Polyhydroxymethacrylates	2–12
Oxirane-acrylic copolymers	0–12
Styrene-divinylbenzene copolymers	1–13
Polyvinylalcohols	1–14
N-acryloyl-2-amino-2-hydroxy-1,2-propane	1–11
PTFE	Unaffected

2. Materials

2.1. Adsorbents for Affinity Bioprocessing

The key to successful affinity separation is the selection of appropriate affinity matrix (*see* **Table 1**). The types of adsorbents to consider include the following.

1. Commercially available broad specificity affinity adsorbents that bind to specific classes of protein molecules and purify different proteins with some similarities and seemingly unrelated proteins. These include the Mimetic range of adsorbents, which are used in purification of a wide range proteins including albumin, interferon, and nucleotide-dependent enzymes. Immobilized heparin is used for isolation of blood coagulation proteins, growth factors, lipoproteins, and steroid receptors. Initial evaluations with these adsorbents can provide a high degree of purification ***(6)***.
2. For greater specificity, ligands with more precise specificity for the target molecule should be evaluated. Examples of specific adsorbents include: immobilized Protein A for purification of immunoglobulins, immobilized specific antibodies for immuno-affinity chromatography, which exploits the specificity and avidity between antigen, and its antibody to purify its antigen ***(7)***.
3. Alternatively, if a highly specific ligand for the target molecule is available, its coupling to support matrices to provide affinity adsorbents can be considered. Available coupling chemistries are summarized in **Table 2**. Because most users of affinity chromatography may not wish to undertake extensive organic synthesis, the most obvious route is use commercial activated gels and follow the vendor provided instructions for coupling of the ligand. The choice of the activated

Table 2
Chemistries for Coupling Ligands to Hydroxylic Supports

Activator	Bonding group of the ligand
Cyanogen bromide	Primary amines
Tresyl chloride	Primary amines, thiols
Tosyl chloride	Primary amines, thiols
Epichlorohydrin	Primary amines, hydroxyls, thiols
1,4-Butanediol diglycidyl ether	Primary amines, hydroxyls, thiols
1,1'-Carbonylimidazole	Primary amines, hydroxyls
Divinylsulphone	Primary amines, hydroxyls
2-Fluoro-1-methylpyridinium toluene-4-sulphonate	Primary amines, thiols
Sodium periodate	Primary amines
Glutaraldehyde	Primary amines

coupling gel will depend on the group available on the ligand for immobilization and by the nature of ligand's interaction with the target molecule.

4. Designed ligands and affinity adsorbents are now available from several suppliers and are developed specifically for large-scale affinity bioprocessing. Contact suppliers of chromatography adsorbents for details.

2.2. Buffer Requirements for Affinity Bioprocessing

Selection of appropriate buffers for use in affinity bioprocessing is a key consideration. Often at the development stage of a downstream process, the tendency is to use expensive zwitterionic buffers such as MES, (2-[*N*-Morpholino]ethanesulphonic acid), MOPS (3-[*N*-Morpholino] propanesulphonic acid), or Tricine (*N*-Tris [hydroxymethyl]methylglycine). This strategy can lead to substantial increase in the process cost and should be avoided if future scale-up is under consideration. Use only cheap reagents for large-scale work ***(8)***.

1. Buffers containing acetate, citrate, glycine, phosphate, and succinate are used for large-scale affinity bioprocessing.
2. The pH and ionic strength of the buffers should be selected on basis of stability of the target protein, and to maximize the interaction with the ligand. Obviously, for elution, the buffer is selected to minimize this interaction.
3. Additives that enhance the stability of the target molecule can be included in the buffers. The stabilizing compounds include metal ions, reducing agents to prevent oxidation of the protein, complexing agents, and glycerol.
4. In downstream processing, large buffer volumes are used, which ensure that the reagents used in buffer preparations are nontoxic and can be safely disposed via normal drainage system.

3. Methods

3.1. Developing Strategy for Implementation of Affinity Chromatography in Downstream Processing

There are four basic initial steps involved in developing a strategy:

- gathering knowledge of the target protein;
- finding an assay to assess the amount and purity (or activity) at each step;
- selecting a source;
- separating and purifying the target protein.

The knowledge of the protein's native environment has been considered to be essential for selection of a separation process of protein purification. Is the target protein intracellular, extracellular, independent or attached to other structural elements, soluble or membrane bound? How stable is the protein to pH, salt concentration, temperature, air, materials of construction? What is its isoelectric point (p*I*) and molecular weight? Are stabilizers, cofactors, substrates, inhibitors, and/or proteases present ***(6)***? Although this represents a daunting list, in practice most information is not necessarily required in the earliest stages of research. Modern technology has now provided a quick screening method that performs well even when the pI and molecular weight are unknown. Consequently, a separation/purification method can be available very rapidly; remaining data can be developed later.

The most crucial element that will affect downstream process is the availability of specific assays for the target protein and the contaminants. Although total protein assays are reasonably accurate, a specific assay is needed to determine the efficacy of each stage of purification. It is not unusual to spend more time developing a suitable assay than designing a separation process.

The properties of the starting material will depend on the source and thus, source selection is rarely an option, but it can often be modified to minimize degradation of the target protein as a means of maximizing yield. For example, from knowledge of the native environment, early removal of proteases; addition of stabilizers; addition of hormones to increase concentration of the target protein; mutation of the producing organism, are all established methods. Consequently, the timing of removal of the target from its native environment is an important parameter. In most situations, there is an optimum point at which separation should take place, usually the point at which the maximum amount of protein at minimum degradation exists. Therefore, the speed of separation is of paramount importance.

Affinity chromatography (**Fig. 2**) is probably the simplest and the rapid of all chromatographic separation techniques. The ligand is an entity that recognizes and binds to the target protein and is coupled to a suitable solid support. A solution containing the target is passed through the column wherein the tar-

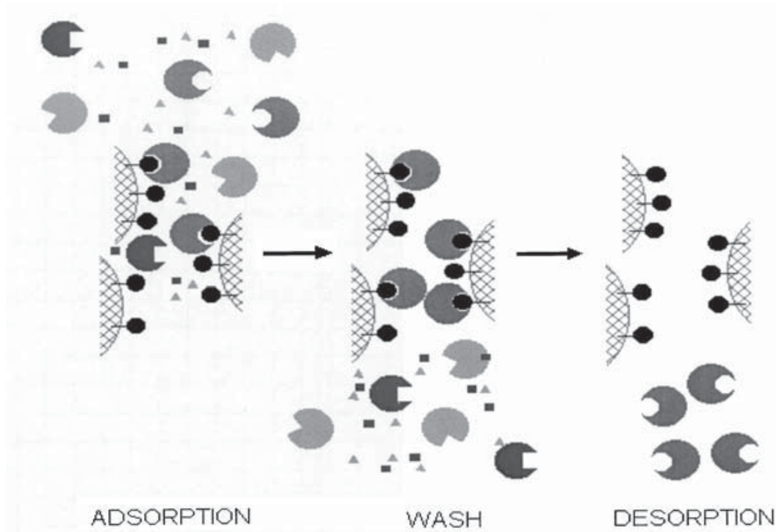

Fig. 2. Schematic representation of principle of affinity chromatography.

get dissolved in the mixture is exclusively recognized and bound by the ligand. Nonretained impurities are washed through, at which point the column can be regenerated by a change in salt concentration/pH/cofactor addition, followed by collection of the now-purified protein. This technique can, of course, be used in reverse; impurities can be adsorbed whereas the pure product is washed through. Typically, purification of several thousandfold are often obtained by affinity chromatography.

In practice, it is convenient to carry out initial evaluations using commercially available group specific affinity adsorbents that bind to molecules containing specific structural features. Certain types of textile dyes, known as reactive dyes, represented by Cibacron Blue F3G-A™ (Ciba Geigy) are often used as group specific ligands for affinity chromatography since they bind a wide variety of proteins in a selective and reversible manner. The use of immobilized reactive dyes to purify a number of different proteins is well documented. A range of adsorbents with reactive dye ligands are now available prepacked in the suitable screening column format for application with automated chromatography workstations (*see* **Subheading 4.1., item 7**).

For greater specificity, a more specific ligand, e.g., cofactor, substrate analog, inhibitor, or specific antibody is often used to provide precise interaction for the target molecule. The specific ligand is covalently attached to the chemically activated support matrix. Chemically activated supports are commercially available. The requirement for diverse specific ligands to bind target protein exclusively is subject of intense research. Thus, rational design using compu-

tational chemistry was used to design a synthetic analog of Protein A, a natural ligand used for purification of immunoglobulins. This is now commercially available (Mimetic A™ Agarose) Affinity Chromotography, Isle of Man, UK. Replacement of a natural ligand by a synthetic mimic then offers, considerably improved stability, low cost, easy availability, consistent separations, and simultaneously retains the ability to achieve highly specific separations.

Where the details of the three-dimensional (3-D) structure are not available, screening of ligand libraries are used to discover new ligands for application in affinity purification. Peptides of less than 50 amino acid residues are highly structured and particularly assessable via phage display screening. Such an approach has provided ligands of considerable specificity for several protein targets. Similarly conventional peptides obtained from solid phase combinatorial synthesis have been shown to be useful as purification tools particularly if solid phase beads are used as chromatography matrix ***(10)***. Although such peptide ligands are proving useful in affinity applications, defined small organic molecules as ligands are practically more useful particularly with respect to GMP manufacture of therapeutic proteins because the stability and safety of peptide ligands is difficult to assess. As mentioned above, these approaches to identifying new ligands and subsequent affinity adsorbent development requires specific approaches and suppliers of such technologies should be consulted for further advise.

Screening strategy for selecting suitable affinity adsorbent is detailed in the companion volume to this series ***(8)*** and will not be described here. Once the suitable affinity adsorbent is identified, the processing of the material can be scaled-up. The binding and elution conditions should be determined from such a screening study. In practice, it is often necessary to perform several optimization experiments. The availability of dedicated chromatography workstations allow several columns to be screened sequentially to arrive at an optimized separation procedure. For coupling own preferred ligands, the starting point is to use commercial activated gels ***(11)***. Many types of activated supports are available from commercial suppliers of chromatography media.

3.2. Preparing for Affinity Separation

1. Commercial affinity adsorbents are often supplied in preservatives that should be removed before packing the column. This is best achieved by copiously washing the adsorbent on a sintered glass funnel with water. Slurry-pack columns by transferring the adsorbent to the column. The method of packing will depend on the column type and size. Always follow manufacturer's instruction with respect to maximum flow rate and pressure. It is preferred that adsorbent slurry is degassed before column packing to avoid formation of air bubbles in the packed bed. Equilibrate the column with at least 10 column volumes of the equilibration buffer.

2. The applied protein sample should be clarified either by microfiltration (0.45 μm filter) or by centrifugation (20,000*g* for 30 min) and be of an equivalent ionic strength and pH as the equilibration buffer. The sample should by dialysed or treated by diafiltration such that it is in equivalent buffer to the equilibration buffer.
3. The column size should be determined by obtaining affinity adsorbent binding capacity values (frontal analysis) by carrying out small scale experiments or from initial screening studies. Scale-up of the chromatography column is achieved by increasing the column diameter while maintaining the bed height and linear flow rate. A bed height of 10–15 cm is optimal.
4. Process-scale separations are often carried out at ambient temperatures. If the target molecule is particularly labile than it necessary to carry out all chromatography at 4°C. The use of jacketed columns and circulating coolant can be used to maintain the column at the desired temperature.

3.3. Affinity Adsorption Step

1. The concentration and pH of the equilibration buffer is selected to maximize binding of the target protein to the affinity adsorbent. In most large-scale applications, the target protein is often present under physiological conditions and therefore neutral pH buffer is appropriate, for example, 10–100 m*M* phosphate, pH 7.0–7.5.
2. The sample volume is applied to the column at predetermined optimal flow rate, which is the function of the matrix bead size, molecular weight of protein, and temperature. For newer high performing agarose gels, linear flow rates of up to 10–30 cm/h have been used in adsorption step. Higher flow rates reduce protein binding capacity. Linear flow rate is obtained by dividing the flow rate mL/h by cross-sectional area of the column (cm^2). On scaling up of affinity chromatography, it is important for the column residence time and column loading per volume of adsorbent to remain constant.
3. After loading, the column is washed with equilibration buffer to remove nonbound material. An on-line ultraviolet (UV) detector is useful to monitor the column effluent and washing is carried out until the absorbance returns to the baseline or stabilizes at a low absorbance level.
4. By changing the composition of buffer used to wash the adsorbent before elution of the bound target protein, it is possible to remove any nonspecifically bound contaminants. This washing step may be performed at higher flow rate to speed the process times.

3.4. Elution of the Bound Target Protein

Affinity adsorbed proteins are eluted either in a selective or nonselective manner. Selective elution is accomplished by using a soluble ligand in the elution buffer that competes for the same ligand binding site on the protein. Nonselective elution is achieved by changes in the pH, ionic strength, or polarity of the elution buffer.

1. For selective elution, the competing ligand should ideally have greater affinity than the immobilized ligand is simply dissolved, at concentration range of 0.1–10 m*M* in the elution buffer and used to elute the bound protein. Potential candidates for competing ligands include inhibitors, cofactors, enzyme substrates and products, or allosteric effectors. Thus, some knowledge of ligand binding properties of the target protein is required to implement this elution strategy. Because specific elution provides higher purity of the eluted product, for affinity bioprocessing, this will add to the cost and may require subsequent polishing step to remove the soluble ligand from the product.
2. Nonselective elution by changing pH or increasing the ionic strength of elution buffer is a preferred method for affinity bioprocessing. Provided the target protein molecule is stable pH change, elution maybe achieved by either lowering the pH or increasing the pH. In many cases, increasing the ionic strength of elution buffer is sufficient to release the adsorbed protein. Combination of a change in both the pH and ionic strength can be used to elute the protein as a sharp elution peak. Where increases in ionic strength or pH changes fail to cause elution, addition of polyols (ethylene glycol or glycerol) at 10–50% in the elution buffer to reduce polarity is often effective.

3.5. Column Cleaning and Regeneration

All chromatography adsorbents used for protein purification become fouled with protein and other contaminants present in the column feedstreams and are not eluted by the usual buffer. The columns used for affinity bioprocessing, therefore, require cleaning and regeneration before they can be used again. Optimal conditions for column regeneration require evaluation and will depend both on the nature of the ligand and the support matrix.

1. Affinity adsorbents containing alkali-stable ligands coupled to polysaccharide matrices, for example, mimetic affinity adsorbents can cleaned and regenerated with 1 *M* NaOH. Most biologically derived molecules are solubilized by NaOH and the industry standard for clean-in-place procedure is to flush the column with 0.5 *M* NaOH.
2. For adsorbents which are unstable to alkali, for example, those containing protein or peptide ligands, mild chaotropic agents such as 3–5 *M* urea, high pH wash (pH 10, buffer) followed by a low pH wash (pH 3, buffer), or buffers containing organic solvents (up to 20% isopropanol) may be considered.

4. Notes

4.1. Quality and Regulatory Aspects of Column Chromatography

1. Any affinity adsorbent used for downstream processing of biopharmaceutical must meet the essential quality standards of its manufacture ***(12)***. Thus, if a process incorporates a chromatography step in the biopharmaceutical manufacture,

this will require submission of qualification data to the regulatory authorities. It is important, therefore, that when considering affinity chromatography scientists should acquaint themselves with the regulatory environment.

2. Selection of appropriate quality of reagents for buffers and solvents should be considered at the research stage of process development. When moving to bulk manufacture there is a strong desire to purchase less expensive materials, but great care must be taken when lower quality materials are substituted. Each substitution has to be tested experimentally, adding an additional layer of expense to development costs. Therefore, for biopharmaceutical manufacture careful consideration of reagents used during the research phase of the program will save valuable development time.
3. For an affinity or any other chromatography adsorbent, a Drug Master File, which defines exactly how a product is manufactured, including the description of the adsorbent manufacturing site, the product stability and toxicity data with storage information should be registered with FDA by the adsorbent supplier and is available upon request. Assurances should be obtained that the product is manufactured in a current Good Manufacturing Practice compliant plant.
4. The base matrix, to which the ligand is coupled via covalent linkage and spacer arm, can have major impact on the performance of affinity chromatography. Most matrices consist of macroporous hydrophilic particles and the ligands are immobilized via surface hydroxyl groups. For downstream processing, matrix performance consistency is crucial and the beaded agarose continue to show excellent properties and is the industry standard (*see* **Table 1**).
5. The type of chemistry used to link ligands to the matrix will determine the stability of the adsorbent selected for affinity bioprocessing (*see* **Table 2**). The correct ligand density must be determined to provide the best result. Similarly, if the correct bonding chemistry is not selected then capacity could be low and leakage can occur. Comparison between various commercially available textile dye products illustrates this problem when operating under sanitization conditions. The trace leakage shown with the Mimetic and perfluorocarbon matrices in ***(13)*** is actually caused by the breakdown of the matrix itself rather than the breaking of the covalent bond. All remaining commercially available textile dye media leak significantly, usually caused by dye impurities and poor bonding technology. It should be noted that for biopharmaceutical proteins, at least two distinct chromatography process stages are preferred, even when high yields and purity are obtained in a one-step operation. This is to minimize any undetectable and uncharacterized contaminants passing through into the product.
6. Recent developments in rational designed ligands to provide robust and highly selective affinity adsorbents to meet the needs in bioprocessing of diverse biopharmaceuticals is redefining the previously perceived limitations of affinity chromatography.
7. Available from many chromatography suppliers, e.g., PIKSI™ Screening Columns from ACL/ProMetic BioSciences (Isle of Man, UK).

4.2. Alternative Affinity Approaches

1. Although affinity chromatography is by far the most used affinity technique, aqueous two-phase systems have been extensively applied to biomolecular purifications. This is achieved by attaching affinity ligands to one of a pair of phase-forming polymers, a method known as affinity partitioning. Unfortunately, few examples exist of commercial applications. Reactive dyes, with their simple and well-defined coupling chemistries, have generally been favoured as the active ligand. The advantages of affinity partitioning are the process is less diffusion controlled, binding capacities are high, and the process operates with fewer theoretical plates than those generated by chromatography columns, making the recovery of bound proteins easier. This technique has also been combined with affinity precipitation, where a homobifunctional ligand composed of two ligand entities connected by a spacer (for example, a bis-dye) is used ***(14)***. This approach, however, suffers from considerable nonspecific binding resulting in less than optimal purification, which suggests, that it may be more suited to low purity bulk products.
2. Alternatively perfluorocarbon emulsion chemistry utilizing mixer-settlers appears to offer more promise. By using a series of mixer-settlers connected in a loop a continuous process has been developed. A ligand (usually a reactive dye) is covalently bonded to a high-density perfluorocarbon emulsion and contacted with the crude protein solution. After settling in the first tank the emulsion is pumped to a second settler and washed before passing to the third settler for elution. This emulsion is regenerated in the fourth settler. The supernatants from each settler, but still containing some unbound target protein, are normally discarded. Although reasonable recoveries and yields are obtained, significant development is needed for this system to become competitive with conventional chromatography column methods ***(15)***.
3. The use ultrafiltration membranes as affinity supports by attaching standard ligands to them is an actively researched area ***(16)***. The main advantage of such a technique is that the membrane structure provides for a high convective transport of the solution through the pore structure by, thus minimizes the normally encountered mass transfer diffusion limitation. High adsorption rates can therefore, be achieved. However, with exception of ion exchange membranes, high-transport adsorption rates are not often observed in the affinity mode.

5. Conclusion

Protein separations can be achieved by a variety of techniques, but separations in the chromatography mode are by far the most widely used. Affinity chromatography is the method of choice for the purification of high-value biopharmaceutical proteins. Many of the commonly used affinity adsorbents are often based on natural high molecular weight ligands which have associated limitations, such as high cost, poor chemical and biological stability and ligand leakage contaminating the product. Recent developments in designed synthetic

ligands has resulted in affinity adsorbents that are resistant to chemical and biological degradation. This will lead to an increase in the use of affinity chromatography in downstream processing of valuable biopharmaceutical proteins.

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9

Immunoaffinity Adsorption in the Large-Scale Isolation of Biomolecules

Mohamed A. Desai

1. Introduction

The efficient separation and isolation of recombinant biomolecules from complex and crude feedstock solutions such as fermentation broths and cell culture supernatants has been a challenge for the purification technologists. This challenge has become even greater with the appropriate stringent regulatory requirements of expected levels of purity (>99%) for the injectable biopharmaceutical drugs. This demanding purity criteria imposed on biomolecules for human use have precluded reliance on traditional, nonselective, physicochemical approaches to protein separation. This has contributed to the increased interest in highly selective adsorption techniques such as immunoaffinity adsorption or chromatography as a purification technology.

Affinity chromatography generally involves unique interactions between biomolecules. For example, the interactions between enzymes and coenzymes, substrates and/or inhibitors, single-stranded DNA and its complement, and hormone and its receptor(s). The interaction between an antigen and antibody may be considered similar to such binding associations, but are much more stronger and specific. The antigen, or a constituent antigenic determinant, forms a tight, noncovalent complex through a combination of electrostatic, hydrophobic, and other interactions ***(1)***.

With the developments in genetic engineering and hybridoma technology, low to intermediate affinity (10^{-4} to 10^{-7} *M*) monoclonal antibodies (MCAB) having identical site recognition and affinity characteristics can now be utilized in the purification of high-value therapeutics. Immunoaffinity chromatography is ideally suited to large-scale industrial applications and the use of

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such technique (using immobilized MCAB) is already in use in industry for the purification of proteins for pharmaceutical use.

Immunoaffinity purification require that the dissociation constant for the antigen–antibody complex (K_d) should neither be too high (resulting in inferior fractionation owing to poor binding) nor too low (leading to diminished recovery of active product and reduced ligand lifetime) ***(2)***. The dissociation constants for antigen–antibody binding reaction range from 10^{-3} to 10^{-14} *M* at 25°C ***(3)***. Those in the range 10^{-6} to 10^{-10} *M* are most suited to immunoaffinity adsorption, whereas lower values are applicable to the irreversible binding required for immunodiagnostic tests.

The operating principles of immunoaffinity technique is summarized in **Fig. 1**. Crude starting material containing the desired product is contacted with an inert and porous adsorbent (solid phase) immobilized covalently with antibodies. These have a specific binding affinity for the product (antigen). The antigen adsorbs to the antibody, and provided the interaction is strong enough, impurities present in the starting heterogeneous crude material is washed free from the adsorbent. The physical and chemical conditions of the loading and washing process may be altered to promote dissociation or desorption of the antigen from the adsorbent. Following desorption, the adsorbent is regenerated and equilibrated for a further cycle of operation. The high selectivity of immunoaffinity chromatography results in excellent purification efficiency (up to 1000-fold in a single step) with recoveries of greater than 90% under appropriate conditions.

A number of important technical and operational aspects have to be considered when an immunoaffinity technique is evaluated for large-scale purification. These include the choice of matrix (solid support), ligand coupling chemistry (activation procedure), ligand coupling density, capacity for the ligand and product, washing and elution agents, column geometry, and flow rate. In order to improve the performance of the immunoadsorbent, all these parameters must be carefully studied and optimized.

This chapter will focus on the practical aspects of immunoaffinity purification technique with emphasis on operational steps for developing a successful purification method for potential optimization and scale-up. The key procedures of the technique are as follows.

1.1. Choice of Matrix (Solid Phase)

A range of matrices have been used in a continuing search for improved immunoaffinity performance, and some general parameters for the "ideal" matrix have been defined ***(4,5)***. The basic requirements of a matrix include mechanical strength, hydrophilic surface, stability to a wide range of solvents,

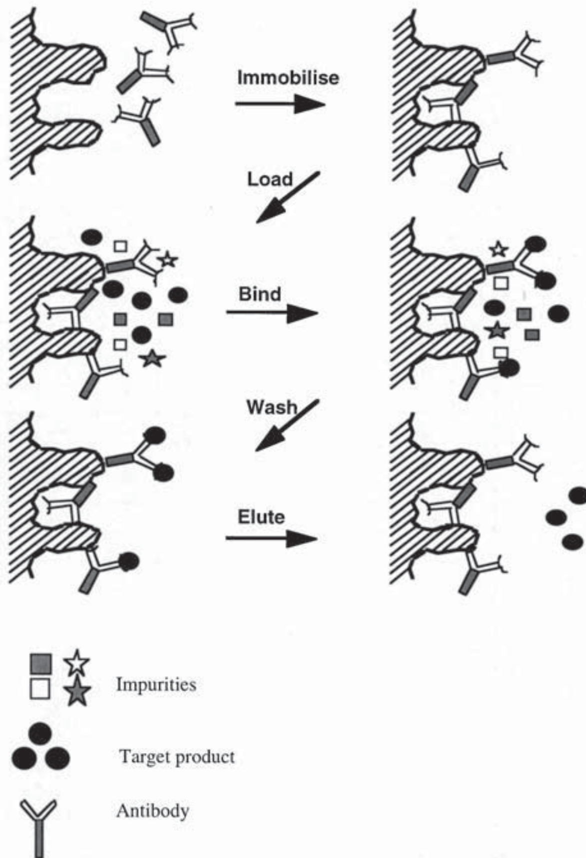

Fig. 1. Schematic of steps in the operation of immunoaffinity adsorption during purification of biomolecules.

high porosity, nontoxic and stable chemical bonding, biocompatibility, low nonspecific adsorption, dynamic capacity, and reproducibilty of performance.

The most commonly used matrix is the beaded cross-linked agarose marketed in various activated forms stabilized for direct coupling to antibodies at chosen ligand densities. Other beaded porous materials suitable for such applications include celluloses, acrylamides, methacrylates, porous glass and silica, and a range of polymer composite matrices. Certain synthetic matrices are also becoming popular in general affinity separations.

1.2. Activation Procedure (Ligand Coupling Chemistry)

The common matrices described above are available and supplied in activated forms, however, cost can be high and activation densities may be vari-

able and inappropriate for the intended application. The practical guide detailing experimental protocols for the assembly of activated matrices could be referred to for further optimization work ***(6,7)***. However, the marketed activated matrices would be adequate and more appropriate for a preliminary evaluation of the technique.

1.3. Antibody Immobilization

A range of antibody immobilization procedures have been documented in the literature ***(8,9)***. The use of a particular procedure depends on the functional group(s) present on the matrix and ligand as well as their stability. The chemically reactive groups which are generally used for the attachment are hydroxyl, amino and carboxyl functions.

Immobilization can be controlled by the use of limiting amounts of antibody, the use of competitive spacer molecules, or the time-based monitoring of ligand uptake using continuous-flow analysis based upon ultraviolet (UV) spectrophotometry. A key drawback of immobilizing antibodies to common matrices is that, although a high degree of ligand substitution can be achieved using conventional immobilization techniques, only a small proportion of the immobilized species attains the appropriate orientation. This results in only a fraction of potential product binding sites being made available.

1.4. Desorption Methods

Efficient elution requires the rapid quantitative desorption of concentrated product. This may be difficult with immunoaffinity adsorption systems because of low K_d values. Specific desorption methods would require expensive immunochemicals or eluents and their subsequent removal from the product after elution. Therefore, such methods are not generally used in immunoaffinity purification applications.

Nonspecific desorption methods, such as the manipulation of pH, ionic strength, or the use of mild denaturants (e.g., urea) or strong chaotropic agents and certain solvents are generally used. Although these may perturb the biochemical conformation of product and/or ligand, such changes are generally reversible. However, the effectiveness of desorption is very much dependent upon individual antigen–antibody system and therefore there will be a requirement to tailor the adsorption/desorption processes for specific systems.

1.5. Biochemical Performance

Characterization of immunoaffinity adsorbents in batch and packed-bed operations can be performed in a variety of procedures ***(9,10)*** that would permit the prediction of performance on larger scales. There is clear benefit in limited characterization to determine effective dissociation constants, effective

maximal capacities, product recoveries, and percentage ligand utilization. Coupled with physical studies of resistance to fluid flow, matrix compression, and band spreading, the limited characterization will decide the stability of an immunoadsorbent and provide a means of monitoring performance during operation.

2. Materials and Reagents

1. Activated solid phases: a) Affi-gel, and Affi-prep (Bio-Rad, Richmond, CA); b) CNBr-activated Sepharose 4FF, NHS-activated Sepharose 4FF (Amersham-Pharmacia, Uppsala, Sweden); c) Toyopearl AF-Tresyl-650M, Toyopearl AF Epoxy-650M (TosoHaas, Montgomeryville, PA).
2. Antibody against the antigen (product) of interest to be purified. The purity of this antibody should preferably be high to minimize nonspecific interactions.
3. Low pressure glass columns for packing immunoaffinity resin: ~1.5 × 10 cm Econo-Column (Bio-Rad).
4. Feedstock solution containing the antigen (product) in the form of fermentation broth, cell culture supernatant or ascites fluid.
5. A liquid chromatography system comprising of a peristaltic pump(s), UV monitor, and a chart recorder.
6. Benchtop centrifuge (Beckman, Fullerton, CA or Kendro Laboratory Products, Newton, CA).
7. UV spectrophotometer for absorbance measurements at 280 nm.
8. A range of common physiological buffers, such as phosphate buffered saline at pH 7.4 (PBS); 20 m*M* Tris-HCl + 200 m*M* NaCl at pH 8.0, and/or 20 m*M* sodium bicarbonate + 200 m*M* NaCl at pH 8.5.
9. 0.5 *M* NaCl in 0.1 *M* $NaHCO_3$ at pH 9.0.
10. 1 *M* NaCl in 0.1 *M* sodium acetate at pH 4.0.
11. 1 *M* ethanolamine in PBS (blocking buffer).
12. 3 *M* potassium thiocyanate in PBS (chaotropic elution buffer).
13. 0.1 *M* glycine/HCl + 200 m*M* NaCl at pH 3.0 (low pH elution buffer).
14. PBS + 0.02% sodium azide.

3. Methods

The procedures for performing a generic immunoaffinity purification technique have been outlined in this section and divided into five distinct steps for ease of operation.

3.1. Antibody Immobilization

1. It is assumed that antibody solution against the antigen (product) will be available at a reasonable level of purity. The purer antibody solution would result in low levels of nonspecific interactions during purification. Dialyze the antibody solution in PBS prior to immobilization (*see* **Note 1**). Measure the absorbance of the antibody solution in PBS prior to initiating immobilization.

2. Weigh out the amount of activated solid phase (matrix), 2 g of freeze-dried activated agarose-based matrix would generally yield approx 6 mL of gel, and resuspend in PBS. This would give enough gel to pack a 2–3 mL column. Pellet the gel in a benchtop centrifuge (2000*g*) and remove the supernatant. Wash the gel with 10 vol of PBS discarding the supernatants after wash.
3. Challenge the activated and washed matrix with 100 mg of antibody solution in 20 mL of PBS. Add the antibody solution to the washed gel in a sealed glass tube and rotate end over end on a rocking reaction platform at room temperature or at 4°C.
4. At intervals, centrifuge the reaction mixture at 2000*g* and measure the absorbance of supernatants at 280 nm until no further change in absorbance is observed (*see* **Note 2**). Determine the concentration of bound antibody per unit volume of gel matrix by a method of difference.
5. Terminate the reaction by successively washing with 10 vol of 0.5 *M* NaCl in 0.1 *M* $NaHCO_3$ at pH 9.0; 10 vol of 1 *M* NaCl in 0.1 *M* sodium acetate at pH 4.0; 10 vol 0.5 *M* NaCl in 0.1 *M* $NaHCO_3$ at pH 9.0 and finally, 10 vol of 1 *M* ethanolamine in PBS to block remaining activated groups on the solid phase (*see* **Note 3**).
6. The immunoadsorbent thus generated is packed in a glass column at a flow rate of 1.0 mL/min using the liquid chromatography system (*see* **Note 4**) and treated with 5 vol of 3 *M* potassium thiocyanate in PBS or 0.1 *M* glycine/HCl + 200 m*M* NaCl at pH 3.0 and 10 vol of PBS in packed bed mode (*see* **Note 5**).

3.2. Sample Loading

1. Equilibrate the column with 5 column volumes of PBS, adjusting the chart recorder baseline at 0% deflection by setting the UV monitor.
2. Apply the clarified feedstock solution (or culture supernatant) containing the antigen (product) on to the column at a flow rate of 0.5 mL/min (*see* **Notes 6** and **7**).
3. Collect the column outlet fractions (2 mL) into glass borosilicate tubes throughout the purification cycle.
4. After a single pass, wash the immunoadsorbent column with 5 column volumes of PBS until the absorbance of the column outlet drops to baseline level on the chart recorder (*see* **Note 8**).

3.3. Elution

1. Elute the product with at least 2 column volumes of either 0.1 *M* glycine/HCl + 200 m*M* NaCl at pH 3.0 (low pH buffer) or the chaotropic buffer (3 *M* potassium thiocyanate in PBS) collecting the fractions (2 mL) into glass borosilicate tubes. The choice of eluting buffer will be dependent on the interactions between the product and the immobilized antibodies as well as the stability of product in eluting agents.
2. Elution may require approximately 2–3 column volumes of the elution buffer (*see* **Note 9**). An absorbance peak obtained will be collected in the tubes. Care

should be taken of the chromatography system during elution with certain types of elution buffers (*see* **Note 10**).

3. As the eluting conditions in immunoaffinity adsorption are generally harsh because of the strength of the antigen–antibody interactions, product stability in such elution buffers is generally a problem. It is, therefore, common to bring the product into a physiological condition soon after elution. This is achieved by either increasing the pH of the product solution to between 7 and 8 using 0.1 *M* NaOH in the case of low pH elution buffer, or desalting the product (*see* **Note 11**) in the case of chaotropic elution buffer.
4. The pH-adjusted or desalted product is stored at 4°C until further analysis.

3.4. Regeneration

1. Regenerate the immunoadsorbent column by washing with 5 column volumes of PBS and finally 2 column volumes of 0.02% sodium azide in PBS.
2. Store the immunoadsorbent at 4°C until required for the next purification cycle.

A typical separation profile during purification of biomolecules using immunoaffinity adsorption is presented in **Fig. 2**.

For large-scale immunoaffinity chromatography operations, it is advisable to have a small precolumn or a guard column for protecting the usually expensive immunoadsorbent column. It is not unusual to expect up to 100 cycles of operation from an immunoaffinity adsorbent if the column is maintained and operated in a sanitary fashion through out its use (*see* **Note 12**). However, the longevity of immunoadsorbents vary widely in the literature ranging from five to hundreds of cycles (*see* **Subheading 4.**).

3.5. Product Analysis

A range of analytical tests commonly used in the analysis of the product and efficiency of the immunoaffinity adsorption are listed here. The level and complexity of analysis will be dictated by the intended use of the product. However, the list here incorporates the generally applied analytical tools for evaluating the purity, integrity, and concentration of the product after a typical immunoaffinity separation. The experimental details of such analytical tests are not covered here and therefore can be accessed from a generic molecular biology or biochemistry methods book.

1. Generally, protein concentration of the start sample, breakthrough and the elution fractions are determined by absorbance measurements at 280 nm using a standard laboratory UV spectrophotometer. The conversion of the absorbance units to product concentration is done using the predetermined extinction coefficients of the product (*see* **Note 13**). Alternatively, a commercially available protein assay such as a Lowry, Bradford, or a BCA total protein assay can be performed using a common standard such as albumin. This will provide a total

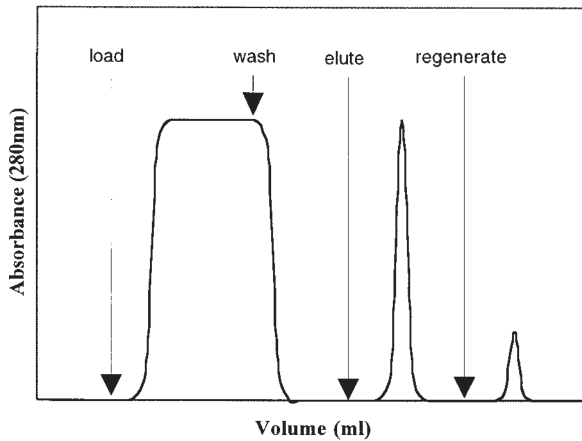

Fig. 2. A typical separation profile during purification of biomolecules using immunoaffinity adsorption.

protein content in the elution fractions. Not all of the protein content will be the result of the product, and will depend on the level of purity during purification.

2. For determining purity of the eluted product, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie or Silver Staining is commonly used. Depending on the nature of the product, a reducing or nonreducing gel will be used. Similarly, if the molecular weight of the product is known, the gel percentage, and selections on gradient or standard gels, can be made. The start sample, breakthrough, and eluted fractions can all be run on an SDS-PAGE to assess the success of the purification.
3. The SDS-PAGE in addition to providing information on purity of the product (absence or presence of other impurity bands), also serves as an identity test for the product if the true molecular weight is known. The eluted peak fractions should only show the product bands, however, the bulk of the impurities should be present in the breakthrough fractions.
4. Western blot analysis on SDS-PAGE gels, in the presence of antibodies against the product already used in the construction of immunoadsorbent, will provide a definitive identity test. This test will also provide information on any cross-reactivity of the antibody to the impurities in the feedstock. This will give an indication of the potential for nonspecific binding to the immunoadsorbent.
5. Finally, an enzyme-linked immunosorbent assay (ELISA) is commonly used to quantitate the product concentration and determine its activity. Conventional ELISA for the product can be developed relatively easily as the antibody to the product is already available. This is a rapid screening technique with potential for testing a large number of fractions (up to 96 samples/microtiter plate) generated through evaluation, optimization, and scale-up of immunoadsorption technique.

4. General Scale-Up Considerations

The capacity of the immunoadsorbent will be affected by the density, points of attachment, and orientation of the immobilized antibodies on to the matrix. Therefore, a range of schemes for achieving oriented coupling such as site-directed immobilization have been reported ***(7,11,12)***. The extent of improvement in capacity through orientated coupling of antibodies to the matrix is unclear, however, generally low density of immobilization results in high efficiencies of antigen binding. Even this is dependent on the size and geometry of antigen. For large-scale separations using immunoaffinity adsorbents, it is significant to have a high capacity immunoadsorbent for economics of large-scale processing, however, this has to be balanced against the cost of increased processing times. The nature of the matrix (rigidity, porosity, and so on) and kinetics of adsorption and desorption processes will also influence the through-put.

Immunoadsorbents are prone to loss of capacity over usage as a result of several processes including ligand leakage and irreversible denaturation of the immobilized antibodies because of harsh elution regimes. It is not always possible to separate these two primary causes. However, studies in the literature indicate that types of antibodies (polyclonal or monoclonal), different panels of antibodies, coupling chemistries and elution regimes all influence the loss in capacity to varying degrees and their impact on certain immunoadsorbents can only be determined for individual systems through detailed evaluations. The cost and stability of immunoadsorbents are important factors to be considered in large-scale applications. Reduced loss of capacity over large number of cycles under the optimum conditions of operation would bring down the overall cost of immunoaffinity separations.

The performance and efficiency of some immunoadsorbents may be influenced by the choice of pH and buffer systems during various stages of the immunoaffinity operation. It is advisable to screen the common pH and buffer systems for immobilization and immunoadsorbent operation processes to select the best and stable set of conditions for specific systems. Choice of pH and buffer systems which minimize loss of capacity, has less impact on stability of immunoadsorbent and the activity of product would prove more attractive for large-scale applications.

General methods used to elute adsorbed products involve altering the physical and chemical properties of the solution in contact with the immunoadsorbent to weaken the antigen–antibody association. Amongst these, altered pH (high or low), chaotropic agents, organic solvents, change of ionic strength, thermal elution, electrophoretic elution, pressure elution, and biospecific elution have all been used with varying degrees of success depending on a specific separation system. However, nonspecific elution regimes such as low pH

(e.g., glycine/HCl at pH 3.0 or below) and high concentrations of chaotropic agents (up to 3 *M* SCN^-) have largely been more successful than others.

The elution regime will have a profound effect on stability, capacity, and longevity of immunoadsorbents. For large-scale applications, it is vital that the elution regime is cost-effective, but also it does not result in ligand leakage, which may result in loss of capacity and have an impact on the product quality. This could have serious consequences on products destined for therapeutic applications. It is, therefore, important to screen appropriate elution regimes and validate the repetitive usage over a large number of cycles by monitoring both capacities and ligand leakage.

The use of membrane-based immunoaffinity adsorbents provide an alternative to the conventional gel-based matrices. Several reports ***(13,14,15)*** indicate that advantages of mechanical strength, high porosities, and lack of diffusional resistances enable high volumetric throughputs, thus reducing processing times. These can be used either in the form of hollow fiber cartridges or filter stacks. For certain applications this approach may be worth exploring for large-scale operations.

For large-scale applications it would be economical in certain processes to incorporate the immunoaffinity purification step in the early stages of the downstream processing scheme to reduce the process volume as early as possible. However, this has to be balanced against the cost and lifetime of the immunoadsorbent column because of its use with very crude feedstreams early in the process.

5. Notes

1. If the antibody solution is not already in PBS, dialyze either using a dialysis bag or a small UF cell with a molecular weight cut off of ~10 kDa.
2. Typically, the reaction would be complete in ~2 h at room temperature to achieve equilibrium when the maximum amount of antibody binding has taken place.
3. Alternatively, 0.1 *M* Tris-HCl at pH 8.0 is also used as a blocking agent in the absence of ethanolamine.
4. It is not necessary to have a liquid chromatography system, a low speed peristaltic pump with a flow-cell UV monitor and a chart recorder will generally be sufficient for preliminary evaluations.
5. As with all chromatography operations involving gradient or stepwise elution, immunoaffinity columns should be washed with final elution buffer prior to equilibration and sample loading. If the resulting packed immunoadsorbent is to be stored for any length of time before use, it is advisable to wash the column with PBS containing 0.02% sodium azide and stored at 4°C.
6. Clarification of the supernatant can be done by either low speed centrifugation (2000*g*) or filtration through a 0.5 μm filter. This will protect the immunoadsorbent from fouling.

7. It is advisable to dialyze the product containing solution with the wash buffer (PBS) prior to loading onto column to obtain similar ionic strengths. This can be done either through a dialysis bag or by diluting the solution 1:4 by PBS.
8. If the product is breaking through in the wash after analysis (*see* later), reduce the loading flow rate from 0.5 mL/min to 0.2 mL/min. Alternatively, recycling of the loading solution is also possible to reduce the loss of the product in the breakthrough.
9. Depending on the desorption mechanism, elution peak may show a tail which requires more than 2–3 column volumes of elution buffer to reduce the absorbance to baseline level. In some cases, particularly in the case of chaotropic elution buffer, true baseline may not be reached due to the background absorbance of the elution buffer itself, in which case a stable baseline is what one should look for.
10. The low pH elution buffer (glycine-HCl pH 3.0 + 0.5 *M* NaCl) can oxidize stainless steel. Always wash the column and the LC system with halide-free buffer (or preferably distilled water) at the end of the day.
11. Desalting is commonly done using a gel filtration column of Sephadex G-15 (Amersham Pharmacia Biotech; Uppsala, Sweden) (2.6 × 40 cm) previously equilibrated with PBS and run at 1 mL/min.
12. It is advisable to regenerate the column with a routine wash of 2 *M* KCl and 6 *M* urea after each use. This will clear the denatured components that have bound nonspecifically to the column and have not been released by either washing or elution buffer.
13. The extinction coefficient of the product can only be determined using a pure product usually by amino acid analysis. However, relative protein concentrations to, for example, albumin standard can be obtained in the preliminary evaluations.

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10

Expanded Bed Adsorption in the Purification of Biomolecules

Allan Lihme, Marie Hansen, Morten Olander, and Elias Zafirakos

1. Introduction

Stabilized fluid bed adsorption also phrased, expanded bed adsorption (EBA), is a recently introduced "whole broth processing" technique that enables the isolation of biomolecules (e.g., proteins and plasmids) directly from crude raw materials such as fermentation broth or extracts from natural sources (**Fig. 1** and **Table 1**). The use of EBA, as an alternative to the traditional methods, may in many instances combine the effects of centrifugation, filtration, concentration and purification into *one* step and hereby save time, increase yields, and cut down processing costs ***(1–9)***. EBA is a new technology that—given room for proper adaptations—may be successfully applied within a number of different industries ranging from the highly sophisticated and highly regulated pharmaceutical industry, through the production of industrial specialty enzymes, to the low-cost high-volume applications characteristic for the food industry. Even certain applications within the field of waste water treatment and valorization of waste materials may be envisioned.

1.1. How EBA Works

Opposed to traditional chromatographic technology, which employs tightly packed beds of adsorbent; EBA involves an expansion of the adsorbent caused by an upward flow of liquid. The expansion allows free passage of crude, nonclarified raw materials through the column without any clogging which is often seen with packed bed columns. The expanded bed thus enables adsorption of the product directly from the primary source (**Fig. 2**). EBA is a special case of fluidized bed adsorption wherein the chromatographic bed is stabilized towards uncontrolled turbulence and back-mixing by an optimal design of both

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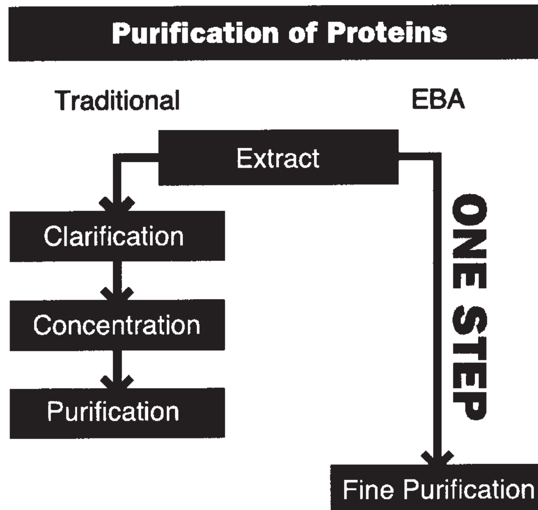


Fig. 1. Expanded-bed adsorption combines the effects of centrifugation, filtration, concentration, and purification into one step.

Table 1
Expanded Bed Adsorption—Potential Areas of Application

Isolation of proteins, peptides, enzymes, plasmids, and other relevant biomolecules from:	Adsorption of unwanted substances from:	Other
Crude fermentation broth	Fruit juices, beer, wine, and other beverages	Immobilized enzymes
Animal tissue extracts	Waste water streams	
Plant extracts	Blood (extra corporal adsorption)	
Blood/plasma/serum		
Milk and whey		
Egg white and egg yolk		

the column and the high-density adsorbent media. An expanded bed has plug flow without back-mixing up through the bed, which ensures an efficient adsorption with a reasonable number of theoretical plates (e.g., a plate number of 100–200 N/m).

Another significant advantage of EBA as compared to packed bed adsorption is the lack of problems with back-pressure and compression of the bed during operation. Because the adsorbent bed is kept nonpacked during operation it is now possible to scale up to very high columns—a problem that for a long time has haunted the industrial application of packed bed adsorption. Still

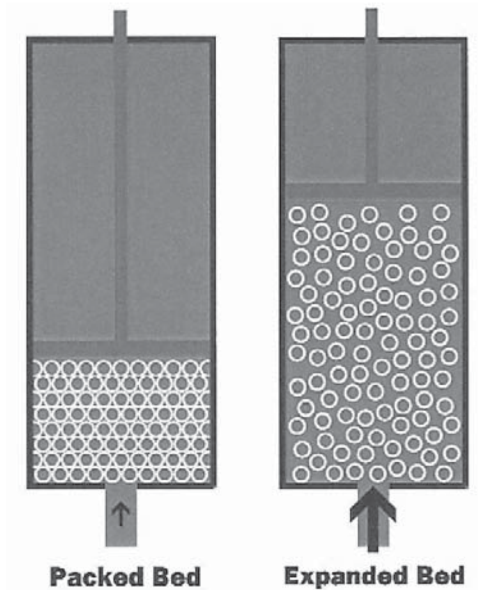


Fig. 2. During EBA, the adsorbent bed is allowed to expand inside the column when an upward flow of liquid is applied. The distance between the adsorbent particles result in an unhindered passage of any particulate impurities in the feed stock. Traditional packed beds work as dead-end filters that will clog up unless the feed is thoroughly clarified.

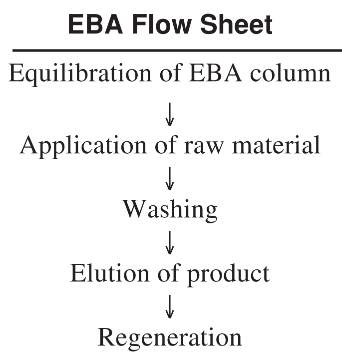
another important consequence of the nonpacked mode of operation is a new degree of freedom in the choice of solid-phase materials. Traditional packed bed adsorption has called for rigid solid phases that do not deform when packed in a column. As this is no longer an issue of the same importance, it is now possible to choose solid-phase materials of lower cost and maybe even with higher binding capacities.

1.2. Overview of the EBA Process

Basically, EBA involves the same steps as a traditional packed bed adsorption. In some instances, EBA may be the only step to achieve an acceptable product, but, in many instances it may be the first "capture step" that provides a fast concentration, clarification, and buffer exchange, which is followed up by one or several polishing steps (*see* the following table).

1.3. EBA Hardware

Successful and robust EBA processes are the result of integrated and optimized solutions for the three determinant parts of EBA: the column, the adsorbent beads, and the ligand chemistry applied to the beads.



1.3.1. Design of EBA Columns

An EBA column must fulfill at least two requirements: (1) facilitate the achievement of a stabilized fluid bed with efficient adsorption characteristics and (2) not give rise to any clogging problems during operation. To facilitate a stable bed, it is necessary to have an efficient distribution of the incoming flow of liquid in the bottom of the column.

The design of the UpFront EBA column has eliminated the customary distribution plate used to distribute the flow, which gives rise to clogging with some types of raw materials. The crude raw material is introduced through an inlet valve placed in one side of the column. A gentle stirring localized at the bottom ensures an efficient distribution of the liquid thus avoiding channeling and turbulence (*see* **Fig. 3**). Because of the very gentle stirring and the optimized design of the stirrer, no vortex is formed, only a local efficiently mixed zone at the bottom of the column is observed. Above the mixed zone there is undisturbed "plug flow." At the top of the UpFront EBA column there is an outlet without any nets or screens that could give rise to clogging during the process. This design ensures that any particulate impurities in the raw material, as well as large air bubbles, will pass freely through the column ***(10–12)***.

After application of raw material and wash, the flow may be reversed and elution performed in fixed-bed mode where the chromatographic beads are packed on the support net at the bottom of the column. At this stage there is no risk for clogging because all the crude raw material has been washed out. Alternatively, the elution may also be performed in the expanded bed state, e.g., at a relatively low degree of expansion to assure a minimal elution volume (*see also* **Notes**).

1.4. Adsorbent Beads

The adsorbent particles or beads that are to be employed in an EBA process must have a significantly higher density than the raw material to allow accept-

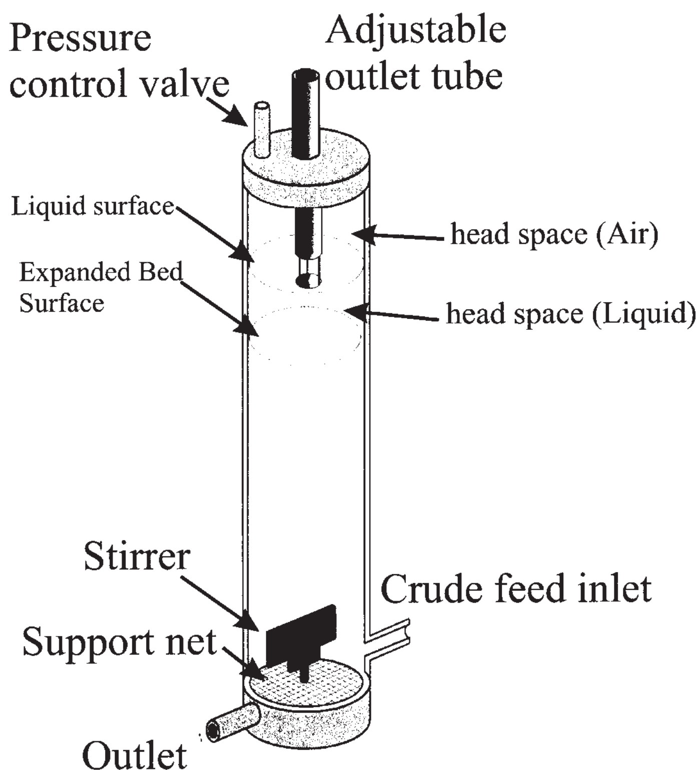


Fig. 3. The UpFront EBA column (Fast Line) has no distribution plates that clog up with crude raw materials. A mechanical stirrer ensures even distribution and plug flow. The support net and the bottom outlet is used for packed-bed elution only. Stirring speed is adjusted according to flow rate and viscosity of the raw material. The liquid head space above the expanded bed is controlled by the position of the adjustable outlet tube and the air head space is controlled via the pressure control valve.

able flow rates during operation. If the density is too low, the beads will be lost in the column effluent. As most raw materials are aqueous this means, in practice, that densities from about 1.1 and upwards are relevant candidates. Robustness of the EBA process is, however, enhanced by the use of relatively high-density EBA adsorbents (e.g., d = 1.3–1.5). An expanded bed of beads with a high density expands less at a given flow rate (*see* **Fig. 4**) and is less vulnerable to variations during the process, e.g., density differences between buffers and raw materials or the occurrence of air bubbles.

As depicted by the Stokes law on terminal sedimentation velocities for particles falling in liquids (*see* **Fig. 5**) the diameter of the particles is also a determinant factor. The larger the diameter, the faster the EBA particle sediments in

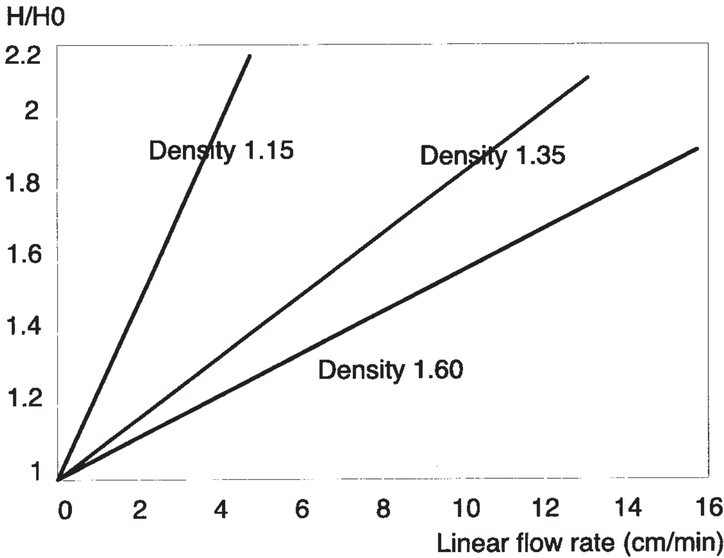


Fig. 4. Expansion curves as a function of adsorbent density. The graph illustrates the dramatic differences in the degree of expansion by varying the density of the adsorbent (*H* = expanded-bed height observed at a given flow rate, H*0* = packed-bed height without flow). A density of 1.3–1.5 seems to be the best compromise between degree of expansion, process robustness, and total binding capacity per liter adsorbent. For certain applications, e.g., involving highly viscous raw materials or ultrahigh flow rates, it may, however, still be relevant to use adsorbents with densities in the range of 1.6–1.8. Note that these data are produced with $\theta = 100 - 300$ µm beads. Other size ranges would produce other curves.

the liquid, and the less the adsorbent bed expands at a given flow rate. However, the applicable flow rate is limited to a very high degree also by the mass-transfer kinetics and the kinetics for binding of the protein to the ligand. Therefore, a compromise in terms of size range and density has to be made between fast sedimentation rates and fast mass-transfer kinetics. This compromise will, of course, depend on the nature of the raw material (e.g., viscosity and product concentration) and it may, therefore, be very relevant to optimize the bead characteristics according to the application to obtain maximal performance.

Apart from having an optimal density and size with respect to sedimentation and throughput, it is also of major importance to have an optimal size *range* of the adsorbent beads. This is to ensure a proper stabilization of the expanded bed, i.e., when a flow is applied to the initially packed bed of adsorbent beads, a stratification of the beads according to their size and density will take place.

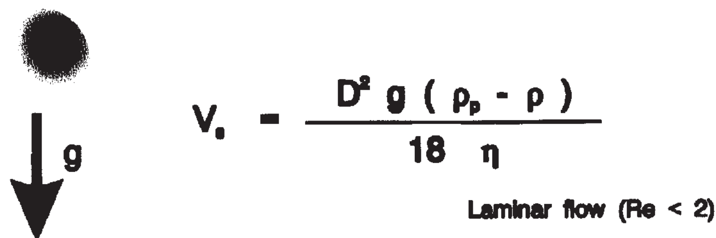


Fig. 5. Stokes law about the terminal sedimentation velocity of a particle falling in a liquid. D = particle diameter, ρp = density of particle, ρ = density of liquid. η = viscosity of the liquid.

The larger and the more dense beads will be at the bottom of the column, whereas the smaller and the less dense beads will position themselves at the top of the column—a gradient of beads sizes and densities is formed up through the column. This phenomenon is self-creating and is stabilizing the bed against back-mixing and unwanted turbulence. As a rule of thumb, the optimal size range is obtained by having a factor of about three between the size of the smallest and the largest beads.

EBA adsorbents should furthermore be relatively stable toward shear forces because they are applied in a dynamic system where the beads are in movement much of the time. Also, the chemical stability should be high owing to the use of harsh regeneration conditions (e.g., 1 *M* NaOH).

Agarose has been used for production of beads for packed-bed adsorption for a long time (e.g., Sepharose [Pharmacia, Uppsala, Sweden, UK]) and is well accepted as a near-optimal material for affinity chromatography. Cross-linked agarose beads are very stable toward shear and harsh chemical regeneration procedures and there are plenty of proven chemical derivation schemes available. The only problem with ordinary agarose beads is that the density is very close to 1.0, which invalids their use in EBA.

One way of solving this problem is to incorporate a solid glass bead *inside* the agarose bead (*see* **Fig. 6**). The density of such an agarose-glass bead will be about 1.3 if the incorporated glass bead takes up 20% of the total bead volume ***(13)***.

As the glass bead is impermeable and has a very low surface area, it does not interact significantly with the biomolecules to be fractionated. By having a central core of glass, (i.e., a pellicular structure), as opposed to having many smaller glass particles dispersed in the agarose bead (i.e., a conglomerate) it is ensured that the beads have maximum mass-transfer kinetics and, therefore, fast equilibration with the surroundings. Also, the physical stability is kept at a maximum by using a pellicular structure.

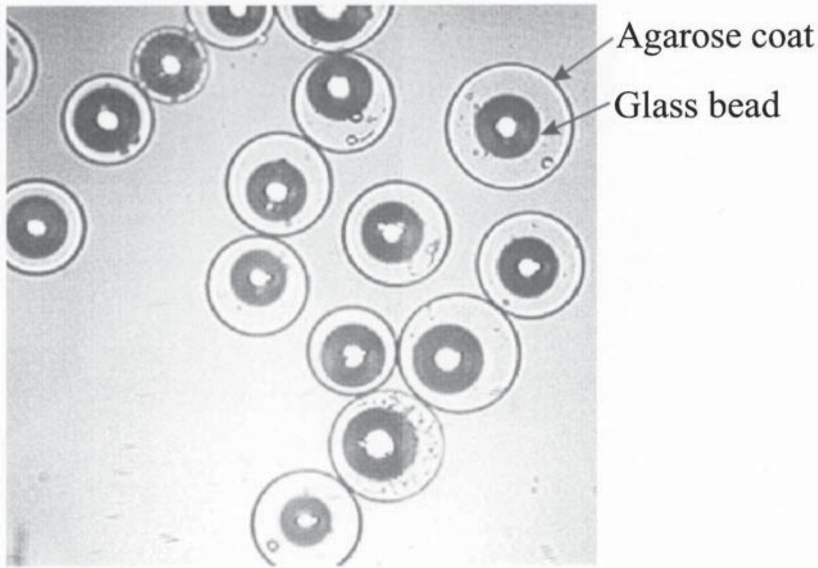


Fig. 6. A pellicular structure of the adsorbent beads ensures optimal mass transfer kinetics and a high stability towards shear forces. UpFront EBA adsorbents are based on cross-linked agarose beads (Ø =100–300 μm) with a central core of high-density glass (20–30 vol %) resulting in a density of 1.3–1.5.

1.5. Ligand Chemistry

In principle, any type of ligand chemistry used for packed-bed adsorption may be applied for EBA as well. The fact that EBA is applied on the crude raw material may, however, give rise to a different set of considerations than those connected to the adsorption from more refined raw materials. A crude raw material typically contains a number of different foulants that may not be present or may be significantly reduced further downstream, e.g., cell debris, colored polymers, and DNA. Therefore, it is of major importance that the ligand chemistry is chosen to minimize interaction with these contaminants and to ensure that it can withstand any harsh regeneration conditions (e.g., 1 *M* NaOH) that may be needed to remove strongly bound contaminants. Typically, the crude raw material also has a relatively low product concentration, which calls for ligands with a high binding constant to ensure a satisfactory dynamic bind-

ing capacity. Ion exchangers, which generally have a high binding capacity, may be a good choice in some applications, but may also suffer from heavy fouling because of the high charge density with decreased capacity and agglomeration of the adsorbent as the result. Furthermore, many crude raw materials have a relatively high ionic strength, which makes dilution or desalting necessary before the EBA step.

Also, the very popular hydrophobic adsorbents based on uncharged alkyl or phenyl ligands may not be the optimal choice in EBA because efficient binding to these ligands typically require the addition of high concentrations of lyotropic salts (e.g., ammonium sulphate) to the raw material. This may be prohibitive in a large-scale production because the cost of the salt and the disposal of the waste adds considerably to the overall cost of the process. In some instances, it is furthermore necessary to use an organic solvent for elution of the bound protein, which also may be prohibitive resulting from denaturation of the protein, increased costs, and safety issues.

Biospecific affinity ligands may be employed in EBA with great success. It is, however, again necessary to carefully consider the chemical stability and the cost of the ligand in each application.

At UpFront, we have revived and optimized the principle of "mixed mode" ligands ***(14–24)*** for optimal capture and partial purification of proteins from crude raw materials. A typical mixed-mode ligand is a stable, low molecular weight, chemical substance with a hydrophobic core onto which different hydrophilic or ionic substituents are attached (*see* **Fig. 7**).

The substituents, and their charge and pKa values, influence the binding specificity and the binding strength in a strongly pH dependent manner. This means that binding will take place at pH values characteristic for the particular "mixed-mode" ligand and the specific protein, whereas efficient elution of the bound protein often can be performed by a simple change of pH. By employing a range of mixed-mode ligands, it is possible to obtain a wide spectrum of group-specific binding patterns from which the optimal binding characteristics can be chosen.

The binding of proteins to mixed-mode ligands is, in many instances, furthermore largely independent of ionic strength (*see* **Fig. 8**), which gives the mixed-mode adsorbents a distinct advantage over standard ion exchangers. The potential advantages of mixed-mode adsorbents compared to traditional ion exchangers and noncharged hydrophobic adsorbents are summarized in **Table 2**.

The following section describes a practical example on the application of EBA for isolation of monoclonal antibodies using a mixed-mode adsorbent optimized for this purpose.

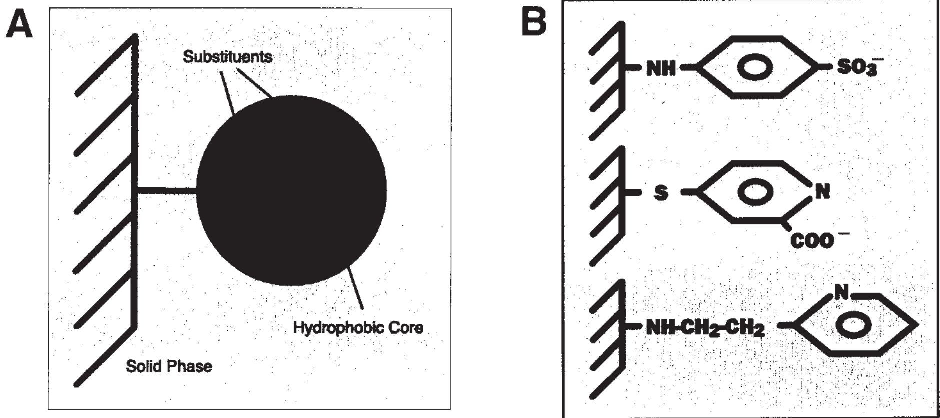


Fig. 7. Mixed mode ligands comprise a hydrophobic, typically aromatic, core structure derivatized with hydrophilic, acidic, or basic groups. **(A)** is a schematic illustration of a mixed-mode ligand with three substituents, whereas **(B)** exemplifies chemical structures, which may be employed as mixed-mode ligands.

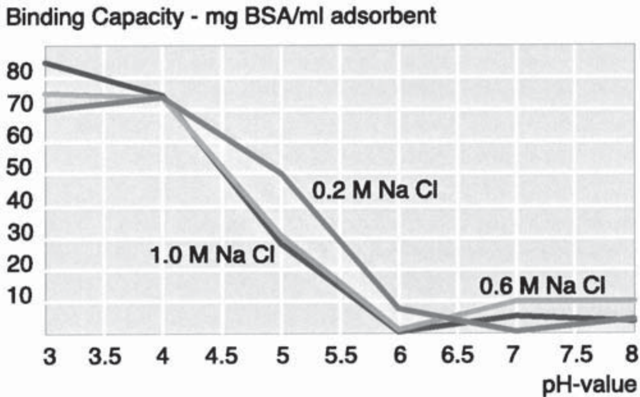


Fig. 8. Mixed-mode matrix. The binding of proteins to a mixed-mode ligand may in many instances be independent of ionic strength in the raw material. The graph illustrates the binding of bovine serum albumin to a specific mixed-mode ligand as a function of pH and salt concentration.

2. Materials

2.1. Raw Material

1. Hybridoma cell culture grown in Dulbecco's Modified Eagle's Medium (DMEM) + 1% fetal calf serum.
2. Monoclonal antibody concentration: 40 µg monoclonal mouse IgG1/mL.

Table 2
Mixed-Mode Adsorbents

Advantages over traditional ion exchanges and hydrophobic adsorbents
No desalting or dilution of the raw material
Avoidance of lyotropic salts in the raw material
Avoidance of organic solvents in the eluent
Individual group specificities are available
Low fouling characteristics

2.2. Adsorbent

®AD, cat. no.: 1502, UpFront Chromatography A/S, Copenhagen, Denmark.

2.3. Buffers

1. Conditioning buffer: 2 *M* acetic acid/NaOH pH 5.1 + 10 mg/mL sodium lauroyl sarcosinate (cat. no.: L5125, Sigma Chemical Co., St. Louis, MO) (also *see* **Note 1**).
2. Washing buffer: 0.01 *M* sodium citrate pH 6.0.
3. Elution buffer: 0.05 *M* potassium phosphate pH 7.0.

2.4. EBA Column

1. FastLine™ 20, cat. no.: 7001-0020 (UpFront Chromatography A/S)
2. UV-monitor and recorder (Amersham-Pharmacia Biotech, Uppsala, Sweden).
3. Peristaltic tubing pump type SF 70 (Verder, Haan, Germany).

2.5. Analyses

1. Mercaptoethanol reduced SDS-PAGE was performed using precast gels (4–12%) from Novex. Scanning densitometry on Coomassie stained gels was performed with CREAM (Kem-En-Tec A/S, Copenhagen, Denmark).
2. Single radial immunodiffusion ***(25)*** for determination of monoclonal mouse IgG concentrations were performed using rabbit anti mouse IgG, Z109, from Dako A/S (Copenhagen, Denmark). The tests were performed on 10 × 10 cm glass slides covered with 15 mL 1% agarose containing 12.5 μL antibody solution.

3. Methods

3.1. The FastMabs System

The FastMabs system is a set of different mixed-mode adsorbents individually optimized for isolation of monoclonal and polyclonal antibodies from different raw materials (**Table 3**). The FastMabs A adsorbent is optimized for isolation of monoclonal antibodies and their fragments from hybridoma cultures as well as genetically engineered *E. coli* fermentations ***(26–29)***. FastMabs A is an agarose matrix derivatized with a low molecular weight mixed-mode ligand, which

Table 3
The FastMabs System

FastMabs adsorbent	Main applications/species bound/Ig's bound
FastMabs A	Monoclonal antibodies from hybridoma cell cultures/ mouse, rat, human/all IgG subclasses, IgM, Fabfragments
FastMabs B	Polyclonal antibodies from serum, ascites, egg yolk, milk, whey/mouse, rat, rabbit, human, horse, goat, sheep, cow, chicken/ IgG
FastMabs C	Polyclonal IgG (IgY) from chicken serum

Table 4
Process Flow Sheet for Purification of Monoclonal Antibodies with FastMabs A

Conditioning of raw material	Addition of conc. acetate binding buffer (1:20), pH adjustment to pH 4.5–5.5
Adsorption	Expanded-bed adsorption, flow rate 2–8 cm/min
Washing	Acetate or citrate buffer pH 4.5–6.0
Elution	Neutral buffer, e.g., phosphate, or carbonate buffer pH 7–9

is stable toward 1 *M* sodium hydroxide. It has a broad-binding specificity for both mouse, rat, and human immunoglobulins including IgM and is recommended for the first capture step using expanded bed adsorption.

3.2. Process Flow Sheet

The binding of antibodies to FastMabs A is performed at pH 4.5–5.5. One of the distinct advantages of FastMabs A is that the binding is largely independent of the ionic strength in the raw material so that dilution or desalting, therefore, is avoided. Elution of the bound antibody is accomplished by changing pH to around neutral pH. **Table 4** illustrates the straightforward purification procedure for hybridoma supernatants.

1. Two-and-a-half liters of the crude hybridoma cell culture (comprising fetal calf serum as well as hybridoma cells) is adjusted to approx pH 5.1 by the addition of 125 mL conditioning buffer. After mixing, check pH, and adjust if necessary to pH 5.1 +/– 0.05 with 1 *M* acetic acid (*see* **Notes 1** and **2**).
2. The UpFront FastLine column is assembled and fitted with the θ = 20 mm column tube. The inlet valve at the bottom of the column is closed and a suspension of 50 mL FastMabs A is poured into the column from the top.
3. Fit on the column cap and start pumping in water, while opening the inlet valve at the same time. Flow rate approx 25 mL/min.

4. Start the mechanical stirring on the EBA column, first at low speed and then at full speed. Ensure that the column is positioned completely vertical. Continue washing the column for about 10 min.
5. Start applying the conditioned raw material by manually changing the inlet tube from the water tank to the raw material tank (you may, of course, also use a valve for this purpose).
6. Collect 100 mL fractions of the run-through manually or with a fraction collector.
7. When all raw material has been applied, change to the washing buffer and wash the column at the same flow rate for 15–20 min or until the UV-recorder shows a stable baseline (*see* **Note 5**).
8. Stop the pump, close the raw material inlet valve, and let the adsorbent settle on the support net. Then, open the outlet valve at the bottom of the column and allow air to enter through the top inlet so that the liquid is drained partially from the column. Draining of buffer is continued until the liquid surface is 3–5 cm above the settled bed (*see* **Note 6**).
9. When the liquid surface is about 5 cm from the settled bed, start applying washing buffer by connecting the top inlet to the washing buffer vessel and start the pump with reversed flow direction of the pump (so that washing buffer is sucked into the column). Flow rate: approx 5–8 mL/min.

   Any air present in the inlet tube will diminish the head space of liquid above the settled bed. Take care that the top of the bed is not running dry. Wash the column with washing buffer until the UV-recorder gives a stable baseline.
10. Elution of the bound antibody is performed by changing the top inlet to elution buffer keeping the flow rate at 5–10 mL/min. Collect the eluate according to the signal from the UV-recorder.
11. After complete elution of the antibody you may perform a fast cleaning procedure: reverse the flow direction again and reexpand the adsorbent by washing the column with elution buffer for 10–20 min followed by washing with water for 20–40 min. The column is now ready for the next adsorption cycle. In cases where you want to clean the column and the adsorbent more exhaustively, you may also wash with 0.1–1.0 *M* NaOH for 20–40 min followed by elution buffer and, finally, water. The adsorbent may be stored (long term) in 0.1 *M* NaCl + 20% ethanol.

3.3. Results

Figure 9 illustrates the break-through curve of a monoclonal antibody grown in DMEM culture medium supplemented with 1% fetal calf serum. As can be seen, the adsorbent binds all the antibody until approx 1500 mL has passed the expanded bed of 50 mL FastMabs A adsorbent. After this point, an increasing concentration of antibody is passing the column unbound. In a real production situation it would probably be preferred to stop the application of raw material at about 2000 mL to avoid a too-large loss in the run through. However, this experiment is typical for the first exploratory tests

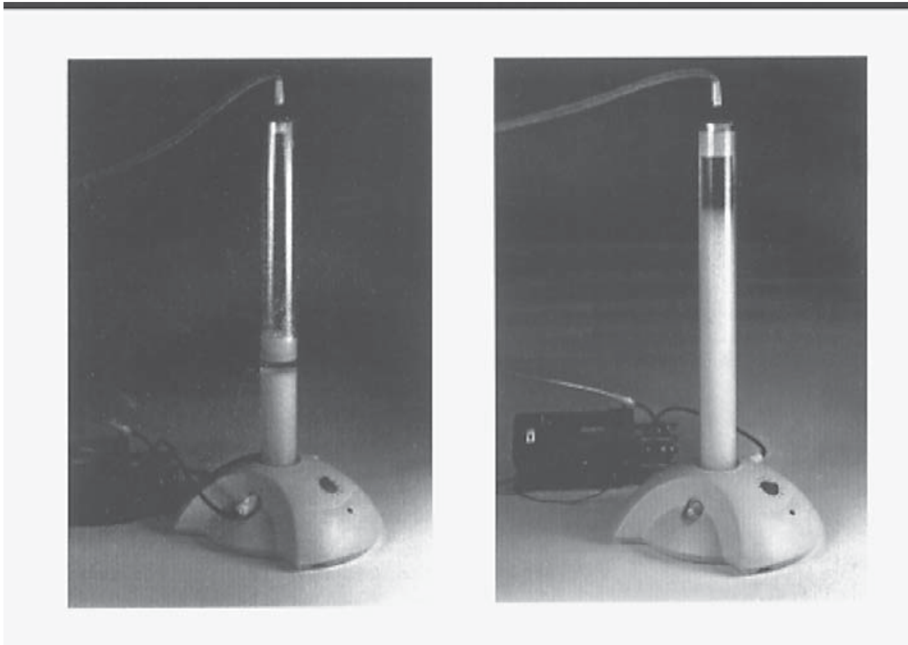


Fig. 9. The left photo shows the UpFront FastLine column without any flow through the column. The FastMabs A adsorbent is sedimented. The right photo shows the expanded bed obtained by applying a flow of washing buffer.

(the method development phase) wherein the dynamic capacity of the adsorbent is determined on the relevant raw material. The figure also illustrates the highly efficient, nontailing elution of the antibody, which is obtained by changing the buffer from pH 5.1 to neutral pH. **Table 5** summarizes the key figures from the experiment.

SDS-PAGE of the raw material and the isolated antibody (*see* **Figs. 10 and 11**) reveals that the one-step expanded-bed adsorption has resulted in a purity (as judged by coomassie staining and scanning densitometry) of approx 91%. The major impurity being fetal calf serum albumin. At the same time, the volume was reduced to 95 mL clear and colorless eluate, a factor of 26 when compared to the volume of the starting material. The yield, i.e., the percentage of applied antibody obtained in the eluate, was relatively low at 85%. The reason being that the column was overloaded in this experiment. The high recovery of 96% indicates that the yield will increase if the amount of raw material applied is adjusted according to the dynamic capacity defined by the break through curve. The total process time was about 2.5 h with only a small fraction of this being hands-on time.

Table 5
Expanded-Bed Adsorption

Raw material		2.5 L (100 mg IgG_1)
Column diameter	cm	2
Bed height	cm	16
Flow rate	cm/h	480
Total process time	min	150
Eluate volume	mL	95
Recovery	%	96
Yield	%	85
Purity	%	91

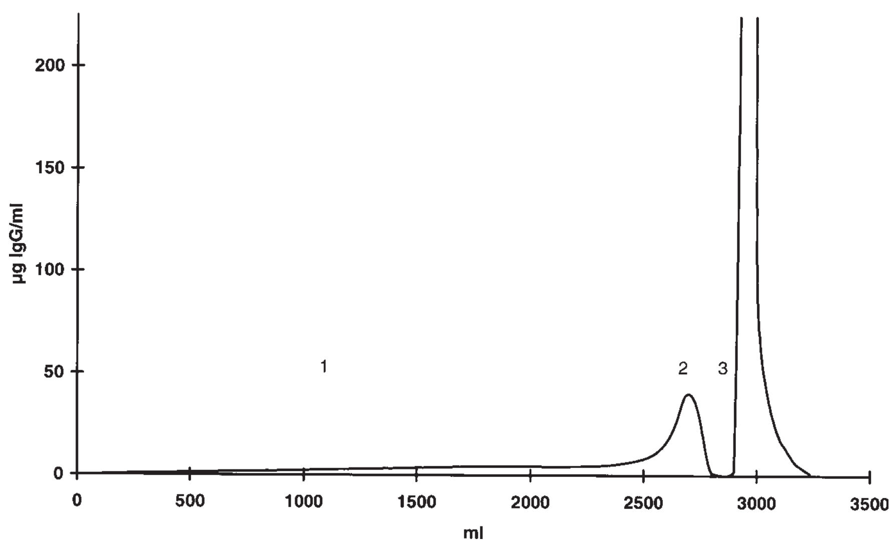


Fig. 10. Breakthrough of monoclonal IgG as a function of applied raw material volume. Determined by single radial immunodiffusion. 1. Application of raw material. 2. Start of washing step. 3. Start of elution step.

4. Notes

1. Conditioning of the raw material. The binding of immunoglobulins to FastMabs A is not entirely specific and it can, therefore, be advantageous to add a mild anionic detergent such as sodium lauroyl sarcosinate to improve the purity of the product. The optimal concentration of detergent is dependent on the concentration of fetal calf serum in the raw material. It is recommended to use in the range of 1–2 mg/mL sarcosinate if the raw material contains 2–5% fetal calf serum. If there is only very low amounts of fetal calf serum present (0–1%) it is recom-

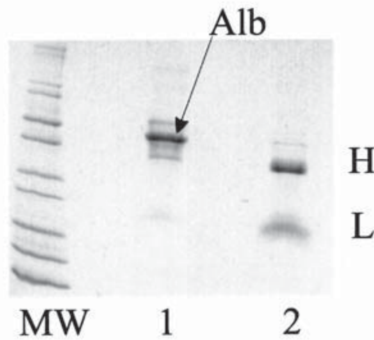


Fig. 11. SDS-PAGE on starting material and eluate from EBA adsorption. MW = molecular weight marker; 1-raw material, 2-eluate.

mended to use 0–0.5 mg/mL sarcosinate. In cases where you only want to use EBA as a high speed first-capture step with high capacity and do not care too much about the purity of the eluate, you may choose to omit the sarcosinate completely and maybe even adjust the binding pH to about pH 4.5–5.0.

2. In certain cases, a slight precipitation has been observed when adjusting pH in the cell culture down to about pH 5. In most instances, this has no effect on the monoclonal antibody and because the adsorption is performed as an expanded-bed operation it has often no practical consequence at all.
3. Care must be taken that the adsorbent is free of adsorbed air bubbles (e.g., originating from washing on a suction filter) before pouring it into the column. You may simply degas the adsorbent by applying a slight vacuum.
4. The flow rates recommended in the procedure can be varied within rather wide limits depending on, e.g., the viscosity of the raw material, the binding kinetics, and the concentration of antibody in the raw material. The binding capacity of FastMabs A will increase significantly with increasing concentration of the antibody in the raw material. In most cases, it will be advantageous to perform a few experiments either in small packed beds using clarified raw material or in small batch incubations of the adsorbent and the crude raw material to determine and optimize binding conditions for maximum capacity and purity.
5. The washing buffer used in this experiment has a pH of 6.0 using a very dilute citrate buffer. Some antibodies do not bind very efficiently to the FastMabs adsorbent under these conditions (i.e., they are released from the column when washing). In such instances, it is advisable to adjust the pH of the washing buffer down to about pH 5.
6. The procedure for elution of the bound antibody is here described as a packed-bed process. It has recently been documented ***(30,31)*** that elution may be performed efficiently and without significant dilution of the product also in the expanded-bed mode. This simplifies and shortens the process time.

4. Acknowledgments

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11

Application of HPLC in the Purification of Biomolecules

Paul Bradley and Mohamed A. Desai

1. Introduction

1.1. HPLC of Biomolecules

High-performance liquid chromatography (HPLC) was introduced commercially in the 1960s for the analysis of small molecules. The use of HPLC for the analysis and purification of macromolecules is a fairly recent development; traditional matrices did not provide the mechanical strength that was required for such an application. More recently, the introduction of semirigid (polystyrene) and rigid (silica) stationary phases has allowed macroporous matrices to be developed with sufficient mechanical strength to allow the use of high flow rates.

Macroporous matrices allow the complete permeation of proteins, thereby allowing a greater surface area to interact with the sample. This property is important for high-resolution separations, particularly at the preparative and commercial scale. Detailed discussions on the effects of particle and pore size on separation are presented in the literature ***(1)***. Currently, the use of HPLC in biotechnological, biomedical, and biochemical research comprises approx 50% of all users ***(2)***.

1.2. Principles of HPLC

Chromatographic separations involve the interaction of the sample (or target) molecule with two phases:

- *Stationary phase (also termed packing material, sorbent, or matrix)*: This usually consists of semirigid or rigid spherical beads of a defined size packed into a metal or synthetic column.
- *Mobile phase (also termed eluent, buffer, or solvent)*: This is a liquid phase, which carries the sample through the column.

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There are two types of interaction that can exist between the sample and stationary phase:

- *Adsorptive*: The protein is initially introduced to the column under conditions where the affinity for the matrix is greater than for the eluent and, as a result, the sample binds to the matrix. Sample components are removed (eluted) from the stationary phase by altering the composition of the eluent. The types of adsorptive interactions are discussed in **Subheading 1.3.**
- *Nonadsorptive*: An eluent is chosen, which is an optimum solvent for the sample, so that no binding occurs. The matrix acts as a physical sieve that retards the elution of sample components on the basis of size. The passage of sample completely through a packed column is termed elution. Two types of elution exist:
- *Isocratic*: In this type of elution, the composition of the eluent does not change. This is most commonly used in nonadsorptive methods.
- *Gradient*: This type of elution involves a stepwise or linear change in the buffer composition to desorb the sample from the matrix. Several types of HPLC exist, each involving a different type of interaction between sample and matrix and a different type of elution. These are introduced in **Subheading 1.3.** and discussed in detail in **Subheading 3.**

1.3. Types of HPLC

Several modes of HPLC are generally applicable, each exploiting different properties of proteins.

1. *Size-exclusion HPLC (SE-HPLC)*: This separates proteins on the basis of their apparent molecular size.
2. *Reversed-phase HPLC (rpHPLC)*: This method utilizes organic solvents to separate proteins on the basis of their degree of hydrophobicity.
3. *Hydrophobic-interaction chromatography (HIC)*: This method is similar in principle to rpHPLC, but uses aqueous solutions to separate proteins on the basis of polarity.
4. *Ion-exchange chromatography*: This method exploits the ionizable characteristics of some amino acid side chains, separating proteins on the basis of charge.
5. Affinity chromatography: This mode utilizes the specific interactions of proteins with other molecules, e.g., enzyme-substrate or antibody-antigen interactions.

1.4. Selecting the Correct Chromatographic Mode

The first and most important step before approaching a chromatographic problem is to clearly define what the objective of the separation is to be. A typical feature of biomolecules, not least proteins, is their great structural and functional diversity, which means that some forms of chromatography are unusable or inappropriate. For example, the use of reversed-phase chromatography for the purification of enzymes may not be appropriate because the harsh

solvents and acidic pH conditions common to the method often result in loss of enzyme activity. This would negate the use of immunosorbent or substrate-based assays and present problems if the step was preparative. Therefore, it is important beforehand to have a well-characterized product in terms of the number of components present, their size, charge, hydrophobicity, affinity characteristics, solubility, and stability, and to also have a clear idea of the overall objectives of the separation.

This chapter will deal predominantly with size exclusion and reversed phase HPLC, which will be discussed in more detail later. Size-exclusion HPLC (SE-HPLC) was chosen for its compatibility with biomolecules and, therefore, prevalence in the biotechnology industry. It will also permit the use of alternative detection modes such as refractive index (RI), viscometry, light scattering, and fluorescence. Reversed-phase HPLC (rpHPLC) is probably the most popular chromatographic technique because of its speed, high resolution, and robustness regarding sample composition or concentration. As a result, a vast array of column technologies are commercially available.

Hydrophobic-interaction chromatography (HIC) will be discussed in brief, with a description of the basis of separation and considerations for method development.

1.5. General Precautions When Performing HPLC

The following rules apply when performing all types of HPLC:

1. Always use HPLC-grade solvents and buffers.
2. Filter all eluents before use (0.45 µm; nylon for aqueous buffers, PTFE for organic solvents).
3. Degas eluents before use to prevent air from becoming trapped in the pump, column, or detector.
4. Flush column regularly with a suitable solvent/regeneration buffer to remove tightly bound sample components (refer to manufacturer's notes).
5. If possible, keep the column temperature below 60°C. The recommended temperature may be much lower than this in many cases (refer to manufacturer's notes).
6. Operate between the recommended pH limits of the column, e.g., three to seven for silica-based columns, and use a guard column when possible to prolong column life.
7. Replace eluents regularly or add a preservative such as sodium azide to prevent bacterial growth.
8. After using a column, flush through with an appropriate storage solvent (see manufacturer's notes) and cap tightly to prevent drying out.
9. Avoid banging or dropping the column, which could cause cracks to form in the matrix bed and worsen column performance.
10. Do not overtighten end fittings, as this can damage the column frits.

2. Materials

A vast selection of HPLC systems are commercially available from a number of manufacturers. Systems are generally modular, with a typical instrument comprising the following:

1. Pump: The purpose of this component is to accurately deliver a pulse-free supply of eluent to the column. When choosing a pump, one should consider the application and the degree of flexibility required for future work. If the system is a designated SEC/GPC system, a single channel, isocratic pump is sufficient and often more suitable. More often, however, binary or quaternary pumps are used, which allow several eluents to be drawn and blended in a predefined manner to form a gradient.
2. Detector (typically a UV detector): This module receives sample and eluate from the column and contains a flow cell through which the eluate passes. The flow cell acts as a cuvet that allows the absorbance of specific UV wavelengths and, therefore, the presence of sample components to be continuously monitored. Several types of UV detectors are available:
   - *Fixed wavelength detector*: To monitor at a single defined wavelength only.
   - *Single wavelength detector*: Only one wavelength can be monitored, but the value can be changed between runs.
   - *Multiple wavelength detector* (MWD): Allows several wavelengths to be monitored simultaneously.
   - Diode array detector: Performs the same function as the MWD, but is also able to perform UV scanning during elution for peak identification.

   Wavelengths from the visible spectrum can be used for the detection of colored compounds (chromophores), e.g., ninhydrin mixing with column eluate to produce a colored compound.
3. Autosampler: This unit usually consists of a removable tray into which vials, containing the sample, can be inserted at defined numbered positions. Each vial is accessible to a needle, which can draw a defined volume of sample and inject it onto the column for analysis.
4. Column holder: Most modern column holders include a heater to maintain the column at a constant defined temperature. Some units include a setting that allows the column to be kept at temperatures below ambient.
5. Eluent tray/degassing unit: The purpose of the eluent tray is to provide safe storage for the eluent bottles during analysis. Degassing of eluents is performed to prevent air bubbles coming out of solution during elution. Two types of degassing units are commonly used:
   - *Sparging with helium*: This displaces less soluble atmospheric gas, thereby reducing the chance of air bubbles occurring.
   - *Vacuum degassing*: This method draws dissolved gas out of solution under vacuum and is generally the preferred method, because it eliminates gas altogether.

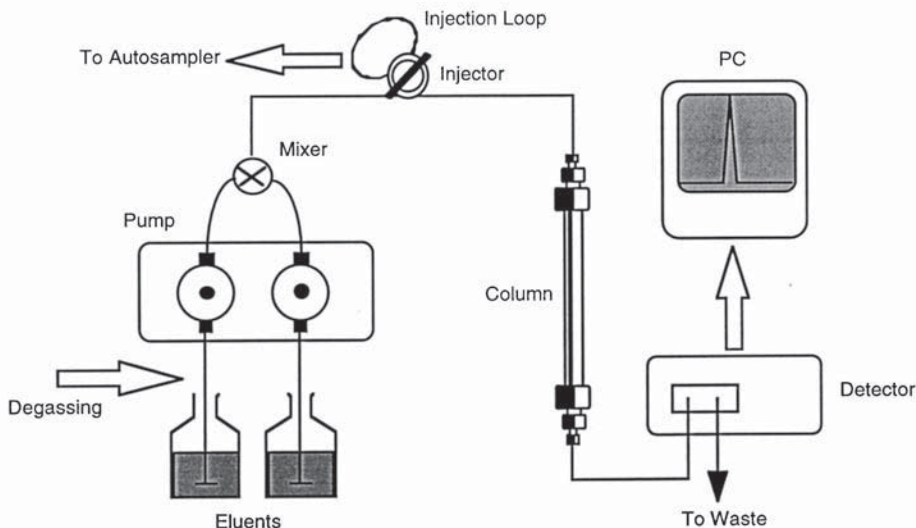

Fig. 1. Schematic diagram showing typical HPLC instrumentation.

6. User interface: This can vary from a simple keypad on the HPLC modules to a Windows™-driven software package with self-diagnosis CD ROM for trouble-shooting problems. It is important to tailor the complexity of the user interface to your specific needs and always bare ease-of-use in mind.

The schematic diagram shown **Fig. 1** represents a "typical" system, giving an indication of the buffer flow path and how the components interconnect.

Most HPLC systems use a flow path, which shows a broad range of solvent and sample compatibility. For dedicated biochromatography instruments, titanium and PEEK (polyethylethylketone) are preferred by many scientists because of their greater biocompatibility. However, choosing a system with a 100% titanium or PEEK flow path will limit choice.

3. Method

In order to make the methods listed as generic as possible, the following assumptions have been made:

1. The mode of detection used in each case is measurement of UV absorbance.
2. The operator has a working knowledge of the HPLC system and user interface being used, including analysis of results.
3. The operator has prior knowledge of the sample to be analyzed, e.g., number and size of components. This will aid in the evaluation of initial results.

Method-specific assumptions are provided with each technique, where appropriate.

3.1. SE-HPLC

3.1.1. Background

Size-exclusion chromatography (SEC), also termed as gel-filtration chromatography, molecular sieving, and gel-permeation chromatography separates proteins on the basis of molecular size. This is achieved by the differential permeation of the various molecular species into matrices of defined porosity. Buffered salt solutions are used as eluents, allowing biological activity to be retained; consequently SEC is often used in the purification of biomolecules. The HPLC mode (SE-HPLC) was made possible in the 1980s when packing materials with sufficient mechanical strength became available.

The chromatographic support consists of rigid spherical particles, which are permeated by a series of pores with a defined range of diameters. Very large proteins that cannot enter any of the pores pass between the matrix beads and elute first at the void volume (V_0) of the column. Sample components of this size are said to be "excluded" from the column. Very small peptides and salts that freely pass in and out of the pores are retained and elute last. The sum of the external (interparticle) and internal (intraparticle) volumes represents the total or included volume (V_t) of the column. Sample components of an intermediate size have varying degrees of access to the pores, depending on their size, and therefore have different elution volumes (V_e).

Retention in SE-HPLC is governed solely by entropy differences between sample and solvent, with no enthalpy differences allowed as this would mean a thermodynamically favorable interaction between sample and matrix (i.e., adsorptive interactions) ***(3)***. This fact is demonstrated by the independence of SE-HPLC retention on flow rate and temperature. Therefore, it is the choice of column that governs the selectivity and resolution of the method, provided optimal buffer conditions have been selected.

Because separation is on the basis of size, it is not necessary to change the composition of the buffer during analysis (isocratic elution). Because elution is only possible between V_o and Vt, the separation capacity of the mode is limited with a molecular-weight difference of 10–20% required for resolution. Consequently, a maximum of only 5–10 sample components can be resolved in a single analysis.

3.1.2. Uses of SE-HPLC

1. The process scale version of the mode is frequently used as a final "polishing" or buffer-exchange step in the purification of biomolecules.
2. Separation on the basis of size allows the visualization of aggregate species, making the mode a useful tool in stability studies.
3. Similarly, breakdown products (e.g., autolysis products) can be visualized as late-eluting peaks.

4. Provided a sufficient change in size is generated, the formation of adducts (e.g., enzyme-substrate binding) can be monitored.
5. SE-HPLC columns with smaller pore diameters (e.g., Superdex manufactured by Pharmacia, Sweden) can be used in the analysis of peptides.
6. Molecular-weight calibration of unknown species can be estimated using a suitable standard curve (*see* **Note 2**).
7. Alternative detection modes such as light scattering, viscometry, and refractive index can be used in series to calculate parameters such as the hydrodynamic volume of a protein.

3.1.3. SE-HPLC of Proteins

Because of the range of buffers and columns available, the following assumptions are made in order to make the method listed as generic as possible:

1. Prior knowledge of the number of sample components and their molecular weights as this will aid column selection. If this information is not available, it can be obtained using techniques such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or mass spectrometry.
2. The sample consists of 10 or less components, that differ in molecular weight by at least 10%. If this is not the case, only partial resolution may be achieved for some or all species.
3. The sample is composed of globular proteins, as most column calibration data is expressed in these terms.

The method listed below provides a “starting point” from which further optimization can be performed. Some general tips before starting a method are listed in **Subheading 3.1.4.** Resulting from the high cost of SE-HPLC columns (typically two to three times that of a reversed phase column), one would strive to achieve a satisfactory separation without changing the column. This reemphasises the importance of prior knowledge of the size and range of molecules in your sample, as well as the type of protein (remember: linear and globular proteins of an identical molecular weight will have different radii of gyration and will, therefore, show different retention by SE-HPLC). If the desired resolution is not achieved by the method listed, *see* to **Notes 7–15** for troubleshooting tips.

For more detailed background and methodology, *see* to **refs. 4–6**.

3.1.3.1. Column Selection

Select an appropriate SE-HPLC column for the molecular-weight distribution of your sample. A list of commonly used columns is provided in **Table 1**. If the molecular weight of your sample has proved unobtainable, choose a column with a broad separation range such as a TSK G4000 SW_{XL}.

Table 1
Commonly Used SE-HPLC Aqueous Columns and Their Properties

Column name	Particle size (µm)	Pore size (nm)	Separation range for globular proteins k(Da)	pH range	Manufacturer
TSK G2000 SWXL	5	12.5	5,000–150,000	2.5–7.5	TosoHaas[a]
TSK G3000 SWXL	5	25	10,000–500,000	2.5–7.5	TosoHaas[a]
TSK G4000 SWXL	8	45	20,000–10,000,000	2.5–7.5	TosoHaas[a]
ZorbaxGF-250	4	15	10,000–250,000	3.0–8.5	Hewlett-Packard
ZorbaxGF-450	6	30	25,000–800,000	3.0–8.5	Hewlett-Packard
PL-GFC 300 Å	8	30	500–500,000	1–13	Polymer Labs
PL- GFC 1000 Å	8	100	100,000–10,000,000	1–13	Polymer Labs
PL- GFC 4000 Å	8	400	500,000–>10,000,000	1–13	Polymer Labs
Superose 12	10	25	1,000–300,000	1–14	Pharmacia
Superose 6	13	40	5,000–5,000,000	1–14	Pharmacia
Superdex 75	13	13	3,000–70,000	1–14	Pharmacia
Superdex 200	13	13	10,000–600,000	1–14	Pharmacia

[a]For an equivalent, see also Progel-TSK columns manufactured by Supelco and BioSep™ columns available from Phenomenex.

Table 2
A List of Common Buffers Used for SE-HPLC of Proteins and Their pK_a Values

Buffer	pK_a values
50 mM Sodium citrate	3.06, 4.76, 5.40
50 mM Sodium (or potassium) phosphate	2.15, 7.20, 12.43
50 mM Sodium (or potassium) carbonate	6.35, 10.33
50 mM Tris(hydroxymethyl)aminomethane	8.06

As a general rule, the buffering capacity is ± 1 pH unit around the pK_a value. The addition of salt (about 100 m*M*) is usually performed to increase the ionic strength and prevent ionic interactions between the sample and stationary phase.

3.1.3.2. Sample and Buffer Preparation

1. Clarify the sample, if necessary, using a syringe filter or by centrifugation. This will prevent column blockage during analysis (*see* **Note 1**).
2. Prepare a set of calibration standards; these will differ depending upon the type of column used and are usually identified in a calibration data sheet, which is provided with the column. The recommended calibration standards for the TSK G4000 SWXL are: Thyroglobulin: (1.00 mg/mL); Ferritin: (2.00 mg/mL); Ovalbumin (1.00 mg/mL); and P-Aminobenzoic acid: (0.01 mg/mL). Dissolve the standards in eluent and mix by gentle vortexing or repeated inversion (*see* **Note 2**).
3. Prepare the eluent; a common starting buffer is 50 m*M* KH_2PO4 (pH 6.5) containing 100 m*M* NaCl. If the sample is not stable in this eluent, substitute for one of the alternative buffers listed in **Table 2**. Note that silica-based matrices are not stable above pH 7.5. Filter the eluent through a 0.2 μm nylon filter.
4. Degas the eluent thoroughly prior to analysis. If using a helium-based degassing system, constant sparging is preferable if possible.

3.1.3.3. System Preparation

1. Prime solvent lines and pump with the degassed solvent, ensuring that the column is off-line. On many HPLC systems, this is done using a "purge" setting (*see* **Note 3**).
2. Switch flow off and install the HPLC column, taking care to note the direction of flow, which is indicated by an arrow on the column.
3. Switch the column in-line and increase the flow rate up to 1 mL/min in 0.1 mL/min increments (*see* **Note 4**).
4. Equilibrate the column using at least one column volume of eluent before analysis. Refer to manufacturer's notes for the value of V_i; the value for a TSK G4000 SW_{XL} column is approximately 12.5 mL (*see* **Note 5**).

3.1.3.4. Analysis of Standards and Samples

1. Create a method file that contains all of the parameters by which standards and samples will be analyzed. The suggested starting conditions are as follows:

flow rate: 1.0 mL/min; injection volume: 100 μL (for a sample concentration of 1 mg/mL); detection wavelength: 280 nm; column temperature: 37°C; run time: Allow one column volume (+25%) to allow for any components which may elute after the solvent front (*see* **Note 11**).
2. Aliquot standards and samples into appropriately sized vials (*see* **Note 6**).
3. Analyze standards and sample by the parameters listed above.

3.1.3.5. Analysis of Eluate Fractions

One advantage of SE-HPLC is that, owing to the aqueous conditions and near-neutral pH that are often used, biological activity of the sample is frequently retained. This allows greater options for postchromatographic analysis than for methods such as rpHPLC. The following analytical techniques are commonly used to analyze eluate fractions produced by SE-HPLC:

1. SDS-PAGE: Reducing and nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) can be used to confirm molecular-weight data and Western blotting can be performed to confirm the identity. Samples may require concentration prior to SDS-PAGE analysis.
2. Dot blotting: Eluate fractions can be dot blotted onto a Western blotting membrane and screened using a range of antibodies.
3. Enzyme-Linked Immunosorbent Assay (ELISA): Eluate fractions can be screened against a range of antibodies by ELISA, which offers high sensitivity, but intolerance to many reagents, especially solvents, detergents, and reducing agents.
4. Mass spectrometry: Analysis of eluate fractions or on-line detection for size and identity data.

3.1.4. Notes for SE-HPLC

1. It should be noted that sample preparation can result in the loss of sample and/or the preferential loss of one or more components and, therefore, may not be appropriate if quantitative results are required.
2. A standard curve can be generated by plotting the logarithm of the molecular weight of each standard against the retention time. This can be used to provide estimates of molecular weight for unknown sample species, but the following should be noted:
   - All standards should elute within the linear range of the column (refer to manufacturer's notes).
   - Elution volume is proportional to apparent molecular size, not weight, so calibrants should be the same type of molecule as the sample. For example, the molecular weight of a globular protein would not be calculated using a standard curve of dextran standards.
3. Vacuum degassing systems have a considerable dead volume, typically in the order of 50 mL. This should be taken into account when calculating purging times.
4. Gradually increasing flow rates prevents column damage resulting from pressure shock. This is particularly important when using costly SE-HPLC columns.

5. Particularly when the column is new, and when feasible during normal use, the column should be equilibrated overnight at 0.1 mL/min to remove fines.
6. Where possible, aliquot a surplus of 10–20 μL of the injectable material to prevent air from being drawn into the needle.

Notes 7–15 detail some troubleshooting tips when performing SE-HPLC. A brief description of the chromatogram is given in italics with suggested remedial action as follows.

7. *Components elute as an exclusion peak:*
   - The exclusion limit of the column could be too low; select a column with a higher separation range.
   - The proteins may have formed aggregates, in which case add a small volume of detergent, such as SDS, or solvent to the mobile phase.
8. *All components elute very late with the solvent front:*
   - The exclusion limit of the column is too high; select a column with a lower molecular-weight separation range.
   - Hydrophobic or ionic interactions may be occurring between the sample and matrix, in which case add detergent or solvent to the mobile phase or increase the eluent salt concentration to 0.5 *M* (for ionic interactions).
   - Use an end-capped matrix (e.g., BioSep™).
9. *Components elute within the linear portion of the curve, but are grouped together and are poorly resolved:*
   - The sample components may not be sufficiently different in molecular size to be separated by SE-HPLC. In this case, it may be necessary to try a different chromatographic mode, e.g., rpHPLC.
   - Alternatively, obtain approximate molecular weights for the flanking peaks from retention volume data and choose a more appropriate column.
10. *Components are well separated but poorly resolved:*
    - The column may be overloaded, dilute the sample twofold and reanalyze.
    - If resolution is still poor, try a longer column (or two identical columns in series).
    - A new column may be necessary, as the column bed could contain cracks or voids that will diminish performance. Run a non-protein standard e.g., P-aminobenzoi acid and calculate asymmetry of the peak.
    - Alternatively, repack the column if you have the correct apparatus.
    - Use a smaller particle size (e.g., if using 10 μm, switch to 5 μm) or switch to smaller bore tubing and column as the dead volume of the system may be too large.
11. *Some components elute after the column volume*: This would suggest interactions between the sample and stationary phase.
    - Increase the eluent salt concentration if interactions are ionic.
    - Add detergent or solvent to the mobile phase if interactions are suspected to be hydrophobic.
    - Use an end-capped matrix.
12. *One or more components elute before the void volume*: This is likely to be carry-over from the previous analysis, suggesting interactions between the sample and stationary phase.
    - Modify the eluent as suggested in **Note 11**.

13. Peak “fronting” or “tailing” is observed:
    - Replace or repack (top up) the column.
    - Modify the mobile phase as suggested in **Note 11**.
    - Reduce the sample load.
14. *No peaks obtained:*
    - Ensure the sample is soluble at the pH being used.
    - Use a more sensitive detection wavelength, e.g., 215 nm.
    - The sample may be too dilute; concentrate the sample or switch to a more sensitive mode of detection such as fluorescence. Note that concentration can result in sample loss or aggregation.
    - The sample has bound to the column; try modifying the mobile phase composition as described in **Note 11**.
    - Use an end-capped matrix.
15. *Split peaks:*
    - Inject standard protein, if peak splitting is not observed in the standard it could be due to coeluting components.
    - Where peak splitting of the standard is observed, backflush the column; replace column frits, repack or replace column, or check the system plumbing for kinks.
    - If the sample has not been injected in one continuous slug, splitting can occur (autosampler problem).

3.2. Reversed-Phase HPLC (rpHPLC) of Proteins

3.2.1. Background

rpHPLC separates proteins on the basis of hydrophobicity. Packing materials are typically silica-based, with the surface silanol groups chemically bonded to a chlorosilane functionality containing a carbon chain of a defined length. The chlorosilane group imparts a specific hydrophobicity to the matrix; increasing the length of the chain increases the hydrophobicity and, therefore, the retentivity towards hydrophobic proteins. Reversed-phase matrices are termed according to the chain length; the most commonly used are C_4, C_8, and C_{18}. For stearic reasons, not all surface silanols can be reacted, so it is usual to “cap” residual silanols with a less bulky chlorosilane derivative. Capping of unreacted silanols is necessary as these can interact with protein and diminish column performance.

Polystyrene-based matrices are also popular, but are not graded in a similar fashion, because the hydrophobicity is inherent in the material itself and not resulting from a separately bonded group. **Table 3** shows some of the commonly used C_{18} rpHPLC columns for biomolecule analysis.

The interaction of proteins with reversed-phase packings is complicated and difficult to predict, but as a simple model it can be assumed that proteins bind to the matrix at a low organic concentration and elute at increasing organic

Table 3
Some Commonly Used Wide-Pore C_{18} rpHPLC Columns and Their Properties

Name	Particle diameter	Pore diameter (nm)	Manufacturer
Poros R2[a]	10 µm	N/A	PerSeptive Biosystems
Synchropak 300 Å	6.5 µm	30	Hewlett Packard
Vydac 300 Å	5 µm	30	Hewlett Packard
Jupiter C18	5 µm	30	Phenomenex

[a]Not technically a C_{18} matrix; Poros R2 columns have an intrinsic hydrophobicity, which is equivalent to that of a C_{18} packing material.

concentrations depending on their hydrophobicity. Therefore, the elution of adsorbed proteins typically involves a gradient of increasing organic concentration. The following parameters affect a reversed phase chromatographic separation:

1. Type of organic solvent: Acetonitrile is the most common, but a range of other solvents can be used, with differing "elution strengths." A list of popular solvents is provided in **Fig. 2**.
2. Gradient: If flow rate is kept constant, decreasing the slope of the gradient increases resolution. Complicated multistep gradients can be used for samples containing components with markedly different retention characteristics.
3. Ion-pairing agents: These are small molecules with both charge and hydrophobic functionality. They are added in small amounts (typically about 0.1%) to the mobile phase and modify charged groups on the surface of the protein. Trifluoroacetic acid (TFA) is the most common ion-pairing agent in the use of silica based stationary phases. It is such a strong acid that it prevents the ionization of residual silanols and helps to solubilize proteins in organic solutions. Other ion-pairing agents include heptafluorobutyric acid (HFBA), hydrochloric, formic, phosphoric, and acetic acid for acidic to neutral pH, or triethanolamine (TEA), ammonium acetate, and NaOH for neutral to basic pH.
4. pH: This affects the charge distribution of proteins and, therefore, the interaction of proteins with reversed-phase packings.
5. Stationary phase: The retentivity of the stationary phase will affect the recovery and selectivity of the separation. Wide-pore packings (300 Å) are preferred, but the use of nonporous packings has been reported.
6. Temperature: Higher temperatures increase the rate of mass transfer and so increase resolution and peak sharpness.

3.2.2 Uses of rpHPLC

1. Because separation is on the basis of hydrophobicity, it is theoretically possible to separate proteins that differ by only a few amino acids (e.g., protein subtypes).

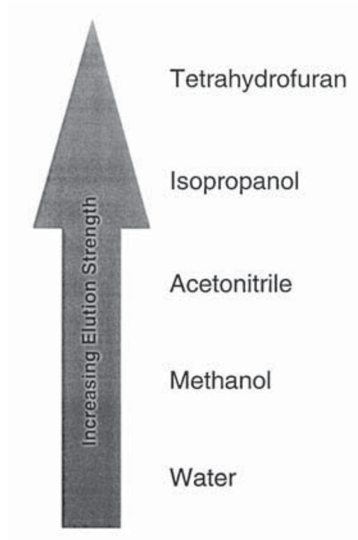

Fig. 2. Some commonly used rpHPLC solvents in order of increasing elution strength.

2. Similarly, the mode can be used to identify altered forms of a protein (e.g., incompletely translated forms or proteins with "ragged" C-termini).
3. Because of the high resolution of rpHPLC, it is frequently used to analyze protein digests to yield a unique peptide "fingerprint."
4. Similarly, analysis of acid hydrolysates can be performed for the determination of amino acid ratios and absolute amino acid content of a protein. These data, along with UV absorbance values, can be used to calculate the extinction coefficient of a protein at specific UV wavelengths.
5. Because rpHPLC is an adsorptive technique, it may be used on very dilute solutions to concentrate and purify a sample in a single step.

3.2.3. RP-HPLC of Proteins

The method given below is a starting point from which to develop a reversed-phase separation. One would not expect the conditions listed to give an "ideal" separation in the first instance; the conditions are designed with the following criteria in mind:

- Maximize the initial adsorbtion of the sample to the matrix, i.e., try to prevent any sample passing through the column unbound.
- Maximize recovery of the sample, i.e., ensure that all of the material is desorbed during elution.

If a satisfactory resolution is not obtained with the starting method, refer to **Notes 6–11** (*see* **Subheading 3.2.4.**). For more detailed background and methodology, *see* **refs. 4–6**.

3.2.3.1. Column Selection

Select a suitable column for the analysis. A good general column is a silica-based C18 column with a pore diameter of greater than 30 nm (*see* **Table 3**).

3.2.3.2. Sample and Buffer Preparation

1. Remove solids from the sample using a syringe filter or by centrifugation. This will prevent the column from becoming blocked during analysis (*see* **Note 1**). Aliquot filtrate/supernatant into glass vials.
2. Prepare a calibration standard solution with which to calibrate the column performance. Reversed-phase protein standard kits are available from Sigma. Details of the composition, concentration and recommended loadings will be included with the kit.
3. Prepare 1 L of the following eluents (*see* **Notes 2** and **3**):
   Buffer A: 5% (v/v) acetonitrile in dH_2O, containing 0.1% TFA.
   Buffer B: 95% (v/v) acetonitrile in dH_2O, containing 0.1% TFA.
4. Filter each eluent through a 0.2-μm teflon filter to remove any solids that may block the column or cause ghost peaks (*see* **Note 4**).
5. If using a helium-based degassing system, degas all solvents thoroughly before use. If possible, sparge with helium continuously.

3.2.3.3. System Preparation

1. Prime HPLC solvent lines and pump with the degassed solvents, ensuring that the column is off-line. This is usually achieved using a "purge" setting (*see* **Note 5**).
2. Decrease flow rate to 0.1 mL/minute and proportion the solvents to 100% A.
3. Switch the column in-line.

3.2.3.4. Sample and Standard Analysis

1. Create an analysis method, which contains all of the parameters by which samples and standards will be analyzed. The typical starting conditions are: flow rate: 1.0 mL/min; injection volume: 100 μL; gradient: 0–100% B over 30 min; column temperature: 40°C; and detection wavelength: 280 nm. At the end of the gradient, the flow should be held at 100% B for approximately five column volumes to remove any tightly bound sample.
2. Analyze the sample according to the parameters listed in **Subheading 3.2.3.4.** ***(1)***.
3. Analyze the standard solution according to the parameters listed in **Subheading 3.2.3.4.** ***(1)***.

3.2.3.5. Analysis of Eluate Fractions

Eluate fractions from reversed-phase analyses are generally less amenable to postchromatographic analysis by virtue of the harsh solvent conditions often used. Not only are the solvents themselves frequently incompatible with many assays, e.g., ELISA assays can be incompatible with solvents, but the denaturation that they cause can negate the use of immunoassays and measurements of enzymic activity.

Although all of the techniques listed in **Subheading 3.1.2., item 5,** may apply, SDS-PAGE and mass spectrometry (MS) are the preferred methods. One should bear in mind that MS is incompatible with SDS, so if a detergent is required in the sample, a nonionic detergent such as octyl glucoside should be used.

3.2.4. Notes for rpHPLC

1. It should be noted that sample preparation can result in the loss of sample and/or the preferential loss of one or more components. Sample preparation may not be appropriate if quantitative results are required.
2. Acetonitrile and water should be measured out separately before mixing to ensure accurate and reproducible buffer composition.
3. All solvents and TFA should be aliquoted in a fume cupboard.
4. To further reduce contamination, glassware can be rinsed with a small amount of concentrated HNO_3, followed with copious amounts of purified water.
5. Vacuum degassing systems have a considerable dead volume, typically in the order of 50 mL. This should be taken into account when calculating purging times.

Notes 6–11 detail some troubleshooting tips when performing rpHPLC. A brief description of the chromatogram is given in italics with suggested remedial action below.

6. *Poor recovery*: Signs of poor recovery include peaks eluting at the very top of the gradient, including during the "100% hold." Low or nonexistent peaks suggest that material is still bound to the column. Pre- and postcolumn samples can be tested by A_{280}, total nitrogen, or radiolabeling with I_{125} to accurately determine recovery. Please note that total nitrogen analysis should not be used with nitrogen-containing solvents, e.g., acetonitrile. The easiest way to alleviate poor recoveries is to use a column with a lower retentivity stationary phase. If recoveries are still low when using a C1 matrix, a stronger solvent can be used in place of acetonitrile such as propanol (*see* **Fig. 2**). Failure to adequately recover sample under these conditions would suggest that rpHPLC is not a suitable mode in this instance.
7. *The sample elutes as a tight group of peaks during the gradient*: Further optimize the gradient by determining the solvent concentration at which the earliest and latest molecules elute. Use these concentrations as the start and end points of the gradient and elute over the same time period. It should be noted, however, that some proteins require a low initial organic concentration to bind. In this case, the sample should be loaded at 100% A and the proportion of buffer B increased over 1 column volume to the new starting concentration.
8. *Peaks are well spread out but do not show baseline resolution*: This is known generically as band spreading and can usually be attributed to one of the following:
   - Overloading of the column; dilute the sample or reduce the load volume and reanalyze.

- Stationary phase particle size; try a smaller matrix size. If already using 5–8 μm it is unlikely that this is the problem.
- High system dead volumes; use smaller bore capillary tubing to minimize the dead volume of the system. It is important to note the flow rate restrictions of using smaller bore tubing.
- Solvent composition; change to a different solvent or ion-pairing agent to increase sample solubility.
- Voids or cracks in the column bed. Repack or replace the column.

9. *Split peaks*: Inject a protein standard; if this does not show peak splitting it is probably because of two coeluting components. If peak splitting is observed, it is commonly resulting from one of the following reasons:
   - Partially blocked column frit. Backwash the column and repeat the analysis. If this does not work, the frits can sometimes be removed and sonicated in 3 *M* nitric acid.
   - Void or crack in the column bed. Repack or replace the column.
   - Check connections and tubing for kinks.
   - Autoinjector problems. If using an autoinjector, perform a service or consult a qualified engineer.
10. *Ghost peaks*: These are spurious, nonsample related peaks that appear even during blank gradients. The problem can usually be attributed to contaminated solvents.
    - Prepare fresh solvents and repeat the analysis.
11. *Drifting baseline*: There are three common causes of baseline drift.
    - Temperature drift: This produces a gradual change in refractive index of the solvent, which alters the absorbance of UV light.
    - Contaminants in one of the buffers. During gradient formation the proportion of contaminant changes, thereby changing the absorbance characteristics of the eluent.
    - TFA can generate baseline drift, as its absorbance characteristics change with organic concentration. Reducing the amount of TFA in buffer B by 5–10% can usually solve this.

3.3. Hydrophobic-Interaction Chromatography

3.3.1. Background

As with rpHPLC, HIC separates proteins on the basis of hydrophobicity. However, while the basic principle of separation is similar, there are two important differences. First, the hydrophobicity and density of the functional groups attached to HIC supports is considerably less than rpHPLC matrices. Consequently, a milder interaction of proteins with HIC stationary phases is achieved that depends more on native features, such as hydrophobic pockets, than the overall content of hydrophobic amino acids. Second, binding in HIC is mediated by salt concentration; adsorbtion of proteins to the matrix is induced by the addition of a lyotropic salt (e.g., ammonium sulphate) to the eluent. This decreases ionic interactions between proteins and induces hydrophobic inter-

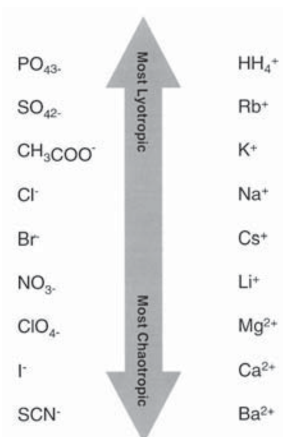

Fig. 3. Some common HIC buffers, ranked according to their tendency to encourage hydrophobic interactions (lyotropic or "salting out" effect) and tendency to break hydrophobic interactions (chaotropic effect).

actions ("salting out"), which would normally cause aggregation, but the presence of a hydrophobic stationary phase induces binding. Desorption is achieved using a gradient of decreasing salt concentration. The concentration at which individual proteins desorb depends upon the hydrophobicity of the protein; the more hydrophobic the protein, the lower the salt concentration required. Because buffered salt solutions are used as eluents in HIC, retention of biological activity is often achieved.

3.3.2. Recommended Starting Conditions

The following list of conditions provide a "starting point" from which to further develop a method: A column with a high retentivity matrix, e.g., TSK gel Phenyl-5PW (Tosobtaas) or Phenyl Superose (Pharmacia). Temperature = 40°C; buffer A = 50 m*M* k_2HPO_4 containing 1.8 *M* ammonium sulphate; buffer B = 50 m*M* k_2HPO_4; flow rate = 1.0 mL/min; loading 100 µg of protein; detection wavelength 280 mn and gradient 0 –100% over 45 min.

3.3.3. Considerations for Method Development

1. The salt concentration at which binding of the protein to the stationary phase occurs can be modulated by:
   - *Changing the bonded phase*: Increasing the hydrophobicity of the bonded phase increases protein retention:
     hydroxypropyl<methyl<benzyl = propyl<phenyl<pentyl.
     One should be aware, however, that excessively strong column–protein interactions may cause denaturation.
   - *Changing the salt*: A list of anions and cations in order of increasing lyotropic ("salting out") and decreasing chaotropic effect is given in **Fig. 3**.

2. Addition of an organic modifier: Highly hydrophobic proteins may not elute from the stationary phase even in the absence of salt. In this instance, addition of solvent such as isopropanol or acetonitrile may be performed to encourage elution. The solvent strengths listed in **Fig. 2** are also applicable in HIC.
3. Temperature and pH: These parameters can have effects on the strength of binding and the elution order, although the effects are unpredictable.
4. Gradient: Similar rules apply to those in rpHPLC (**Subheading 3.2.1.**).

4. Scale-Up

When performing scale up of HPLC methods it is important to initially optimize on a small scale to prevent wastage of valuable sample and stationary phase. The methods by which scale-up can be performed are discussed in **Subheading 4.1.**

4.1. Increase Packed-Bed Volume

An increased bed volume enables a greater quantity of sample to be applied and purified. The column dimensions must be increased to accommodate a greater volume of stationary phase and can be achieved by two methods:

1. Increase column length: This is often the cheapest way to increase the column volume, but can be limiting because of an increase in back pressure. Consequently, larger diameter martrices may be required to allow for increased flow rates. Column lengthening generally results in loss of resolution, with increased loads, as the sample is applied over a greater depth of column (band spreading).
2. Increasing column diameter: Although this method is generally the most expensive, resolution and back pressure are unaffected by scale up. Provided that even sample and buffer application is achieved, chromatographic performance is generally unaffected by scale-up. This eliminates the uncertainty that usually accompanies the procedure.

4.2. Cycling

This is the simplest form of scale-up and involves repeat analyses at the analytical scale, coupled with pooling of the fractions containing the purified component of interest. Cycling can often prove the most cost effective method in terms of scale-up development time, space, and purchase of new equipment and stationary phase. However, throughput is limited and the very nature of the technique can pose problems with validation. For example, a reversed phase analysis may require blank gradients or regeneration runs to be performed after each run to show that no sample has remained bound to the matrix. This would add time and cost to the process.

4.3. Considerations When Performing Scale-Up

1. What level of scale-up is required? This will help determine if an increase in column size is necessary or if cycling would be sufficient.

2. What are the validation and system suitability requirements? If additional steps are required with each run, e.g., blank runs, regeneration washes, or HETP tests it may be better to limit the number of purification runs, i.e., opt for apparatus scale-up over cycling.
3. How important is resolution? If the retention characteristics of product and impurity are markedly different, a lower cost scheme that compromises on resolution may be adequate. Conversely, higher grade matrices, lower loadings, and longer run times may be necessary if high resolution is required.
4. Scale-up in a linear fashion: increase load volumes in proportion to packed-bed volume; keep the linear flow velocity constant by adjusting the flow rate with column diameter; keep the sample and buffer compositions constant, and maintain the elution parameters in terms of column volume.
5. Matrix diameter: It is preferable to keep the matrix-particle diameter as small as possible, but larger matrices may be necessary if back pressure is limiting and high resolution is not important.

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Countercurrent Chromatography of Proteins with Polymer Phase Systems

Yoichiro Shibusawa, Kazuhiro Matsuda, and Yoichiro Ito

1. Introduction

Polymer phase systems were first introduced by P. Å. Albertsson in the 1950s for partitioning macromolecules and cell particles ***(1)***. These two-phase solvent systems consist of either one polymer component such as polyethylene glycol (PEG) and a high concentration of salt such as potassium phosphate, or two different polymers such as PEG and dextran in water. Being free from the organic solvent, the system can preserve a natural structure of proteins if the pH of the system is kept within a physiological range. In the past, polymer phase systems composed of PEG and potassium phosphate have been most successfully used for the protein separation. In these polymer phase systems, proteins are distributed according to their partition coefficients, which provide the basis for their purification. For example, relatively hydrophobic proteins distribute more into the PEG-rich upper phase and hydrophilic proteins into the phosphate-rich lower phase. Thus, repeating this partition process in a chromatographic column will result in separation of proteins according to their partition coefficients: the hydrophilic proteins will elute earlier than the hydrophobic proteins when the lower phase is used as the mobile phase.

Chromatographic partitioning of the proteins with polymer phase systems can be performed by countercurrent chromatography (CCC) ***(2)*** using two types of centrifuge devices: the cross-axis coil-planet centrifuge (cross-axis CPC) ***(3–8)*** is mainly used for the preparative-scale separation (20-mL sample size) and a toroidal-coil centrifuge ***(9–13)*** for the small-scale separation (1-mL sample size). Because of the protective effects by high polymer-salt concentrations, proteins can maintain their integrity at room temperature for a relatively

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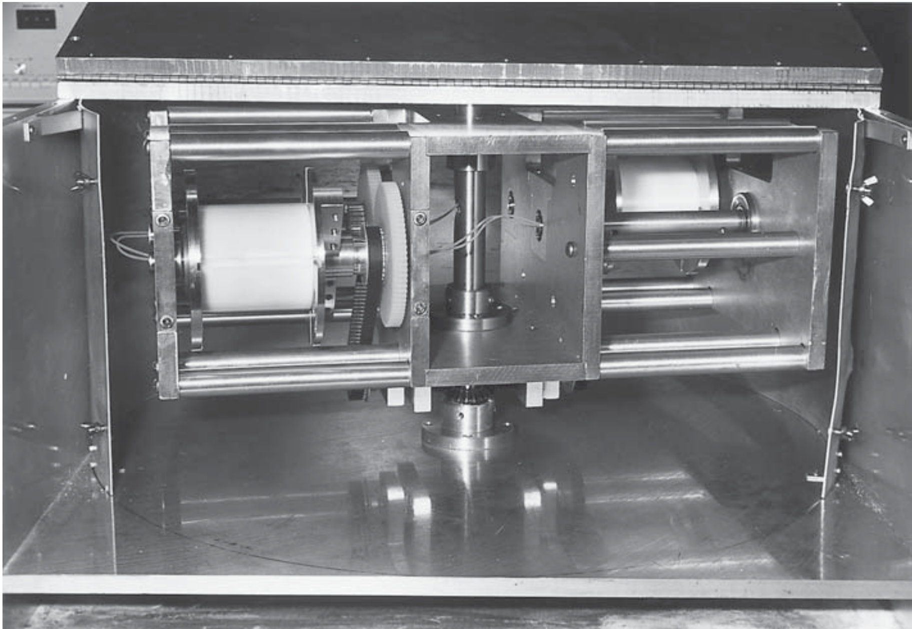

Fig. 1. Cross-axis coil planet centrifuge equipped with a pair of multilayer coils.

long period of time and usually the purification can be performed without cooling the column or collected fractions.

Successful separation of proteins in these CCC systems primarily depends on their partition coefficients in the polymer phase system, which determines the retention times of these proteins. Optimization of the partition coefficient of the target proteins at the best range between 0.5–2 may be accomplished by a simple test tube experiment by varying the pH and/or a molecular weight of PEG in the solvent system. Once the suitable two-phase solvent system is found, the target protein may be purified directly from a crude lysate in one step operation usually within 10 h at a high recovery rate.

2. Materials

2.1. Apparatus

Two types of flow-through centrifuge systems can be used for CCC purification of proteins: the cross-axis CPC for the preparative-scale separation (**Fig. 1**) and the toroidal-coil centrifuge for the analytical-scale separation. In both centrifuges, the mobile phase can be eluted through the rotating column without the use of a conventional rotary seal device, thus eliminating various complications such as leakage and clogging.

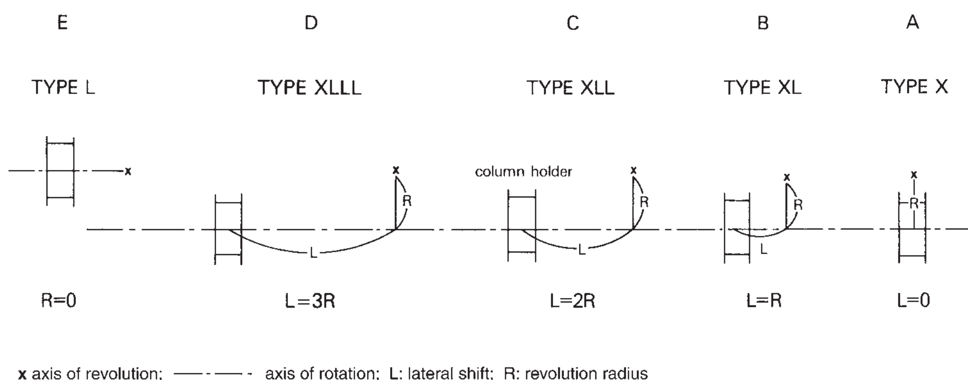

Fig. 2. Orientation of the column holder in five different types of the cross-axis coil planet centrifuge.

The cross-axis CPC can be obtained through Pharma-Tech Research Corp. (Baltimore, MD), Shimadzu Corp. (Kyoto, Japan), and Beijing Institute of New Technology Application (Beijing, China). There are several types of the apparatus such as XL, XLL, XLLL, and L according to the position of the column holder on the rotary frame (**Fig. 2**) *(7)*. Among those, either XL or XLL may be effectively used for separating proteins with PEG-potassium phosphate biphasic systems. The apparatus is usually equipped with a pair of multilayer coils of 2.6-mm inner diameter (ID) serially connected to provide a total capacity of about 300 mL. Some models are also equipped with an analytical coil for the small-scale purification *(8)*. The commercial model of the toroidal-coil centrifuge is currently available only from Pharma-Tech Research Corporation. It is equipped with an analytical column of 0.4–1.0 mm ID teflon tubing with a 5–10 mL capacity. There are two types of coils: helical column and twisted-pair column. The latter can retain a larger volume of the stationary phase at relatively lower hydrostatic pressure, while it produces less efficient separation per unit length of the column.

2.2. Reagents

PEG with various molecular weights is available from Sigma Chemical Co. (St. Louis, MO). PEG 1000 is most often used for separation of proteins. Anhydride potassium phosphates of reagent grade (monobasic and dibasic) are available through many sources including Sigma Chemical Co.; J. T. Baker Chemical Co. (Phillipsburg, NJ); Fisher Scientific Co. (Pittsburgh, PA); and so on. Also, it is safe to use the water of chromatographic grade supplied from the above companies unless double-distilled deionized water is available in the laboratory.

3. Methods

3.1. Preparation of Solvent System

The composition of the polymer phase system is conventionally specified according to the percentage (%) on a weight/weight basis such as 12.5% (w/w) PEG 1000–12.5% (w/w) dibasic potassium phosphate. As an example the preparation of this solvent system (100 g) is described as follows:

1. Place a beaker (250-mL capacity) on the balance and set the reading to 0.
2. Put 12.5 g of PEG 1000 in the beaker (total weight reading: 12.5 g).
3. Add 12.5 g of dibasic potassium phosphate (total weight reading: 25.0 g).
4. Add 75 g of water (total weight reading: 100 g).
5. Cover the beaker with aluminum foil and gently stir the contents with a magnetic stirrer until all solids are dissolved.
6. Pour the contents into a separatory funnel. After the contents reach room temperature, thoroughly mix the contents and leave it until two clear layers are formed.
7. Shortly before use, gently deliver each phase separately into a glass container by discarding the particulate trapped at the liquid interface.

The volume of the solvent system may be proportionally varied according to the capacity of the separation column. A much smaller volume is required for the preliminary test for the partition coefficient *(K)*. In order to find a suitable pH range of the solvent system, it is convenient to prepare a pair of acidic and basic systems using dibasic and monobasic phosphates, respectively. These solvent systems can be combined in various ratios to obtain a series of two-phase solvent systems with a wide pH range. **Table 1** lists an example of these solvent systems together with *K* values of various proteins in these systems. Many recombinant enzymes can be efficiently purified by choosing proper pH in the above solvent system and/or a slight modification of its polymer composition.

3.2. Determination of Partition Coefficients

Optimization of the solvent composition to adjust the partition coefficient *(K)* of the target analyte(s) is essential for successful separation (*see* **Note 1**). This can be done by a simple test tube experiment. When a standard pure sample is available, the measurement is easily performed as follows:

1. Prepare the two-phase solvent system in a small separatory funnel.
2. Deliver 1 mL of each phase, total of 2 mL, into a test tube.
3. Add a small amount of dried standard sample.
4. Thoroughly mix the contents and separate the two phases. If necessary, apply centrifugation at 1000*g* for 5 min.
5. Take 0.5 mL of each phase separately into a test tube and dilute the contents of each tube with 2 mL of water.

Table 1
Partition Coefficients of Proteins in Aqueous Two-Phase Solvent Systems

	pH	9.2	7.2	9.4	7.3	7.0	6.6
PEG 1000	(g/100g)	12.5	12.5	16.0	16.0	16.0	16.0
K_2HPO_4	(g/100g)	12.5	9.4	12.5	9.5	8.3	6.3
KH_2PO_4	(g/100g)	—	3.4	—	3.1	4.2	6.3
Protein							
BSA (68,000)		1.95	0.58	10.8	0.82	0.48	0.24
Ovalbumin (45,000)		1.26	0.96	3.16	1.36	1.21	0.91
Cytochrome C (12,000)		0.02	0.08	0.04	0.01	1.36	2.10
Hemoglobin (67,000)		25.0	1.16	33.7	2.10	0.97	0.36
Myoglobin (17,000)		0.59	0.30	0.71	0.13	0.09	0.09
γ-globulins		112	14.9	4.13	17.0	52.0	26.0
Trypsinogen (24,000)		1.79	1.02	5.41	1.46	1.32	1.24
Trypsin Inhibitor (20,100)		9.45	10.5	20.3	19.8	17.5	16.3
α-Chymotrypsinogen A (25,635)		6.00	5.01	29.1	11.8	9.50	6.48
apo-Transferrin		0.17	0.04	0.12	0.06	0.05	0.03
Carbonic Anhydrase (29,000)		4.46	2.53	9.88	5.00	3.67	1.83
Lactalbumin (14,200)		4.06	1.59	5.94	3.50	2.12	1.33

Partition coeffficients were calculated from the absorbance of the upper phase divided by that of the lower phase.

6. Mix the contents and measure the absorbance at an appropriate wavelength, typically 280 nm, using a spectrophotometer.
7. Obtain the blank absorbance value for each phase free of sample (0.5 mL each phase mixed with 2 mL of water) and subtract each blank value from the corresponding absorbance value.
8. Obtain the partition coefficient *(K)* by dividing the adjusted absorbance value in the upper phase with that of the lower phase.

If the standard sample for the above procedure is not available, the partition coefficient *(K)* of the target proteins should be obtained from the crude sample solution in the following steps:

1. Dilute crude sample solution (1–2 mL), potassium phosphate, and PEG are mixed in a test tube at a desired w/w % ratio.
2. After the salt and PEG are completely dissolved, the contents stand still to form two layers. Apply centrifugation if necessary.
3. Then, an aliquot of each layer is subjected to a specific assay to determine the concentration of the target proteins.

4. The final assay of the target proteins should be carefully performed by considering the entirely different compositions between the upper and lower phases. The upper phase contains a large amount of PEG and a small amount of phosphate, whereas the lower phase consists of high concentration of phosphate with a small amount of PEG. This difference of the phase composition in the sample solution may be compensated by adding new counterphase to each sample, that is, the sample from the upper phase is mixed with the same volume of the sample-free lower phase, and the sample from the lower phase with the same volume of sample-free upper phase. Then diluting each sample with a relatively large volume of buffer solution is often sufficient for the quantitative analysis to obtain the concentration ratio of the target protein between the two phases. However, if the polymer and/or salt in the sample solution strongly interferes with the assay of the target protein, they should be eliminated by microdialysis before analysis.

The partition coefficient *(K)* of the target protein is usually expressed by dividing the amount of the protein in the upper phase by that of the lower phase ($K = C_U/C_L$). If the K value of the protein is between 0.5 and 2.0, the solvent system is suitable for the separation. Otherwise, the measurement is repeated by changing the pH and/or phase composition. The pH of the solvent system is adjusted by the ratio (weight) between monobasic and dibasic potassium phosphates.

The effect of pH on the partition coefficient of four stable proteins are illustrated in **Fig. 3** *(7)* and partition coefficients of various protein samples in a series of solvent compositions are listed in **Table 1** *(8)*.

The retention volume of the target protein can be predicted from the partition coefficient *(K)*, which eases the analysis of the collected fractions (*see* **Note 2**).

3.3. Preparation of Sample Solution

The sample solution should have nearly the same composition as that of the polymer phase system used for the separation. The supernatant of crude *E. coli* lysate may be used for the sample preparation directly or, if necessary, after suitable concentration using a device such as “Centricon.” The proper amounts of PEG and potassium phosphate are added gradually to the crude sample solution while gently mixing the solution. After all the additives are dissolved, the solution is centrifuged at 1000*g* for 10–15 min. Any precipitation, if noted, may be eliminated from the sample solution. If a large amount of the material is precipitated, it is important to analyze the precipitates to see whether it contains a significant amount of the target protein. If so, the sample solution may be diluted or the solvent composition may be modified to improve the solubility of the target protein. The sample solution thus prepared usually consists of nearly equal volumes of each phase and can be directly injected into the separation column through the sample port.

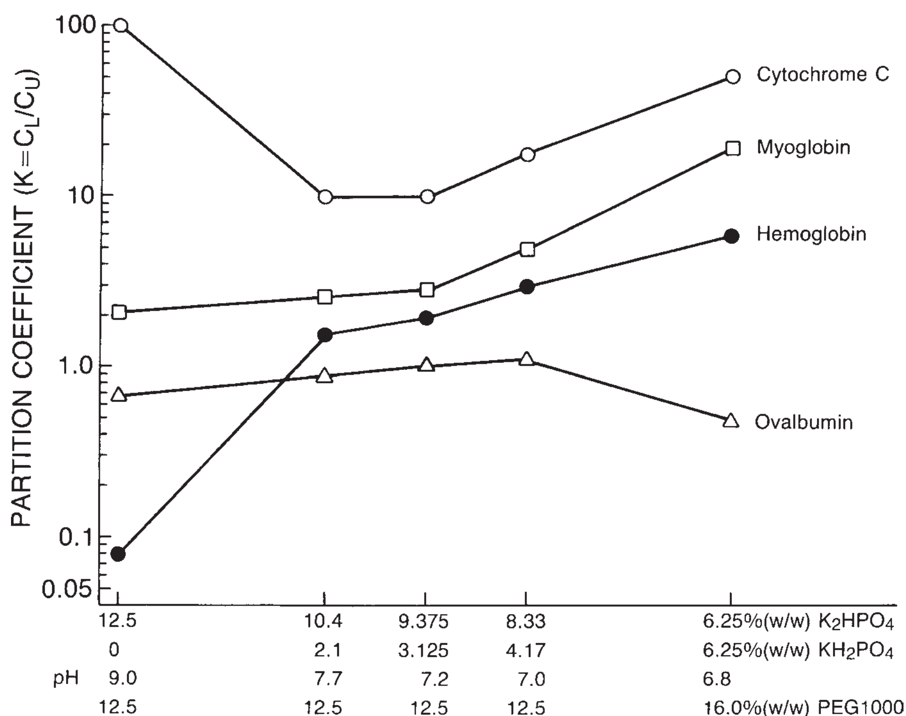

Fig. 3. Partition coefficients *(K)* of four stable proteins in various polymer phase systems composed of PEG 1000 and potassium phosphate. The partition coefficient is expressed as the solute concentration in the lower phase divided by that in the upper phase.

3.4. Purification Procedure

As mentioned earlier, CCC purification of the proteins can be carried out by two types of flow-through centrifuges: the cross-axis CPC can separate a relatively large amount of sample up to 20 mL and the toroidal-coli centrifuge purifies a small volume of sample typically 1 mL in the total volume. However, the separation procedure of these two instruments are quite similar except for a few points (*see* **Note 3**).

1. In both instruments, each separation is initiated by entirely filling the column with the stationary phase, either upper PEG-rich phase or lower phosphate-rich phase.
2. This is followed by injection of the sample solution through the sample port.
3. Then, the other phase is pumped into the column at a desired flow rate, while the column is rotated at an optimum speed (40–50*g* for the cross-axis CPC and 120–250*g* for the toroidal-coil centrifuge). Any chromatographic pump can be

used, where typical flow rates range between 0.5–2 mL/min for the toroidal-coil centrifuge. Under these flow rates, the maximum column pressure measured at the outlet of the pump usually does not exceed 200 psi.

4. The effluent from the outlet of the column is continuously monitored with a UV monitor at a suitable wavelength and then collected into test tubes with a fraction collector. In order to avoid trapping the stationary phase in the flow cell, the effluent should be introduced from the bottom of the flow cell if the mobile phase is the lower phase and introduced from the top of the flow cell if the mobile phase is the upper phase. In the latter case, care should be taken to avoid a trapped bubble in the flow cell. Attaching a length of narrow-bore teflon tubing (typically 0.4 mm ID × 1 m) at the outlet of the monitor will raise the pressure in the flow cells to prevent bubble formation (*see* **Note 4**).
5. After the separation is completed, the column rotation is stopped and the column is emptied by connecting the inlet to an N_2 cylinder (ca 80 psi). The column is washed by passing water several times and dried by N_2 for the next run.

3.5. Analysis of fractions

1. CCC fractions can be analyzed in various ways including high-performance liquid chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, specific enzymatic assays, mass spectroscopy, and so on.
2. Some of those may be interfered with a high concentration of PEG and/or potassium phosphate. In this case microdialysis may be used to alleviate the problem.
3. **Figure 4** shows a typical chromatogram of a recombinant enzyme, uridine phosphorylase with a PEG-potassium phosphate biphasic system where the enzyme is detected by the specific enzymatic assay *(7)*.
4. **Figure 5** shows the mass spectrometric analysis of N^{13}-labeled KSI purified by the toroidal-coil centrifuge using a polymer phase system ***(11)***. Before analysis, the CCC fraction was subjected to dialysis to avoid interference of PEG and salt.

4. Notes

1. In many cases, the K value of the target protein can not be easily determined directly from the crude sample solution. In this case, one may determine the average K value of the total proteins by measuring the absorbance of the diluted sample solution at 280 nm. If this value ranges between 0.5 and 2, the total protein mass will be distributed rather evenly through the chromatogram and by rechromatographing the fractions containing the target proteins with a suitable polymer phase system will lead to successful purification of the final products.
2. In **Fig. 6**, the retention volume (V_R) of the analyte is computed from its partition coefficient *(K)* and the retention volume of the mobile phase front (V_m) from the following equation:

$$V_R = V_m + K(V_c - V_m) \quad (1)$$

where V_c indicates the column capacity and K is defined as the solute concentration in the stationary phase divided by that of the mobile phase.

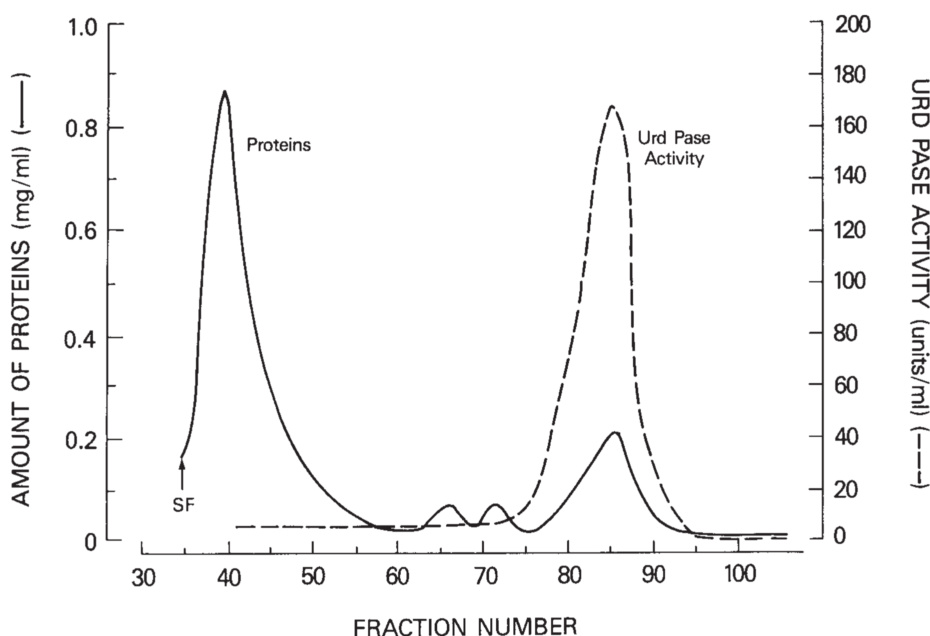

Fig. 4. Purification of recombinant uridine phosphorylase (UrdPase) from a crude *E. coli* lysate by polymer phase countercurrent chromatography. Experimental conditions: column: a pair of 2.6 mm ID teflon multilayer coils with a total capacity of 250 mL; sample: 2 mL of crude *E. coli* lysate in 4 mL of solvent; solvent system: 16% (w/w) PEG 1000 and 6.25% (w/w) each of KH_2PO_4 and K_2HPO_4 (pH 6.8); mobile phase: phosphate-rich lower phase; flow rate: 0.5 mL/min; revolution: 750 rpm.

3. Once the solvent and the partition coefficient of the target protein are determined, the next step is to choose the correct elution mode of the instruments, i.e., the proper choice of the inlet-outlet and the direction of the rotation especially for the cross-axis CPC.

   Operation of the cross-axis CPC utilizing the planetary motion of the coil requires a proper elution mode involving a combination of three parameters, i.e., direction of the revolution, handedness of the multilayer coil, and flow direction through the coil ***(6)***. When the organic/aqueous solvent systems are used, the proper combination of all three parameters should be determined for maximizing the partition efficiency. However, for the separation of proteins using the polymer phase systems, the direction of the elution through the coil becomes the most important parameter. In short, the lower mobile phase should be introduced from the proximal end of the coil toward the peripheral end (along the action of the centrifugal force) and the upper mobile phase should be introduced from the peripheral end of the coil toward the proximal end (against the action of the centrifugal force). The other two parameters play only a minor role in both partition

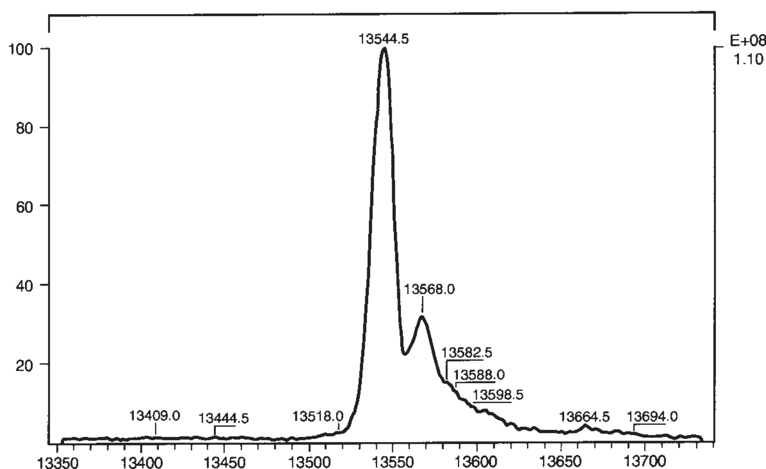

Fig. 5. ES/MS analysis of CCC-purified KSI (20% 15N-labeled KSI with molecular weight of 13568.0) from a crude *E. coli* lysate. The separation was performed with a polymer phase system composed of 12.5% (w/w) PEG 3350 and 12.5% (w/w) potassium phosphate system composed of 12.5% (w/w) PEG 3350 and 12.5% (w/w) potassium phosphate (pH 7.0) using the lower phase as the mobile phase. A large amount of impurities eluted near the solvent front while the KSI was totally retained in the column. After stopping the centrifugation, the enzyme was recovered from the column contents. Desalting of fractions was performed by dialysis against water. Then, a short ion-exchange column was used to remove the PEG. The toroidal coil centrifuge used in this separation is equipped with a 1.07 mm ID and 36 mL capacity twisted column. Sample size: 6 mL.

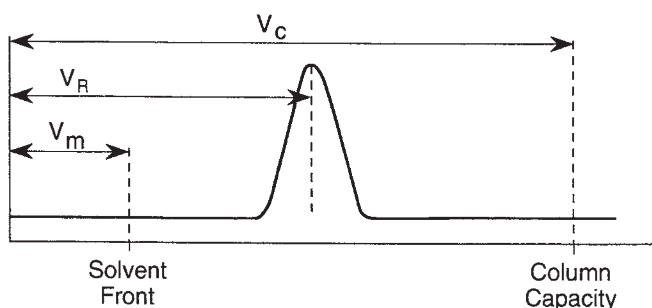

Fig. 6. Computation of the retention volume (VR) of the target analyte peak from the partition coefficient (*see* **Note 2**).

efficiency and retention of the stationary phase in the present application. In the toroidal-coil centrifuge utilizing a simple rotation, the choice of the inlet and outlet can also affect the partition efficiency, but to a lesser extent compared with

the cross-axis CPC. Recently, we found that the Coriolis force produces some effect on the partition process of the polymer phase system in the toroidal coil. In order to maximize the partition efficiency, the upper phase should be eluted through the toroidal coil toward the direction of the revolution and the lower phase should be eluted toward the opposite direction ***(12)***.

4. The polymer phase system under a subtle phase equilibrium is highly thermolabile and tends to develop turbidity in the flow cell disturbing the stable recording of the elution curve. In addition, a steady carryover of a small volume of the stationary phase also adversely contributes to the problem. One way to solve this problem is to introduce water into the flow line to dilute the effluent before it reaches the flow cell. This can be done by inserting a tee connector and a mixer on the flow line near the inlet of the monitor. Using a pump, a small volume of water (typically one-fifth of the flow rate of the main pump) is consistently introduced into the flow line through the tee to dilute the effluent. Although the system requires an additional pump, it gives the advantage of not only improving the tracing of elution curve, but also allowing introduction of the effluent from the bottom of the flow cell regardless of the choice of the mobile phase so that the complication caused by an air bubble is eliminated.

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Practical Aspects and Applications of Radial Flow Chromatography

Denise M. Wallworth

1. Introduction

1.1. Radial Versus Axial Chromatography

Traditionally, axial flow columns have been utilized for protein purification: samples are applied to the top of the column and separated down the vertical length. A piston presses against the top of the resin bed to ensure an evenly packed bed and to remove dead spaces that develop during resin compression. This forms part of the head plate of the column, which generally has to be removed before packing the column.

Uniform application of the sample is important for all chromatographic processes: a compromise in separation performance owing to inconsistencies and the inefficient use of the resin bed could otherwise result. All axial column designs, therefore, incorporate a distribution plate at the top of the column where horizontal flow channels distribute the flow to the outer diameter of the column.

The evolution of slurry packed axial columns has proved a great advance and has removed some of the most costly aspects of chromatography in terms of time, wastage, and safety. Radial flow chromatography, however, provides an effective alternative to axial columns in respect of simple packing techniques, and has the additional benefits arising from the much lower pressure drops observed for these columns and their small footprint.

1.2 Principles of Radial Flow Chromatography

Radial flow chromatography columns (**Fig. 1**) utilize two concentric cylindrical porous frits that hold the resin between them. Eluent and sample flow from the outer cylinder to the inner cylinder, across the radius of the column,

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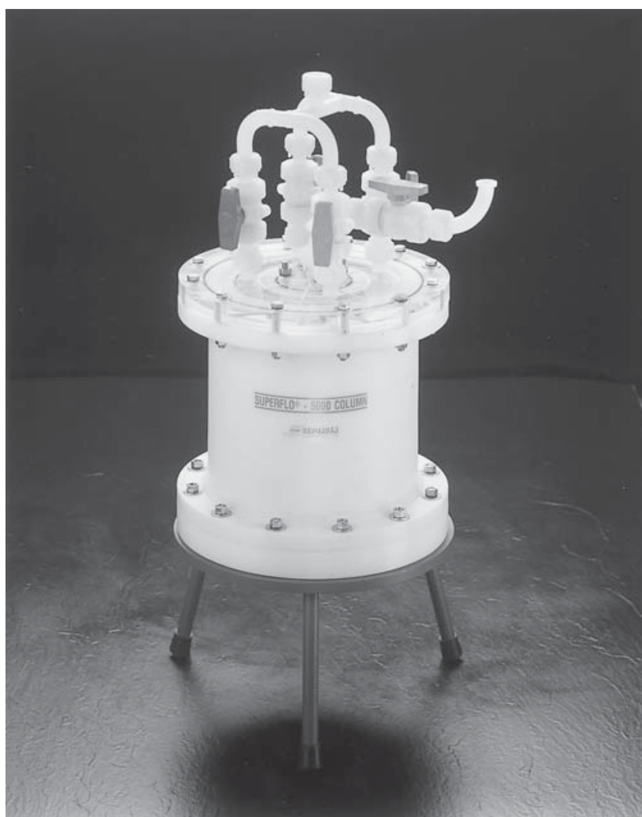


Fig. 1. Radial flow chromatography column.

which is now the effective bed height. The center solid core supports the inner frit that collects eluent and product before they exit the column. The outer cylindrical frit in radial flow columns is, therefore, the inlet of the column, and as such has an extremely large surface area in contact with the resin, making radial flow chromatography ideally suited to adsorptive type separations, such as ion-exchange, affinity, hydrophobic interaction, reversed phase, and any other adsorption–desorption type of separation. However, apart from low-resolution separations, they are generally unsuitable for bed-depth dependent, isocratic separations such as size-exclusion chromatography.

A consequence of the high inlet surface area (outer frit) and short bed depth is that column back pressures are typically very low, enabling exceptionally high flow rates, often as high as one or two column volumes per minute. It is almost equivalent to having an axial column with the ideal geometry of a wide diameter and shallow bed depth combined with a perfect distribution head. The

use of radial flow technology can provide access to new opportunities in downstream chromatography, whereby some of the process rate determining steps such as equilibration and washing may be speeded up considerably. The loading of large volumes of dilute feedstock from some bioprocesses can often be the slowest stage in a process: in radial flow columns this can also be speeded up considerably, enabling the process to become economically viable. In addition, for those products that have a high-binding constant, it is possible to load and adsorb in the forward direction and desorb by reversing the flow in a radial flow column, thereby reducing product dilution and speeding purification further. For labile products, this technique could potentially increase the yield considerably simply by reducing time spent on the column.

One key aspect of the design of the radial flow column is that it need not be taken apart either for packing or for unpacking. While not a critical factor for laboratory columns, it becomes very important at the process level. The hazardous aspects of removing a large and heavy inlet plate and the manual removal of spent resin, along with the handling of drying solids, can be avoided. Radial flow columns are slurry packed, and as a result, can be packed faster and under highly sanitary conditions, because neither resin nor column need ever be exposed. The column can be subsequently unpacked also as a slurry straight into a disposal or regeneration tank.

Scale up to the process level using axial columns frequently leads to problems with high column back-pressures resulting in low throughput and gel compression. In addition, for axial columns, scale-up involves an increase in column diameter, with the aim of maintaining the same linear flow rate between laboratory and production columns. As a result, a compromise is frequently reached between increasing the column diameter and increasing column length, often simply because the physical size of the column cannot be accommodated in a process area. Radial flow columns tackle this issue by maintaining the same bed depth in all process column sizes. A process that uses a 10- L column with a bed depth of 10 cm, can be very simply scaled up to a 100- L process column (which will also have a 10 cm bed depth) by using ten times the flow rate and feedstock volume. Scale-up, loading studies and troubleshooting can now also be accomplished using a Wedge™ column (Sepragen Corp., San Leandro, CA). This column is essentially a scaled-down version of a process radial flow column and mimics effectively the effects of using radial flow technology at the production scale (*see* **Note 1**).

2. Materials

2.1. Radial Flow Column Construction

Figure 2 shows the overall construction of the column. The flow path of a radial flow column begins at the column inlet at the top of the column. Capil-

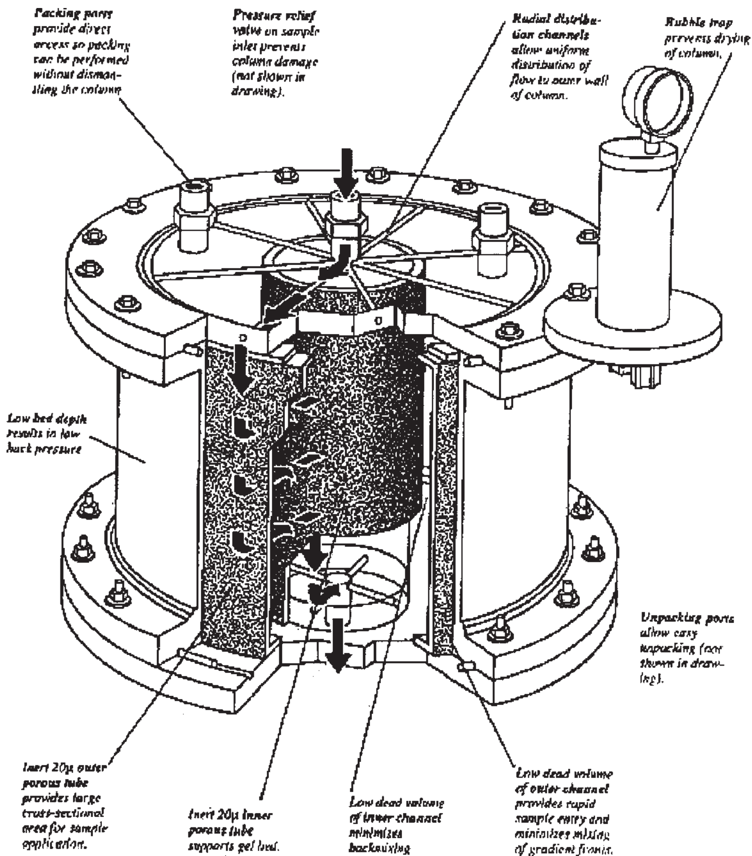


Fig. 2. Diagram of radial flow column.

lary channels take the flow rapidly to the perimeter of the column where it fills the capillary space that lies just outside the outer frit. When sample is applied to a radial flow column, the entire surface area of the outer frit is utilized giving even distribution. Flow then proceeds through the frit and across the resin bed. After passing through the inner frit (the equivalent of the bottom frit in an axial column), the flow path proceeds down the capillary channel to the exit port of the column.

Figure 2 also shows two additional ports at the top of the column. These are designed for packing the column: a packing manifold is attached at this point and a slurry of resin in buffer is pumped into the column (*see* **Subheading 3.2.**). To empty the column, the reverse procedure is followed, and in pilot and process columns, further ports on the base of the column are available for rapid column emptying into a waste or recycling tank.

Table 1
Compatibility Chart for Radial Flow Columns

Column material	Acrylic	Polycarbonate	Polyethylene	Stainless steel
Maximum pressure	50 psi	50 psi	50 psi	50 psi
pH range	2–12	2–12	2–12	2–12
Strong acids	Up to 2 *M*	Up to 2 *M*	Up to 2 *M*	Up to 1 *M*, short duration
NaOH, KOH	Up to 2 *M*	Up to 1 *M*, short duration	Up to 2 *M*	Up to 1 *M*, short duration
Alcohols	No	Yes	Yes	Yes
Organic solvents	No	Limited	Limited	Yes
Autoclavable	No	Yes	No	Yes

2.2. Conditions of Use

In the commercially available product (Sepragen Corp.), the columns are manufactured from acrylic, polyethylene, polycarbonate, or stainless steel, the choice depending on the eluent conditions to be used, the sanitization procedures to be used and their compatibility with the biological molecules to be purified (*see* **Table 1**). Solvents systems in use with radial flow chromatography columns are typically buffers and buffer-organic mixes, depending on the biomolecule to be purified.

Inner and outer frits are normally manufactured from either porous polyethylene (standard in acrylic, polyethylene, and polycarbonate columns) or scintered stainless steel (standard in stainless steel columns and in all process columns). Under some circumstances, porous polyethylene, however, can block, and scintered stainless steel filters can be installed in all columns in these cases (*see* **Note 2**).

2.3. Feedstock Preparation

Radial flow columns can process feedstocks with up to fairly high levels of solids, provided that they are smaller than 40 µm, the pore size of the standard frits installed in the columns. Other frit porosities can be utilized, and these include 10 and 20 µm. The actual maximum solids content will be dependent also on the resin in use. Because of the increase in velocity of the flow across the column bed depth (*see* **Note 3**), particulate materials such as precipitates and cell debris are carried through the column and rarely cause any increase in back pressure or blockages (*see* **Note 4**). For example, a feedstock comprising 10” cell density *E. coli* has been successfully routinely applied to a radial flow column.

3. Method

3.1. Choice of Resin

Radial flow columns can be packed with any type of adsorptive resin—ion exchange, hydrophobic interaction or affinity. This includes resins based on cellulose, agarose or silica beads and includes resins from most manufacturers. As a result, the choice of resin will rest almost entirely on that most suitable for the product to be isolated, or with cost and availability.

Resins used in chromatography columns shrink and swell with changing buffer strength, which can cause a reduction in flow through the column: subsequent loss of bed integrity results in a low-cycle rate before the column has to be repacked. Axial columns incorporate a piston pressed against the top of the resin bed to remove dead spaces that develop during resin compression. Current resins that have been crosslinked for additional strength go some way to solving this problem, although this is at the expense of ion exchange capacity.

The packing process for radial flow columns (*see* **Subheading 3.2.**) allows for the amount of shrinkage and swelling expected for the resin under the conditions that it will be used. By packing in salt buffers of a higher concentration than will be used at any stage in the method or process, bed integrity is maintained and a piston is not required, keeping column design simple.

3.2. Packing Radial Flow Columns

To pack the radial flow column, the column is placed in a closed-loop system as shown in **Fig. 3A**: the packing manifold is attached to the top of the column and to the pump; generally, a peristaltic pump that has both forward and reverse flow capabilities. The first stage in the process is that the column is filled with packing buffer, a buffer that has the same (or higher) concentration than that of highest ionic strength buffer to be used in any stage of the process: a high buffer concentration will ensure that the resin is packed in its fully compressed form. For some resins that have a high shrinkage, this buffer can be increased to 0.5 *M* higher than the highest ionic strength that the column will experience in the process. This stage of the packing process appears to be easiest if the column is operated in reverse, pumping buffer into the column from the outlet port. Once all the air is expelled from the column, the flow path is reversed to allow the resin slurry to be pumped into the column through the packing ports (**Fig. 3B**). Generally, a 25–30% slurry of the resin in packing buffer is used ***(1)*** at a flow rate of one half column volume per minute. Excess buffer exits to the buffer tank through the inlet and outlet ports. Less compressible, or more rigid bead resins generally benefit from packing at a 10% slurry concentration and a reduced flow rate.

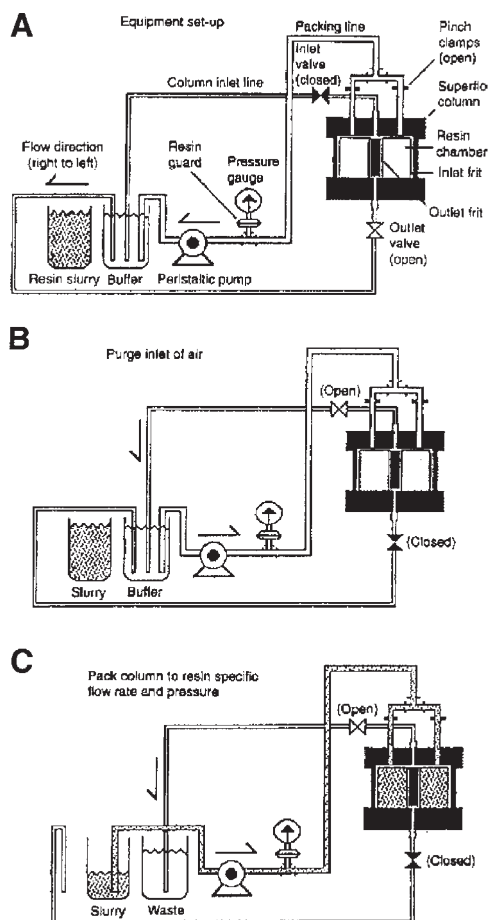


Fig. 3. Column-packing procedure for radial flow columns

When the column is almost packed, a rapid increase in back pressure is observed. Once the pressure has reached the specified pressure for the resin shown in **Table 2** (lowering the flow rate towards the end of the packing process can assist in determining this end point) the flow of resin slurry is stopped. These pressures have been empirically derived ***(2)***, and allow for a highly reproducible packed bed and possible automation of the column packing process.

The packing manifold is removed and flushed with fresh buffer to remove all excess resin. At the same time, packing port plugs are inserted into the packing manifold inlets: these press directly onto the column bed, removing any possible dead zones. In the final stage of column packing, the bed is conditioned by backflushing the column with packing buffer. Equilibration of the

Table 2
Packing Pressure Data for Radial Flow Columns

Resin	Maximum packing pressure
Sepharose 4B[a]	0.5 psi
Cellulose	1.0–3.0 psi
Agarose	5.0–8.0 psi
Trisacryl	5.0–8.0 psi
Sepharose CL-4B[a]	5.0–8.0 psi
Sepharose Fast Flow[a]	12.0–15.0 psi
Fractogel[b], polydextran beads, Macroprep	12.0–15.0 psi
Silica	25.0 psi

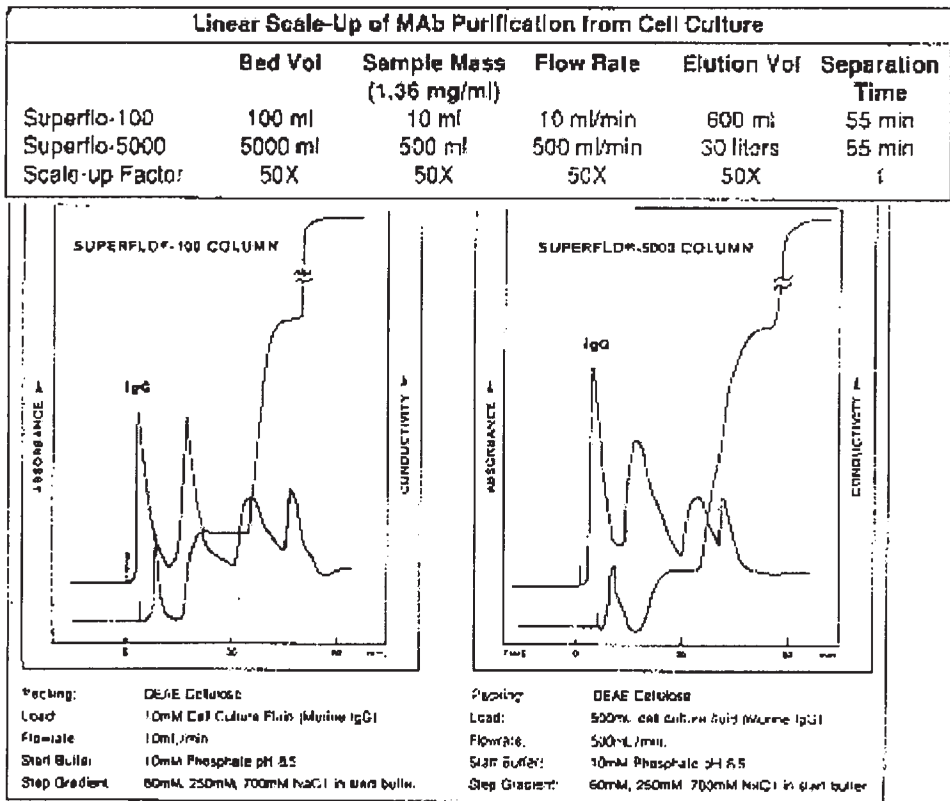
Trademarks: [a]Pharmacia. [b]ToyoSoda.

column ready for the first stage of the purification process can then be carried out, generally at a flow rate of between one third to one column volume a minute. This packing process is similar whether the column is of a laboratory, pilot, or process size, and appears to provide for a highly stable column bed ***(1)***.

3.3. Chromatography

Chromatographic processes on radial flow columns follow the same equilibration, load, wash, and elute stages that are typical of most processes. For example, the isolation of a monoclonal antibody from mouse ascites utilized a 100-mL radial flow column packed with DEAE cellulose. After equilibrating with 10-m*M* phosphate buffer (pH 8.5) 10-mL of a 1.36-mg/mL sample was applied at a flow rate of 10 mL/min. A step gradient of 60 m*M*, 250 m*M*, and 700 m*M* NaCl in 10 m*M* phosphate buffer (pH 8.5) was used to elute the product. The total cycle time was 55 min. A 50-fold scale up of the procedure was also carried out on a 5-L column utilizing identically the same method (*see* **Fig. 4**).

Products in dilute feedstocks are often best isolated by radial flow chromatography. The expression of large quantities of eukaryotic proteins in bacteria involves in vitro renaturation, optimal conditions at low protein concentrations (generally below 100 mg/mL), generating large volumes of dilute proteins ***(2)***. The use of radial flow chromatography was used since a 4.0-L sample could be processed in less than 2 h by utilizing the high flow rates possible. Two 100 mL radial flow columns were packed with ion exchange resins Q-Sepharose FF and S-Sepharose FF, connected in tandem and equilibrated with buffer, pH 7.0. Refolded protein was applied to the columns at a flow rate of 50 mL/min and the absorbance of the eluate monitored at 280 nm. The protein was eluted using the loading buffer with 1 *M* NaCl added and the peak eluted from the



Linear Scale-Up of MAb Purification from Cell Culture

	Bed Vol	Sample Mass (1.36 mg/ml)	Flow Rate	Elution Vol	Separation Time
Superflo-100	100 ml	10 ml	10 ml/min	600 ml	55 min
Superflo-5000	5000 ml	500 ml	500 ml/min	30 liters	55 min
Scale-up Factor	50X	50X	50X	50X	1

Fifty fold scale-up of MAb purification from cell culture fluid using a 100 ml and 5000 ml radial flow column.

Fig. 4. Mab purification on radial flow columns.

S-Sepharose column in about 60 mL of buffer. SDS-PAGE studies showed that the Q-Sepharose column removed most of the contaminating material, whereas the S-Sepharose column efficiently bound the recombitant protein—in this case, providing a 67-fold concentration step in less than 2 h. A subsequent "polishing step" utilizing a hydrophobic interaction resin completed the purification in less than one day.

In a comparative study with axial chromatography for factor IX purification, an immunoaffinity resin was packed into a 50-mL laboratory scale radial flow column and also into a 4.8-cm diameter glass axial column ***(3)***. The antibody was monoclonal and was coupled to Sepharose CL4B. After equilibration with five column volumes of buffer (10 m*M* magnesium chloride, 100 m*M* sodium chloride, 20 m*M* phosphate, pH 7.0), the lyophilized coagulation factor IX was loaded. Antibody capacity appeared to be identical for both

columns, suggesting that radial dispersion, mass transfer, and intraparticular diffusion do not have a significant impact on immunoaffinity chromatography.

The initial step in the purification of the enzyme uridine phosphorylase, used to catalyze the synthesis of a number of pyrimidine nucleosides, involves the break up of the cells and removal of cell debris by diafiltration. This and other enzymes from *E. coli* were purified without this diafiltration step with an increased yield by utilizing a radial flow column packed with Q Sepharose ***(4)***. Because of the high flow rates possible through the radial flow column, it was possible to force cell debris through the resin without clogging the column: the back pressure remained at 10 psi during a 3-h loading step on a 10-L column using a flow rate of 1.3 L/min. Recycling the feedstock through the column ensured a high product adsorption of 95% after 3 h. No channeling was evident under these conditions and, over some 60 cycles, no reduction in resin binding capacity or stability was observed.

One method for the purification of Factor IX from human plasma has utilized an uncrosslinked cellulose (Whatman DE52). In the existing process, the cryogenic precipitate is adsorbed onto the resin in a batch process because the amount of precipitate present in the supernatant prevents the use of normal column techniques. In an alternative method ***(5)***, the DE52 was packed into a 100-mL radial flow column and 5 L of the crude unfiltered cryoprecipitate loaded onto the column. Binding was achieved efficiently and the precipitate cleared the column without causing any increase in column back pressure.

4. Notes

1. A recent development in radial flow technology is the Wedge™ column (Sepragen Corp.). This mimics a large scale process column for process development (especially resin choice), viral clearance studies and troubleshooting. It is, literally, a slice out of the process column (*see* **Fig. 5**) and can be used at column volumes as low as 50 mL. It is packed, used and unpacked in identically the same way as a conventional radial flow column and provides the same flow characteristics as the process version. Linear scale-up studies from 1:100 are possible.
2. There are occasions where too dirty a feedstock has been used and the filters in a radial flow column become blocked. Sometimes a protein can have an extremely high affinity for the resin and adsorbs to the first resin surface it meets. In these cases, backflushing of the radial flow column is recommended. By simply reconnecting the buffer inlet to the column outlet and running the flow through to the column inlet to a waste tank, adhered product or debris can often be successfully removed. If using an acrylic column where the resin can be clearly seen, it will be noticed that the resin bed becomes disrupted during the backflushing process: this is reversible and does not affect the performance of the column in any way. If the radial flow column has been left running with no buffer in the supply reservoir, this backflushing procedure is also a very effective way of regenerating the column. Unlike axial columns, where such an event would require the column to

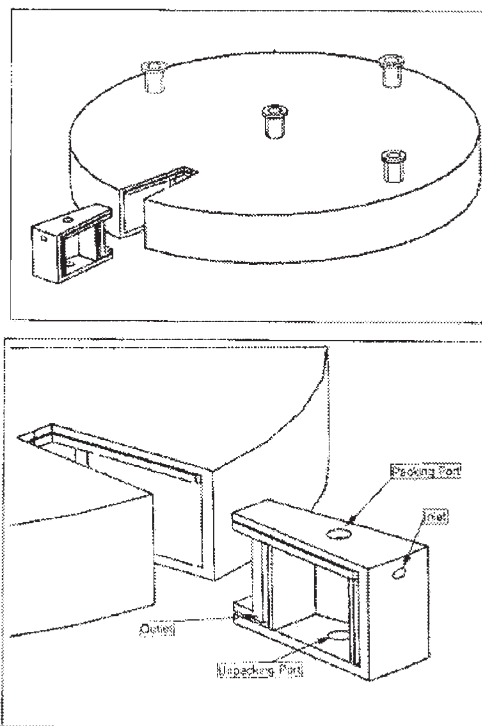


Fig. 5. Wedge™ column diagram.

be completely repacked, radial columns rarely require further remedies other than simple backflushing. In affinity chromatography, the hydrophilic nature of the polyethylene frits can sometimes cause a gradual buildup of product within the frit. This will be seen as a gradual buildup of backpressure. In these cases, changing the frit to the scintered stainless steel type, even for acrylic or polyethylene radial flow columns, removes the problem.

To install replacement frits, the head plate of the column is removed by undoing the bolts around the rim of the column. After lifting this off, the outlet frit can be pulled out, followed by the inlet frit, gentle pulling to release it from the O-ring seal at the base of the center core. Place the new or alternative frits in position and replace the column head plate, tightening the bolts opposite one another, as you would a car wheel, to ensure that the head plate sits level.

3. As the flow travels from a large surface area inlet to a smaller surface area outlet across the column bed in a radial flow column, the velocity of the eluent increases. The main implication in practice is that the linear flow rate quoted for axial columns is generally not used for radial flow columns. When transferring methods from axial to radial, it is usual to start by using the same flow rate as used in the axial process, then to increase it until method optimization is complete. Because

of the low back pressure of these columns, processes such as washing and equilibration can often be carried out at flow rates of one to two columns volumes per minute, greatly speeding up the method. Sample loading flow rates will also generally be higher than conventional axial columns, and will need to be determined by checking for breakthrough of product.

4. A consequence of the increased velocity across the column is that generally precipitates and cell debris successfully travel through the radial flow column without becoming deposited on frits. It is likely that the increase in velocity across the column assists their removal.

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Sterile Filtration and Virus Filtration

Ralf Kuriyel and Andrew L. Zydney

1. Introduction

Drug sterility is a critical requirement in the pharmaceutical industry. Virus removal or inactivation is also required for biologically derived therapeutics (e.g., monoclonal antibodies, recombinant gene proteins, and plasma components). Membrane filtration is attractive for both bacterial removal and viral clearance because it is simple to operate and causes minimal damage, even for products that are highly labile to heat, radiation, or chemical treatment.

Sterile filtration (i.e., bacterial removal) is used for aseptic processing to produce a sterilized product solution that can be directly added to a presterilized container. Sterile filters are also used to remove bacteria (and particles) from feed stock solutions, and to reduce the overall bioburden (and maintain low pyrogen levels) in processes where the product will be subjected to a terminal sterilization step (typically, steam or an autoclave).

Sterile filtration is usually performed in the through-flow (dead-end) filtration mode using disposable cartridges. Through-flow filtration is effected by passing the feed solution directly through the membrane with particles larger than the membrane pores being retained while the product is collected in the permeate. Filtration performance is evaluated in terms of three parameters: flux, particle retention, and capacity (throughput). Flux is defined as the ratio of the filtrate flow rate to the surface area of the membrane. The flux increases with increasing pressure differential between the feed and filtrate sides of the membrane and decreases with increasing solution viscosity. As the filtration proceeds, the resistance to flow increases because of fouling. Fouling can occur on the upper surface of the membrane, either by pore blockage or by formation of a cake or deposit consisting of retained particles and biomolecules. Fouling can also occur within the membrane pores through adsorption or deposition of

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biomolecules. Fouling increases the differential pressure for constant flow filtration and causes a decay in flux for constant pressure operation.

Bacterial retention for sterile filtration is characterized in terms of the log reduction value (LRV), which is defined as the logarithm (base 10) of the ratio of the microbiological concentration prior to and after processing. The capacity of the filter is the volume that can be processed per unit filter area before the pressure differential exceeds a specified limit (for constant flow-rate operation) or before the flow rate falls below a specified value (for constant pressure operation). Filtration capacity can be determined by accelerated tests as discussed in **Note 4**. Sterile filters are generally thought of as operating via a predominantly size-based (size exclusion) mechanism, with complete removal of all microorganisms larger than the membrane pore size. Filters rated as 0.22 μm were typically considered as sterilizing grade. However, it is now recognized that bacteria can also be removed by adsorption onto the membrane surface. This is particularly true for larger pore size (e.g., 0.45 μm) filters. Sterilizing grade filters are currently defined by the FDA as a filter that produces a sterile filtrate when challenged by 10^7 colony-forming units (cfu) of *Brevundimonas diminuta* (formerly classified as *Pseudomonas diminuta*) per cm^2 of membrane area ***(1)***. This test method uses an acepted model bacteria, which is small and monodispersed, at a concentration designed to effectively challenge the membrane surface area. Validation should be performed at the same pH, ionic strength, and chemical environment as the process solution to ensure equivalent adsorptive characteristics and bacterial properties (including size).

Recent examples of viral contamination (e.g., hepatitis A from contaminated Factor VIII) have attracted considerable attention to the removal of viruses from bioprocess streams. Viruses can be introduced through genetically engineered cell lines, contaminated raw materials (e.g., serum), operator contact, process gases, and so on. Viral removal/inactivation must be accomplished using a combination of steps involving different (independent) mechanisms, e.g., heat, chemicals, adsorption, radiation, and filtration. Virus filtration is typically performed toward the end of the downstream purification train. The relative purity of the solution at that stage renders the filtration operation easier. Virus filters can also be used as protective barriers for bioreactors through the filtration of media and buffer solutions.

FDA regulations require validation of viral removal and/or inactivation. The clearance rate is again reported in terms of the LRV. The total required LRV depends on the nature and potential for viral contamination of the starting material. For example, biologicals produced from cell lines containing retroviruses will typically require higher LRV. Viral clearance studies are performed by spiking high titer infectious viruses (with different physical characteristics) into scaled-down production steps and evaluating the ability of each step to

remove or inactivate the virus. Viral filtration is generally considered to operate on a sieving mechanism and is thus complimentary to other processes that often depend on specific electrostatic and chemical interactions. Membranes with nominal molecular weight cut-offs ranging from 100–500 kDa are capable of removing viruses as small as 18 nm.

Viral filtration can be performed in either through-flow or tangential-flow mode. Through-flow filtration is preferred for most applications because of its ease of operation and minimal capital costs. In tangential-flow filtration, the feed flow sweeps the surface of the membrane reducing the accumulation of retained species on the membrane surface (*see* **Note 6**). This can be a distinct advantage for highly fouling solutions. The design and operation of tangential flow filters is discussed in Chapter 4 in this volume.

Integrity testing is critical for all sterile and viral filters to insure that the system operates at the required level of performance ***(2)***. The real test for a filter would be to challenge with *B. diminuta* or selected viruses. However, such tests are destructive (i.e., the filter cannot be used after the test). Thus, a number of surrogate nondestructive integrity tests have been developed. The most common integrity tests in bioprocessing are pressure decay, bubble point ***(3)***, and liquid intrusion. Each of these tests is based on the displacement of a fluid from the pores by a second fluid (or gas), with the rate of displacement providing a measure of the membrane retention characteristics. For the gas or intrusion liquid to expel the wetting liquid out of the pore, the pressure force on the feed side has to exceed the capillary force within the pore. The bubble point is defined as the pressure at which a liquid-filled pore is first intruded by a gas ***(2)***. The bubble point can be observed experimentally by a vigorous stream of bubbles exiting the membrane on the filtrate side. Because the capillary force varies inversely with pore diameter, membranes with smaller pores have higher bubble points. The bubble point pressure *(P)* can be related to the membrane pore diameter *(d)* and the surface tension (γ) by the modified Young-Laplace equation:

$$P = K4\mathrm{g}\cos\theta/d \qquad (1)$$

where K is correction factor that accounts for the tortuosity of the pores and θ is the contact angle between the membrane and the solution ***(4)***. The solution properties, membrane chemistry, and temperature all affect the surface tension and contact angle and, therefore, the bubble point. The bubble point for sterilizing grade filters is correlated to the LRV of *B. diminuta*. Filters with water bubble points of 45 psi or greater typically yield the necessary LRV to be qualified as sterilizing grade filters. Filters exhibiting bubble points lower than the manufacturer's specification are considered nonintegral and should not be used. Bubble-point tests cannot be used for viral filters because the bubble-point

pressure would exceed the maximum pressure limits of the membrane/cartridge. Liquid intrusion tests have been correlated to viral LRV.

Integrity tests should be performed both prior to, and immediately after, filtration of the solution. Integrity tests performed prior to filtration must not affect the sterility of the connections downstream of the filter. Some postfiltration integrity tests are based on particle challenge. Particle challenge tests are usually performed by processing a solution containing particles of an appropriate size through the filter and determining the retention of particles. The particle retention is typically correlated to the retention of the biological contaminant. Colloidal gold particles, polyvinyl pyrrolidone (PVP), or dextrans are typically used for this type of integrity test.

2. Materials

Filtration systems for virus or bacterial removal consist of a tank containing the feed solution, a tank for the filtrate solution, a pump, the filter housings, valves, and associated instruments. Information on the selection of tanks, valves, and pumps is presented in the Chapter 3 in this volume.

2.1. Membrane

The membranes used for sterile filtration have to demonstrate absolute retention of *B. diminuta* according to the Food and Drug Administration's criteria. These membranes typically have a symmetric (isotropic) structure with pores that are smaller than 0.2 μm. The membranes must be steam-sterilizable or autoclaveable, have minimal particle shedding during operation, have low extractables, and must pass USP Class VI toxicity testing. In addition, the membranes should have low protein adsorption characteristics to minimize product loss during filtration. Most manufacturers use surface-modifications to reduce protein-binding capacity of the base polymer (typically polyvinylidene fluoride, polyamide, or polysulfone).

Virus removal membranes generally have an asymmetric structure, with a thin skin layer providing the membrane its retention characteristics, whereas the more open porous substructure provides the necessary mechanical stability. These membranes are designed to be "void-free" to eliminate defects that can arise from the penetration of fingerlike voids from the substructure up and through the skin layer *(4)*. Typical polymers include hydrophilic polyethersulfone, hydrophilic polyvinylidene fluoride, and regenerated cellulose.

2.2. Modules

Most sterilizing and virus removal filters use pleated membranes in cartridge form. The membranes are supported on a nonwoven polyester, folded to form pleats, wrapped around an inner core, and sealed by the use of two end

caps. Support cages are usually placed around the membrane to protect it against mechanical damage. Cartridges are available in a variety of sizes (e.g., 2, 5, 10, 20, 30, and 40 in.). The cartridge is placed inside a stainless steel housing prior to use. Self-contained cartridges, called capsules, are attractive because they do not require a housing and are easier to use. Small volumes can also be processed with cartridges containing a stack of membrane disks. Filters are sized using either the flow-decay or V_{max} method as described in **Note 4**.

Tangential-flow filtration for virus removal is typically performed using hollow-fiber modules or flat-sheet cassettes. Hollow-fiber cartridges use an array of narrow bore, self-supporting, fibers potted at the ends in an epoxy or polyurethane resin and housed within a cylindrical cartridge of plastic or steel. Flat-sheet cassettes use a sandwich arrangement of a permeate screen, membrane, and retentate screen. The screens define the flow paths above and below the membrane, promote mixing, and increase mass transport.

2.3. Filter Housings

Filter-cartridge housings are made of stainless steel. Surfaces in contact with the feed solution are typically 316L stainless. The internal surface is finished and electropolished to create a smooth sanitary design. Triclamp fittings are used for the inlet and outlet. The housings, O-rings, and gaskets should all be steam sterilizable. The system should be designed to allow steam-in-place.

2.4. Instruments

Pressure, temperature, and flow rates are typically measured during both through-flow and tangential-flow filtration. Flow rates can be measured with rotameters, magnetic meters, or turbine flow meters. Flow meters should be calibrated using the actual process fluids. Temperatures are measured using resistance temperature detectors. Standard pressure gauges or transmitters are used. Liquid level in the retentate tank is typically measured during tangential-flow filtration using displacement floats.

2.5. Compressed Gas

Compressed nitrogen or air is used for integrity tests and for providing the required pressure during constant pressure filtration. The gas should be sterile filtered prior to its contact with the solution.

2.6. Chemical Solutions

Chemical solutions are used to sanitize, condition, and integrity test the cartridges. Membrane conditioning is performed with appropriate buffer solution. Sterilizing grade filters are integrity tested using the diffusion or bubble-point methods, with the membrane initially wet with water for injection. Virus

removal membranes can be integrity tested by liquid-intrusion porosimetry. This is typically done using the two equilibrium phases produced by a mixture of ammonium sulfate, water, and polyethylene glycol (PEG). Tangential-flow filtration devices also require solutions for cleaning and storage. Cleaning is typically done with sodium hydroxide (0.1–0.5 *N*) or sodium hypochlorite (300–500 ppm), with the cartridges stored in sodium hydroxide (0.1 *N*), formaldehyde (1–2%), or sodium bisulfite (1%).

2.7. Integrity Testers

Automated integrity testers are widely used in the biotechnology industry because of their consistency and reliability. The tester typically connects to the upstream side of the membrane and can perform bubble-point pressure-decay, or diffusion tests. These are available directly from most membrane manufacturers.

3. Methods

Filters used for sterilization or virus removal are integrity tested before and after filtration to insure that the filter will and has achieved the required level of performance. Filters are installed into their housings, flushed with water, steam sterilized, and preintegrity tested prior to processing. They are then rinsed and integrity tested postfiltration.

3.1. System Assembly

Triclover fittings are typically used for all connections. Fill the system with purified water while keeping the vent in the filter housing open to allow air to escape. Close the vent and flush the cartridge with water-for-injection using the flow rates (typically 10 L/min per m^2 effective filtration area) and volumes (typically 50–100 L/m^2) recommended by the manufacturer.

3.2. Steam Sterilization

Steam sterilization is generally performed at 15 psi and 121°C for a minimum of 30 min.

1. Open the drain valve in the steam line, and open the vent and drain valve in the housing to allow the fluid to drain (*see* **Fig. 1**).
2. Partially close the drain and vent valves.
3. Gradually increase the steam pressure to the desired value and maintain throughout the sterilization. Do not exceed the manufacturer's specification for the maximum pressure difference across the membrane (typically 5 psi).
4. Close the drain and vent valves. Then, close the steam valve and immediately introduce sterile-grade air or nitrogen to cool the system and prevent damaging back pressure across the membrane. Allow the system to cool to room temperature, and then close the cooling gas valve.

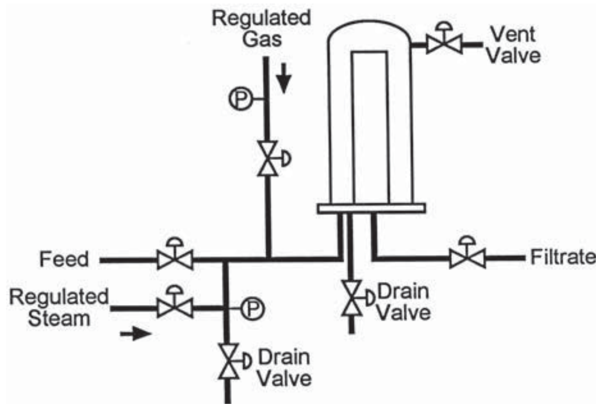

Fig.1. Steaming cartridge filters.

3.3. Integrity Test

Bubble-point diffusion (also known as forward flow) and pressure decay are typically used for prefiltration testing. In all cases, the membrane and cartridge are initially filled with sterile water.

1. The filter should be flushed with enough sterile water to completely wet the membrane (typically 50–100 L/m^2) prior to the test.
2. After flushing the membrane, the feed region is drained and the upstream side of the membrane is pressurized with a gas (typically air or nitrogen) to a level recommended by the manufacturer. When the desired pressure is reached, the system is allowed to stabilize for approx 5 min.

In the pressure-decay test, the feed-side pressure decay is measured as a function of time (typically for 5–10 min). The duration of the test will depend on the upstream volume because the decay will be a function of that volume. A drop in gas pressure greater than manufacturer's specification indicates a compromise in the filter. In the diffusion test, the air pressure is maintained constant (at approximately 80% of the minimum bubble-point pressure) and the air flow rate through the wetted membrane is measured. Check the measured flow rate against the manufacturer's specification. In the bubble-point test the pressure is adjusted to 80% of the bubble point and then increased at typically 1 psi increments. The system is allowed to stabilize for a few minutes at each level. A steady stream of bubbles in the filtrate side indicates that the bubble point has been reached or exceeded (*see* **Notes 1** and **2**).

Liquid-intrusion tests using two-phase systems of alcohol—water or ammonium sulfate, polyethylene glycol, and water—are often used to test the integrity of viral filters.

1. Mix the liquid components and allow them to phase separate.
2. Thoroughly flush the cartridge with one of the liquid phases.
3. Drain the upstream side of the cartridge.
4. Introduce the second fluid phase, and increase the transmembrane pressure drop to the desired level.
5. Measure the filtrate flow rate.
6. Flush the device, refill with buffer, and then measure the buffer filtrate flow rate at the same transmembrane pressure and feed rate.

The CorrTest value (CTV) is defined as the logarithm (base 10) of the ratio of the buffer flow rate to the CorrTest fluid flow rate. CTV below a critical level are indicative of membrane failure.

3.4. Filtration

Cartridge filters can be operated at either constant flow rate or constant differential pressure. Constant pressure is more commonly used with typical differential pressures of 20 psi. In this mode, the feed solution is typically pressurized with compressed gas. For constant flow operation, the pump is connected upstream of the filter and the system is sized so as not to exceed the maximum pressure difference (typically 20 psi) when operated at the required flow rate (*see* **Note 3**).

1. After completing the integrity test, flush the filter with a suitable (sterile) buffer.
2. Connect the feed tank to the compressed gas source and process the solution at the manufacturer's recommended pressure.
3. When all the solution is filtered close the compressed gas source. If the filtration is conducted at constant flow rate, shut off the pump when the level in the feed tank reaches its minimum acceptable value. Buffer can be used to recover the remaining product if product dilution is not an issue.

Viral filtration can also be done using tangential-flow filtration. A two pump system is used, with the feed pump providing the desired tangential flow while the filtrate pump maintains the constant filtrate flux. The filtrate flux is typically chosen to provide the maximum product mass flux through the membrane. (The mass flux is the product of the volumetric flux and the product sieving coefficient). The system should be started with the permeate pump closed.

1. Start the feed pump and slowly increase to the desired flow rate.
2. Recirculate for 5 min to equilibrate the system.
3. Turn on the permeate pump and gradually ramp the flux to the desired level.
4. Direct the permeate line to the permeate tank, and continue operation until desired recovery of the product is obtained. A diafiltration mode can be used to increase product recovery. In this case, a diafiltration pump is used to simultaneously add buffer to the feed tank while permeate is removed.

The design and operation of a diafiltration system is discussed in Chapter 3 in this volume.

3.5. PostIntegrity Test

A second integrity test is performed after filtration to verify that the filter achieved the desired bacterial or viral removal. This integrity test is not constrained by concerns about sterility and does not need to be nondestructive. Procedures for the bubble point, diffusion, pressure decay, and CorrTest are identical to those described in **Subheading 3.3.** Particle challenge tests are often used postfiltration. For virus membranes these employ well-defined particles such as colloidal gold or PVP (polyvinyl pyrrolidone). The challenge solution is usually supplied by the filter manufacturer. The particle passage, as determined by a spectrophotometer, is compared to the manufacturer's specification.

4. Notes

1. The bubble-point test can produce false failures for large-area filters because of the diffusive flow through the pores. Therefore, the diffusion test is preferred for large-area filters. Some users perform the diffusion test by measuring the air flow rate required to keep the upstream pressure constant.
2. Integrity tests can be affected by the surface tension of the wetting fluid, membrane fouling, and temperature. Care should be taken to maintain the temperature constant and use purified solutions. It is particularly important to eliminate surface active agents, which can reduce capillary forces in the pores. Integrity-test failures can also be caused by improper wetting of the membrane. If an integrity test fails, the filter should be rewetted using twice the original wetting pressure, flush volume, and contact time, before retrying the integrity test.
3. There have been reports in the literature that mycoplasma can penetrate 0.22 μm membranes ***(5)***, possibly because of the lack of a cell wall. Validated 0.1 μm filters are available for removal of mycoplasma.
4. Filters should be sized to permit processing of a particular volume of solution in a given time. Through-flow filters are sized by flow decay or Vmax methods. Both methods use small, typically 47 mm, membrane disks. The actual process solution is filtered at the process temperature and pressure. In the flow-decay method, measure the cumulative filtrate volume and the filtrate flow rate. The filtration is continued until the flow rate decays to 20% of its initial value. The filter size required for the large-scale process is calculated by taking the ratio of the process volume to the volume measured in the flow decay test and multiplying it by the area of the filter disk.

   In the *V*max test ***(6)***, the constant pressure filtration is performed for a period of 10 to 15 min with the cumulative filtrate volume recorded as a function of time. The maximum volume that can be filtered can be obtained from the slope of

a plot of *t/V* vs *t*, where *V* is the cumulative filtrate volume measured at time *t*. The advantage of the *V*max test is that it requires less time and less process solution.

5. Validation of sterilizing grade and virus removal filters is crucial. Sterilizing grade filters are validated at small scale by challenging the filters with the process solution spiked with 107 cfu/mL of *B. diminuta*. Virus removal filters are validated by spiking the process solution with different marker viruses. These should include expected viral contaminants, as well as model viruses with different physical characteristics, e.g., both enveloped and nonenveloped viruses. Typical model viruses are X174, PPV, and MVM.
6. Tangential-flow filtration (TFF) devices used for virus removal must be properly optimized. The effect of cross-flow rate and filtrate flux on product-mass flux should be determined at small scale. Operating conditions are generally chosen to maximize the product mass flux. Diafiltration can be used to improve product recovery (see discussion in Chapter 4 in this volume).
7. Product retention can also be reduced by optimizing buffer conditions. Protein transmission is generally greatest at pH near the protein isoelectric point and at high salt concentrations, conditions that minimize electrostatic exclusion of the protein from the membrane pores.
8. Membrane fouling can cause reduced flux and unacceptable product retention. Fouling can be reduced by using prefilters to remove large particles prior to the sterile or virus filtration.

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15

Design and Interpretation of Viral Clearance Studies for Biopharmaceutical Products

Allan J. Darling

1. Introduction

Substrates and raw materials used in the manufacturing of biological products are known to harbor adventitious agents including viruses and mycoplasma. In the past, some products derived from these processes, including vaccines, hormones, and blood-clotting factors, have resulted in incidents of the transmission of infectious disease.

A number of commonly employed cell substrates, although free of overt viral contamination, can be shown by electron microscopy to contain retrovirus particles. Endogenous retroviruses are widespread in animal populations and have been described in a variety of species. If the substrate is of human or nonhuman primate origin, additional concerns for zoonotic agents arise. Also, regulatory agencies have voiced concerns regarding raw materials, especially serum, and the potential for bovine virus and prion contamination. The safety of biopharmaceutical products is closely regulated worldwide by the relevant regulatory authorities (Food and Drug Administration, European Medicines Evaluation Agency, and the Japanese Ministry of Health and Welfare) and various guidelines exist that detail the requirements necessary to ensure these products are free from viral contamination ***(1–5)***.

Several steps can be taken to assure that infectious agents do not copurify with the biological product. These include: (1) careful selection and characterization of the cell seed system of master, working, and end of production cells; (2) thorough examination of media components and raw materials; (3) evaluation of the purification process to remove and/or inactivate potential contaminants; and (4) final product safety evaluation. A summary of the worldwide testing requirements is given in **Tables 1** and **2**. Because many production cell

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Table 1
Summary of Virus Testing Performed on Cell Banks

	MCB	WCB	Cells at in vitro limit
Tests for retroviruses			
and other endogenous viruses			
Infectivity	+	–	+
Electron microscopy	+(1)	–	+(1)
Reverse transcriptase	+(2)	–	+(2)
Other virus-specific tests	As appropriate(3)	–	As appropriate(3)
Tests for nonendogenous			
or adventitious virus test			
In vitro assay	+	–(4)	+
In vivo assay	+	–(4)	+
Antibody production tests	+(5)	–	–
Other virus-specific tests	+(6)	–	–

MCB - Master Cell Bank. WCB - Working Cell Bank Cells at in vitro limit - cells at the limit of in vitro age used for production. End of production cells.

(1) This technique can also detect other contaminants.

(2) Not necessary if positive by retrovirus infectivity test.

(3) Tests for viruses known to have been infected by these agents, e.g., EBV testing for cell lines immortalized by EBV infection.

(4) For the first WCB, this test should be performed on cells at the limit of in vitro cell age, generated from that WCB; for WCB's subsequent to the first WCB, a single in vitro and in vivo test can be done either directly on the WCB or on cells a the limit of in vitro cell age.

(5) For example MAP, HAP, RAP testing for rodent cell lines.

(6) For example testing for human viruses such as HIV, HTLV, Hepatitis B, and so on, on human cell lines.

lines cannot be certified to be free of endogenous viruses, and adventitious agents can enter the production process through any number of portals in the production process, purification process evaluation studies are essential in assuring the safety of biological products. The aim of this chapter is to outline the principles, methods, and concerns pertinent when performing viral clearance studies as part of an overall safety testing strategy.

2. General Design

A purification-process evaluation study is a documented program that provides a high degree of assurance that specific steps in a purification process can eliminate potential infectious viruses that may be present. The basic steps in performing such studies include: (1) the scale down of the purification process; (2) the choice of appropriate viruses; (3) the evaluation for cytotoxicity and viral interference; (4) the virus spike of selected steps in the process;

Table 2
Summary of Virus Testing Performed on Unprocessed Bulk and Purified Bulk Product

	Unprocessed bulk	Purified bulk
Tests for retroviruses and other endogenous viruses		
Infectivity	+/–(1)	+/–(2)
Electron microscopy	+(3)	–
Reverse transcriptase(4)	–	–
Other virus-specific tests	As appropriate(5)	–
Tests for nonendogenous or adventitious virus test		
In vitro assay	+(6)	–
In vivo assay	+/–(7)	–
Antibody production tests	+/–(8)	–
Other virus-specific tests(6)	+/–(9)	+/–(9)

(1) For murine hybridomas, cocultivation assays are important if MCB or end of production cells are positive

(2) Where infectious virus has been identified during cell line or unprocessed bulk testing. Highly sensitive assays such as *Mus dunni* amplification assays with various end points should be used for murine retrovirus for at least three lots.

(3) TEM usually performed on at least three lots to quantify viral load in unprocessed bulk as a starting point for viral clearance evaluation.

(4) RT can be used as an end point for amplification assays performed in ***(2)***.

(5) Tests for viruses known to have been infected by these agents, e.g., EBV testing for cell lines immortalized by EBV infection.

(6) On every lot.

(7) Usually only performed once.

(8) On ascites only.

(9) Specific, sensitive tests for infectious viruses identified during cell-line testing may be required if infectious virus other than retrovirus is detected. If human infectious agent is detected during cell-line characterization, every lot should be tested and regulatory authorities consulted. If virus is nonpathogenic for humans, then testing of at least three lots is sufficient.

(5) the determination of physical removal vs inactivation; and (6) the overall calculation of the reduction factor expressed as the sum of the individual steps.

When performing a process-evaluation study, the starting material for each step of the process to be evaluated is “spiked” with large amounts of infectious virus. The virus spike is added in a volume that is less than 10% v/v of the total volume of the starting material to be spiked. Determination of the spiking ratio requires a balance between maximization of the amount of virus used to spike the starting material and the necessity of maintaining the nature of the material with respect to pH, ionic strength, and protein concentration. The virus-spiked

material is taken through the purification/inactivation step and various fractions are collected and assayed for viral infectivity. By assessing virus load before and after a particular step a viral reduction or clearance factor (usually expressed in logarithmic terms) can be calculated.

3. Selection of Steps for Virus-Spiking Studies

A number of factors influence which steps should be evaluated for a virus-clearance study. The main consideration is whether the step will be expected to contribute to the safety of the product by removing or inactivating significant levels of infectious virus. Information on the efficacy of various steps can be found in the public domain or from the different contract biosafety testing companies. The robustness of any particular step is defined by the value obtained for viral clearance, the reproducibility of this step in the process, and the validity of scale down. The more reproducible the step and the easier it is to scale down, the more confidence can be attributed to extrapolating the virus-clearance results from the laboratory to the actual production scale. Steps such as pH inactivation, heat treatment, solvent detergent treatment, and physical removal by virus-removal filters are very effective steps as they generate good virus-clearance values and validation and performance of the scale down is relatively simple to accomplish. At the opposite end of the spectrum, steps such as centrifugation, precipitation, and other types of filtration are viewed very critically.

Column chromatography lies somewhere in the middle of these two extremes, especially where no buffers are used in the purification that can inactivate virus. Column chromatography can generate a wide variety of different results even when the same virus and resin are used. These differences probably reflect the variety of different conditions and buffers used to perform the chromatography and demonstrate that column chromatography should not be relied on as an absolute guarantee of providing safety. Comparative studies on new and used resins may be necessary, particularly at later stages of product development when the chromatography step contributes significantly to the overall log reduction calculated for the process. For columns that are run more than once, it is essential to validate the effectiveness of the column resin cleaning/sanitization regime to inactivate viruses.

It is highly desirable to have at least one inactivation step in a production process. This can be a specific inactivation step introduced into the process or can be, as in the case of low pH elution from Protein A columns, incidental, occurring as part of the purification process. For blood products, at least two "robust" virus clearance steps are recommended to ensure an adequate margin for safety. Examples of steps commonly used in virus clearance studies are given in **Table 3**.

Table 3
Examples of Common Virus Removal and Inactivation Technologies

Virus inactivation methods		Virus removal methods	
Chemical methods	Organic solvents Disinfectants Enzymic digestion Alcohol pH extremes Detergent Solvent detergent	Precipitation	Ammonium sulfate, and so on
Physical methods	Heat treatment (dry heat or pasteurization UV radiation Ionizing radiation	Column chromatography	Ion exchange Gel filtration Affinity Reverse phase Hydrophobic interaction
		Membrane filtration	e.g., Omega, Planova, Viresolve, DV50

4. Scale Down of the Purification Process

Validation of the scale down remains one of the essential prerequisites for performing virus-clearance evaluation studies. Obviously, cGMP considerations prohibit the spiking of the actual production steps and, therefore, viral-clearance studies are normally performed on a scaled-down version of the production step in a laboratory equipped to handle large amounts of infectious virus safely under GLP conditions. In order to accurately extrapolate the results of the virus-spiking experiments to the production scale, it is essential that the scaled-down steps mimic as accurately as possible the full-scale manufacturing process. For example, the scale down of a chromatography column should consider factors such as the bed size, flow rate to bed size ratio, HETP values and peak asymmetry, buffer types, pH, and product yield and purity. Equivalency between the manufacturing scale and the scale down must be demonstrated and any deviations must be discussed along with their potential impact on the virus clearance. Certain steps are inherently more difficult to scale down than others and this should be considered in the selection of steps to be spiked. An example of various scale-down calculations is given in **Table 4**.

There is no specific guideline for the size of the scale-down, but generally a 1/50 to 1/200 scale-down factor generates a suitable laboratory-sized process. The scale-down process should be designed to minimize volumes of output

Table 4
Calculation of Ion-Exchange Chomatography Scale-Down Values

	Linear flow rate	Production scale	Calculated values for scale down		
Column size		100/20	XK 50/20	XK 26/20	XK 16/20
Column radius		5 cm	2.5 cm	1.3 cm	0.8 cm
Column surface area		78 cm^2	19.62 cm^2	5.31 cm^2	2.01 cm^2
Column height		17.8 cm	17.8 cm	17.8 cm	17.8 cm
Column volume		1.39 L	349.24 mL	94.51 mL	35.78 mL
Equilibration flow rate	250 cm/h	330 mL/min	81.75 mL/min	22.13 mL/min	8.38 mL/min
Load flow	250 cm/h	19.50 l/h	81.75 mL/min	22.13 mL/min	8.38 mL/min
Wash flow rate	300 cm/h	23.40 l/h	98.10 mL/min	26.55 mL/min	10.05 mL/min
Elution flow rate	76 cm/h	5.93 l/h	24.85 mL/min	6.73 mL/min	2.55 mL/min
Load volume		21.52 L	5406.94 mL	1463.21 mL	553.95 mL
Approximate scale factor		1:1	1:4	1:15	1:39
Output volume		2.36 L	592.95 mL	160.46 mL	60.75 mL

samples. Because an aliquot of the total volume of each sample is generally used for titration (with the remainder saved as backup), the failure to detect virus in the aliquot does not rule out the possibility of a low amount of infectious virus being present in the total sample. To take such instances into consideration, a theoretical minimum detectable level of virus must be assumed based on sampling effects; this is represented statistically by the Poisson distribution. Greater reduction factors can be obtained by using a larger number of replicates and increased inoculation volumes. This procedure increases the probability of detection and lowers the theoretical minimum detectable level of virus that must be assumed.

5. Selection Criteria of Viruses for Evaluation of Viral Clearance

The selection of viruses for purification-process evaluation studies is critical. Generally, the selection should take into account the nature and origin of the starting material and the raw materials used in production. Viruses known to contaminate the starting material should be used, for example, retroviruses and retroviral particles found in murine hybridomas and some CHO lines. Where this is not possible, models for those viruses should be considered. Agents possessing a range of biophysical and structural features should also be included to test the production process for its ability to inactivate or remove any virus in the event that novel or unpredictable virus contamination occurs and to determine the robustness of the process. Two additional important aspects in the selection of any virus are its ability to grow a high-titer stock in serum-free or low-protein medium and its ease of detection in a sensitive and reliable assay.

For Phase I and II clinical trials, one or two viruses are often sufficient for the process evaluation. Later stages of clinical trials and for product licensure require a broad range of virus types to be tested. For example, it might be appropriate to use DNA and RNA enveloped and nonenveloped viruses with different sizes and resistance to inactivating agents. Typically, four viruses are required, including an appropriate retrovirus. The number and type of viruses considered are determined on a case-by-case basis. A common virus selection for a product derived from a murine monoclonal antibody is shown in **Table 5**.

Biological products derived from bovine, and ovine tissues raise concerns about the possible contamination by agents of transmissible spongiform encephalopathy (TSE), such as bovine spongiform encephalopathy (BSE) and scrapie. TSE agents are highly resistant to inactivation and no suitable diagnostic test exists for the identification of the agents. At present, the definitive diagnosis of TSE depends upon pathological study of a brain biopsy. Generally recognized systems for evaluating a step in a purification process for its ability to remove or inactivate TSE are, therefore, in vivo models. Two animal models for TSE are routinely used: a Syrian hamster model utilizing the 263K (hamster

Table 5
Viruses Used to Validate Products Derived from Murine Hybridomas and Cell Lines

Virus	Genome	Size	Enveloped	Resistance
MVM	ss-DNA	18–26 nm	No	Very high
Reo-3	ds-RNA	60–80 nm	No	High
MuLV	ss-RNA	80–130 nm	Yes	Low
PRV	ds-DNA	150–200 nm	Yes	Low-medium

MuLV, murine leukemia virus; Reo-3, reovirus type 3; MVM, minute virus of mice; PRV, pseudorabies virus.

adapted) strain of scrapie, and a C57BL/6N mouse utilizing the ME7 (mouse adapted) strain of scrapie.

6. Cytotoxicity and Viral Interference

Product and buffers that will ultimately be generated and tested for the presence of infectious virus should first be evaluated independently for cytotoxicity and interference in the assays used to determine virus titer. If cytotoxicity of the indicator cells (cells used in the assay system) is observed, dilutions, pH adjustments, or in some cases dialysis of the product/buffers may be necessary. Viral interference in the assay system caused by a buffer or a product can give an overestimation of clearance for a specific process step. Virus may still be present in the sample, but the ability of the assay to detect infectious virus may be impaired. This is demonstrated in **Table 6**. In this example, the test article at undilute, 1:3, and 1:9 dilutions clearly interfered significantly with the ability of the virus to replicate in the indicator cells. However, no toxicity was seen; thus, experiments based on cytotoxicity alone would not demonstrate the true effect of the test article on the virus/indicator cell system.

7. Performing the Spiking Experiments and Collection of Samples for Assay

By the time the actual spiking experiments are initiated, a large amount of work should have already been performed to ensure the accuracy and validity of the study. This should ensure that the actual spiking experiments, sample collection, and titration are relatively straightforward. The number and nature of samples taken for collection depends on the type of step being studied. For inactivation experiments, samples are taken from the spiked load material prior to treatment and, at various times, posttreatment to examine the kinetics of inactivation of the virus for the particular treatment. For partitioning steps, the distribution of virus must be examined. In addition to the spiked load and prod-

Table 6
Cytotoxicity and Interference Results of Test Article on Porcine Parvovirus/PK13 Cell Titration System

Sample identification	Sample dilution	% Cytotoxicity	Average plaque count
Negative Control	Undilute	0	0
Positive Control	Undilute	0	105
Test Sample 1	Undilute	0	33
Test Sample 1	1:3	0	54
Test Sample 1	1:9	0	74
Test Sample 1	1:27	0	110
Test Sample 1	1:81	0	106
Test Sample 1	1:243	0	106

uct-containing fractions, all other fractions must also be collected and tested for the presence of virus.

Samples from the spiking studies should be titrated immediately upon collection. If this is not possible, and it is necessary to freeze samples prior to titration, then appropriate controls should be employed. In this respect, an aliquot of the stock virus frozen alongside the samples should not be considered an appropriate control. This is because the survival of virus frozen and thawed in tissue-culture medium potentially will be different from virus present in samples generated during spiking studies, which will be in a wide variety of buffers containing different concentrations of protein affording different degrees of "protection" from freeze-thaw damage. Slow freezing can also cause significant solute and pH changes in certain buffers, which could cause inadvertent inactivation of virus. Similarly, any other manipulations that have to be performed on the samples that are not part of the production process should be controlled to ensure that the virus titers obtained in the samples are accurate.

8. Assay Titration Methods—Accuracy and Validation

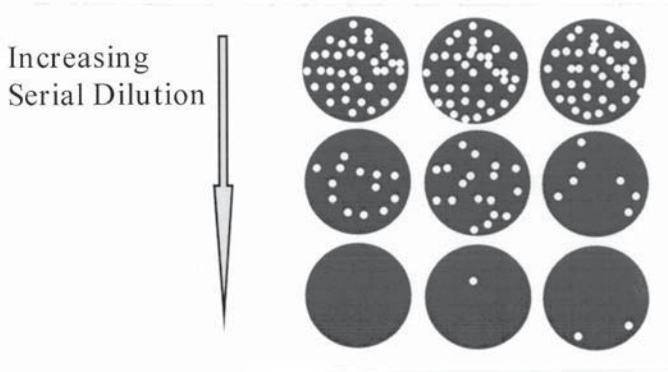
The FDA in the "Points to Consider," the EMEA in the "Notes for Guidance" and the ICH virus safety document emphasize clearly the need for accuracy and statistical evaluation in the results obtained from studies designed to show the effectiveness of the production process to remove potential viral contaminants. Assays for the detection of viral contamination can result in highly variable results owing to the biological nature of the assay systems. Test data generated using virus titration methods in viral clearance evaluation studies must provide a reliable estimation of process-reduction factors and, therefore, methods must provide accurate and reproducible quantitation of virus concentration. Virus titers are normally expressed with 95% confidence limits that

should not exceed 0.5 log of the stated titer. Historical data can give a picture of the variability of a particular assay and, thus, to assess the significance of current test results, but is no substitute for comprehensive validation. Accuracy, reproducibility, repeatability, linearity, limit of quantitation, and limit of detection are essential test-method performance characteristics and successful assay validation provides the data to assess these validation parameters. Test methods must also demonstrate reasonable sensitivity for low-level virus concentrations in order to maximize reduction factors for process steps capable of full viral inactivation.

A wide variety of assay types can be used to detect and quantify virus titer. Each assay type has specific advantages and disadvantages. The two main in vitro assay methods used to quantitate infectious virus in virus-clearance studies are the plaque (or focus) formation assay and the cytopathic effect (CPE) assay. Both assay types have been successfully validated and are used reliably for the quantitative determination of virus titer and process-reduction factors.

Plaque assays offer the specific advantage of producing a countable event, i.e., plaque formation, vs virus dose (**Fig. 1**). In this example, virus titer/mL is obtained by dividing the total number of plaques by the total volume of original sample tested. This method of computation is an averaging procedure, which gives equal weight to equal volumes of the original suspension at different dilutions. In order to determine the standard error (SE) and 95% confidence interval (CI) for a sample, the standard deviation is calculated at each dilution. From the standard deviation, the variance is then calculated (the square of the standard deviation) and the SE in the plaque counts is then calculated from the square root of the sum of the variances multiplied by the number of replicates per dilution. Dividing this figure by the overall volume tested gives the standard error of the titer. Since these values are normally expressed in logarithmic terms, the SE is transformed into $\log_{10}$ by dividing the standard error by the titer and multiplying by the constant, 0.434 (the log of e). To determine the 95% CI, the number of replicates is totaled (n) to calculate the degrees of freedom ($n - 1$) and this value is used to look up the critical t-value for a 95% CI from t-statistic tables. The standard error is then multiplied by the critical t-value to give the 95% confidence limits for the plaque titer. Increasing the number of replicates per dilution or decreasing the dilution interval results will result in an increase in the number of plates where plaques can be accurately counted and thus, to an increase in the accuracy of the titers calculated.

The second method used to quantitate infectious virus is the CPE or the 50% tissue culture infectious dose ($TCID_{50}$) assay. This method is useful to determine the titer of viruses that do not produce plaques, but do cause a change in cellular morphology. This assay is a quantal assay, i.e., wells are scored either positively or negatively for the presence of infectious virus in samples serially



Dilution factor	Total amount of original sample tested	Plaques per dish	Standard deviation	Variance
10^{-6}	$3 \times 0.2 \times 10^{-6}$ mL	144,126,173	23.71	562.16
10^{-7}	$3 \times 0.2 \times 10^{-7}$ mL	13,18,7	5.51	30.33
10^{-8}	$3 \times 0.2 \times 10^{-8}$ mL	0,1,2	1.00	1.00
Totals	6.66×10^{-7} mL	484		593.49

Titer of the sample is $484/(6.66 \times 10^{-7}) = 7.27 \times 10^{8} = 8.86\ \log_{10}$ pfu mL^{-1}

SE in plaques counted = $\sqrt{(593.49 \times 3)}$

SE in original sample = $42.2/(6.66 \times 10^{-7}) = 6.34 \times 10^{7}$.

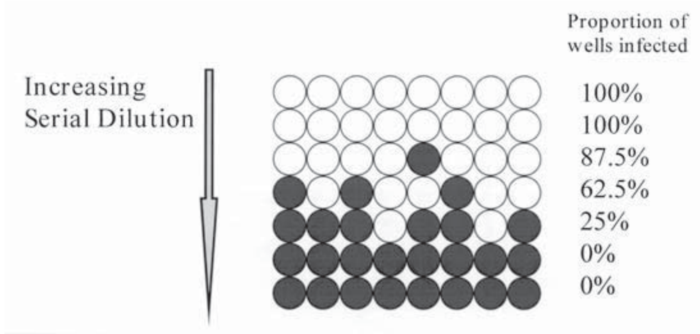
SE in $\log_{10} = \dfrac{6.34 \times 10^{7}}{7.27 \times 10^{8}} \times 0.434 = 0.0378$

From the student's *t* tables, the critical *t*-value for 3 replicates (2 degrees of freedom) is 4.303. The 95% confidence interval is $0.0378 \times 4.303 = 0.16$ in $\log_{10}$ or $6.34 \times 10^{7} \times 4.303 = 2.73 \times 10^{8}$

The titer with 95% confidence limits is $8.86 \pm 0.16\ \log_{10}$ pfu/ mL

Fig. 1. Calculation of virus titers by plaque assay.

diluted to end point and the dilution of the sample needed to infect 50% of the culture wells is calculated (**Fig. 2**). The accuracy of this assay is dependent on how accurately the infection rate at each dilution is determined. For this reason, a larger number of replicates at each serial dilution leads to more accurate titer determinations. Ensuring that several serial dilutions infect between 10 and 90% of the inoculated cell cultures also increases accuracy. Care must also be taken in the method used for calculation of the titers. Although the Spearman–Kaerber method is widely used ***(6)***, the methodology has an absolute requirement that serial dilution's giving 100% and 0% infectivity are demonstrated. These criteria are often not met in virus titrations (for example, when only low levels of virus are present) and under these conditions an alternative method of calculation such as the probit method or modified Spearman–Kaerber methods should be used to ensure accurate determination of viral titers.



The formula for the final titer calculation of $TCID_{50}$ is based on the Spearman–Kaerber method using the following formula:

$$m = X_k + (d/2) - d\Sigma p_{\mathrm{i}}$$

where
m = the logarithm of the dilution at which half the wells are infected relative to the test volume
Xk = the logarithm of the smallest dosage which induces infection in all cultures
d = the logarithm of the dilution factor
p_i = the proportion of positive results at dilution i
Σp_i = sum of p_i (starting with the highest dilution producing 100% infection)

The standard deviation σm, is calculated using the following formula:

$$\sigma_m{}^2 = d_f{}^2\Sigma[p_i(1 - p_i)/(n_i - 1)]$$

where
d_f = the logarithm of the dilution factor
p_i = the proportion of positive results at dilution i
n_i = number of replicates at dilution i
Σ = denotes the summation over dilutions beginning at the kth dilution

The 95% confidence limit is calculated as m ± $1.96\sigma_m$.

Fig. 2. Calculation of virus titers by $TCID_{50}$ assay.

9. Calculation of Virus-Reduction Factors

Virus-reduction factors, R, for an individual inactivation/removal step are calculated as follows:

$$R = \log (V_1 \times C_1/V_2 \times C_2)$$

where
R is the reduction (clearance) factor
V_1 is the volume of the starting material

C_1 is the concentration of virus in the starting material
V_2 is the volume of the post-processing material
C_2 is the concentration of virus in the postprocessing material

Guidelines specifically state that confidence intervals should be calculated for all studies of relevant and specific viruses and that the confidence intervals for both the preprocessing titer and the postprocessing titer should be included in the confidence interval for the process reduction factor. Specifically, the confidence interval for reduction-factor calculations should be calculated with confidence intervals equal to $\sqrt{(s^2 + a^2)}$ where s is the 95% CI for the preprocess material and a is the 95% CI for the postprocess material.

Having calculated the individual reduction factors for each step of the process, the next stage in analysis of the results is to calculate the reduction factor for the process as a whole. If each of the individual steps in the process is deemed to be independent (i.e., removes or inactivates virus by a separate mechanism) then the log clearance values for each step can be added together. Summation of reduction factors for repeated and similar process steps can result in a significant overestimation of the ability of the purification scheme to effectively remove viral contaminants and should be avoided. It should be noted that the use of orthogonal and robust processing procedures ensures the greatest probability of virus removal/inactivation and for this reason clearance factors associated with robust steps should contribute most significantly to calculated overall reduction

10. Interpretation of Clearance Results

Having obtained the overall clearance factor for the process, the final step is to try to put this number into the context of risk assessment of the final product. This is approached differently depending on the type of product being studied and on the virus of concern. For blood products, the 1994 Paul Ehrlich recommendations state that for enveloped viruses, at least two robust steps should be demonstrated in a process, each of which should be able to remove or inactivate at least four logs of enveloped virus with the whole process able to clear at least 10 logs of virus ***(7)***. For nonenveloped virus such as hepatitis A, one step should be able to clear at least four logs of this class of virus with the whole process able to generate at least a six-log clearance. These requirements were modified in the 1996 CPMP guidelines to place less emphasis on the actual clearances to be achieved and more emphasis on demonstrating the robustness of the individual steps and of the process ***(1,2)***.

The CPMP guidelines also emphasize robustness of steps rather than clearance values to be achieved for products derived from cell lines. This approach is different from the ICH guidelines and the 1997 “Points to Consider” docu-

ment which, although they include the same requirements for incorporation of robust steps, give specific recommendations for the level of murine retrovirus clearance that has to be achieved ***(4,5)***. In this case, the level of clearance demonstrated should be substantially in excess of the potential virus load in one dose of the final product as calculated from the virus particle count obtained by transmission electron microscopy on the unprocessed bulk material. For example, TEM analysis on the unprocessed bulk may have shown a particle count of 10^9 per mL (the sensitivity of this technique is 10^6 per mL) and 1 L of unprocessed bulk may be required to produce one dose of the final product. If the process validation study has been shown to remove 10^{18} infectious retroviruses, then the number of virus particles that may be present in one dose of the final product is:

$$\frac{(10^3 \text{ mL per dose}) \times (10^9 \text{ virus particles per mL})}{\text{Clearance factor} \geq 10^{18}} = <10^{-6} \text{ particles per dose/}$$

Therefore, on average, less than one virus particle per million doses would be expected, which is an adequate margin for safety. This calculation is relevant only to those viruses for which an estimate of the starting numbers can be made, as is the case for endogenous retroviruses. The figure of six logs excess clearance is not an absolute figure as each study is looked at on a case-by-case basis by the regulatory authorities.

11. Limitations of Virus-Clearance Studies

Although virus-clearance evaluation remains an essential component in ensuring that biopharmaceutical products are free from viral contamination, it should be remembered that these studies have certain limitations. These studies are performed on a scale-down process—not on the full manufacturing scale—and even with accurate scale down, there is no guarantee that virus partitioning and inactivation will be identical at both scales. The processes themselves are loaded with extremely large amounts of infectious virus, which is, in most cases, totally different from the natural situation where virus contamination, if present, may only be at a low level. The viruses that are used may not behave the same as those viruses found in the manufacturing environment because they are laboratory-adapted isolates and may differ in their susceptibility to removal and inactivation. Summation of the individual clearance values to obtain an overall clearance value for the process can also lead to overstating the clearance capacity if virus is removed or inactivated by similar mechanisms in apparently independent processing steps. Variations in the process may also impact on the clearance values obtained, and for this reason the spiking studies should always be performed using worst-case conditions where this can be identified.

Various parameters of the study design including virus titers, cytotoxicity, interference, volumes, limits of detection of assays, and so on, all have a significant impact on the clearance values obtained, which can potentially lead to understating the potential clearance capacity of the overall process. Given all these limitations, careful study design and experience is essential in the interpretation of the results.

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16

Validation of Sterilizing Filters in the Biotechnology Industry

Manohar Kalyanpur

1. Introduction

Parenteral drugs have been manufactured on an industrial scale for many years and drugs that could resist heat were sterilized by terminal heat sterilization. However, some parenteral drugs were heat sensitive and could not be sterilized by heat. This was the beginning of the search for alternative methods of sterilization. Filtering out contaminating organisms from the drug product was obviously an excellent idea and filters made of a variety of materials such as porcelain, asbestos cellulose, and polymeric membranes have been developed over the years. Today, only the polymeric membrane filters are used as sterilizing grade filters.

The porcelain filters suffered problems of adequate cleaning and posed the consequent risk of cross-contamination of production batches of the drugs. The asbestos cellulose filters fell out of favor because of the inherent risk of shedding of asbestos fibers by such filters and the fibers then ending up in the product being filtered. The polymeric membrane filters were first manufactured on a commercial scale in 1929 and were only available as disc filters. However, these filters were impractical for filtering large batches of drug solutions and a need for a truly practical version of the filters was felt. The 1970s saw the development of the first pleated cartridge filters for this application in the biopharmaceutical industry. Such filters are in use today in the industry.

A great majority of the products of the biotechnology industry are heat sensitive proteins and cannot be sterilized by heat. Therefore, filter sterilization is the only suitable method for sterilizing these products. The proper validation of these filtration processes is the topic of this chapter.

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In 1987, the U.S. Federal Drug Administration (FDA) ***(1)*** issued the "Guidelines for sterile drug products produced by aseptic processing," which recommended that all sterilizing grade filters used in the production of parenteral drugs must be properly validated for total bacterial retention. By the FDA definition, a filter is deemed sterilizing if it can retain a challenge of 10^7 colony forming units (CFUs) of *Brevundomonas diminuta*, hitherto known as *Pseudomonas diminuta* ***(2)***, per square centimeter of the filter surface and yield a sterile filtrate. These FDA guidelines further recommend that the validation must be properly performed by challenging the filter with an appropriate number of cells of *B. diminuta* suspended in the drug product and the filtration during validation must be performed under conditions that mimic as closely as possible, the actual process conditions employed during manufacture of the drug product.

Another important aspect of filter validation is the confirmation of the integrity of the filter, especially at the end of the filtration process. Nondestructive methods of integrity testing such as the bubble point or forward flow (diffusive flow) measurement are recommended by the filter manufacturers. The integrity test value obtained can also be correlated to the bacterial retention capacity of the filter. Although these tests can be performed manually, automatic integrity testers today are commonly used in the pharmaceutical and biotechnology industries. Proper validation of these integrity testers, which contain computer software, therefore, becomes a critical component of the task of the validation of the sterilizing filters.

More recently in 1994, the FDA issued a supplementary guideline to further ensure the safety of aseptically processed drug products. The drug manufacturers are asked to validate that the filtration process does not add any extractables to the filtrate by leaching out extractables from the filter components made of organic polymers. They also need to confirm that the sterilizing filter does not remove any components, especially active ingredients and antibacterial agents present in the drug formulation. Such removal of drug components changes its composition and can render the formulation less efficacious and also prone to bacterial contamination during storage.

Thus, the validation of the sterile filtration process confirms the following:

1. That the filter can remove a bacterial challenge of 10^7 CFUs of *B. diminuta* (suspended in the drug product) per square centimeter of its surface area under the actual process conditions.
2. That the filter remains integral at the end of the filtration.
3. That the integrity tester used for the test is properly validated.
4. That the filter does not release toxic extractables into the drug product.
5. That it does not remove by absorption or adsorption, any important components of the formulation.

The regulatory authorities in Europe and Japan have issued guidelines similar to those issued by the FDA.

2. Responsibility for Filter Validation

Both the filter manufacturers and drug producers share this responsibility. The filter manufacturers perform intensive qualification of the filter production and quality control testing of the filters prior to their release to the pharmaceutical industry. This enables them to assure the filter users the following:

1. The filters they choose are capable of retaining the recommended level of the challenge of *B. diminuta*.
2. The filters are integral at the time they leave the manufacturer's warehouse.
3. The filters can be properly sterilized by autoclaving or steaming in place, according to their recommended methods for the sterilization.
4. They do not release high levels of endotoxins or other toxic materials including extractables into the filtered drug product.
5. They can withstand certain processing conditions such as filtration flow rates and pressure drops.

The filter manufacturers provide comprehensive validation guides for the filters that give extensive data and describe the test methods employed for collecting the data pertaining to the above points. Filter users are therefore advised to refer to the validation guides for filters to assure themselves that the filters selected for their process will perform according to their expectations. The sections that follow in this chapter will describe individual components of the validation of sterilizing filtration including methods utilized to achieve this.

3. Methods

3.1. Validation of Bacterial Retention

The validation of bacterial retention is performed following the ASTM-F 838-83 ***(3)*** or a comparable method. The filter manufacturers provide evidence that the filter can retain the challenge made in a suitable aqueous medium under a certain set of process conditions. The drug manufacturer is obliged to confirm that the filter can retain the challenge suspended in the drug product (or a suitable substitute) under his normal process conditions.

Bacterial retention by filters is affected by several factors which include the type of filter used, components of the drug product being filtered and their properties such as pH, viscosity, osmolarity and ionic strength, process conditions used, and characteristics of the challenge organism. It is important to consider the interaction between the challenge organism and the drug product under "worst case" conditions that make it possible for the bacteria to pass the filter barrier. The validation must be performed under "worst case" scenarios

in triplicate with the membrane chosen for the process. Membranes from three different manufacturing lots are selected and their integrity test values determined before the test. The values must exceed the minimum integrity test value recommended by the filter manufacturer for that membrane.

The challenge organism recommended by the regulatory authorities for the validation studies is the strain of *B. diminuta* bearing the American Type Culture Collection number ATCC 19146. The culture conditions recommended by ASTM in 1983 are employed. Both the saline lactose broth (SLB) or the frozen cell paste (FCP) methods are valid. The need for the challenge level of at least 10^7 CFUs per cm^2 of filter area comes from the observation of Bowman et al. ***(4)*** that a *B. diminuta* challenge at less than 10^7 CFUs per cm^2 may not penetrate a 0.45 μm filter and they therefore established the minimum challenge level at (10^7 CFUs per cm^2 surface area of a sterilizing grade filter which is normally a 0.2 μm filter.

Also, a monodispersed suspension of the challenge organism is required for the validation test. Aggregrated cells are less likely to penetrate defective larger membrane pores and a challenge of such cells, therefore, does not represent a "worst case" challenge. Therefore, the challenge culture should be screened by optical microscopy to ensure the absence of aggregrates and dispersed in an ultrasonic bath if aggregration is noticed. The lack of aggregation and the necessary size control is confirmed by filtering the challenge through a 0.45-μm rated filter, which is, therefore, a positive size control. The viability of the challenge organism is confirmed by growth on either trypticase soy agar or Muller–Hinton agar plates. Such plates should also be used to determine the titer of viable organisms just prior to the challenge test and at the end of the procedure. Similar titers before and after the test confirm the viability of the challenge organism during the duration of test. Agar plates of a similar nutrient medium are also used for cell counts downstream of the sterilizing filter. The regulatory guidelines call for the challenge organism to be inoculated in the drug product whose sterile filtration is being validated. It, therefore, becomes very important to confirm that the organism remains viable in the drug product during the entire duration of the test. The cells must be exposed to the product for such a period and if at the end of this exposure, the bacterial cell count diminishes by *less* than one log, the product is deemed *nonbactericidal*. The method of *direct inoculation* of the organism into the drug product can then be employed. A schematic for this procedure is illustrated in **Fig. 1**.

3.2. Tests with Bactericidal Drug Products

If the bacterial cell count drops by greater than one log during the viability test in the drug product, the product is deemed bactericidal. In this case, the

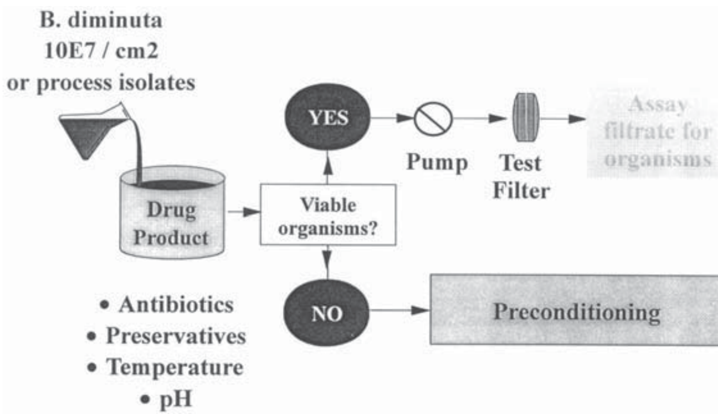

Fig. 1. General schematic for test procedure for validation of bacterial retention by sterilizing filters.

challenge organism must not be inoculated into the drug product, but in a suitable surrogate fluid that matches the drug product as closely as possible in terms of its physical and chemical properties and also does not affect the viability of the challenge organism.

3.3. Modification of the Test Procedure

As per the guidelines given by the regulatory authorities, the validation of sterile filtration must confirm the interaction between the three components of the system: the drug product, the challenge organism and the filter under actual process conditions (*see* **Fig. 2**). Simply, a challenge performed in the surrogate fluid therefore does not sufficiently meet the guidelines. A modified test procedure is followed in such cases, following the schematic shown in **Fig. 3**. First, the test filter is preconditioned with the drug product by circulating the product through the filter according to the process conditions of temperature, flow rate, pressure, and time. The test filter is then rinsed with a suitable solution to remove the bactericidal substance from the filter. The viability of the challenge organism in the last few milliliters of the collected rinse solution confirms that the bactericidal has been completely rinsed off the membrane. The preconditioned filter is then challenged with the organism inoculated into the surrogate fluid. **Figure 3** illustrates the procedure for the validation of processes for sterile filtration of bactericidal substances.

3.4. Validation with Resistant Test Organisms

Sometimes the drug product under the validation can be bactericidal to *B. diminuta*, the standard challenge organism under the process conditions.

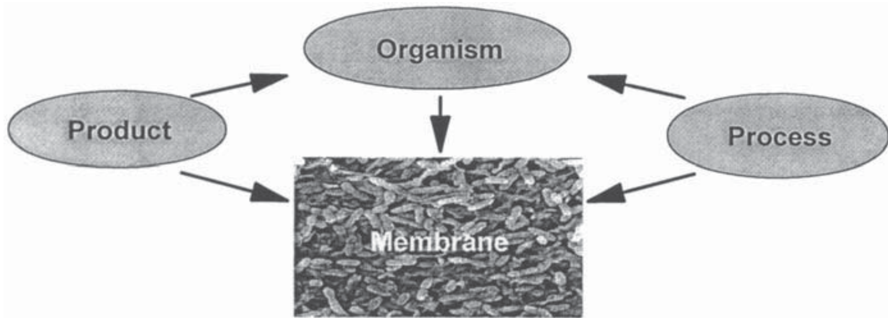

Fig. 2. Interacting components of sterile filtration process.

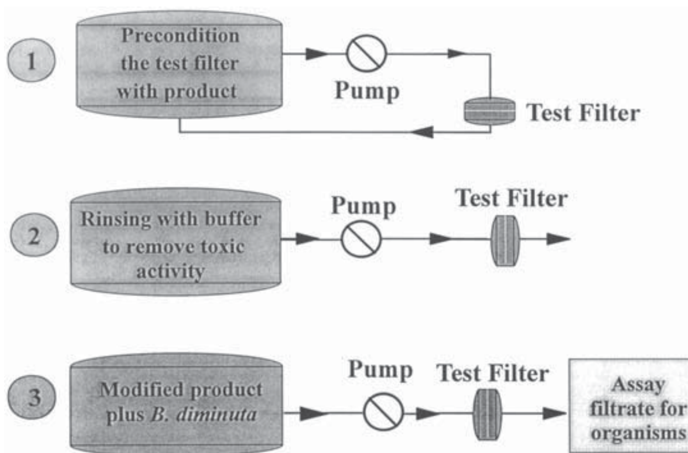

Fig. 3. Modified test procedure for validation of bactericidal drug products after preconditioning the filter.

But other organisms, especially those isolated in the work environment where the drug production takes place, may be resistant to the drug and also the process conditions. This brings another modification to the test. The resistant organism is isolated in a pure form and propagated in a medium containing the actual product since the product can have a significant effect on the morphology and physiology of the organism. The individual cells thus cultivated can be smaller than those of the standard *B. diminuta* cells and penetrate the pores of the 0.2 µm filter. The resistant organism is cultured in the modified medium to a high cell density and a suspension in the product at the desired concentration is then used for the challenge test, which takes place under actual process conditions. If the filter retains the challenge of the organism, the test validates the process.

3.5. Validation Under Modified Process Conditions

3.5.1. Temperature

Sometimes the sterilizing filtration in the manufacturing process is performed at an elevated temperature which makes it difficult for the challenge organism to remain viable during the course of the test. In this instance, the filter is first preconditioned to the drug product as per the standard process *including temperature*, and subsequently challenged with the organism inoculated into the drug product. The filtration is performed according normal process conditions except at ambient temperature to permit the challenge organism to survive and remain viable.

3.5.2. Pressure Differential and Flow Rates

The challenge test must be performed at the highest pressure differential observed in the actual process. However, care must be taken to keep this under the maximum value recommended for the filter in the filter manufacturer's specifications. The test pressure used in the validation study must be well documented in the test protocol and must never be exceeded during the test. It must also stay within the filter specifications during actual drug production. Sometimes it is not possible to simulate both the pressure differential and flow rate at the same time during the validation study. In this case, the drug product manufacturer must decide which of the two process conditions is specific to the actual process and this condition is maintained during the test. He must have a sound rationale to justify the decision to make such a change in the validation test.

3.5.3. Duration of the Challenge Test

The duration of a sterile filtration process can have some effect on the ability of the filter to retain the bacterial challenge. For example, the exposure time can have an effect on the overall compatibility of the filter to the drug product, especially to a specific component or components of the formulation. This can affect the filter integrity and permit the time-dependent penetration of the test filter by the organisms. Also, the composition of the drug formulation can affect the morphology of the challenge organism after prolonged exposure. If the bacterial cells shrink, the change in their dimensions can help them to penetrate the filter membrane. Thus, cells that are normally retained by the filter can pass the filter barrier and end up in the filtrate giving a nonsterile filtrate. This phenomenon is sometimes referred as the *grow through effect* and is discussed in detail by several authors ***(5–8)***. Therefore, the validation process must continue for at least as long as the actual process.

3.5.4. Analysis of the Effluent in the Challenge Study

The entire effluent from the challenge test must be screened for the presence of bacterial colonies to confirm complete retention by the filter over the full duration of the test. To accomplish this, the effluent is passed through a second analytical membrane filter of a 0.2- or 0.45-μm rated pore size. This filter is referred to as the collection filter. The presence or absence of bacterial colonies on the collection filter is confirmed by placing the filter on an agar plate of a suitable nutrient medium and incubation of the plates for up to seven days. The bacterial cells, if stressed because of exposure to the drug product or owing to the process conditions during the filtration, can take long to grow and show up on the plate. Absence of CFUs on the plate confirms a successful sterile filtration.

3.6. Interpretation of Results

A total reduction of bacterial challenge by all three test filters resulting in a sterile effluent validates the process of sterile filtration. The use of three filters from different manufactured lots of the membrane is considered sufficient replication and assures repeatability of the filtration process. If even one of the collection filters shows bacterial colonies, the reason for their presence on the filter needs to be determined and the test is repeated with three filters from the suspected membrane lot.

3.7. Future Changes in the Process and Revalidation

A validated process of sterile filtration confirms that subsequent changes do not call for revalidation provided the process conforms to the following conditions.

1. The filter membrane is not changed, especially because of changes introduced during its manufacture. Manufacturers are expected to notify filter users of any changes in the manufacturing process, especially if any of the materials of construction are replaced by new ones.
2. The filtration flow rate per unit area of the filter is maintained by increasing or decreasing membrane area in proportion to changes in the volume of the processed batch during scale up and scale down of the process.
3. None of the process conditions, e.g., pressure differential and process time exceed those in the validated process.
4. The extractables data for the chosen configuration is available from the filter manufacturer as part of the supporting documents.

3.8. Integrity Testing of Sterilizing Filters

The bacterial challenge test is a destructible test of its bacterial retention capacity because a filter thus tested cannot be used for a sterile filtration. It is also not practical to perform this test on each and every filter used in the envi-

ronment of drug production. Therefore, nondestructive physical integrity tests are recommended by the filter manufacturers. These are well accepted by the regulatory agencies and drug industry; The tests are easy to perform but the operators handling these critical processes must be properly trained and periodically retrained. The documentation to prove such training must be maintained by the drug company. The bubble point test and the forward or diffusive flow test are the two most commonly performed tests. The physical integrity test values can be correlated to the actual bacterial retention by the membrane and filter manufacturers provide correlation data in the validation guides for the filters. Using this data, the filter user can predict the eventual bacterial retention capacity of a filter from the physical integrity test value he obtained while performing the bubble point or the diffusive flow test.

3.9. Product Wetted Integrity Tests

An important filter specification provided by their manufacturers is the integrity test value for filters when they are wetted with a standard liquid, typically water. However, drug formulations, which have a viscosity and surface tension different from those of water, tend to alter these values ***(9)***. Quite often the values are depressed sufficiently to make an operator doubt that the filter is not integral. An easy solution to this problem is to perform, ahead of time, integrity tests with the filters wetted with water and again with the drug product. A ratio of the two values is calculated and used in subsequent tests at the production level where product wet values are determined and multiplied by the ratio to obtain the water wet value. The resulting value must always be above the minimum manufacturer's specification to be sure of the filter being integral.

3.10. Extractables from Sterilizing Filters

Most sterilizing filters are made of plastic materials and the manufacturers provide data showing the levels of extractables leached out of these filters after soaking them in water. They also provide proof that the extractables at the obtained level are not toxic. Filter users look at such data critically before choosing the filter for a specific filtration application. But the data with a standard solvent such as water is not always sufficient to assure the filter user and the regulatory agencies that the filtration of the actual drug product does not leach out toxic organic compounds into the filtered drug product. The recommendation published in the FDA's Human Drug cGMP notes ***(10)*** adds a new dimension of safety to filter sterilized drug products. Filter users are asked to keep on hand data showing the identity, quantity, and toxicity of filter extractables. Additionally, data should be made available on the extractables leached out from the filters during their normal use in the filtration of products,

especially those containing solvents other than water and the analytical methods used for determining the levels of such extractables.

The regulatory agencies are aware of the extreme difficulty of accurately measuring the low levels of extractables in the presence of the drug product. Individual components of the drug formulation interfere with the detection and quantification of these extractables. To get around this technical impasse, filter manufacturers have come up with different approaches ***(11–13)*** to estimate the extractables leached out by a product from a filter. The approach of Millipore ***(11)***, one of the filter manufacturers consists of the following steps.

1. Components of the drug formulation are first classified into solutes and solvents. Since the solvents are responsible for leaching out extractables, these are selected for the study and the solutes are left out because even small quantities of these solutes interfere with the detection and quantification of the extractables.
2. The filters are soaked in the selected solvents for at least as long as the maximum process time to reach the "worst case" conditions, at the temperature of the process.
3. The filters are removed from the solvent containers and the extractables are measured by several analytical procedures:
    a. Nonvolatile residue. An aliquot of the extract is evaporated to dryness and the residue weighed to ascertain the quantity of total extractables leached out from the filter.
    b. Total oxidizable carbon. The residue is analyzed to obtain the TOC level.
    c. The residue is also analyzed by *analytical HPLC* to look at the profile obtained by the chromatographic separation.
    d. Infrared spectroscopy. Particularly large peaks from the HPLC are collected and are further analyzed by infrared spectroscopy for identification. The spectra are compared to those of the known plastic components of the filter for identification of the extractable component. This procedure leads to unequivocal identification of the components of the extractables residue and confirms their source. As long as the level of these extractables is below the known toxic limit, the extractables report helps the user to assure himself of the suitability of the filter for the specific application.

4. Conclusions

The validation of sterile filtration in the biotechnology industry has helped to bring a very high level of safety to parenteral drugs. The general acceptance by the regulatory authorities in Europe and Japan of the guidelines initially set in place by the FDA, has ensured an almost uniform level of product safety in all these countries. The awareness of product safety and quality among both large and small biopharmaceutical houses has led to the use of state-of-the-art purification technologies including the use of filters and other accessories to remove bacterial contaminants from parenteral drugs. As the industry has perfected the methods of removing bacterial contaminants from process streams,

it faces new challenges of removing viruses and prions from biotechnology products derived from bacterial and cell culture processes as well as those derived from starting materials of animal origin.. The filter manufacturers, always aware of the new challenges facing the biotechnology industry, have come up with new membrane devices to remove these contaminants from the drug products. These removal techniques are an excellent complement to older viral inactivation methods such as heat and solvent/detergent treatments and the drug industry can continue to offer safe products to the consumers. In short, the filter manufacturers and the biotechnology industry are combining their talents and efforts to provide the sick population with novel medications that are both safe and effective.

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