4 Production and Purification of Recombinant Proteins

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INTRODUCTION

The growing therapeutic use of proteins increases the need for practical and economical processing techniques. As a result, biotechnological production methods have advanced significantly over the past three decades. Also, single-use production technology that has the potential to mitigate many of the economical and quality issues arising from manufacturing has evolved rapidly (Hodge 2004; Luitjens et al. 2012).

When producing proteins for therapeutic use, a number of issues must be considered related to the manufacturing, purification, and characterization of the products. Biotechnological products for therapeutic use have to meet strict specifications especially when used via the parenteral route. The regulatory agencies both in Europe (EMA: European Medicines Agency) and in the United States of America (FDA: Food and Drug Administration) play a pivotal role in providing legal requirements and guidelines (www.ICH.org, www.FDA.gov).

In this chapter the focus is on the technical aspects of production (upstream processing) and purification (downstream processing) of recombinant therapeutic proteins. However, a majority of the techniques discussed can also be applied to vaccines and viral vector production. For further details, the reader is referred to the literature mentioned.

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UPSTREAM PROCESSING

Expression Systems

General Considerations

Expression systems for proteins of therapeutic interest include both pro- and eukaryotic cells (bacteria, yeast, fungi, plants, insect cells, mammalian cells) and transgenic animals. The choice of a particular system will be determined to a large extent by the nature and origin of the desired protein, the intended use of the product, the amount needed, and the cost.

In principle, any protein can be produced using genetically engineered organisms, but not every type of protein can be produced by every type of cell. In most cases, the protein is foreign to the host cells that have to produce it, and although the translation of the genetic code can be performed by the cells, the post-translation modifications of the protein might be different compared to the native product.

About 5% of the mammalian proteome is thought to comprise enzymes performing over 200 types of posttranslation modifications of proteins (Walsh 2006). These modifications are species and/or cell-type specific. The metabolic pathways that lead to these modifications are genetically determined by the host cell. Thus, even if the cells can produce the desired post-translation modification, such as glycosylation, still the resulting glycosylation pattern might be different from that of the native protein. Correct N-linked glycosylation of therapeutically relevant proteins is important for full biological activity, immunogenicity, stability, targeting, and pharmacokinetics. Prokaryotic cells, such as bacteria, are sometimes capable of producing N-linked glycoproteins. However, the observed N-linked structures differ from the structures found in eukaryotes (Dell et al. 2011). Yeast cells are able to produce recombinant proteins such as albumin, and has been engineered to produce glycoproteins with human-like glycan structures including terminal sialylation (reviewed by Celik and Calik 2012).

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Still, most products on the market and currently in development use cell types that are, if possible, closely related to the original protein-producing cell type. Therefore, for human-derived proteins, typically mammalian cells are chosen for production as prokaryotic cells are less effective in producing post-translational modifications. Those are often essential when it comes to complex protein structures such as monoclonal antibodies. However, driven by the increasing demand for inexpensive products, especially for costly antibody therapies, two trends opened new opportunities to produce antibody fragments in *E. coli*; (i) generation of improved engineered *E. coli* strains and (ii) new knowledge in using biologically functional antibody fragments. Based on this, two *E. coli* derived antibody fragments (Ranibizumab and Certolizumab pegol) have been approved by the regulatory bodies. It is expected that in the near future more antibody fragments will be launched. Therefore, although still to be further developed, bacteria and yeast may keep on playing a role as future production systems given their ease and low cost of large-scale manufacturing.

Generalized features of proteins expressed in different biological systems are listed in Table 4.1 (see also Walter et al. 1992; Yao et al. 2015). However, it should be kept in mind that there are exceptions to this table for specific product/expression systems.

Transgenic Animals

Foreign genes can be introduced into animals like mice, rabbits, pigs, sheep, goats, and cows through nuclear transfer and cloning techniques. Using milk-specific promoters, the desired protein can be expressed in the milk of the female offspring. During lactation the milk is collected, the milk fats are removed, and the skimmed milk is used as the starting material for the purification of the protein.

The advantage of this technology is the relatively cheap method to produce the desired proteins in vast quantities when using larger animals such as cows. Disadvantages are the long lead time to generate a herd of transgenic animals and concerns about the health of the animal, food safety and ethics (see: report Bundesministerium für Gesundheit, Familie und Jugend, Sektion IV http://www.science-art.at/uploads/media/report_transgenic_animals_02.pdf). Some proteins expressed in the mammary gland leak back into the circulation and cause serious negative health effects. An example is the expression of erythropoietin in cows. Although the protein was well expressed in the milk, it caused severe health effects and these experiments were stopped.

The purification strategies and purity requirements for proteins from milk can be different from those derived from bacterial or mammalian cell systems. Often the transgenic milk containing the recombinant protein also contains significant amounts of the nonrecombinant counterpart. To separate these closely related proteins poses a purification challenge. The "contaminants" in proteins for oral use expressed in milk that is otherwise consumed by humans are known to be safe for consumption.

The transgenic animal technology for the production of pharmaceutical proteins has progressed within the last few years. The FDA and EMA approved recombinant antithrombin III (ATryn®, GTC Biotherapeutics) produced in the milk of transgenic goats, as well as recombinant human C1 esterase inhibitor (Ruconest®,

Protein feature	Prokaryotic bacteria	Eukaryotic yeast	Eukaryotic mammalian cells	Eukaryotic plant cells	Transgenic animals
Concentration	High	High	High	Low	Medium-High
Molecular weight	Low	High	High	High	High
S-S bridges	Limitation	No limitation	No limitation	No limitation	No limitation
Secretion	No	Yes/no	Yes	Yes/no	Yes
Aggregation state	Inclusion body	Singular, native	Singular, native	Singular, native	Singular, native
Folding	Risk of misfolding	Correct folding	Correct folding	Correct folding	Correct folding
Glycosylation (human-like)	Limited	Limited	Possible	Limited	Possible
Contamination risk	Possible (endotoxin)	Low	Possible (virus, prion, oncogenic DNA)	Low	Very possible (virus, prion, endotoxin)
Cost to manufacture	Low	Low	High	High[a]	Medium-high

[a]Due to current limited scalability (Shukla et al. 2017)

Table 4.1 ■ Generalized features of proteins of different biological origin

Pharming Group N.V.) produced in the milk of transgenic rabbits. More details about this technology are presented in Chap. 9.

Plants

Therapeutic proteins can also be expressed in plants and plant cell cultures (see also Chap. 1). For instance, human albumin has been expressed in potatoes and tobacco. Whether these production vehicles are economically feasible has yet to be established. The lack of genetic stability of plants was sometimes a drawback. Stable expression of proteins in edible seeds has been obtained. For instance, rice and barley can be harvested and easily kept for a prolonged period of time as raw material sources. Especially for oral therapeutics or vaccines, this might be the ideal solution to produce large amounts of cheap therapeutics, because the "contaminants" are known to be safe for consumption. However, challenges are the presence of high endotoxin levels, a relatively low expression level of the product, and secretion of proteases limiting the shelf life of plant extracts (Shukla et al. 2017). A better understanding of the plant molecular biology together with more sophisticated genetic engineering techniques and strategies to increase yields and optimized glycan structures resulted in an increase in the number of products in development including late-stage clinical trials (reviewed by Orzaez et al. 2009, and Peters and Stoger 2011). Biosafety concerns (such as pollen contamination and immunogenicity of plant-specific glycans) and costly downstream extraction and purification requirements, however, have hampered moving therapeutic protein production in plants from the laboratory to industrial size application (Yao et al. 2015).

More details about the use of plant systems for the production of pharmaceutical proteins are presented in Chap. 9.

Cultivation Systems

The remainder of this chapter will focus on mammalian cell-based expression systems. Non-mammalian expression systems will only briefly be discussed.

General

Cells can be cultivated in vessels containing an appropriate liquid growth medium in which the cells are either immobilized and grow as a monolayer, attached to microcarriers, in suspension, or entrapped in matrices. The culture method will determine the scale of the separation and purification methods. Production-scale cultivation is commonly performed in fermentors, used for bacterial and fungal cells, or bioreactors, used for mammalian and insect cells. Bioreactor systems can be classified into four different types:

- Stirred tank (Fig. 4.1a)
- Airlift (Fig. 4.1b)
- Fixed bed (Fig. 4.1c)
- Membrane bioreactors (Fig. 4.1d)

Because of its reliability and experience with the design and scaling up potential, the stirred tank is still the most commonly used bioreactor. This type of bioreactor is not only used for suspension cells like CHO, HEK293, and PER.C6® cells, it is also used for production of adherent cells like Vero and MDCK cells. In the latter case the production is performed on microcarriers (Van Wezel et al. 1985).

Bioreactor Processes

The kinetics of cell growth and product formation will not only dictate the type of bioreactor used but also how the growth process is performed. Three types of bioreactor processes are commonly employed and discussed below:

- Batch
  In a batch process, the bioreactor is filled with the entire volume of medium needed during the cell growth and/or production phase. No additional supplements are added to increase the cell growth or production during the process. Waste products, such as lactate and ammonium, and the product itself accumulate in the bioreactor. The product is harvested at the end of the process. Maximum cell density and product yields will be lower compared to a fed-batch process.
- Fed-batch
  In a fed-batch process, a substrate is supplemented to the bioreactor. The substrate consists of the growth-limiting nutrients that are needed during the cell growth phase and/or during the production phase of the process. Like the batch process, waste products accumulate in the bioreactor. The product is harvested at the end of the process. With the fed-batch process, higher cell densities and product yields can be reached compared to the batch process due to the extension of production time that can be achieved compared to a batch process. The substrate used is highly concentrated and can be added to the bioreactor at certain points in time or as a continuous feed. The fed-batch mode is currently widely used for the production of proteins. The process is well understood and characterized.
- Perfusion
  In a perfusion process, the media and waste products are continuously exchanged and the product is harvested throughout the culture period. A membrane device is used to retain the cells in the bioreactor, and waste medium is removed from the bioreactor by this device (Fig. 4.2). To keep the medium level constant in the bioreactor, fresh

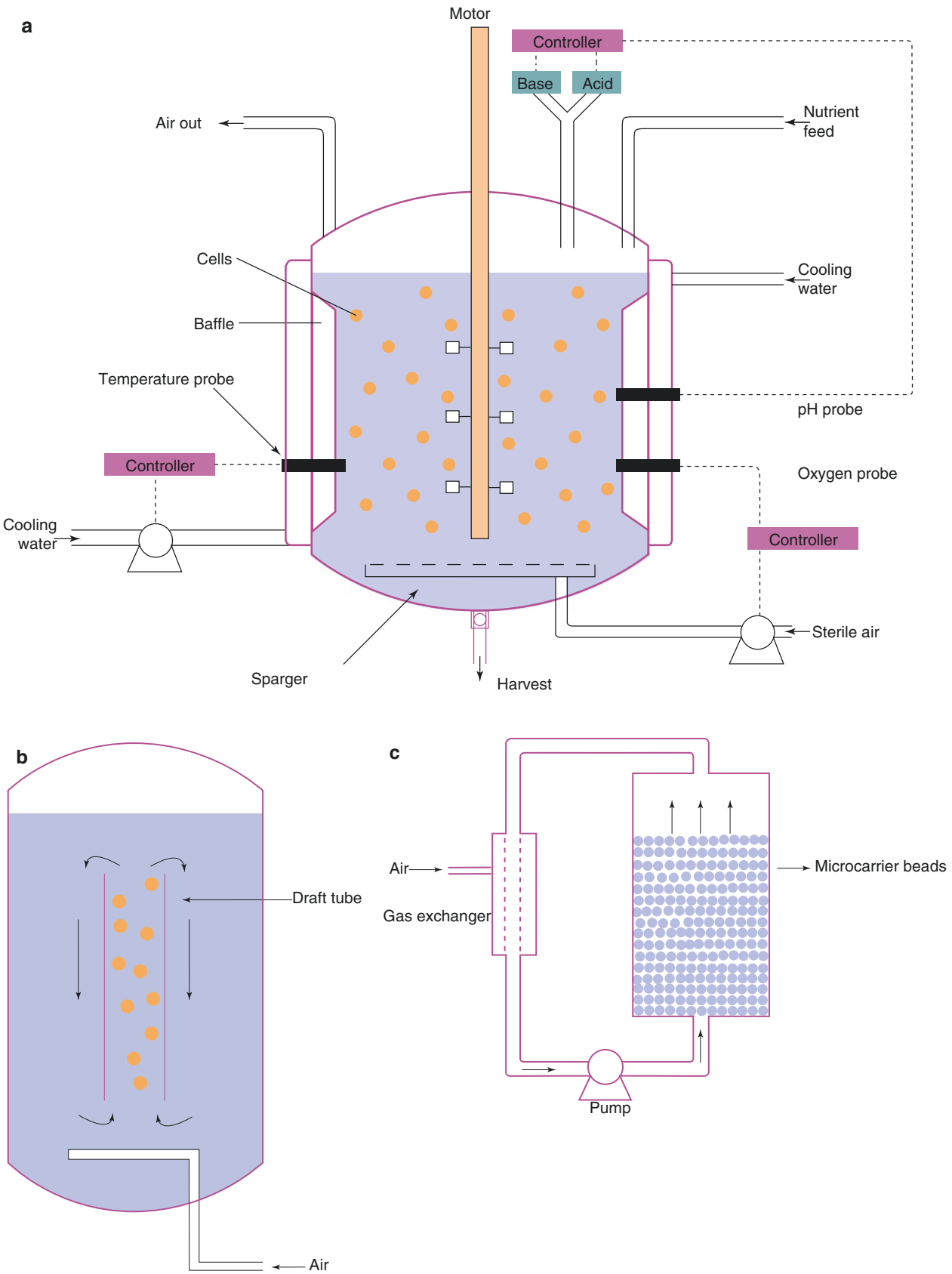

Figure 4.1 ■ (**a**) Schematic representation of stirred-tank bioreactor. (**b**) Schematic representation of airlift bioreactor. (**c**) Schematic representation of fixed-bed stirred-tank bioreactor. (**d**) Schematic representation of hollow fiber perfusion bioreactor. All schematics are adapted from Klegerman and Groves (1992)

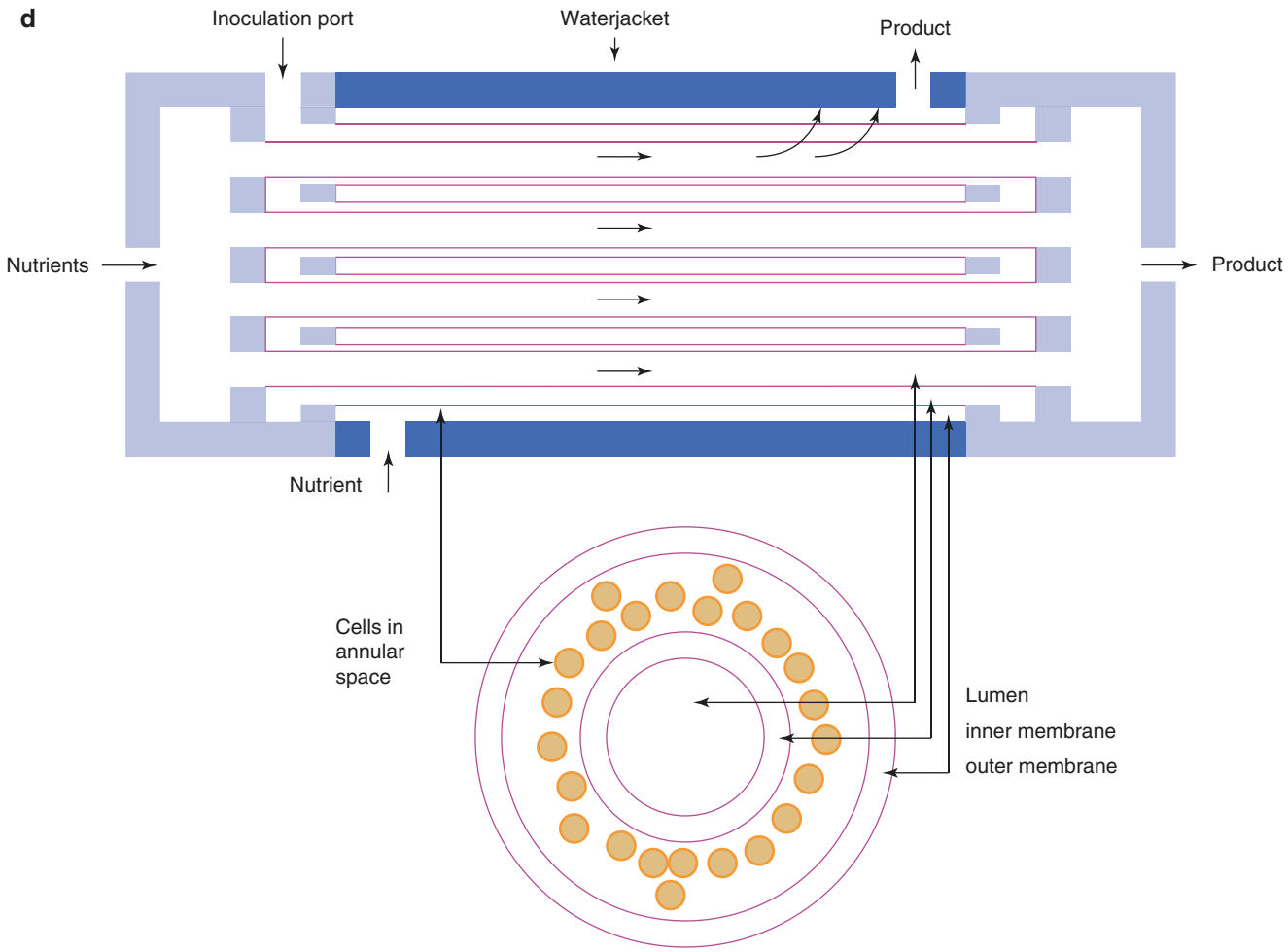

Figure 4.1 ■ (continued)

medium is supplemented to the bioreactor. By operating in perfusion mode, the level of waste products will be kept constant and one generates a stable environment for the cells to grow or to produce (see below). With the perfusion process, much higher cell densities can be reached and therefore higher productivity (Compton and Jensen 2007).

In all these three protocols, the cells go through four distinctive phases:

1. Lag phase
   In this phase the cells are adapting to the conditions in the bioreactor and do not yet grow.
2. Exponential growth phase
   During this phase, cells grow in a more or less constant doubling time for a fixed period. However under the right process conditions mammalian cell doubling time is dependent on the cell type used, and will usually vary between 20 and 40 h. Plotting the natural logarithm of cell concentration against time produces a straight line. Therefore, the exponential growth phase is also called the log phase. The growth phase will be affected by growth conditions such as temperature, pH, oxygen pressure, and external forces like stirring and baffles that are inserted into the bioreactor. Furthermore, the growth rate is affected by the supply of sufficient nutrients, buildup of waste products, etc.
3. Stationary phase
   In the stationary phase, the growth rate of the cells slows down since nutrients are depleted and/or build up of toxic waste products like lactate and ammonium. In this phase, constant cell concentrations are found because a balance between cell growth and cell death has been reached.

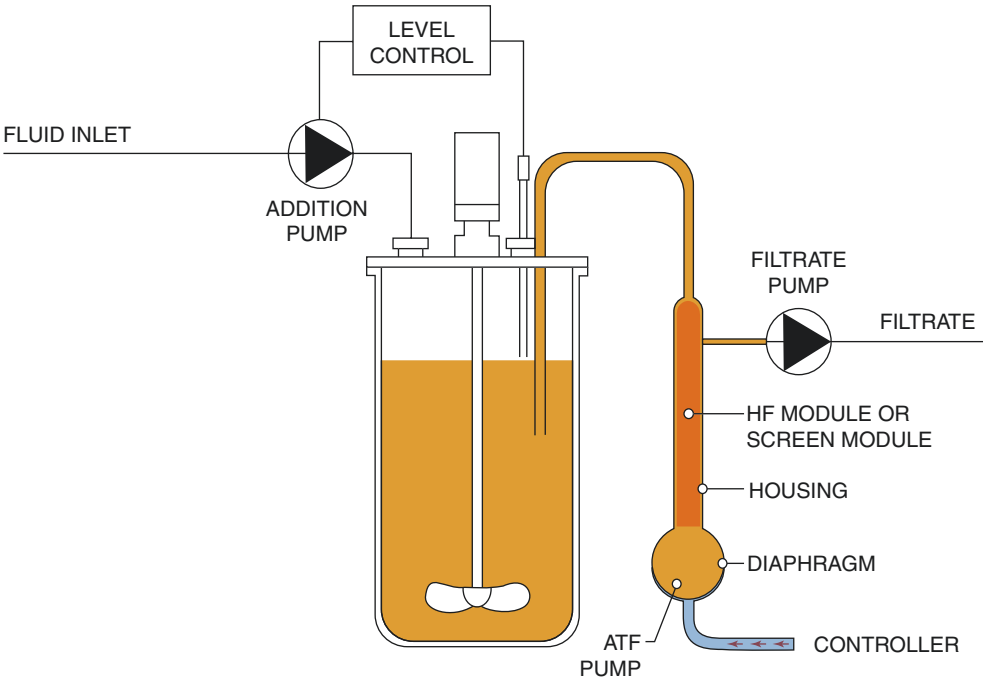

Figure 4.2 ■ Schematic representation of perfusion device coupled to a stirred-tank bioreactor. *ATF* alternating tangential flow

4. Death phase
   Cells die due to depletion of nutrients and/or presence of high concentrations of toxic products such as lactate and ammonium.

Examples of animal cells that are commonly used to produce recombinant proteins of clinical interest are Chinese Hamster Ovary cells (CHO), immortalized human embryonic retinal cells (PER.C6® cells), baby hamster kidney cells (BHK), lymphoblastoid tumor cells (interferon production), melanoma cells (plasminogen activator), and hybridized tumor cells (monoclonal antibodies).

The cell culture has to be free from undesired microorganisms that may destroy the cell culture or present hazards to the patient by producing endotoxins. Therefore, strict measures are required for both the production procedures and materials used (WHO 2010; Berthold and Walter 1994) to prevent a possible contamination with extraneous agents such as viruses, bacteria, and mycoplasma. Furthermore, strict measures are needed, especially with regard to the raw materials used, to prevent contaminations with transmissible spongiform encephalopathies (TSEs).

■ Cultivation Medium

In order to achieve optimal growth of cells and optimal production of recombinant proteins, it is of great importance not only that conditions such as stirring, pH, oxygen pressure, and temperature are chosen and controlled appropriately but also that a cell growth and protein production medium with the proper nutrients are provided for each stage of the production process.

The media used for mammalian cell culture are complex and consist of a mixture of diverse components, such as sugars, amino acids, electrolytes, vitamins, fetal calf serum (caveat, see below), and/or a mixture of peptones, growth factors, hormones, and other proteins (see Table 4.2). Many of these ingredients are pre-blended either as concentrate or as homogeneous mixtures of powders. To prepare the final medium, components are dissolved in purified water before sterilization. The preferred method for sterilization is heat (≥15 min at 121 °C). However most components used in cell culture medium can not be sterilized by heat, therefore filtration is used. Then, the medium is filtrated through 0.1 µm (to prevent mycoplasma and bacterial contamination) or 0.2 µm filters (to prevent bacterial contamination). Some supplements, especially fetal bovine serum, contribute considerably to the presence of contaminating proteins and may seriously complicate purification procedures. Moreover, the composition of serum is variable. It depends on the individual animal, season of the year, processing differences between suppliers, etc. The use of serum may introduce adventitious material such as viruses, mycoplasma, bacteria, and fungi into the culture system (Berthold and Walter 1994). Furthermore, the possible presence of prions that can cause transmissible spongiform encephalitis almost precludes the use of materials from animal origin. However, if use of this material is inevitable, one must follow the

Type of nutrient	Example(s)
Sugars	Glucose, lactose, sucrose, maltose, dextrins
Fat	Fatty acids, triglycerides
Water (high quality, sterilized)	Water for injection
Amino acids	Glutamine
Electrolytes	Calcium, sodium, potassium, phosphate
Vitamins	Ascorbic acid, -tocopherol, thiamine, riboflavine, folic acid, pyridoxin
Serum (fetal calf serum, 'synthetic' serum)	Albumin, transferrin
Trace minerals	Iron, manganese, copper, cobalt, zinc
Hormones	Growth factors

Table 4.2 ■ Major components of growth media for mammalian cell structures

relevant guidelines in which selective sourcing of the material is the key measure to safety (EMA 2011). A measure to prevent the contaminations mentioned above is gamma irradiation of the fetal bovine serum at 25 kGy and use sourcing from countries that have a TSE/BSE free status (Australia, New Zealand, Tasmania, USA, etc.). Many of these potential problems when using serum in cell culture media led to the development of chemically defined medium, free from animal components and material derived from animal components. These medium formulations were not only developed by the suppliers. There is the trend that the key players in the biotech industry develop their own chemically defined medium for their specific production platforms. The advantage of this is that manufacturers are less dependent on medium suppliers, and have full knowledge on the composition of the medium used for their products. The chemically defined media have been shown to give satisfactory results in large-scale production settings for monoclonal antibody processes. However, hydrolysates from non-animal origin, such as yeast and plant sources, are more and more used for optimal cell growth and product secretion (reviewed by Shukla and Thömmes 2010).

DOWNSTREAM PROCESSING

■ Introduction

Recovering a biological reagent from a cell culture supernatant is one of the critical parts of the manufacturing procedure for biotech products, and purification costs typically outweigh those of the upstream part of the production process. For the production of monoclonal antibodies, protein A resin and virus removal by filtration can account for a significant part, e.g., 40%, of the cost (Gottschalk 2006, Sinclair et al. 2016).

In the 1980s and early 1990s, the protein of interest was produced in bioreactors at low concentrations (e.g., 10–200 mg/L). At the most concentrations up to 500–800 mg/L could be reached (Berthold and Walter 1994). Developments in cell culture technology through application of genetics, proteomics, medium compositions and increased understanding of bioreactor technology resulted in product titers well above 1 g/L. Product titers above 20 g/L are also reported (Monteclaro 2010). These high product titers pose a challenge to the downstream processing unit operations (Shukla and Thömmes 2010).

With the low-yield processes, a concentration step is often required to reduce handling volumes for further purification. Usually, the product subsequently undergoes a series of purification steps (Fig. 4.3). The first step in a purification process is to remove cells and cell debris from the process fluids ('clarification'). This process step is normally performed using centrifugation and/or depth filters. Depth filters are often used in combination with filter aid/diatomaceous earth. Often the clarification step is regarded as a part of the upstream process. Therefore, the first actual step in the purification process is a capture step. Subsequent steps remove the residual bulk contaminants, and a final step removes trace contaminants and sometimes variant forms of the molecule. Alternatively, the reverse strategy, where the main contaminants are captured and the product is purified in subsequent steps, might result in a more economic process, especially if the product is not excreted from the cells. In the case where the product is excreted into the cell culture medium, the product will generally not represent more than 1–5% of total cellular protein, and a specific binding of the cellular proteins in a product-specific capture step will have a high impact on the efficiency of that step. If the bulk of the con-

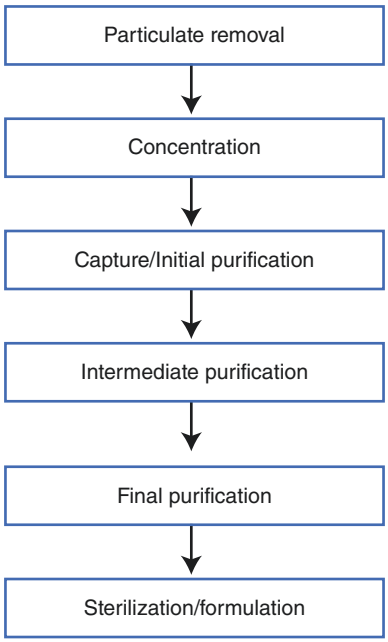

Figure 4.3 ■ Basic operations required for the purification of a biopharmaceutical macromolecule. For monoclonal antibody processes the concentration occurs within the capture step. Final purification is often called "polishing"

taminants can be removed first, the specific capture step will be more efficient and smaller in size and therefore more economic. Subsequent unit operation steps (e.g., chromatography columns) can be used for further purification.

After purification, additional steps are performed to bring the desired product into a formulation buffer in which the product is stabilized and can be stored for the desired time until further process steps are performed. Before storage of the final bulk drug substance, the product will be subjected to a bioburden reduction step. Normally this will be performed by a 0.2 µm filtration step. Formulation aspects will be dealt with in Chap. 5).

When designing an upstream and purification protocol, the possibility for scaling up should be considered carefully. A process that has been designed for small quantities is most often not suitable for large quantities for technical, economic, and safety reasons. Developing a process to purify the desired product is also called the downstream process (DSP). Two stages can be defined: *design* and *scale-up*.

As mentioned above, separating the impurities from the product protein requires a series of purification steps (*process design*), each removing some of the impurities and bringing the product closer to its final specification. In general, the starting feedstock contains cell debris and/or whole-cell particulate material that must be removed. Defining the major contaminants in the starting material is helpful in the downstream process design. This includes detailed information on the source of the material (e.g., bacterial or mammalian cell culture) and major contaminants that are used or produced in the upstream process (e.g., albumin, serum, or product analogs). Moreover, the physico-chemical characteristics of the product versus the known contaminants (stability, isoelectric point, molecular weight, hydrophobicity, density, specific binding properties) largely determine the process design. Processes used for production of therapeutics in humans should be safe, reproducible, robust, and produced at the desired cost of goods. The DSP steps may expose the protein molecules to high physical stress (e.g., high temperatures and extreme pH) which can alter the protein properties possibly leading to loss in efficacy. Any substance that is used by injection must be sterile. Furthermore, the endotoxin concentration must be below a certain level depending on the product. Limits are stated in compound specific compendial monographs (e.g., European Pharmacopoeia: <0.2 endotoxin units per kg body mass for intrathecal application). Aseptic techniques have to be used wherever possible and necessitate procedures throughout with clean air and microbial control of all materials and equipment used. During validation of the purification process, one must also demonstrate that potential viral contaminants are inactivated and removed (Walter et al. 1992). The purification matrices should be at least sanitizable or, if possible, steam-sterilizable. For depyrogenation, the purification material must withstand either extended dry heat at ≥180 °C or treatment with 1–2 M sodium hydroxide. If any material in contact with the product inadvertently releases compounds, these leachables must be analyzed and their removal by subsequent purification steps must be demonstrated during process validation, or it must be demonstrated that the leachables are below a toxic level. The increased use of plastic film-based single-use production technology (e.g. sterile single-use bioreactor bags, bags to store liquids and filter housings) has made these aspects more significant in the last decade. Suppliers have reacted by providing a significant body of information regarding leachables and biocompatibility for typical solutions used during processing. The problem of leachables is especially hampering the use of affinity chromatography (see below) in the production of pharmaceuticals for human use, in which the removal of any leached ligands well below a toxic level has to be demonstrated. Because leached affinity ligands will bind to the product, the removal might be cumbersome.

Scale-up is the term used to describe a number of processes employed in converting a laboratory procedure into an economical, industrial process. During the scale-up phase, the process moves from the

laboratory to the pilot plant and finally to the production plant. The objective of scale-up is to reproducibly produce a product of high quality at a competitive price. Since the costs of downstream processing can be as high as 50–80% of the total cost of the final drug product, practical and economical ways of purifying the product should be used. Superior protein purification methods hold the key to a strong market position (Wheelwright 1993).

Basic operations required for a downstream purification process used for macromolecules from biological sources are shown in Fig. 4.3.

As mentioned before, the design of downstream processing is highly product dependent. Each product requires a specific multistage purification procedure. The basic scheme as represented in Fig. 4.3 becomes complex. Two typical examples of a process flow for downstream processing are shown in Fig. 4.4. These schemes represent the processing of a monoclonal antibody (about 150 kDa) and another glycosylated protein (recombinant interferon; about 28 kDa) produced in mammalian cells. The aims of the individual unit operations are described in the figure as well.

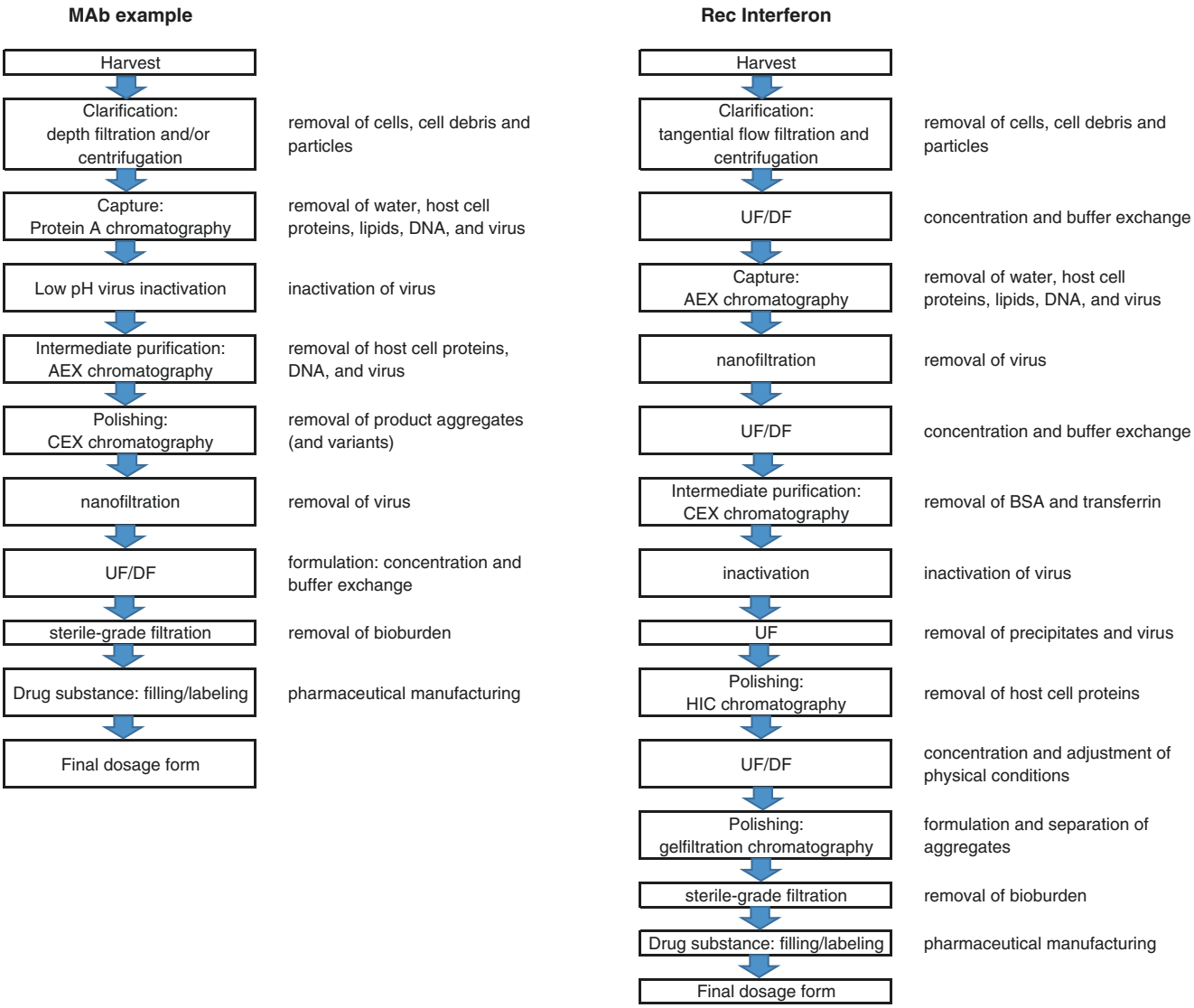

Figure 4.4 ■ Downstream processing of a monoclonal antibody (MAb) and a glycosylated recombinant interferon, describing the purpose of the inclusion of the individual unit operations. (*F* filtration, *TFF* tangential flow filtration, *UF* ultrafiltration, *DF* diafiltration, *A* adsorption; Rec Interferon adapted from Berthold and Walter 1994). For MAbs, the sequence of anion exchange chromatography (AEX), cationic exchange chromatography (CEX), and nanofiltration steps can change. Also, instead of ion exchange ligands, hydrophobic interaction chromatography (HIC) or mixed mode ligands are used (Shukla et al. 2017)

Separation technique	Mode/principle	Separation based on
Filtration	Microfiltration	Size
	Ultrafiltration	Size
	Nanofiltration	Size
	Dialysis	Size
	Charged membranes	Charge
	Depth filtration	Size
Centrifugation	Isopycnic banding	Density
	Non-equilibrium setting	Density
Extraction	Fluid extraction	Solubility
	Liquid/liquid extraction	Partition, change in solubility
Precipitation	Fractional precipitation	Change in solubility
Chromatography	Ion exchange	Charge
	Gel filtration	Size
	Affinity	Specific ligand-substrate interaction
	Hydrophobic interaction	Hydrophobicity
	Adsorption	Covalent/non-covalent binding

Table 4.3 ■ Frequently used separation processes and their physical basis

A number of purification methods are available to separate proteins on the basis of a wide variety of different physico-chemical criteria such as size, charge, hydrophobicity, and solubility (Table 4.3). Detailed information about some separation and purification methods commonly used in purification schemes is provided below.

■ Centrifugation

Recombinant protein products in a cell harvest must be separated from suspended particulate material, including whole cells, lysed cell material, and fragments of broken cells generated when cell breakage has been necessary to release intracellular products. Most downstream processing flow sheets will, therefore, include at least one unit operation for the removal ("clarification") of particulates. Most frequently used methods are centrifugation and filtration techniques. However, the expense and effectiveness of such methods is highly dependent on the physical nature of the particulate material, of the product and the scale of the unit operation. Various clarification technologies are summarized in Turner et al. (2017).

Besides the use of centrifugation for clarification also subcellular particles and organnelles, suspended in a viscous liquid (for example the particles produced when cells are disrupted by mechanical procedures) can be separated by centrifugation. However, subcellular particles and organelles are difficult to separate either by using one fixed centrifugation step (or by filtration), but they can be isolated efficiently by centrifugation at different speeds. For instance, nuclei can be obtained by centrifugation at 400 × g for 20 min, while plasma membrane vesicles are pelleted at higher centrifugation rates and longer centrifugation times (fractional centrifugation). In many cases, however, total biomass can easily be separated from the medium and classified by a simple centrifugation step (e.g., a continuous disc-stack centrifuge). Buoyant density centrifugation can be useful for separation of particles as well. This technique uses a viscous fluid with a continuous gradient of density in a centrifuge tube. Particles and molecules of various densities within the density range in the tube will cease to move when the isopycnic region has been reached. Both techniques of continuous (fluid densities within a range) and discontinuous (blocks of fluid with different density) density gradient centrifugation are used in buoyant density centrifugation on a laboratory scale. However, for application on an industrial scale, continuous centrifuges (e.g. tubular bowl centrifuges) are only used for discontinuous buoyant density centrifugation of protein products. This type of industrial centrifuge is mainly applied to recover precipitated proteins or contaminants.

■ Filtration

Filtration is often applied at various stages during downstream processing. The most successful set ups being normal flow depth filtration, membrane filtration

and tangential flow filtration (TFF, also referred to as "cross flow"). Separation is achieved based on particle size differences. Below the main types of filtration are described.

Depth Filtration

Depth filters are often applied in the clarification of cell harvest to remove cells and cell debris. Depth filters consist of a complex porous matrix of materials, often including charged components and filter aids such as diatomaceous earth, enabling cellular debris and other contaminants to be retained at both the surface and internal layers of the depth filter (Turner et al. 2017). Issues at large manufacturing scale are usually the large membrane area needed to prevent clogging/fouling, and the large hold up volumes. For large harvest volumes depth filters are also used in combination with centrifugation.

Membrane Filtration

Depth filters retain contaminants within the filter structure, while membrane filters have defined pore size ranges (e.g., in the micrometer or nanometer range) that trap supra-pore size particles on the membrane surface while allowing passage of smaller particles. The main membrane filters are either used in a dead-end mode in which the retained particles collect on the surface of the filter media as a stable filter cake that grows in thickness and increases flow resistance, or in a tangential flow mode in which the high shear across the membrane surface limits fouling, gel layer formation and concentration polarization. Important applications of membrane filters within pharmaceutical processes are described below.

Tangential Flow Filtration

Tangential flow filtration (TFF) is often used for the concentration and buffer exchange of purified product and used sometimes within clarification processes. Depending upon the molecules/particles to be separated or concentrated, ultrafilter or micro membranes are used. Mixtures of molecules of highly different molecular dimensions are separated by passage of a dispersion under pressure across a membrane with a defined pore size. In general, ultrafiltration achieves little purification of protein product from other molecules with a comparable size, because of the relatively large pore size distribution of the membranes. As mentioned, this technique is widely used to concentrate macromolecules, and also to change the aqueous phase (e.g. re buffer components) in which the particles are dispersed or in which molecules are dissolved (diafiltration) to one required for the subsequent purification steps.

Sterilizing-Grade Filtration

Bioburden reduction filters are an essential part of most pharmaceutical processes. These dead-end filters consist of a membrane with an average pore size of 0.1 or 0.2 μm and a narrow size distribution). They are very effective in the removal of (possible) bioburden, and as such used at various steps in the purification process, e.g. at hold steps, and at the final steps to produce drug substance or drug product.

Virus Filtration

As mentioned later in this chapter, removal of potential contaminating viruses is essential in a pharmaceutical process. Nanofiltration is an elegant and effective technique and the validation aspects of this technology are well described (PDA technical report 41 2005). Filtration through 15 nm pore membranes can remove even the smallest non-enveloped viruses such as bovine parvovirus (Maerz et al. 1996). Nanofilters are a major contributor to the costs of the downstream process.

Charged Membranes

A type of membrane that is increasingly used within the biopharmaceutical industry is the charged membrane (Zhou and Tressel 2006; Etzel and Arunkumar 2017). As for ion exchange chromatography (see below), negatively (sulphonic, S) or positively (quaternary ammonium, Q) charged ligands are attached to the multilayer membranes, enabling the removal of residual impurities such as host cell DNA, viruses or host cell proteins from the recombinant protein product. In contrast to ion exchange chromatography, the open structure of the charged membranes enables relatively high diffusion rates of product/contaminants, thus a fast process step. A downside is the lower capacity. Charged membranes are often used in a flow-through mode in e.g. monoclonal antibody production processes, as such replacing the Q-based chromatography columns.

■ Extraction

Extraction, including liquid-liquid extraction, is a technique often used in the chemical industry, but rarely used for biopharmaceuticals. Liquid-liquid extraction basically separates molecules on solute affinity due to differences in the molecule's physical-chemical properties in a mixture of two immiscible phases (reviewed by Dos Santos et al. 2018). Traditionally the phases consist of an aqueous and an organic phase. Upon phase separation, the target molecules are extracted to one of the two phases allowing its concentration and sometimes purification. Due to the possible impact of organic solvents on the structure and biological activity of biopharmaceuticals as well as the environmental impact, this traditional extraction method is rarely used.

To overcome the main concerns, aqueous two phase systems are developed. The compounds enabling separation of biopharmaceuticals encompass polymers, salts, surfactants, amino acids and ionic liquids. Compared to chromatography the operational costs of the two phase systems are relatively low, scale up is straightforward and the technique can be easily integrated in the early steps of a downstream process. However, two phase systems are rarely applied in biopharmaceutical processes due to in general relatively low recovery values and limited purification abilities (reviewed by Dos Santos et al. 2018). A better understanding of the partitioning processes may reduce these limitations in the future.

■ Precipitation

The solubility of a particular protein depends on the physicochemical environment, for example, pH, ionic species, and ionic strength of the solution (see also Chap. 5). A slow continuous increase of the ionic strength (of a protein mixture) will selectively drive proteins out of solution. This phenomenon is known as "salting out." A wide variety of agents, with different "salting-out" potencies are available. Chaotropic series with increasing "salting-out" effects of negatively (1) and positively (2) charged molecules are given below:

1. SCN^-, I^-, CLO_4^-, NO_3^-, Br^-, Cl^-, CH_3COO^-, PO_4^{3-}, SO_4^{2-}
2. Ba^{2+}, Ca^{2+}, Mg^{2+}, Li^+, Cs^+, Na^+, K^+, Rb^+, NH_4^+

Ammonium sulfate is highly soluble in cold aqueous solutions and is frequently used in "salting-out" purification.

Another method to precipitate proteins is to use water-miscible organic solvents (change in the dielectric constant). Examples of precipitating agents are polyethylene glycol and trichloroacetic acid. Under certain conditions, chitosan and nonionic polyoxyethylene detergents also induce precipitation (Cartwright 1987; Homma et al. 1993; Terstappen et al. 1993). Cationic detergents have been used to selectively precipitate DNA.

Precipitation is a scalable, simple, and relatively economical procedure for the recovery of a product from a dilute feedstock. It has been used for the isolation of proteins from culture supernatants. Unfortunately, with most bulk precipitation methods, the gain in purity is generally limited and product recovery can be low. Moreover, extraneous components are introduced which must be eliminated later. Finally, large quantities of precipitates may be difficult to handle. Despite these limitations, recovery by precipitation has been used with considerable success for some products.

■ Chromatography

In preparative chromatography systems, molecular species are primarily separated based on differences in distribution between two phases: one is the stationary phase (mostly a solid phase) and the other moves. This mobile phase may be liquid or gaseous (see also Chap. 3). Nowadays, almost all stationary phases (fine particles providing a large surface area) are packed into a column. The mobile phase is passed through by pumps. Downstream protein purification protocols usually have at least two to three chromatography steps. Chromatographic methods used in purification procedures of biotech products are listed in Table 4.3 and are briefly discussed in the following sections.

Chromatographic Stationary Phases

Chromatographic procedures often represent the rate-limiting step in the overall downstream processing. An important primary factor governing the rate of operation is the mass transport into the pores of conventional packing materials. Adsorbents employed include inorganic materials such as silica gels, glass beads, hydroxyapatite, various metal oxides (alumina), and organic polymers (cross-linked dextrans, cellulose, agarose). Separation occurs by differential interaction of sample components with the chromatographic medium. Ionic groups such as amines and carboxylic acids, dipolar groups such as carbonyl functional groups, and hydrogen bond-donating and bond-accepting groups control the interaction of the sample components with the stationary phase, and these functional groups slow down the elution rate if interaction occurs.

Chromatographic stationary phases for use on a large scale are evolving over time. An approach to the problems associated with mass transport in conventional systems is to use chromatographic particles that contain some large "through pores" in addition to conventional pores (see Fig. 4.5). These flow-through or "perfusion chromatography" media enable faster convective mass transport into particles and allow operation at much higher speeds without loss in resolution or binding capacity (Afeyan et al. 1989; Fulton 1994).

The ideal stationary phase for protein separation should possess a number of characteristics, among which are high mechanical strength, high porosity, no nonspecific interaction between protein and the support phase, high capacity and mass transfer rate, biocompatibility, and high stability of the matrix in a variety of solvents. The latter is especially true for columns used for the production of pharmaceuticals that need to be cleaned, depyrogenized, disinfected, and sterilized at regular intervals.

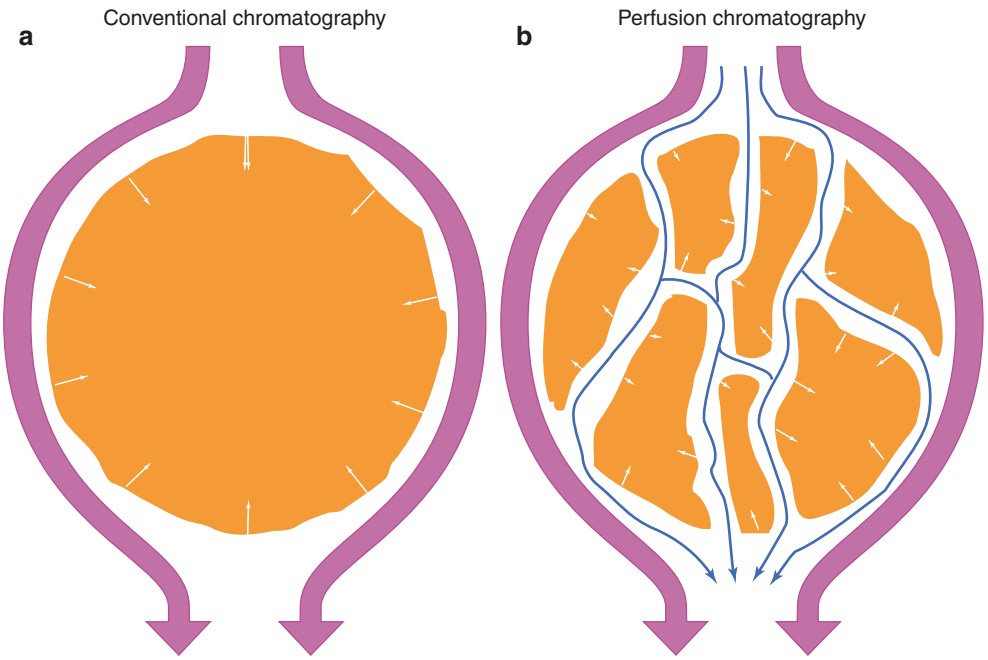

Figure 4.5 ■ The structure of conventional chromatographic particles (**a**) and the perfusion of flow through chromatographic particles (**b**) (adapted from Fulton 1994)

In production environments, chromatography columns which operate at relatively low back pressure are often used. These can be made of stainless steel. But the low back pressure allows the introduction of disposable (plastic) columns in a GMP manufacturing environment. Unlike conventional stainless steel, plastic columns are less sensitive to e.g. salt corrosion. A disadvantage can be leaching of plastic components into the product stream. Disposable plastic columns permit the efficient separation of proteins in a single batch, making this an attractive unit operation in a manufacturing process. A new development is the use of chromatography equipment with fully disposable flow paths that resists almost all chemicals used in protein purification including disinfection and sterilization media.

Adsorption Chromatography

In adsorption chromatography (also called "normal phase" chromatography), the stationary phase is more polar than the mobile phase. The protein of interest selectively binds to a static matrix under one condition and is released under a different condition. Adsorption chromatography methods enable high ratios of product load to stationary phase volume. Therefore, this principle is economically scalable.

Ion-Exchange Chromatography

Ion-exchange chromatography can be a powerful step early in a purification scheme. It can be easily scaled up. Ion-exchange chromatography can be used in a negative mode, i.e., the product flows through the column under conditions that favor the adsorption of contaminants to the matrix, while the protein of interest does not bind (Tennikova and Svec 1993). The type of the column needed is determined by the properties of the proteins to be purified (e.g., isoelectric point and charge density). Anion exchangers bind negatively charged molecules and cation exchangers bind positively charged molecules. In salt-gradient ion-exchange chromatography, the salt concentration in the perfusing elution buffer is increased continuously or in steps. The stronger the binding of an individual protein to the ion exchanger, the later it will appear in the elution buffer. Likewise, in pH-gradient chromatography, the pH is changed continuously or in steps. Here, the protein binds at one pH and is released at a different pH. As a result of the heterogeneity in glycosylation (e.g., a varying number of sialic acid moieties), glycosylated proteins may elute over a relatively broad pH range (up to 2 pH units).

In order to simplify purification, a specific amino acid tail can be added to the protein at the gene level to create a "purification handle". For example, a short tail consisting of arginine residues allows a protein to bind to a cation exchanger under conditions where almost no other cell proteins bind. However, this technique is useful for laboratory-scale isolation of the product and generally not at production scale due to regulatory problems related to the removal of the arginine or other specific tags from the protein.

(Immuno)Affinity Chromatography

Affinity Chromatography

Affinity chromatography is based on highly specific interactions between an immobilized ligand and the protein of interest. Affinity chromatography is a very powerful method for the purification of proteins. Under physiological conditions, the protein binds to the ligand. Extensive washing of this matrix will remove contaminants, and the purified protein can be recovered by the addition of ligands competing for the stationary phase binding sites or by changes in physical conditions (such as low or high pH of the eluent) that greatly reduce the affinity. Examples of affinity chromatography include the purification of glycoproteins, that bind to immobilized lectins, and the purification of serine proteases with lysine binding sites, that bind to immobilized lysine. In these cases, a soluble ligand (sugar or lysine, respectively) can be used to elute the required product under relatively mild conditions. Another example is the use of the affinity of protein A and protein G for antibodies. Protein A and protein G have a high affinity for the Fc portions of many immunoglobulins from various animals. Protein A and G matrices can be commercially obtained with a high degree of purity. Protein A resins are often used in the capture of biotherapeutic monoclonal antibodies at large scale, and these resins are also one of the most expensive parts of the production process. In the last decade the amino acid composition has been modified to generate Protein A ligands that are more resistant to hydroxide, allowing better cleaning of the resin. Also, the coupling chemistry has been improved to allow re-use of the resin for over a hundred cycles, and by that reducing the cost of goods.

For the purification of, e.g., hormones or growth factors, the receptors or short peptide sequence that mimic the binding site of the receptor molecule can be used as affinity ligands. Some proteins show highly selective affinity for certain dyes commercially available as immobilized ligands on purification matrices. When considering the selection of these ligands for pharmaceutical production, one must realize that some of these dyes are carcinogenic and that a fraction may leach out during the process.

An interesting approach to optimize purification is the use of a gene that codes not only for the desired protein but also for an additional sequence that facilitates recovery by affinity chromatography. At a later stage the additional sequence is removed by a specific cleavage reaction. As mentioned before, this is a complex process that needs additional purification steps.

In general, the use of affinity chromatography in the production process for therapeutics may lead to complications during validation of the removal of free ligands or protein extensions. Consequently, except for monoclonal antibodies where affinity chromatography is part of the purification platform at large scale, this technology is rarely used in the industry.

Immunoaffinity Chromatography

The specific binding of antibodies to their epitopes is used in immunoaffinity chromatography (reviewed by Abi-Ghanem and Berghman 2012). This technique can be applied for purification of either the antigen or the antibody. The antibody can be covalently coupled to the stationary phase and act as the "receptor" for the antigen to be purified. Alternatively, the antigen, or parts thereof, can be attached to the stationary phase for the purification of the antibody. Advantages of immunoaffinity chromatography are its high specificity and the combination of concentration and purification in one step.

A disadvantage associated with immunoaffinity methods is the sometimes very strong antibody-antigen binding. This requires harsh conditions during elution of the ligand. Under such conditions, sensitive ligands could be harmed (e.g., by denaturation of the protein to be purified). This can be alleviated by the selection of antibodies and environmental conditions with high specificity and sufficient affinity to induce an antibody-ligand interaction, while the antigen can be released under mild conditions. Another concern is disruption of the covalent bond linking the "receptor" to the matrix. This would result in elution of the entire complex. Therefore, in practice, a further purification step after affinity chromatography as well as an appropriate detection assay (e.g., Enzyme-Linked Immuno Sorbent Assay, ELISA) is almost always necessary. On the other hand, improved coupling chemistry that is less susceptible to hydrolysis has been developed to prevent leaching.

Scale-up of immunoaffinity chromatography is often hampered by the relatively large quantity of the specific "receptor" (either the antigen or the antibody) that is required and the lack of commercially available, ready-to-use matrices. The use of immunoaffinity in pharmaceutical processes will have major regulatory consequences since the immunoaffinity ligand used will be considered by the regulatory bodies as a "second product", thus will be subjected to the nearly the same regulatory scrutiny as the drug substance. Moreover, immunoaffinity ligands can have a significant effect on the final costs of goods.

Examples of proteins of potential therapeutic value that have been purified using immunoaffinity chromatography are interferons, urokinase, epoetin, interleukin-2, human factor VIII and X, and recombinant tissue plasminogen activator.

Hydrophobic Interaction Chromatography

Under physiological conditions, most hydrophobic amino acid residues are located inside the protein core, and only a small fraction of hydrophobic amino acids is exposed on the "surface" of a protein. Their exposure is suppressed because of the presence of hydrophilic amino acids that attract large clusters of water molecules and form a "shield." High salt concentrations reduce the hydration of a protein, and the surface-exposed hydrophobic amino acid residues become more accessible. Hydrophobic interaction chromatography (HIC) is based on non-covalent and non-electrostatic interactions between proteins and the stationary phase. HIC is a mild technique, usually yielding high recoveries of proteins that are not damaged, are folded correctly, and are separated from contaminants that are structurally related. HIC is ideally placed in the purification scheme after ion-exchange chromatography, where the protein usually is released in high ionic strength elution media (reviewed by Chen et al. 2015).

Gel-Permeation Chromatography

Gel-permeation or size-exclusion chromatography, also known as gel filtration, separates molecules according to their shape and size (see Fig. 4.6). Inert gels with narrow pore-size distributions in the size range of proteins are available. These gels are packed into a column. The protein mixture is loaded on top of the column and the proteins diffuse into the gel. The smaller the protein, the more volume it will have available in which to disperse. Molecules that are larger than the largest pores are not able to penetrate the gel beads and will therefore stay in the void volume of the column. When a continuous flow of buffer passes through the column, the larger proteins will elute first and the smallest molecules last. Gel-permeation chromatography is a good alternative to membrane diafiltration for buffer exchange at almost any purification stage, and it is often used in laboratory design. At production scale, the use of this technique is usually limited, because it is a slow process and only relatively small sample volumes can be loaded on a large column (up to one-third of the column volume in the case of "buffer exchange"). It is therefore best avoided or used late in the purification process when the protein is available in a highly concentrated form. Gel filtration is sometimes used as the final step in the purification to bring proteins in the appropriate buffer used in the final formulation. In this application, its use has little if no effect on the product purity characteristics.

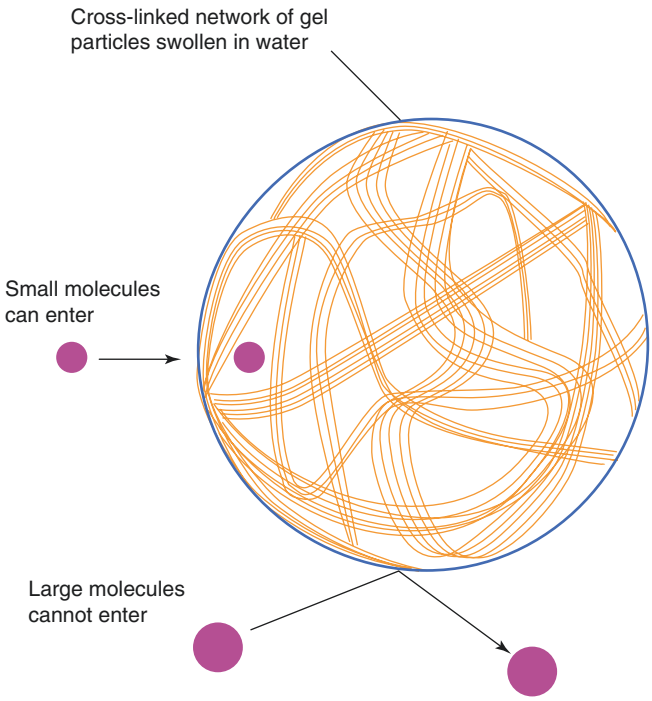

Figure 4.6 ■ Schematic representation of gel filtration (Adapted from James 1992)

Expanded Beds

As mentioned before, purification schemes are based on multistep protocols. This not only adds greatly to the overall production costs but also can result in significant loss of product. Therefore, there still is an interest in the development of new methods for simplifying the purification process. Adsorption techniques are popular methods for the recovery of proteins, and the conventional operating format for preparative separations is a packed column (or fixed bed) of adsorbent. Particulate material, however, can be trapped near the bed, which results in an increase in the pressure drop across the bed and eventually in clogging of the column. This can be avoided by the use of pre-column filters (e.g., 0.2 μm pore size) to save the column integrity. Another solution to this problem may be the use of expanded beds (Chase and Draeger 1993; Fulton 1994), also called fluidized beds (see Fig. 4.7). In principle, the use of expanded beds enables clarification, concentration, and purification to be achieved in a single step. The concept is to employ a particulate solid-phase adsorbent in an open bed with upward liquid flow. The hydrodynamic drag around the particles tends to lift them upwards, which is counteracted by gravity because of a density difference between the particles and the liquid phase. The particles remain suspended if particle diameter, particle density, liquid viscosity, and liquid density are properly balanced by choosing the correct flow rate. The expanded bed allows particulates (e.g., cells and cell debris) to pass through, whereas molecules in solution are selectively retained (e.g., by the use of ion-exchange or affinity adsorbents) on the adsorbent particles. Feedstocks can be applied

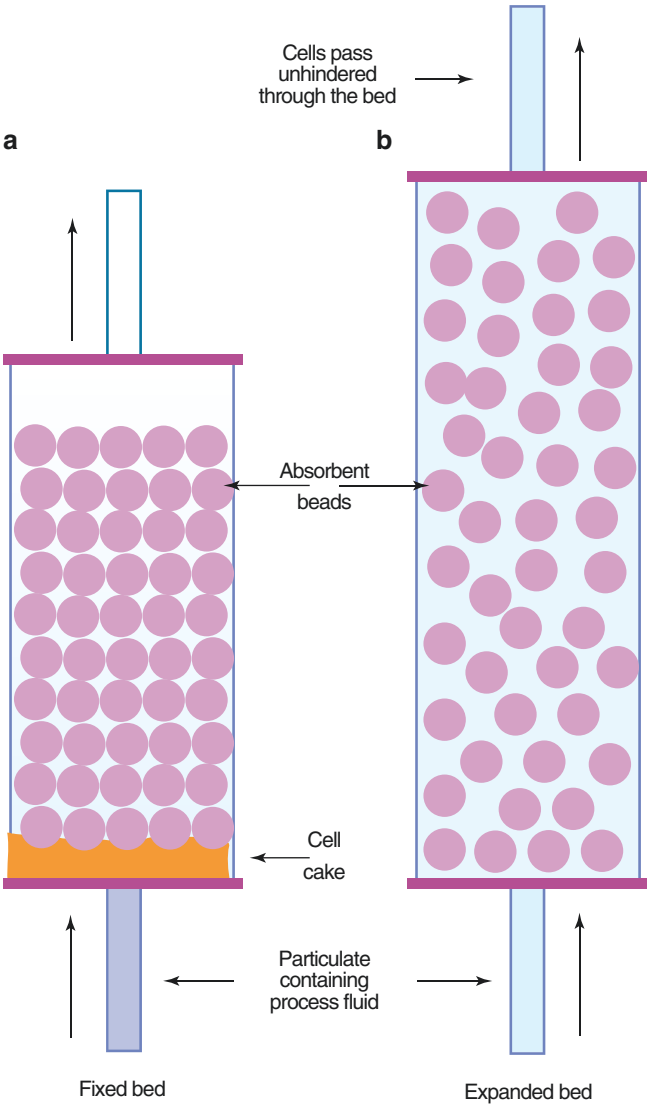

Figure 4.7 ■ Comparison between (**a**) a packed bed and (**b**) an expanded bed (adapted from Chase and Draeger 1993)

to the bed without prior removal of particulate material by centrifugation or filtration, thus reducing process time and costs. Fluidized beds have been used previously for the industrial-scale recovery of antibiotics such as streptomycin and novobiocin (Fulton 1994; Chase 1994). Stable, expanded beds can be obtained using simple equipment adapted from that used for conventional, packed bed adsorption and chromatography processes. Ion-exchange adsorbents are likely to be chosen for such separations.

SINGLE-USE SYSTEMS

In the last decade the development of single-use production systems has been boosting. This is reflected by the growing number of single-use systems available for mammalian cell culture and microbial cultures (see below). Single-Use systems are currently not only developed for culturing, but also for downstream unit operations such as the filtration (depth, membrane) and chromatography steps. It is currently possible to produce proteins with only single-use systems.

Single-use bioreactors for mammalian cell culture and protein production applications are characterized by a low power input, low mixing capabilities, limited oxygen transfer, restrictive exhaust capacity and limited foam management. Therefore, transferring these single-use bioreactors into single-use fermentors that can be used for microbial production is a challenge. The present generation of single-use fermentors is only used in the production of the least challenging five percent of microbial fermentations (Jones 2015).

Single-use bioreactors are used for the manufacturing of products in development and on the market. Shire (Dublin, Ireland) was the first company that used single-use bioreactors up to 2000 L for the manufacturing of one of its products. The advantages of the single-use technology are:

- Cost-effective manufacturing technology
  By introducing single-use systems, the design is such that all items not directly related to the process can be removed from the culture system, such as clean-in-place (CIP) and steam-in-place (SIP) systems that are critical within a stainless steel plant. Furthermore, a reduction in capital costs (CAPEX) is achieved by introducing single-use systems. In a case study that compares the costs for a single-use versus multi-use stainless steel 2 × 1000 L new facility, the single-use facility reduces CAPEX significantly, while operating costs (OPEX) are increased. Overall these studies show that investing in a flexible single-use facility is beneficial compared to a fixed stainless steel facility (Eibl and Eibl 2011; Goldstein and Molina 2016). It must be noted that investment decisions on new production facilities must be taken before the product is licensed by regulatory bodies.
- Increases the number of GMP batches
  By introducing single-use systems, it is possible to increase the number of GMP batches that can be produced within a manufacturing campaign since cleaning and sterilization of the equipment is not needed anymore. The turnover time needed from batch to batch is shortened.
- Provides flexibility in GMP facility design
  When stainless steel systems are used, changes to the equipment might have an impact on the design of the stainless steel tanks, piping, etc. These equipment changes will influence the overall validation status of the facility. By using single-use systems,

equipment changes can easily be incorporated as the setup of the single-use process is flexible. As with the stainless steel systems, in case a change will influence the validated process, the validated status of the process must be reconsidered and a revalidation might be needed.

- Speeds up implementation and time to market
  Due to the great flexibility of the single-use systems, the speed of product to market is less influenced by process changes that might be introduced during the different development stages of the production process than 'traditional' stainless steel equipment. However, the process needs to be validated before market introduction. When changes are introduced after process validation, a revalidation might be needed. Here again, there is no difference in this respect to the traditional stainless steel setup.
- Reduces water and wastewater costs
  Since the systems are single-use, there will be a great reduction in the total costs for cleaning. Not only through a reduction in water consumption but also a reduction in the number of hours needed to clean systems and to set them up for the next batch of product.
- Reduces validation costs
  No annual validation costs for cleaning and sterilization are needed anymore when single-use systems are used.

A disadvantage of the single-use system is that the operational expenses will increase and storage facilities for single-use bags and tubing are needed. Moreover, the dependence of the company on one supplier of single-use systems is a factor to consider. Finally, leachables and extractables from the single use plastics may end up in your product, causing potential safety and efficacy issues.

The advantages of the stainless steel bioreactors are obvious as this traditional technology is well understood and controlled, although the stainless steel pathway had and still has major disadvantages such as expensive and inflexible design, installation and maintenance costs combined with significant expenditures of time in facilities and equipment qualification and validation efforts. For very large volume products stainless steel is still the most economically viable option due to limited scalability of current single-use bioreactors (i.e., 2000 L max).

CONTAMINANTS

Parenteral product purity mostly is ≥99% (Berthold and Walter 1994; ICH 1999a). Purification processes should yield potent proteins with well-defined characteristics for human use from which "all" contaminants have been removed to a major extent. The purity of the drug protein in the final product largely depends upon the applied purification technology.

Origin	Contaminant
Host-related	Viruses
	Bacteria (mycoplasma)
	Host-derived proteins and DNA
	Endotoxins (from gram-negative bacterial hosts)
Product-related	Glycosylation variants
	Amino acid substitution and deletion
	Denatured protein (loss of secondary, tertiary, quaternary structure)
	Oxidized variants
	Conformational isomers
	Dimers and aggregates
	Disulfide pairing variants
	Succinimide formation
	(De)amidated species
	Protein fragments
Process-related	Viruses
	Bacteria
	Cell culture medium components
	Purification reagents
	Metals
	Column materials/leachables
	Leachables from single-use system (tubes, bags, etc.)

Table 4.4 ■ Potential contaminants/variants in recombinant protein products derived from bacterial and mammalian hosts

Table 4.4 lists potential contaminants and product variants that may be present in recombinant protein products from bacterial and mammalian sources. These contaminants can be host-related, process-related and product-related. In the following sections, special attention is paid to the detection and elimination of contamination by viruses, bacteria, cellular DNA, and undesired proteins.

■ Viruses

Endogenous and adventitious viruses, which require the presence of living cells to propagate, are potential contaminants of animal cell cultures and, therefore, of the final drug product. If present, their concentration in the purified product will be very low and it will be difficult to detect them. Viruses such as retrovirus can be visualized by (nonsensitive) electron microscopy. For retroviruses, a highly sensitive RT-PCR (reverse-transcriptase polymerase chain reaction) assay is available, but for other viruses, a sensitive in vitro assay might be lacking. The risks of some viruses (e.g., hepatitis virus) are known (Walter et al. 1991; Marcus-Sekura 1991), but there are other viruses whose risks cannot be properly judged because of lack of solid

Category	Types	Example
Inactivation	Heat treatment	Pasteurization
	pH extremes	Low pH
	Radiation	UV-light
	Dehydration	Lyophilization
	Cross linking agents, denaturating or disrupting agents	β-propiolactone, formaldehyde, NaOH, organic solvents (e.g., chloroform), detergents (e.g., Na-cholate)
	Neutralization	Specific, neutralizing antibodies
Removal	Chromatography	Ion-exchange, immuno-affinity chromatography
	Filtration	Nanofiltration, Q-charged membranes
	Precipitation	Cyroprecipitation

Table 4.5 ■ Methods for reducing or inactivating viral contaminants

experimental data. Some virus infections, such as parvovirus, can have long latent periods before their clinical effects show up. Long-term effects of introducing viruses into a patient treated with a recombinant protein should not be overlooked. Therefore, it is required that products used parenterally are free from viruses. The specific virus testing regime required will depend on the cell type used for production (Löwer 1990; Minor 1994).

Viruses can be introduced by nutrients, by an infected production cell line, or they are introduced (by human handling) during the production process. The most frequent source of virus introduction is animal serum. In addition, animal serum can introduce other unwanted agents such as bacteria, mycoplasmas, prions, fungi, and endotoxins. Appropriate screening of cell banks and growth medium constituents for viruses and other adventitious agents should be strictly regulated and supervised (Walter et al. 1991; FDA 1993; ICH 1999b; WHO 2010). Validated, orthogonal methods (cf Chap. 5) to inactivate and remove possible viral contaminants during the production process are mandatory for licensing of therapeutics derived from mammalian cells or transgenic animals (EMA 1996; ICH 1999b). Viruses can be inactivated by physical and chemical treatment of the product. Heat, irradiation, sonication, extreme pH, detergents, solvents, and certain disinfectants can inactivate viruses. These procedures can be harmful to the product as well and should therefore be carefully evaluated and validated (Walter et al. 1992; ICH 1999b). As mentioned in the filtration section removal of viruses by nanofiltration is an elegant and effective technique and the validation aspects of this technology are well described (PDA technical report 41 2005). A significant log reduction of even the smallest non-evelopped viruses such as bovine parvovirus can be obtained by filtration through 15 nm membranes (Maerz et al. 1996). Another common, although less robust, method to remove viruses in antibody processes is by ion-exchange chromatography and Q-charged membranes (Zhou and Tressel 2006). A number of methods for removing or inactivating viral contaminants are mentioned in Table 4.5.

In general, a protein production process should contain two or more orthogonal virus reduction steps. As mentioned, virus validation studies need to be performed on the developed production process and they should show sufficient removal or inactivation of spiked model viruses before the start of clinical studies. The choice of viruses to be spiked depends upon the production cell line, the ease of growing model viruses to high titers, and should include various types of virus (large vs small, enveloped vs non-enveloped, DNA vs. RNA). These types of studies are performed in specialized laboratories.

■ Bacteria

Bacterial contamination may be a problem for cells in culture or during pharmaceutical purification. Usually the size of bacteria allows simple filtration over 0.2 μm (or smaller) filters for adequate removal. Special attention is given to potential contaminations with mycoplasma, a genus of bacteria having no cell wall around their cell membrane. Some mycoplasma species are pathogenic to humans, and hundreds of mycoplasma species infect animals (Larsen and Hwang 2010). Testing for mycoplasma is a regulatory requirement for human biopharmaceuticals.

In order to further prevent bacterial contamination during production, the raw materials used have to be sterilized, preferably at 121 °C or higher, and the products are manufactured under strict aseptic conditions wherever possible. Production most often takes place in so-called clean rooms in which the chance of environmental contamination is reduced through careful control of the environment, for example,

filtration of air. Additionally, antibiotic agents can be added to the culture media in some cases but have to be removed further downstream in the purification process. However, the use of beta-lactam antibiotics such as penicillin is strictly prohibited due to oversensitivity of some individuals to these compounds. Because of the persistence of antibiotic residues, which are difficult to eliminate from the product, appropriately designed manufacturing plants and extensive quality control systems for added reagents (medium, serum, enzymes, etc.) permitting antibiotic-free operation are preferable.

■ Pyrogens

Pyrogens are compounds that induce fever. Humans are sensitive to pyrogen contamination at very low concentrations (picograms per mL). Exogenous pyrogens (pyrogens introduced into the body, not generated by the body itself) can be derived from bacterial, viral, or fungal sources. Bacterial pyrogens are mainly endotoxins shed from gram-negative bacteria. They are lipopolysaccharides, and Fig. 4.8 shows the basic structure. The conserved structure in the full array of thousands of different endotoxins is the lipid-A moiety. Another general property shared by endotoxins is their high, negative electrical charge. Their tendency to aggregate and to form large units with M_W of 10^4 to over 10^6 Daltons in water, and their tendency to adsorb to surfaces indicate that these compounds are amphipathic in nature. Sensitive tests to detect and quantify pyrogens are commercially available.

They are stable under standard autoclaving conditions but break down when heated in the dry state. For this reason equipment and container are treated at temperatures above 160 °C for prolonged periods (e.g., 30 min dry heat at 250 °C). Removal is complicated because pyrogens vary in size and chemical composition. Pyrogen removal of recombinant products derived from bacterial sources should be an integral part of the preparation process. Ion exchange chromatographic procedures (utilizing its negative charge) can effectively reduce endotoxin levels in solution.

Excipients used in the protein formulation should be essentially endotoxin-free. For solutions "water for injection" (compendial standards) is (freshly) distilled or produced by reverse osmosis. The aggregated endotoxins cannot pass through the reverse osmosis membrane. Removal of endotoxins immediately before filling the final container can be accomplished by using activated charcoal or other materials with large surfaces offering hydrophobic interactions. Endotoxins can also be inactivated on utensil surfaces by oxidation (e.g., peroxide) or dry heating (e.g., 30 min dry heat at 250 °C).

■ Cellular DNA

The application of continuous mammalian cell lines for the production of recombinant proteins might result in the presence of oncogene-bearing DNA fragments in the final protein product (Walter and Werner 1993; Löwer 1990). A stringent purification protocol that is capable of reducing the DNA content and fragment size to a safe level is therefore necessary (Berthold and Walter 1994; WHO 2010; ICH 2017). A number of approaches are available to validate that the purification process removes cellular DNA and RNA. One such approach involves incubating the cell line with radiolabeled nucleotides and determining radioactivity in the purified product obtained through the purification protocol. Other methods are dye-binding fluorescence-enhancement assays for nucleotides and PCR-based methods. If the presence of nucleic acids persists at significant levels in a final preparation, then additional steps must be introduced in the purification process. The question about a safe level of nucleic acids

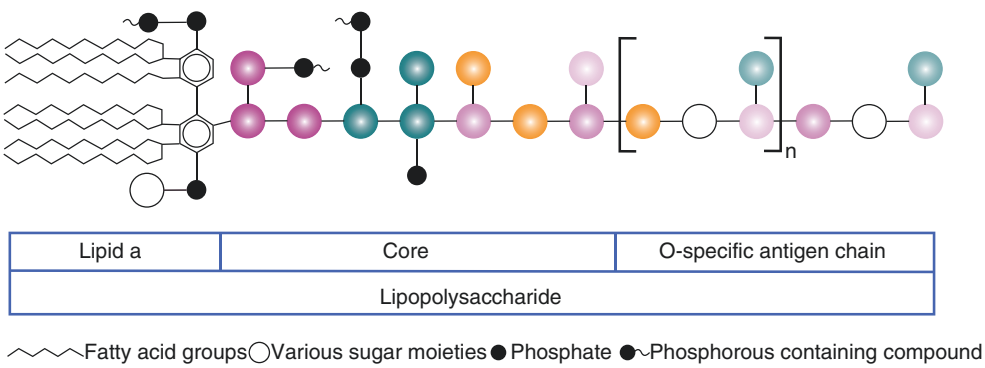

Figure 4.8 ■ Generalized structure of endotoxins. Most properties of endotoxins are accounted for by the active, insoluble "lipid A" fraction being solubilized by the various sugar moieties (circles with different colors). Although the general structure is similar, individual endotoxins vary according to their source and are characterized by the O-specific antigenic chain (adapted from Groves 1988)

in biotech products is difficult to answer. Transfection with so-called naked DNA is very difficult and a high concentration of DNA is needed. Nevertheless, it is agreed for safety reasons that final product contamination by nucleic acids should not exceed 100 pg or 10 ng per dose depending on the type of cells used to produce the pharmaceutical (WHO 2010; European Pharmacopoeia 2011).

■ Protein Contaminants and Product Variants

As mentioned before, minor amounts of host-, process-, and product-related protein contaminants will likely be present in biotech products. These types of contaminants are a potential health hazard because, if present, they may be recognized as antigens by the patient receiving the recombinant protein product. On repeated use the patient may show an immune reaction caused by the contaminant, while the protein of interest is performing its beneficial function. In such cases the immunogenicity may be misinterpreted as being due to the recombinant protein itself. Therefore, one must be very cautious in interpreting safety data of a given recombinant therapeutic protein. Some contaminants may also affect efficacy of the product, for example if they bind to an epitope important for the product to exert its function. Hence, careful control is needed.

Generally, the sources of host- and process-related protein contaminants are the cell culture medium used and the host proteins of the cells. Among the host-derived contaminants, the host species' version of the recombinant protein could be present (WHO 2010). As these proteins are similar in structure, it is possible that undesired proteins are co-purified with the desired product. For example, urokinase is known to be present in many continuous cell lines. The synthesis of highly active biological molecules such as cytokines by hybridoma cells might be another concern (FDA 1990). Depending upon their nature and concentration, these cytokines might enhance the antigenicity of the product.

"Known" or expected contaminants should be monitored at the successive stages in a purification process by suitable in-process controls, e.g., sensitive immunoassay(s). Tracing of the many "unknown" cell-derived proteins is more difficult. When developing a purification process, other less-specific analytical techniques such as SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) are usually used in combination with various staining techniques.

Product-related contaminants may pose a safety issue for patients. These contaminants can, for example, be aggregated, deamidated or oxidized forms of the product. And, importantly, one has to keep in mind that recombinant proteins produced in cells are inherently variable, for example at the level of glycosylation. Such molecules are generally considered product variants. Some of these contaminants/variants are described in the following paragraphs.

N- and C-Terminal Heterogeneity

A major problem connected with the production of biotech products is the problem associated with the amino (NH2)-terminus of the protein, e.g., in E. coli systems, where protein synthesis always starts with methylmethionine. Obviously, it has been of great interest to develop methods that generate proteins with an NH2-terminus as found in the authentic protein. When the proteins are not produced in the correct way, the final product may contain several methionyl variants of the protein in question or even contain proteins lacking one or more residues from the amino terminus. This is called the amino-terminal heterogeneity. This heterogeneity can also occur with recombinant proteins (e.g., α-interferon) susceptible to proteases that are either secreted by the host or introduced by serum-containing media. These proteases can clip off amino acids from the C-terminal and/or N-terminal of the desired product (amino- and/or carboxy-terminal heterogeneity). Amino- and/or carboxy-terminal heterogeneity is not desirable since it may cause difficulties in purification and characterization of the proteins. In case of the presence of an additional methionine at the N-terminal end of the protein, its secondary and tertiary structure can be altered. This could affect the biological activity and stability and may make it immunogenic. Moreover, N-terminal methionine and/or internal methionine is sensitive to oxidation (Sharma 1990).

C-terminal lysine clipping is often observed in monoclonal antibodies produced in mammalian cells. This does not have to be an issue, since the C-terminal lysine is clipped off rapidly in the blood upon injection in humans. The glutamine on the N-terminus of monoclonal antibodies can be converted in pyro-glutamate, increasing the acidity of the antibody. These types of posttranslational modifications should be controlled within a certain range to ensure a robust production process.

Conformational Changes/Chemical Modifications

Although mammalian cells are able to produce proteins structurally equal to endogenous proteins, some caution is needed. Transcripts containing the full-length coding sequence could result in conformational isomers of the protein because of unexpected secondary structures that affect translational fidelity (Sharma 1990). Another factor to be taken into account is the possible existence of equilibria between the desired form and other forms such as dimers. The correct folding of proteins after biosynthesis is important because

it determines the specific activity of the protein). Therefore, it is important to determine if all molecules of a given recombinant protein secreted by a mammalian expression system are folded in their native conformation.. Apart from conformational changes, proteins can undergo chemical alterations, such as proteolysis, deamidation, and hydroxyl and sulfhydryl oxidations during the purification process (cf. Chaps. 2 and 3) These alterations can result in (partial) denaturation of the protein. Vice versa, denaturation of the protein may cause chemical modifications as well (e.g., as a result of exposure of sensitive groups).

Glycosylation (also cf. Chap. 2)

Many therapeutic proteins produced by recombinant DNA technology are glycoproteins of which the majority are monoclonal antibodies. The presence and nature of oligosaccharide side chains in proteins affect a number of important characteristics, such as the proteins' serum half-life, solubility, and stability, and sometimes even the pharmacological function (Cumming 1991). Darbepoetin, a second-generation, genetically modified erythropoietin, has a carbohydrate content of 80% compared to 40% for the native molecule, which increases the in vivo half-life after intravenous administration from 8 h for erythropoietin to 25 h for darbepoetin (Sinclair and Elliott 2005). Antibody-dependent cell cytotoxicity (ADCC) is dependent on the degree of fucosylation of the antibody product (Hossler et al. 2009; reviewed by Krasnova and Wong 2016). As a result, the therapeutic profile may be "glycosylation" dependent. As mentioned previously, protein glycosylation is not determined by the DNA sequence. It is an enzymatic modification of the protein after translation and depends on the metabolic state of the cell (Hossler et al. 2009). Although mammalian cells are very well able to glycosylate proteins, it is hard to fully control glycosylation. Carbohydrate heterogeneity can occur through the size of the chain, type of oligosaccharide, and sequence of the carbohydrates. This has been demonstrated for a number of recombinant products including monoclonal antibodies, interleukin-4, chorionic gonadotropin, erythropoietin, and tissue plasminogen activator. Carbohydrate structure and composition in recombinant proteins may differ from their native counterparts, because the enzymes required for synthesis and processing vary among different expression systems, e.g. glycoproteins from insect cells are frequently smaller than the same glycoproteins expressed in mammalian cells or even from one mammalian system to another.

Proteolytic Processing

Proteases play an important role in processing, maturation, modification, or isolation of recombinant proteins. Proteases from mammalian cells are involved in secreting proteins into the cultivation medium e.g. by cleaving of a signal peptide. Proteases are released if cells die and undergo lysis during production in the bioreactor and at harvest. It is therefore important to control growth and harvest conditions in order to minimize this effect. Another source of proteolytic attack is found in the components of the medium in which the cells are grown. For example, serum contains a number of proteases and protease zymogens that may affect the secreted recombinant protein. If present in small amounts and if the nature of the proteolytic attack on the desired protein is identified, appropriate protease inhibitors to control proteolysis could be used. It is advised to document the integrity of the recombinant protein after each purification step.

Proteins become much more susceptible to proteases at elevated temperatures. Purification strategies should be designed to carry out all the steps at 2–8 °C (Sharma, 1990) if proteolytic degradation occurs. Alternatively, Ca^{2+} complexing agents (e.g., citrate) can be added as many proteases depend on Ca^{2+} for their activity. From a manufacturing perspective, however, cooling large-scale downstream process unit operations, although not impossible, is a complicating and expensive factor.

BACTERIA: PROTEIN INCLUSION BODY FORMATION

In bacteria soluble proteins can form dense, finely granular inclusions within the cytoplasm. These "inclusion bodies" often occur in bacterial cells that overproduce proteins by plasmid expression. The protein inclusions appear in electron micrographs as large, dense bodies often spanning the entire diameter of the cell. Protein inclusions are probably formed by a buildup of amorphous protein aggregates held together by covalent and non-covalent bonds. The inability to measure inclusion body proteins directly may lead to the inaccurate assessment of recovery and yield and may cause problems if protein solubility is essential for efficient, large-scale purification (Berthold and Walter 1994). Several schemes for recovery of proteins from inclusion bodies have been described. The recovery of proteins from inclusion bodies requires cell breakage and inclusion body recovery. Dissolution of inclusion proteins is the next step in the purification scheme and typically takes place in extremely dilute solutions, thus increasing the volumes of the unit operations during the manufacturing phases. This can make process control more difficult if, for example, low temperatures are required during these steps. Generally, inclusion proteins dissolve in denaturing agents such as sodium dodecyl sulfate (SDS), urea, or guanidine hydrochloride. Because bacterial systems generally are incapable of forming disulfide bonds, a protein containing these

bonds has to be refolded under oxidizing conditions to restore these bonds and to generate the biologically active protein. This so-called renaturation step is increasingly difficult if more S-S bridges are present in the molecule and the yield of renatured product could be as low as only a few percent. Once the protein is solubilized, conventional chromatographic separations can be used for further purification of the protein.

Aggregate formation at first sight may seem undesirable, but there may also be advantages as long as the protein of interest will unfold and refold properly. Inclusion body proteins can easily be recovered to yield proteins with >50% purity, a substantial improvement over the purity of soluble proteins (sometimes below 1% of the total cell protein). Furthermore, the aggregated forms of the proteins are more resistant to proteolysis, because most molecules of an aggregated form are not accessible to proteolytic enzymes. Thus the high yield and relatively cheap production using a bacterial system can offset a low-yield renaturation process. For a non-glycosylated, simple protein molecule, this production system is still used.

QUALITY BY DESIGN

The current expectations of regulatory agencies, particularly in implementing the twenty-first century's risk-based GMPs, is to employ the principles of risk analysis, design space (see below), control strategy and Quality by Design (QbD). Implementing QbD should result in a manufacturing process that consistently delivers a high quality product. Furthermore, it ensures that the critical sources of variability are identified and controlled through appropriate control strategies. A detailed end-to-end assessment of the product, its manufacturing process and raw materials will result in the definition of:

1. Critical quality attributes (CQAs)
   The definition of a CQA according to ICH Q8 (R2), 2009 is as follows: "a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality". The CQAs of biologics are basically assessed by measuring their impact on safety and efficacy.
2. Critical process parameters (CPPs)
   CPPs are according to ICH Q8(R2) (2009) "process parameters whose variability has an impact on a critical quality attributes". They are identified by sound scientific judgement and based on prior knowledge, development, scale-up and manufacturing experience. CPPs should be controlled and monitored to confirm that the product quality is comparable to or better than historical data from development and manufacturing. Quality attributes that should be considered in defining CPPs are for example purity, qualitative and quantitative impurities, microbial quality, biological activity and content.
3. Critical material attributes (CMAs)
   CMAs are materials used in the process that affect the quality attributes. They are judged as described above for the CPPs. CMAs should be controlled and monitored by validated incoming goods assays.
4. Control strategy
   The control strategy for the product is defined by controlling CPPs and the CMAs. Based on the risks related to the CPPs/CMAs an appropriate control strategy should be designed. A proper control strategy will decrease the probability/likelihood of out of range CQA and increase the detectability of CPP/CMA failure. During the lifecycle of the product the control strategy should be adjusted based on new knowledge. The control strategy will be assessed by means of a risk assessment (e.g., failure mode effects analysis, FMEA).

Above mentioned analysis must be performed during various stages of process development. However, the starting point for a QbD excercise is to study the (potential) CQAs that are defined in early stage discovery. The analysis should continue during early and late development and commercial scale manufacturing. Prior knowledge, analytical development, comparability studies, and (non-) clinical study results contribute to the understanding of CQAs. By performing this analysis during various stages, the QbD principles will be continuously updated as they are based on increased know-how during product development and commercial scale manufacturing.

Although not obligatory, the authorities encourage to implement a design space in the processes. ICH Q8 defines the design space of a process as follows: "the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality. Working within the design space is not considered as a change. Movement out of the design space is considered to be a change and would normally initiate a regulatory postapproval change process. The design space is proposed by the manufacturer. The advantage of the design space that it is usually broader than the operating ranges".

Based on these assessments the CPPs and CMAs are specified for:

(a) Normal operating ranges (NOR)
   A process range that is representative of historical variability in the manufacturing process

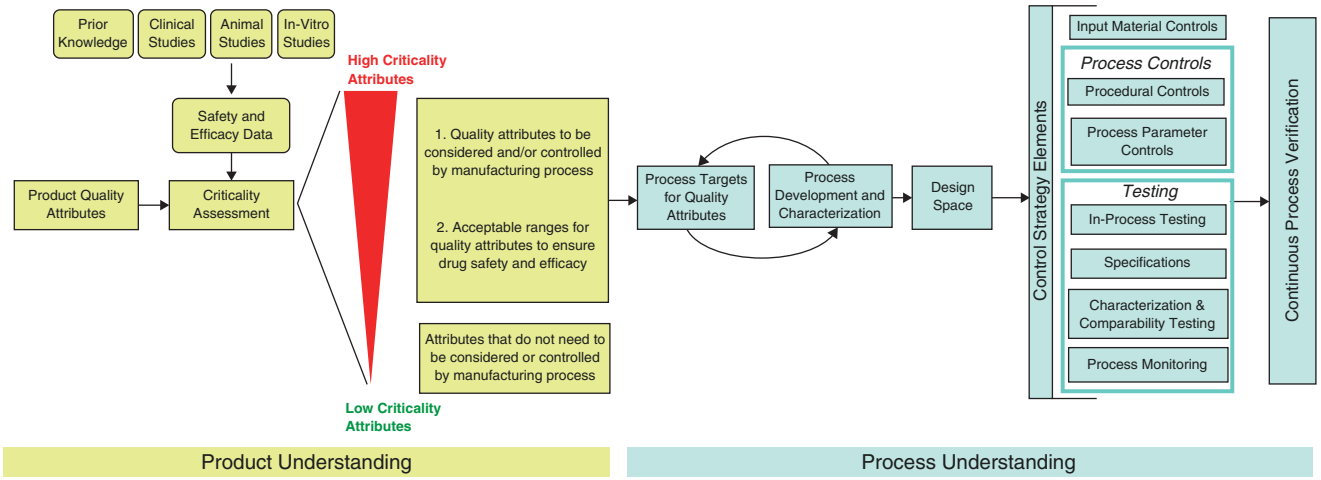

Figure 4.9 ■ The overall approach for A-Mab product realization illustrates a sequence of activities that starts with the design of the molecule and spans the development process ultimately resulting in the final process and control strategy used for commercial scale manufacturing (adapted from Berridge et al. 2009)

(b) Acceptable operating ranges
A range that is specified in the manufacturing batch record
(c) Proven acceptable range (PAR)
A characterized range of the process which will result in producing a product meeting the relevant quality criteria.

Company representatives of the Biotechnology Industry were brought together in 2008 helping to advance the principles which are contained in ICH Q8 (R2), Q9 and Q10, focusing on the principles of Quality by Design. The outcome of this collaboration resulted in a unique document: "A-Mab: A case study in BioProcess Development" (Berridge et al. 2009). The case study is a must read for people involved in the biotechnology industry. Figure 4.9 shows the overall approach for A-Mab product realization.

COMMERCIAL-SCALE MANUFACTURING AND INNOVATION

A major part of the recombinant proteins on the market consist of monoclonal antibodies produced in mammalian cells. Pharmaceutical production processes have been set up since the early 1980s of the twentieth century. These processes essentially consist of production in stirred tanks bioreactors, clarification using centrifugation, and membrane technology, followed by protein A capture, low-pH virus inactivation, cation-exchange and anion-exchange chromatography (or an alternative chromatographic ligand), virus filtration, and UF/DF for product formulation (Shukla and Thömmes 2010). Such platform processes run consistently at very large scale (e.g. multiple 10,000 L bioreactors and higher volumes). Product recovery is generally very high (>70%). Since product titers in the bioreactors have increased to a level where further increases have no or a minimal effect on the cost of goods, the focus of process development in companies having these large-scale manufacturing plants working at full capacity is shifting to understanding the process fundamentals of the current platform (Kelley 2009). However, it is also anticipated that the monoclonal antibody demands for some disease indications may decrease due to the introduction of more efficacious products such as antibody-drug conjugates (e.g. Adcetris®, Seattle Genetics) and increased competition with biosimilar products (e.g., Celltrion's Remsina®/Infectra® as biosimilar of the Johnson & Johnson blockbuster Remicade®), and the introduction of new products with (much) smaller market sizes, including those used in personalized medicines approaches. A lower demand together with the increase in recombinant protein titers and yields will lead to a decrease in bioreactor size, an increase in the need for flexible facilities, and faster turnaround times leading to a growth in the use of disposables and other innovative technologies as discussed above (Shukla and Thömmes 2010). Such innovative technologies and capabilities encompass process intensification, in which production is intensified by using highly concentrated product and reactants, and in which process steps are combined into single units. Innovation is also seen in the introduction of continuous processing strategies in the pharmaceutical industry, as well as steps towards fully automated facilities, enabling a fast response to capacity demands at lower costs and higher quality. Facilities will become modular and mobile, allowing standardized "plug and play" manufacturing systems to be configured, assembled and relocated quickly. A further introduction of

process analytical technology (PAT) is expected, allowing in-line process monitoring and real time drug product release. This includes development of software enabling multivariate data analysis, predictive models and closed feedback control loops (BioPhorum Operations Group 2017).

SELF-ASSESSMENT QUESTIONS

Questions

1. Name the four expression systems mentioned in this chapter?
2. What is the main reason to use eukaryotic mammalian cells as expression system?
3. What are the main reasons for manufacturing companies to change from stainless steel system to single-use systems?
4. Which bioreactor processes are generally used for production of biopharmaceuticals?
5. Membrane filters are frequently used within the purification process of biotech products. Name four different membrane filter types.
6. Compared to other chromatographic methods, what is in general the most significant advantage and disadvantage of affinity purification chromatography?
7. Name at least six different product-related variants.
8. What is the difference between a NOR and PAR? Which of these two parameters gives most flexibility in a process?
9. What are the major safety concerns in the purification of cell-expressed proteins?
10. Glycosylation may affect several properties of the protein. Mention at least three possible effects in case of changing a glycosylation pattern.
11. What is in general the expectation of the size of future GMP manufacturing facilities? What is the reasoning behind this?

Answers

1. Prokaryotic bacteria, eukaryotic yeast, eukaryotic mammalian cells, and eukaryotic plant cells.
2. For biopharmaceutical products used in human health care the glycosylation process is the most important reason. The glycosylation pattern should be human-like which is possible with the eukaryotic mammalian cell system.
3. The main reasons are the speed to market, possibility to increase the number of batches produced per year in a manufacturing facility, providing flexibility in facility design, reduction of water consumption and reduced validation costs.
4. The main bioreactor processes are batch, fed-batch and perfusion.
5. Sterilizing-grade filters, tangential flow filters, virus removal filters, charged filters.
6. Advantage: high degree of purity can be obtained; disadvantage: usually very costly, and extra regulatory burden due to characterization of affinity ligand.
7. Glycosylations variants, amino acid substitution and deletion, denatured protein, oxidized variants, conformational isomers, dimers and aggregates, disulfide paring variants, succinimide formation, (de)amidated variants, protein fragments.
8. The Normal Operating Range (NOR) is a process range that is representative of historical variability in the manufacturing process, while a Proven Acceptable Range (PAR) is a characterized range of the process which will result in producing a product meeting the relevant quality criteria. The PAR gives most flexibility since it allows operation beyond the NOR.
9. Removal of viruses, bacteria, protein contaminants and cellular DNA.
10. Solubility, pKa, charge, stability and biological activity.
11. GMP manufacturing facilities will become smaller, modular and mobile. Rationale: manufacturing volumes will become smaller due to process intensification and the generation of products with a smaller market capture.

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