1 Introduction to Biopharmaceutical Processes

1.1 Context

Thanks to major progress in the understanding of the biological processes involved in human diseases, protein-based drugs have emerged as an important class of therapeutics in the 1980s [1, 2]. The main asset of proteins is rooted in their ability to perform highly specific and complex sets of biological functions that can hardly be mimicked by traditional chemical drugs [2, 3]. The development and commercialization of protein-based drugs have been fostered by significant advances in protein and genetic engineering to such an extent that they are nowadays mostly produced by living organisms [1, 4, 5]. To name just a few examples, therapeutic monoclonal antibodies, hormones, and blood factors are produced at the commercial scale using the so-called *recombinant technology*. Besides recombinant proteins, a few proteins are still extracted from their native source, such as pancreatic enzymes or plasma proteins [2]. A third strategy to produce protein-based drugs is chemical synthesis, which is nevertheless limited to rather small proteins and peptides [2, 6]. Therapeutic peptides represent a kind of hybrid class of drugs, which bridge the gap between small-molecule and protein drugs in terms of physical properties, clinical applications, and means of production [3]. In the following, they will be placed under the umbrella "therapeutic proteins" due to their amino acid content. In any production process, whether based on the recombinant technology, on the extraction of a protein from its native source, or on chemical synthesis, the target protein has to be isolated from a complex mixture, and efficient purification processes are crucial to reach the high purity grades required for medical use.

In this chapter, we provide a succinct description of typical biopharmaceutical processes in order to contextualize the three main aspects that will be addressed in this book: chromatography, protein conjugation, and protein aggregation. In a first part, we briefly present the main unit operations encountered in the production of therapeutic proteins, and then we discuss *if* and *how* the biopharmaceutical industry could benefit from the use of continuous technologies.

1.2 Single-Unit Operations

Biopharmaceutical production processes are commonly divided into two parts: *upstream processing*, where the protein is produced by living organisms, and *downstream*

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processing, where a series of purification steps are performed to meet certain purity specifications. Although the terms *upstream* and *downstream* are usually reserved for the production of recombinant proteins, they could in principle be extended to synthetic proteins, where the term *upstream* would designate the chemical synthesis step.

There exist a wide variety of biopharmaceutical production processes, which greatly differ both in their upstream and downstream parts. Over the past years, there were some attempts to develop a universal protein purification platform [7, 8]. However, the number of available protein sequences rises dramatically with time, and protein databases now contain more than 20 million different amino acid sequences [9]. The classification of these proteins into comprehensive domain families led to the identification of about 16,000 different families, among which about two-thirds are singletons (i.e., single-member families) [10, 11]. Because the choice of the purification process is strongly dictated by the protein structure, this undoubtedly annihilates the vision of a universal purification platform. It is thus impossible to describe here a general production process applicable to any therapeutic protein.

Nevertheless, for some families of proteins, it is possible to identify rather general production platforms, as it is the case for monoclonal antibodies (mAbs) [12]. This is related to the fact that mAbs all share a common structure, with one constant domain (Fc) that is identical within a class of immunoglobulin (Ig) and two antigen-binding domains (Fab) that are specific to the antibody of interest. Besides their industrial relevance [13], mAbs therefore represent a good didactic example to introduce general concepts about biopharmaceutical processes in general and about downstream processes in particular, which is at the core of this book. Accordingly, we will take mAbs as a reference in the following. A representative example of a mAb production process is represented in Figure 1.1, and we will largely follow this scheme throughout this section. Deviations from this general scheme will be pointed out in the text, and appropriate references for nonantibody proteins will be indicated when possible.

1.2.1 Cell Culture

As mentioned earlier, a great number of tailor-made therapeutic proteins are nowadays produced by living organisms. Recombinant proteins can be expressed in a wide variety of systems including bacteria, mammalian cells, yeasts, fungi, as well as transgenic plants and animals [5].

Among these systems, *Escherichia coli* (*E. coli*) bacteria are particularly attractive due to their well-characterized genetics, great versatility, rapid growth, low media cost, and high expression level [14]. However, *E. coli* bacteria suffer from the inability to perform complex posttranslational modifications, such as glycosylation, and from the difficulty to achieve proteolytic protein maturation and disulfide bond formation. Moreover, proteins expressed by *E. coli* are often produced as inclusion bodies, which are intracellular insoluble deposits containing the protein at high concentration in an inactive aggregated state. In this case, it is necessary to dissolve these inclusion bodies and to properly refold the protein of interest *in vitro*, which may turn out to be a very challenging operation [15].

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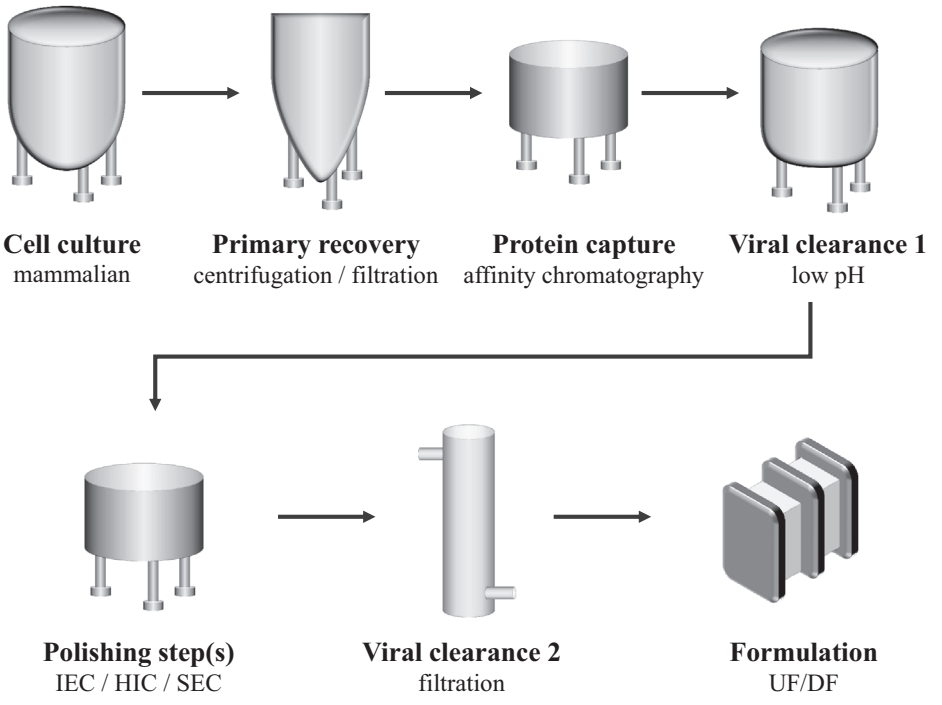

Figure 1.1 Schematic example of a process for the manufacturing of mAbs.

The aforementioned limitations prohibit bacteria from expressing some pharmaceutically relevant proteins, such as mAbs. The latter are largely produced by mammalian cell culture, and in particular with Chinese hamster ovary (CHO) cells [4]. Despite their high cost and complex genetics, mammalian cells are the system of choice for expressing large proteins (above 100 kDa) whose biological activity is strongly affected by both folding and posttranslational modifications [16].

Cell culture falls out of the scope of this book, and the reader interested in biochemical engineering is referred to the following references [17–19].

1.2.2 Primary Recovery

After the cell culture step, the protein of interest is retrieved either by collecting the culture broth supernatant (if the protein is expressed extracellularly) or after supernatant removal and cell disruption (if the protein is expressed intracellularly). In both cases, the first step consists in separating the supernatant from the cells and cell debris formed during cell autolysis, which always occurs to some extent during the cultivation step. This solid–liquid separation step is usually performed with centrifugation or filtration [20, 21] and is relatively simple due to the large size of cells and cell debris as compared to proteins.

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In the case where the supernatant is collected and the cells are discarded, as it is typically the case for mammalian cell culture, this first purification step is termed *clarification*, and the recovered liquid is usually referred to as the *harvested cell culture fluid*.

1.2.3 Protein Capture

The fluid obtained after primary recovery contains a wide range of impurities, which include components secreted by the living cells and intracellular components released during cell lysis (e.g., proteins, nucleic acids, lipids, and endotoxins), unused components from the cell culture media (e.g., salts, sugars, amino acids, surfactants, growth factors), and viruses that potentially infected the cells [21]. Among all these species, endotoxins and viruses are extremely hazardous and thus need to be thoroughly eliminated.

At this level, the objective is to extract the maximum amount of target protein from the biological feedstock and to remove as much as possible of the aforementioned impurities (although not necessarily satisfying the final purity requirement). Appropriately, this step is commonly called the *protein capture step*. During this operation, it is also particularly important to recover the protein at a sufficiently high concentration in order to reduce the volume to be treated during the subsequent purification steps. One of the most powerful techniques to perform protein capture is certainly affinity chromatography, which relies on the highly specific reversible interaction between the protein of interest and a natural or synthetic ligand covalently linked on the solid support. The majority of antibody purification platforms employ Protein A as affinity ligand, which interacts specifically with the Fc domain of immunoglobulin G (IgG) [21–23]. Other specific ligands include antibodies, which are used in the industrial purification of recombinant factor VIII for instance [24], heparin that is widely employed in the purification of plasma proteins [25], as well as substrates, co-factors, and inhibitors that are typically selected for enzyme purification [26]. The high price of affinity chromatographic media promotes the development of alternative methods to perform the capture step [22, 27], and two notable exceptions of antibody purifications using ion exchange chromatography are Zenapax® (Daclizumab) and Humira® (Adalimumab) [22]. More details about affinity chromatography and other types of chromatography will be given in Chapter 2, while multicolumn chromatographic processes relevant for the capture step will be presented in Chapter 4.

Although protein chromatography is traditionally performed with packed beds, membrane chromatography has also been examined as an alternative [20, 28–31]. In addition, aqueous two-phase partitioning, which was popular in the 1980s for enzyme purification [32], has recently regained substantial interest in the biotechnology industry as a potential strategy to perform the capture step [20, 33–35]. Finally, precipitation, either of the impurities or of the protein of interest, has often been used as a first step in protein purification processes [20, 36–38]. A noteworthy example among mAbs is Orthoclone OKT®3 (Muromomab CD3), which is recovered from the clarified supernatant by ammonium sulfate induced precipitation [22].

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1.2.4 Polishing Steps

After the capture step, a number of *polishing steps* aim at removing the residual impurities in order to obtain the product at the required purity. The expression "intermediate purification steps" is sometimes used to reserve the term *polishing* to the very last purification operation. In this case, the purification process is divided into three parts: a capture step, one or several intermediate purification steps, and a polishing step. There is nevertheless no conceptual difference between intermediate and polishing steps, and we will therefore use the term *polishing* to denote any purification step subsequent to the capture step.

Typical impurities that need to be removed after the capture step include the residual host cell proteins and nucleic acids that were not entirely eliminated during the capture step, potential affinity ligands that leached from the chromatographic medium, as well as fragments, aggregates, and variants of the protein of interest [20]. The latter correspond to proteins that vary slightly in their primary sequence as compared to the target protein. These variations are typically the result of posttranslational modifications, for example due to protein oxidation, deamidation, or different glycosylations. Protein variants are usually difficult to remove due to their high similarity with the target protein.

Ion-exchange chromatography (IEC) or hydrophobic interaction chromatography (HIC) are typically employed during the polishing steps [22], exploiting differences in charge and hydrophobicity, respectively, between the solutes to be separated [21]. The use of size exclusion chromatography (SEC) has also been reported for the large-scale purification of antibodies and notably for the removal of aggregates [22]. The fundamental principles underlying the separation mechanisms by chromatography will be described in Chapter 2, while the multicolumn processes relevant for the polishing steps will be presented in Chapter 5.

As for the capture step, nonchromatographic techniques can also be envisaged for the polishing steps. For instance, the purification process of recombinant insulin involves the precipitation of impurities by ethanol addition followed by insulin crystallization [39].

1.2.5 Viral Clearance

As mentioned earlier, the removal of viruses is a major concern for the biopharmaceutical industry, and conventional purification processes typically target a reduction of the level of retroviruses by 12–18 orders of magnitude [40]. Several methods are available for *viral clearance*, such as low-pH inactivation, heat inactivation, filtration, solvents/detergents treatment, and gamma irradiation [40, 41]. Chromatographic steps have also been shown to reduce the content of viruses by several orders of magnitude [40].

The European Medicines Agency recommends "to investigate the contribution of more than one production step for virus reduction and at least two orthogonal steps should be assessed" [42]. For mAbs, a low-pH hold after the capture step and a viral filtration operation after the polishing steps are typically sufficient to meet the

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specifications in terms of virus clearance [22]. Specific process development is needed to extend these methods to continuous manufacturing [43].

1.2.6 Formulation

After the purification process, a *formulation step* is necessary to obtain the therapeutic protein at the desired concentration and in the selected formulation solution, which usually contains a buffering agent to regulate the pH as well as various excipients (e.g., salts, polyols, amino acids, surfactants). A proper formulation solution is essential to ensure proper drug delivery into the organism and to guarantee a sufficiently long shelf life of the biopharmaceutical product [44–47]. With this respect, the strong tendency of proteins to form aggregates represents a major issue because the presence of aggregates may compromise drug efficacy and drug safety. The impact of formulation conditions on protein stability will be discussed in Chapter 7.

Diafiltration (DF) and ultrafiltration (UF) are commonly used to perform buffer exchange and to increase protein concentration, respectively [22, 48, 49]. UF and DF processes are typically performed with the tangential flow filtration (TFF) technology, where the feed stream flows parallel to the filter surface in order to prevent fouling or clogging of the system [48, 49].

Finally, the manufacturing process ends with a sterile filtration step and fill/finish operations, which are sometimes performed by specialized contractors [22, 50].

To date, the development of antibody-based drugs has focused primarily on injectable routes of administration, thus favoring the choice of liquid formulations. These are in general cheaper and faster to develop than alternative formulation types. However, proteins in solution are prone to physical and chemical modifications (e.g., unfolding, aggregation, fragmentation, oxidation, deamidation), which prompted the use of lyophilized formulations in several cases including some blood factors, growth hormones, antibodies, and PEGylated interferon [44, 51, 52].

1.2.7 Additional steps

Protein Refolding

As mentioned in Section 1.2.1, high expression levels of proteins in bacteria often result in the formation of inclusion bodies containing the protein of interest in an inactive aggregated state. In this case, an additional step is required in the production process so as to solubilize and properly refold the therapeutic protein [15]. Protein refolding is performed by exchanging the buffer used to solubilize the protein with a buffer favoring native protein conformations. It has been shown that this step is facilitated by the use of chromatographic columns, although the underlying mechanisms of matrix-assisted refolding has not been fully elucidated yet [21, 53].

Protein Conjugation

Protein-based drugs have emerged as a major class of pharmaceuticals due to their outstanding targeting properties. However, therapeutic proteins often suffer from a rapid

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in vivo clearance, a low solubility, and a limited stability. A possibility to overcome these limitations is to attach a suitable chemical molecule to the protein. The idea is to benefit from the targeting properties of the protein, while further enhancing its therapeutic action by a controlled chemical modification [54]. For example, the attachment of polyethylene glycol (PEG) chains to small proteins has been shown to improve the *in vivo* circulation half-life of the drug [55]. Moreover, the conjugation of antibodies targeting cancer cells to cytotoxic drugs, the so-called antibody drug conjugates (ADC), has led to significant improvements in the field of oncology [56]. Protein conjugation is thus highly important for the pharmaceutical industry, and Chapter 6 will be dedicated to this topic, addressing both reaction and purification aspects.

1.3 Overview of the Impurities to Be Removed

1.3.1 Process-Related and Product-Related Impurities

In Section 1.2, we have enumerated a number of impurities that need to be removed during the downstream processing of recombinant proteins. Figure 1.2 presents an overview of these impurities for a typical mAb production process. Following the nomenclature of the international council for harmonization (ICH), impurities may be classified as *product-related impurities* and *process-related impurities* [57].

Product-related impurities are similar to the target protein and include protein aggregates, protein fragments, and various protein variants such as glycoforms, deamidated proteins, and oxidized proteins. Among these impurities, protein aggregates are of

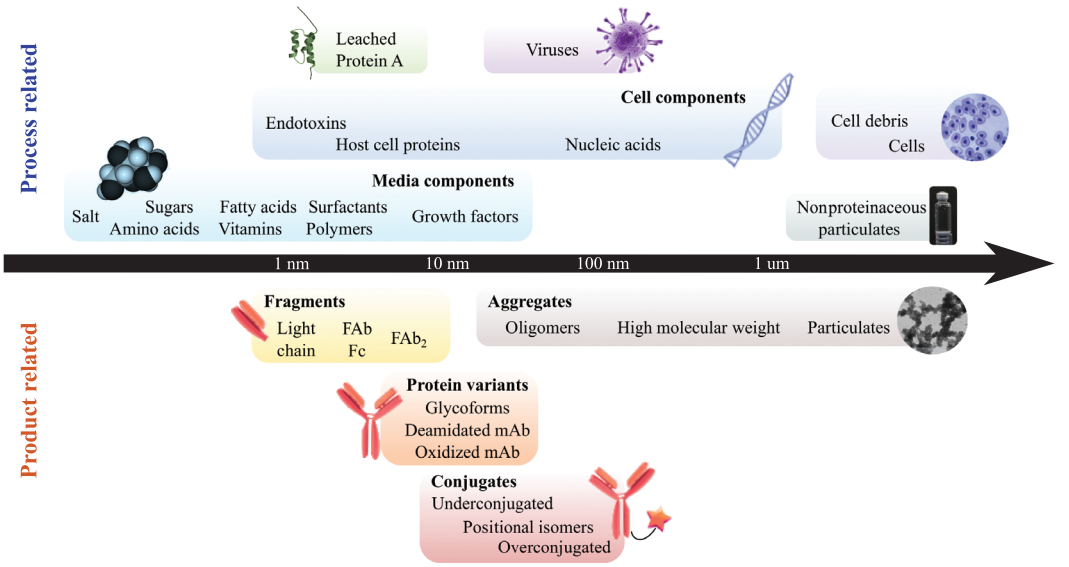

Figure 1.2 Summary of the main process-related impurities and product-related impurities encountered in the manufacturing of mAbs.

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particular concern because they may affect drug efficacy and safety [58, 59]. The formation of protein aggregates and their removal are topics covered in Chapter 7.

When dealing with conjugated proteins, the situation is possibly more complex because the conjugation reaction typically leads to a mixture of conjugates, which differ in terms of both number and position of the grafted molecules. This implies that additional impurities need to be removed, such as unconverted proteins, residual chemical reactants, by-products of the reaction, and under-, over- and misconjugated proteins. Strategies to purify conjugated proteins will be addressed in Chapter 6.

On the other hand, process-related impurities do not share common features with the target protein. They derive from the manufacturing process and include host cell proteins, nucleic acids, viruses, and leached affinity ligands.

Overall, when considering all the aforementioned impurities, two main requirements of the purification process can be identified: *(i)* it should remove numerous and diverse impurities in a limited number of steps, *(ii)* it should separate molecules that differ only by slight variations in their size, charge, or hydrophobicity.

Chromatography is currently the technique of choice for the purification of biopharmaceuticals as it is a versatile, selective, and efficient separation technique satisfying these two points. Chromatography is extensively used to purify mAbs [20, 38], a wide range of other recombinant proteins [21, 60], nonrecombinant proteins (e.g., purification of plasma proteins) [25, 61, 62] as well as synthetic peptides [63].

1.3.2 Purity Specifications

The purity requirements for biopharmaceuticals are specific to each product as they depend on various criteria, such as the therapeutic indication or the dose administrated. With regard to viruses, ICH guidelines recommend to review viral clearance studies on a case-by-case basis according to a risk assessment analysis [64, 65]. There is also no general rule on the maximum limit for the aggregate content in biopharmaceuticals because the toxicity of aggregates may vary from one protein to another [66]. Nevertheless, in order to give a rough idea of the purity specifications of biopharmaceuticals, some indications for few relevant impurities are summarized in Table 1.1 [21].

As mentioned earlier, recombinant proteins are rarely produced as individual molecular entities, but rather as a large number of similar variants, also called isoforms, which vary by subtle differences only. Despite a growing understanding of the protein structure–function relationship, the impact of various isoforms on the biological and pharmaceutical activity of a drug remains largely unclear and can only be assessed

Table 1.1. Examples of indications for purity specifications.

Aggregates	<1.0%
HCP	<100 ng/mg
Ligand leakage	<1 ppm
Viruses	<1 particle per million doses
DNA	<10 pg per dose

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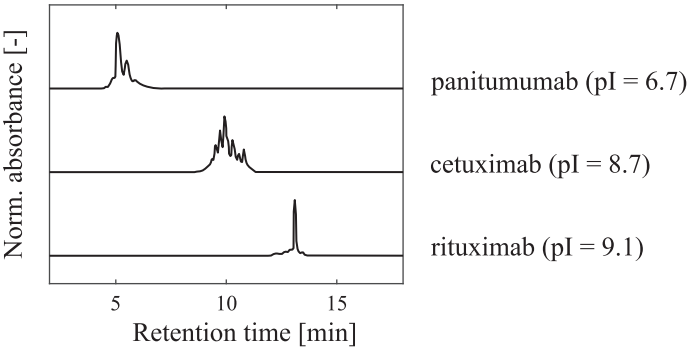

Figure 1.3 Chromatograms of three commercial mAbs obtained by analytical cation exchange chromatography using a pH gradient, illustrating the heterogeneity of biopharmaceutical products in terms of charge variants. The three selected mAbs elute at different times due differences in their isoelectric points (pI). Adapted from [67], copyright 2015, with permission from Elsevier.

by experimentation [16]. In order to guarantee drug quality, efficacy, and safety, it is paramount to maintain the distribution of these isoforms within established acceptable ranges in agreement with the ICH guidelines [57]: "An inherent degree of structural heterogeneity occurs in proteins due to the biosynthetic processes used by living organisms to produce them; therefore, the desired product can be a mixture of anticipated post-translationally modified forms (e.g., glycoforms). [. . .] The manufacturer should define the pattern of heterogeneity of the desired product and demonstrate consistency with that of the lots used in preclinical and clinical studies." For illustrative purposes, the degree of heterogeneity of some marketed biopharmaceutical products can be appreciated in Figure 1.3, which shows the chromatograms of three mAbs obtained by analytical cation exchange chromatography [67]. Each peak can be associated with at least one mAb variant.

Although it is challenging to control precisely the distribution of product variants, it is possible to influence the type and amount of these variants by acting both on the upstream and downstream parts of the production process. These considerations become particularly relevant in the case of biosimilars, where it is crucial to demonstrate that there are "no clinically meaningful differences" with respect to the reference product [68, 69].

1.4 Continuous Production Processes

In the previous sections, we described briefly the unit operations that are commonly used in the production of protein-based drugs. These considerations are sufficiently general to hold true for both batch and continuous manufacturing processes. The goal of this section is to compare these two modes of production, first in general terms and then with specific reference to biopharmaceutical processes.

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1.4.1 Definition of Batch and Continuous Processes

A discussion on batch and continuous processes requires first to give clear definitions of these two terms, which is probably less obvious than one would expect. Generally, a process is termed *continuous* if it is constituted by open units with uninterrupted inlet and outlet material flows. *Batch processes* are instead constituted by units that are prevalently closed, and the feed materials/final products are loaded/withdrawn according to some time schedule. A domestic dishwasher represents a common example of a batch installation, where a given quantity of dishes is introduced in the device, undergoes successive operations (such as washing, rinsing, and drying) and is then taken out before another washing program is started. If the operations are repeated over regular periods (for example, the washing program is started every morning), the process is termed *periodic*. On the other hand, the continuous equivalent of the domestic dishwasher would be a conveyor washing machine used in large dining facilities. Importantly, while in the batch process, cleaning operations are performed one after the other in a single unit; they are performed simultaneously but at different locations along the conveyor belt in the continuous process, allowing constant inlet/outlet of dirty/clean dishes.

It is worth noting that the notion of constant inlet/outlet depends on the scale of observation and on the level of averaging. For example, the number of dishes cleaned per minute with the conveyor washing machine is constant during the working hours of the restaurant. However, if one looks at the number of dishes cleaned per minute over a week, one observes a discontinuous process, with peaks at lunch and dinner times. On the other hand, even though the overall cleaning process in a domestic dishwasher is discontinuous, single operations may be regarded as continuous at the time scale of the considered operation and for some material flows. This is, for example, the case for water (but of course not for the dishes) during the rinsing step if water is injected and evacuated at constant flow rates. It is thus clear that the distinction between "batch" and "continuous" processes is necessarily associated with a relevant time scale and an observed variable.

Moreover, it is important to avoid confusion between the concepts of being continuous, which as mentioned earlier characterizes a system with uninterrupted inlet and outlet flows, and being at *steady state*, where all the internal variables of the units (e.g., temperature, pressure, reactant concentration) are constant in time.

Considering the particular case of cell culture, several reactor types can be envisaged [19]. *Batch bioreactors* are rather rare because the depletion of nutrients eventually causes a decrease in cell viability, which is defined as the ratio of the number of viable cells over the total number of cells. Therefore, *fed-batch bioreactors*, where some feed solution is introduced intermittently, are usually preferred. On the other hand, two types of continuous bioreactors can be considered, namely *chemostats* and *perfusion bioreactors*. In both cases, the cells are introduced in the bioreactor before starting to operate the process, so that the continuous inlet flow contains only the culture medium and no cells. However, the two bioreactors differ regarding their outlet flows. Chemostats are characterized by a single continuous outlet flow containing the supernatant and the cells in the same proportions as inside the bioreactor. On the other hand, perfusion bioreactors

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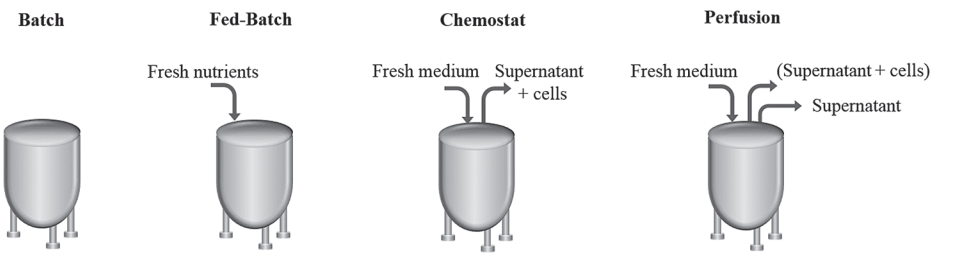

Figure 1.4 The four most common bioreactor types.

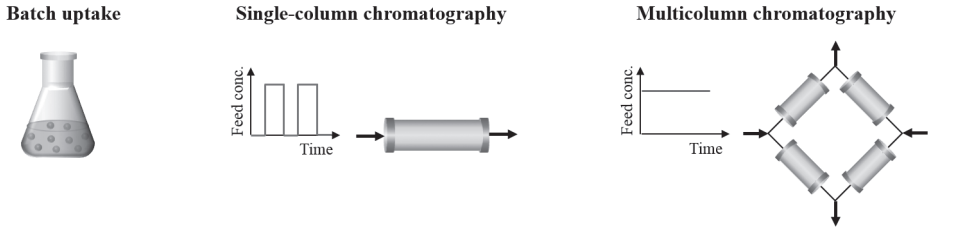

Figure 1.5 Schematic representations of batch uptake, single-column, and multicolumn chromatography.

are equipped with a cell-retention device that allows removing the supernatant, while keeping the cells inside the bioreactor [70, 71]. If one wishes to reach steady state, it is necessary to maintain a constant cell density in the bioreactor. To compensate the increase in cell density due to cell growth, a second outlet stream referred to as "bleed" is required. This outlet flow contains the supernatant and the cells in the same proportions as inside the bioreactor. The four types of reactors mentioned earlier are schematically represented in Figure 1.4.

Let us now analyze the case of chromatography, for which there is often confusion with the terms *batch* and *continuous*. By strictly applying the definitions given earlier, batch chromatography corresponds to a process in which the chromatographic medium, solutes, and eluent are mixed together in a closed vessel until thermodynamic equilibrium is reached. This process is also known as *batch uptake chromatography* and is commonly used to determine solute partitioning between the liquid and solid phases at equilibrium (see Chapter 2). However, in the literature, the term batch chromatography also designates a process in which a defined volume of the mixture to be separated is injected into a chromatographic column and eluted before the next injection starts, while the flow of eluent through the column is maintained constant. To avoid confusion with batch uptake chromatography, we prefer the term *single-column chromatography* in this book. As we will see in Chapter 2, single-column chromatographic processes are not capable of separating feed mixtures in a continuous manner. On the other hand, *multicolumn chromatography* makes it possible. Figure 1.5 illustrates schematically the three types of chromatography aforementioned: batch uptake, single-column, and multicolumn chromatography. In particular, it is seen that the feed mixture is introduced

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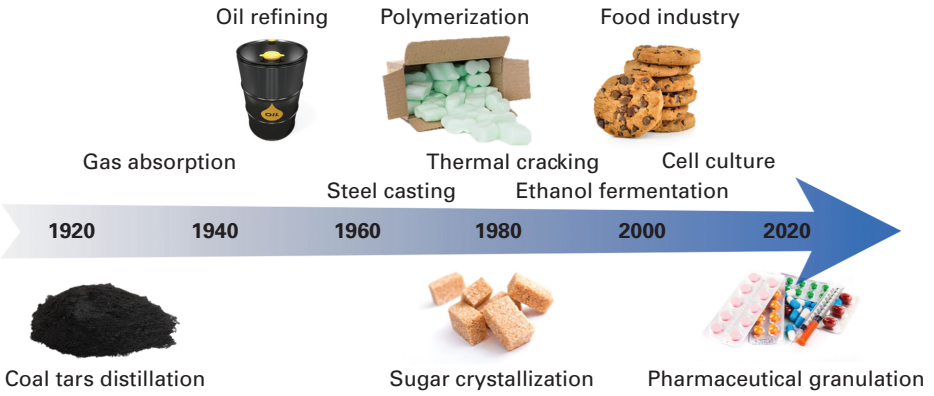

Figure 1.6 Timeline of the establishment of continuous production technologies in various industrial sectors.

by periodic injections in single-column chromatographic processes, while it can be introduced at a constant flow rate in multicolumn chromatographic processes. Fundamental concepts of multicolumn chromatography will be introduced in Chapter 3, while practical applications for the capture and polishing steps will be presented in Chapters 4 and 5, respectively.

1.4.2 Industrial Context

Continuous manufacturing has been successfully used for decades in a number of industries [72, 73], from the petrochemical to the polymer and the food industries. For illustrative purposes, Figure 1.6 shows the timeline for the establishment of continuous production processes in various industrial sectors.

These successes have driven a lot of expectations in the (bio)pharmaceutical industry for the production of both small-molecule [74–77] and protein-based drugs [78–93]. Among those expectations, one can mention a decrease of the capital expenditure (CAPEX) and a decrease of the operational expenditure (OPEX). Such prospects are often attributed to an expected reduction of the turnaround times, equipment size, solvent consumption, and manpower.

However, one needs to realize that the economic challenges faced by various industries may differ greatly, and it would thus be illusive to believe that continuous manufacturing is a one-size-fits-all solution. A careful analysis of the objectives and constraints of the particular process under consideration should be carried out in order to evaluate the pros and cons of the batch and continuous production modes, which may actually differ depending on several factors, such as the scale considered (i.e., laboratory, clinical, or commercial). Although capital and operational costs are critical for the massive production of low-value products such as chemical commodities or sugars, one can wonder what is the contribution of production costs in the manufacturing of biopharmaceuticals, where the costs associated with research and clinical tests are

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significant. The economic pressure may increase in the near future due to the loss of patent protection for several commercialized drugs and the subsequent emergence of biosimilars and biobetters [68]. Another relevant aspect in this context is the growing importance of alternative industrial approaches, such as the Indian pharmaceutical industry that strives to provide therapeutics at the lowest possible cost [94]. This new economic situation may motivate (bio)pharmaceutical companies to reconsider their current production strategies, and the transition from batch to continuous processes could therefore represent a "paradigm shift" in the words of Thomas Kuhn [95], meaning a necessary transformation to adapt to an ever-changing environment.

Finally, the regulatory requirements with respect to product quality control require some comments. The uncertainty regarding the definition of a "lot" is sometimes put forth as an obstacle for the implementation of continuous biopharmaceutical processes. However, the definition of *lot* given in section 210.3 of the code of federal regulations refers to an uniform amount of product, applicable to both batch and continuous production modes: "Lot means a batch, or a specific identified portion of a batch, having uniform character and quality within specified limits; or, in the case of a drug product produced by continuous process, it is a specific identified amount produced in a unit of time or quantity in a manner that assures its having uniform character and quality within specified limits." Therefore, agencies such as the FDA are definitely not an obstacle to the transition from batch to continuous processes and rather recommend "*cleaner, flexible, more efficient continuous manufacturing*" [96, 97].

1.4.3 Some Engineering Considerations

Let us now address the question of continuous pharmaceutical processes from an engineer point of view. For this, we first analyze separately the upstream and downstream parts of the production process. Then, we discuss their possible integration in a unique continuous process.

Upstream Processes

Before considering the specific and complex case of cell culture, we compare the intrinsic performances of batch and continuous reactors. This is a classic theme in chemical reaction engineering [98, 99] that we review here for the case of a simple homogeneous reaction involving only one reactant A and characterized by the reaction rate $\mathcal{R}_A = k_A C_A^{n_A}$, where C_A is the reactant concentration, k_A the reaction rate constant, and n_A the reaction order. We consider first a perfectly mixed reactor and analyze whether it would be more advantageous to operate it in batch or in continuous mode. These two types of reactors are commonly referred to as *batch stirred tank reactor* (BSTR) and *continuous stirred tank reactor* (CSTR), respectively, and are illustrated in Figure 1.7(a).

It can be easily shown with mass balance equations that a BSTR performs better than a CSTR for any positive value of n_A, which corresponds to the most common case where an increase in the reactant concentration leads to an increase in the reaction rate. This is illustrated in Figure 1.7(b) for $n_A = 1$, where the conversion is reported as a

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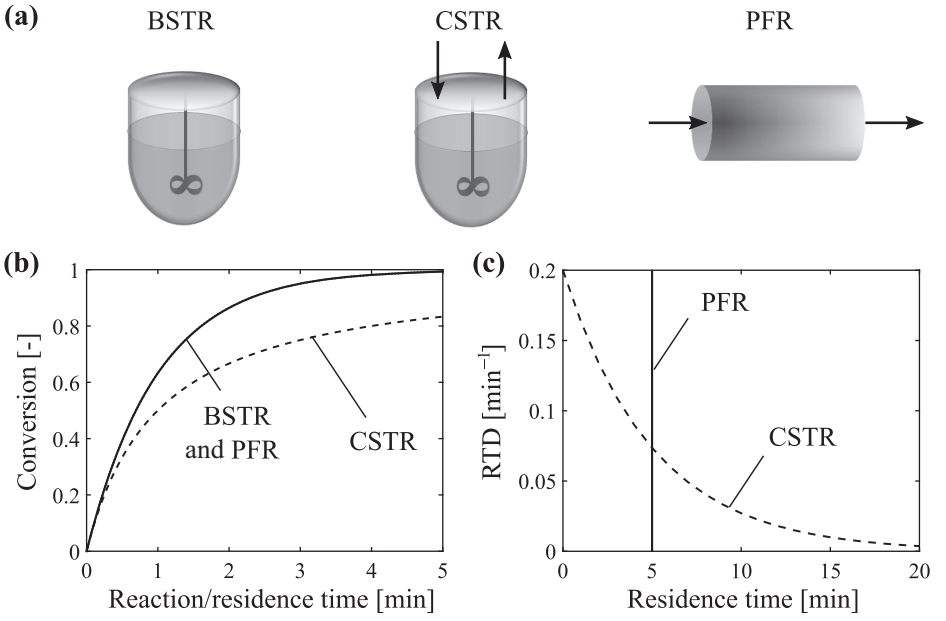

Figure 1.7 (a) Schematic representation of the most common ideal reactors. (b) Time necessary to reach a given conversion in the three ideal reactors considering a first-order reaction kinetics with a reaction rate constant of 1 min^{-1}. The term *time* refers to the reaction time in the case of the batch reactor (BSTR) and to the average residence time in the case of the continuous reactors (CSTR and PFR). (c) Residence time distribution (RTD) for a CSTR and a PFR. The average residence time, defined as the ratio between the reactor volume and the volumetric flow rate, was set to 5 min in both cases.

function of the "time" spent by the reactant inside the reactor. This "time" corresponds to the reaction time in the case of the batch reactor. On the other hand, it corresponds to the average residence time inside the reactor in the case of the continuous reactor, which is defined by the ratio between the reactor volume and the volumetric flow rate. It appears clearly in Figure 1.7(b) that the CSTR is less efficient than the BSTR at any conversion, meaning that more time is required to obtain a certain conversion as compared to the BSTR.

However, we can now consider another ideal continuous reactor called the *plug flow reactor* (PFR). It is represented as a tube, where the fluid is flowing as a series of infinitely thin plugs. It is assumed that each plug is perfectly mixed in the radial direction, while no mixing occurs in the axial direction. In such a reactor, all the molecules spend exactly the same time in the unit, which is given by the ratio between the reactor volume and volumetric flow rate. This is in contrast with the CSTR, where different molecules may follow different paths inside the reactor and thus exit at different times. The residence time distribution of a CSTR is given by a decreasing exponential function, as shown in Figure 1.7(c) for an average residence time of 5 min. As a comparison, the residence time distribution of a PFR is a Dirac function, which is also shown for an average residence time of 5 min in Figure 1.7(c).

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It is important to realize that there is a strong analogy between the behavior of a PFR in space and the behavior of a BSTR in time. This is evidenced in Figure 1.7(b), where it is seen that the conversion profile in a PFR is identical to that obtained in a BSTR, provided that the reaction time is replaced by the residence time in the reactor. It results that the continuous plug flow reactor performs better than the continuous stirred tank reactor, at least for this example. This conclusion can in fact be generalized to any positive reaction order. However, it must be emphasized that the situation may change for more complex reacting systems. Overall, this simple example shows that moving from a batch to a continuous process does not necessarily bring any improvement and may actually even decrease process performances. It is thus crucial to consider the nature of the reactions and their kinetics to select the most appropriate reactor type [98, 99].

In Chapter 6, we will apply these elementary chemical reaction engineering considerations to the particular case of protein conjugation. In this context, we will show how to design both batch and continuous reactors for the production of conjugated proteins. We will then analyze the performances of these reactors in terms of conversion, yield, and selectivity.

Here, we focus on the case of cell culture bioreactors. In light of the preceding discussion, batch or fed-batch bioreactors would be expected to perform better than chemostat and perfusion bioreactors, which can somehow be regarded as CSTRs. In the following, we examine what still makes CSTRs attractive in the particular case of cell culture.

At first, it is essential to keep in mind that running a bioreactor is a rather delicate task because cultivating living organisms requires maintaining a well-defined and well-controlled environment within the whole reactor volume and all along the protein expression step. Small variations in the operational conditions may in fact be detrimental to cell viability and cell productivity. Such variations may also affect the quality of the product, such as the level of aggregates, the distribution of charge variants or the glycosylation pattern [100]. The objective to have a cell culture medium characterized by a composition that is constant in time and uniform in space guides the choice of the bioreactor toward a CSTR. In contrast, in batch or even fed-batch bioreactors, the nutrients from the culture media are depleted during time, and inhibitory or toxic species produced by the cells accumulate. This leads to a changing environment, which may impact cell viability and cell productivity and promote the expression of heterogeneous products [101, 102]. A simplistic theoretical model has been used to compare the kinetics of protein expression in batch and continuous stirred tank reactors [103, 104], illustrating that the latter is a good candidate to maintain the constant environment necessary for cell culture.

Two types of continuous bioreactors may be envisaged to obtain conditions uniform in space and constant in time, namely chemostat and perfusion bioreactors. The possibility to reach a high cell density in perfusion bioreactors thanks to the cell retention device makes the latter very attractive for upstream processing [101]. Perfusion bioreactors are not only appealing as protein production units, but also as seed train reactors to achieve a high inoculum cell density for a subsequent fed-batch production bioreactor [105, 106]. On the other hand, perfusion bioreactors bring new technological challenges

[70, 71, 82, 107]. For example, the fouling of the membrane of the cell retention device is especially troublesome and may cause the premature termination of the perfusion process. It is also necessary to control cell culture conditions in the external loop and to develop aeration strategies suitable to high cell densities, which may be challenging at the large scale. In addition, the cells should be genetically stable, resistant to shear stress and to aggregation. Finally, guaranteeing sterility during the entire production period may be complicated by the continuous inlet/outlet flows of material.

When these obstacles can be overcome, it is possible to cultivate cells at a high density for extended periods of time and to reach steady state, which implies that the composition of the bioreactor remains constant. To illustrate this, Figure 1.8 shows the time evolution of the percentage of glycoforms of a mAb expressed in a perfusion bioreactor (closed symbols). It is observed that, after around seven days, a constant distribution of mAb variants is produced. As a comparison, the percentage of glycoforms produced at a given time by the same cell line in a fed-batch bioreactor is shown with open symbols. It is seen that the distribution of glycoforms significantly changes during time in the fed-batch cell culture experiment.

At steady state, not only the cell density, protein concentration, and isoform distribution remain constant in time, but also the intracellular concentrations. This has been shown by analyzing the concentrations of amino acids, metabolites, and proteins both in the supernatant and in the cells as a function of time [102, 110–112]. These constant conditions are reached after a transient period during which the product specifications are rather unlikely to be met. The duration of the transient period is impacted by two distinct processes [108, 109]. The first one refers to the hydraulics of the system, which depends on the inlet/outlet flow rates, reactor volume, and mixing. Like in any other stirred vessel, the duration of this process is in the order of a few times the average residence time. The second process is more complex and refers to the cell metabolism, independent of the reactor scale [113]. This process seems to be slightly slower than the first one, as indicated by the data of Figure 1.8, where the glycoform distribution achieves steady state in about seven days against the two or three that are expected based on the reactor hydraulics [109, 114].

In the following, we further compare (fed)-batch and perfusion bioreactors in terms of protein concentration, medium consumption, protein residence time, and reactor size. For the sake of simplicity, we assume that steady state is reached in the perfusion process.

Protein Concentration

Let us start with the protein concentration reached in the harvested cell culture supernatant, which is a key parameter for the economic viability of both the upstream and downstream parts of the production process. Nowadays, mAb concentrations in the order of few g/L are typically reached in fed-batch bioreactors, while concentrations two to five times lower are often reported for perfusion bioreactors [82, 85, 88]. However, this difference in concentration is most likely due to a sub-optimal operation of perfusion processes. Extensive time and efforts have in fact been devoted to optimize cell lines and culture media for fed-batch processes, which do not necessarily correspond to the

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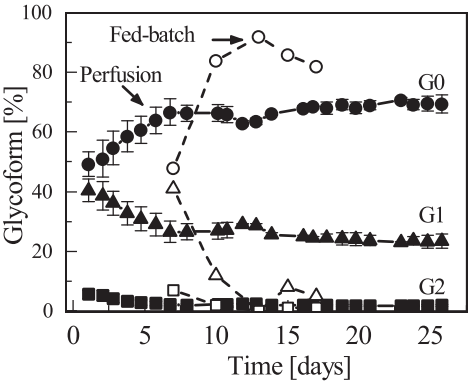

Figure 1.8 Percentage of mAb glycoforms produced by mammalian cell culture in a fed-batch bioreactor (open symbols) and in a perfusion bioreactor (closed symbols) as a function of time. Circles, triangles, and squares correspond to the G0, G1, and G2 glycoforms, respectively. In the case of the fed-batch experiment, the instantaneous distributions of glycoforms are reported (in contrast to cumulative distributions that would also account for the distributions produced at earlier times of the cell culture experiment). Adapted from [108], copyright 2017, with permission from Elsevier and from [109] by permission of John Wiley & Sons, Inc.

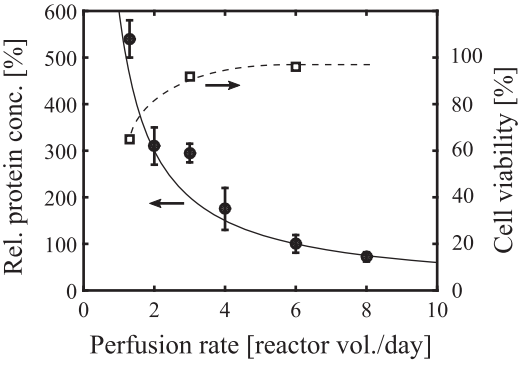

Figure 1.9 Influence of the perfusion rate on cell viability and protein concentration. The dashed line is a guide to the eyes, while the solid line represents the prediction of the decrease in protein concentration with the reciprocal of the perfusion rate. The values of the protein concentration are normalized with respect to the value corresponding to a perfusion rate of 6 reactor volume per day. Adapted from [115], with permission from Springer.

best candidates to run continuous operations. Minimizing cell growth at steady state (to reduce the cell discard rate) and improving the longtime stability of the cells against genetic mutations and clumping are examples of specific objectives to be reached in order to make perfusion bioreactors more competitive.

Figure 1.9 shows experimental results acquired during the production of a mAb by perfusion cell culture [115]. In these experiments, the volumetric flow rate of fresh medium (also called the perfusion rate) was varied between 1.3 and 8 volumes of bioreactor per day, while maintaining the cell density at 20×10^6 cells/mL by adjusting

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the bleed flow rate. It is observed that a high perfusion rate leads to the dilution of the protein inside the bioreactor and that the protein concentration is proportional to the reciprocal of the perfusion rate. In addition, it is seen that the cell viability is close to 100% for perfusion rates above 3 reactor volumes per day, but drops for lower perfusion rates. This indicates that the flow rate of fresh medium is not sufficient to ensure the minimum supply of nutriments and removal of inhibitory species necessary to maintain a good cell viability. The development and selection of proper cell lines and culture media specific to perfusion bioreactors is expected to allow reducing the perfusion rate while maintaining a high cell viability, thus alleviating the protein concentration difference between fed-batch and perfusion modes.

Medium Consumption

Another critical component in the economical evaluation of protein production by cell culture is medium consumption, often expressed in volume of cell culture medium per amount of produced protein. For a fed-batch bioreactor, the medium consumption is given by the reciprocal of the protein concentration at harvest. For a perfusion bioreactor, it is given by the reciprocal of the protein concentration at steady state if there is no bleed stream. Therefore, the lower protein concentrations currently reached in perfusion bioreactors result in higher medium consumption as compared to fed-batch processes. In addition, the loss of depleted medium in the bleed stream to compensate for the cell growth rate further contributes to the large medium consumption. Nevertheless, the development of dedicated cell lines and culture media mentioned earlier is expected to improve the protein titer and limit the growth rate, thus reducing the medium consumption in perfusion bioreactors.

Residence Time

A relevant process variable to be considered is the average residence time of the protein in the bioreactor. In a batch cell culture, proteins produced at the beginning of the cultivation step spend the whole production time inside the bioreactor, while proteins produced later on only spend a fraction of it. To give an order of magnitude, proteins spend on average roughly one week in a fed-batch bioreactor. In the case of perfusion bioreactors instead, the average residence time is given by the ratio between the volume of the bioreactor and the volumetric flow rate of fresh medium. For instance, proteins spend on average half a day in a bioreactor whose perfusion rate is two reactor volumes per day. This reduction in the residence time may be particularly useful for unstable proteins, which are, for example, prone to aggregation, fragmentation, deamidation, or oxidation. An emblematic example is the production of recombinant factor VIII, which is used in the treatment of hemophilia A. Indeed, the high aggregation propensity of this protein encouraged the development of the first worldwide licensing of a recombinant protein produced by perfusion mammalian cell culture (Kogenate®) [24]. The average residence time was reduced from 12 days in the case of the batch process to 2–3 h in the perfusion process. Nevertheless, it has to be noted that a short residence time is associated with a high perfusion rate, which means a high medium consumption and a low protein concentration. There is, therefore, a compromise to be found between protein degradation, medium consumption and protein concentration.

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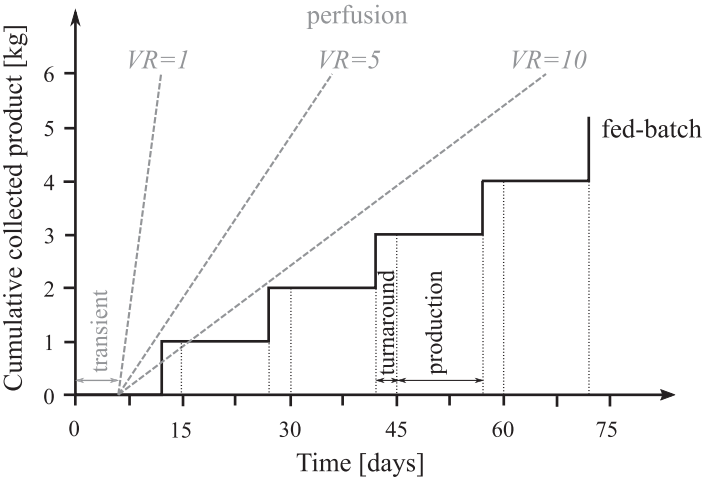

Figure 1.10 Comparison between fed-batch and perfusion cell culture in terms of reactor size. For the fed-batch bioreactor, a production time of 12 days and a turnaround time of 3 days are considered. For the perfusion bioreactor, it is assumed that 6 days are needed to reach steady state. The cell culture supernatant is collected at a protein concentration of 1 g/L in both cases. The perfusion rate is set to one reactor volume per day. The volume of the fed-batch reactor is set to 1000 L, while three different volumes are considered for the perfusion bioreactor, namely 1000 L, 200 L, and 100 L, corresponding to volume ratios (*VR*) equal to 1, 5, and 10, respectively.

Bioreactor Volume

Finally, let us compare fed-batch and perfusion cell cultures in terms of bioreactor size. To do so, we consider the illustrative example represented in Figure 1.10, which shows the cumulative quantity of product collected as a function of time for a typical process (see numerical values in the caption). In the case of the fed-batch bioreactor, no product is collected during the cell culture, and it is only at the end of the culture that the reactor is harvested and the product collected. Then, a turnaround time is necessary for cleaning and start-up operations before a new cell culture run can be started. This leads to the stepwise cumulative collected product profile represented by the black solid line in Figure 1.10. Regarding perfusion bioreactors, we assume that no product is collected during the transient period, i.e., the time before reaching steady state. Then, the quantity of collected product is linearly increasing during the whole time of operation. This is shown by the dotted straight lines in Figure 1.10, which correspond to different values of the volume ratio *VR* defined as the ratio of the volume of the fed-batch bioreactor over that of the perfusion bioreactor.

If the two reactors (fed-batch and perfusion) have the same volume and are operated at the same protein concentration, it is seen in Figure 1.10 that the quantity of product collected from the perfusion bioreactor is always much higher than the one collected from the fed-batch bioreactor, indicated as $VR = 1$ in Figure 1.10. The size of the perfusion bioreactor can therefore be significantly reduced while collecting similar quantities of product. This does not only have a positive impact on the investment costs, but also allows achieving a better mixing, i.e., a more uniform distribution of nutrients

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Table 1.2. Examples of marketed proteins produced by perfusion cell culture. The dates correspond to the years of approval.

Name	Trade name	Company	Year
Abciximab	Reopro®	Janssen Biologics B.V.	1994
β-glucocerebrosidase	Cerezyme®	Genzyme	1994
^{111}In capromab pendetide	ProstaScint®	Cytogen	1996
Follicle-stimulating hormone	Gonal-f®	Merck-Serono	1997
^{99m}Tc votumumab	Humaspect®	Organon Teknika	1998
Interferon β-1a	Rebif®	Merck-Serono	1998
Basiliximab	Simulect®	Novartis	1998
Infliximab	Remicad®	Janssen Biotech / Merck & Co	1998
Factor VIII	Kogenate-FS®	Bayer	1998
Activated protein C	Xigris®	Eli Lilly	2001
Agalsidase β	Fabrazyme®	Genzyme	2003
Alglucosidase α	Myozyme®	Genzyme	2006
Golimumab	Simponi®	Johnson & Johnson / Merck & Co	2009
Ustekinumab	Stelara®	Janssen Biotech	2009
Siltuximab	Sylvant®	Janssen Biotech	2014

and oxygen in the bioreactor. For illustrative purposes, it is shown in Figure 1.10 that a reduction of the reactor volume by a factor 10 can be achieved to obtain a similar productivity in the perfusion process as compared to the fed-batch process (indicated as $VR = 10$) in our example. The actual volume reduction depends on the values of the process variables, such as the turnaround times and protein concentration. For instance, the volume reduction would be even more pronounced for a longer turnaround time between consecutive batches. On the other hand, if the perfusion bioreactor were operated at a protein concentration lower than in the fed-batch bioreactor, as it is still often the case, the volume reduction would be lower.

Overall, the choice of the bioreactor should be dictated by the constraints and objectives of the process, taking into account several factors such as cell stability, protein degradation, medium consumption and productivity. In the literature, the comparison between fed-batch and perfusion technologies has been widely discussed, also in economic terms, indicating the variety of scenarios that can be encountered at the industrial level [82, 85, 86, 88]. Several marketed biologics are currently produced by perfusion cell culture, including blood factors, enzymes, and mAbs [82]. Examples are listed in Table 1.2.

Downstream Processes

Let us now consider the downstream part of the manufacturing process. Chromatography is a crucial technique in the purification of therapeutic proteins, and it is thus important to understand if and how continuous multicolumn chromatography performs better than single-column chromatography. We have seen with simple reaction engineering considerations that moving from a batch to a continuous process does not necessarily lead to improved process performances. The same holds true for chromatography. Indeed, a continuous chromatographic process can be obtained by properly parallelizing

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multiple columns whose loading phases are simply shifted in time so as to allow a continuous feed of the material to be treated and a continuous recovery of the products. This situation is, for example, typical for wastewater treatment, where the feed stream comes from the outlet of a continuous production unit. A peculiar realization of this parallelization is annular chromatography, where the feed is introduced on top of a rotating chromatographic bed packed in between two concentric cylinders [116–119]. Due to the negligible mixing in the radial direction, the contact between the liquid and solid phases in annular chromatography is the same as in a single-column process [120, 121]. Therefore, even though continuous, parallelized and annular chromatographic processes do not bring any improvement in terms of separation performances as compared to single-column processes.

In fact, rather than their continuous nature, it is their ability to simulate a countercurrent movement between the fluid and solid phases that makes multicolumn chromatographic units particularly efficient. The fundamentals of countercurrent chromatography will be presented in Chapter 3, while here we simply show a schematic representation of countercurrent contact in Figure 1.11 for illustrative purposes. This example is inspired from the aquatic respiration of fishes, whose gills allow the uptake of oxygen dissolved in water. Briefly, fish gills are composed of lamellae densely irrigated with blood vessels that are permanently put in contact with water pumped through the fish mouth. The orientation of the gill lamellae is such that the water flows in the opposite direction to the blood [122]. In order to better understand how this countercurrent flow helps the aquatic respiration, it is easier to first take a look at the opposite case, i.e., when

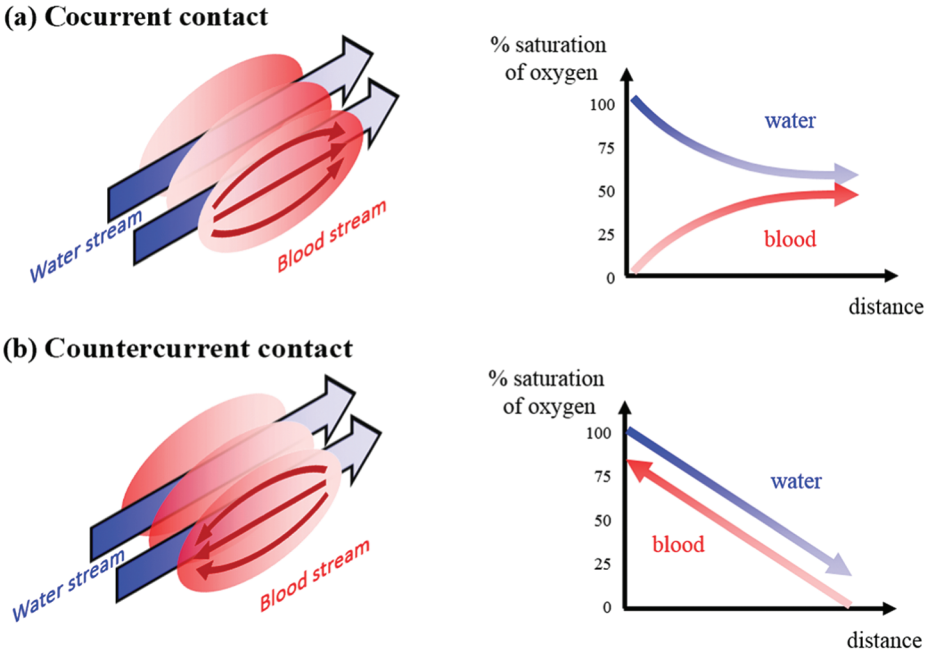

Figure 1.11 Illustration of the oxygen transfer from water to blood in fish gills. (a) Hypothetic cocurrent configuration. (b) Countercurrent configuration.

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the water and the blood flow cocurrently. To do so, a hypothetic cocurrent gas exchange system is represented in Figure 1.11(a). In this cocurrent oxygen exchange system, the inlet water, which is saturated in oxygen (represented in dark blue) comes into contact with the bloodstream which contains very little oxygen (represented in light red). In this configuration, the oxygen transfer from the water to the bloodstream is very fast at the entrance of the gill due to the large difference in oxygen concentration. All along the lamella, the oxygen concentration in the water diminishes (light blue) and is transferred to the bloodstream (dark red). Eventually, the gas transfer stops once the concentrations in the two streams are at equilibrium, as schematically shown on the right-hand side of Figure 1.11(a). On the other hand, when the water flows in the opposite direction than the blood, i.e., the two streams are flowing countercurrently as shown in the left-hand side of Figure 1.11(b), gas transfer occurs all along the lamella. In this configuration, the oxygen-rich blood is brought in contact with the inlet water stream, which is saturated in oxygen. Oxygen uptake by the oxygen-rich blood is still possible because it is in contact with the water stream that contains oxygen at the highest possible concentration. The outlet water stream, which is already partly depleted in oxygen, still contains enough gas to ensure transfer with the oxygen-poor blood. This is schematically represented in the diagram on the right-hand side of Figure 1.11(b), where it is seen that the oxygen concentration in the blood can reach higher values as compared to the cocurrent case. This simple example illustrates how countercurrent exchangers outperform cocurrent ones, which will be particularly important to develop efficient separation processes, as discussed in the following chapters.

The evolution of multicolumn countercurrent chromatography over the years has been quite rich [79]. It started with the invention of the simulated moving bed (SMB) process by Broughton for the separation of xylene isomers in the middle of the twentieth century [123]. Since then, SMB processes found applications in the food industry, and in particular in the production of sugars, as well as in the pharmaceutical industry, especially for the separation of optical isomers [79]. More recently, several variations of the SMB process were developed in order to adapt to the needs of the biopharmaceutical industry [83]. The classical SMB process will be introduced in Chapter 3, while other countercurrent chromatographic processes suitable for the protein capture and polishing steps will be presented in Chapters 4 and 5, respectively.

Although various countercurrent multicolumn chromatographic systems are available for the continuous purification of therapeutic proteins at the laboratory and pilot scales, these processes are not well established at the commercial scale yet. The first applications of continuous chromatographic systems for therapeutic protein purification were reported in the patent literature approximately in the mid-2000s, which is more than ten years after the first marketed mAb was produced continuously by perfusion cell culture. In fact, Table 1.2 shows that continuous perfusion bioreactors are already well implemented in the biopharmaceutical industry. In addition, technologies for continuous centrifugation and continuous filtration are also well established [81]. Therefore, continuous chromatography appears to be the bottleneck for the implementation of integrated continuous processes in the biopharmaceutical industry, which is one of the motivations for writing this book.

Integrated Processes

After having analyzed the unit operations separately, considering now the complete process as a whole offers additional perspectives. Exploiting the unity of the process and the connections between the different units is referred to as *process integration* and is applicable to both batch and continuous modes [124–126].

The choice between batch or continuous mode for the individual unit operations is largely case-specific. However, it is important to realize that ensuring a proper continuity between the various units is key to an effective process integration. In particular, turning only one unit from batch to continuous mode implies to invest either in hold tanks to store inlet and outlet streams or in more units to parallelize the rest of the process and match the different residence times. Therefore, the transition from batch to continuous production technologies may necessitate a holistic redesign of the manufacturing process to achieve end-to-end integration.

The integration of reaction and purification steps will be further discussed in the context of protein conjugation in Chapter 6. A few examples of integrated continuous processes for the production of mAbs, integrating the upstream and downstream operations, have been published in the literature [127–129]. For illustrative purposes, we report two of such examples in the following. These two examples deal with the continuous production of mAbs with perfusion bioreactors equipped with an alternating tangential flow (ATF) system and mainly differ in their downstream part.

In the first example [129], the protein capture step was performed with affinity chromatography using a two-column process, namely the CaptureSMB, which is further described in Chapter 4. A surge bag between the bioreactor and the CaptureSMB was necessary because the feed flow rate of the selected chromatographic unit is periodically changing in time, while the outlet flow rate of the perfusion bioreactor is constant. It is also worth mentioning that sterile filters were added at the inlet and outlet of the surge tank in order to minimize the risk of contamination. The Protein A eluate was kept in a retention device at pH 3.2 for 30 min. Such low pH hold is a standard viral inactivation procedure, as described in Section 1.2.5. Then, two polishing steps were implemented: the first one using a two-column solvent gradient purification unit (MCSGP, further described in Chapter 5) packed with a cation exchange chromatographic medium; and the second one using a single-column unit packed with an anion exchange medium. Before loading the content of the viral inactivation device in the MCSGP, the pH of the solution was adjusted thanks to inline dilution to approximately 5.5 to ensure sufficient binding on the cation exchange medium. Due to the functioning mode of the MCSGP, the mAb was recovered only intermittently at the outlet of the first polishing step. This explains that operating conditions could be found to perform the second polishing step with only one single-column unit. In the case where a continuous stream would exit the preceding unit, several single-column processes in parallel would need to be implemented to avoid intermediate storage (or one should consider using another continuous unit).

The reactor was operated for 18 days continuously, and in the last 4 days it was connected to the downstream units. The concentration of the mAb and of three representative impurities (HCP, DNA and leached Protein A ligands) at steady state at different

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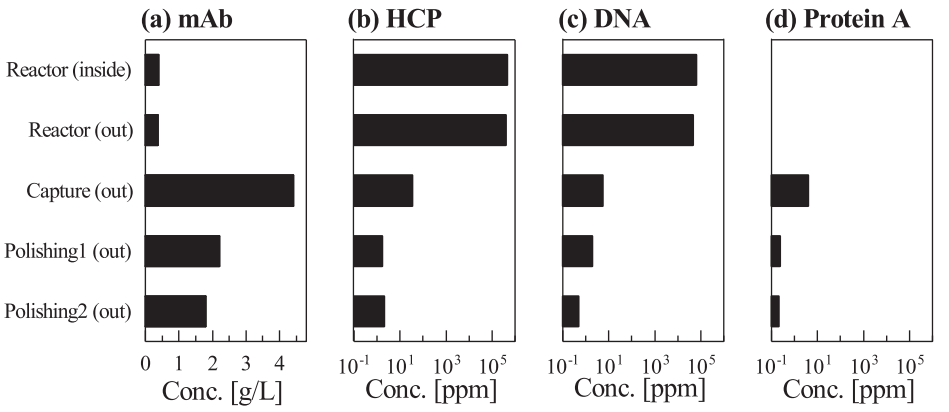

Figure 1.12 Illustrative example of an integrated process for mAb production. Concentrations of (a) mAb, (b) HCP, (c) DNA, (d) leached Protein A at steady state at different locations. Adapted from [129] by permission of John Wiley & Sons, Inc.

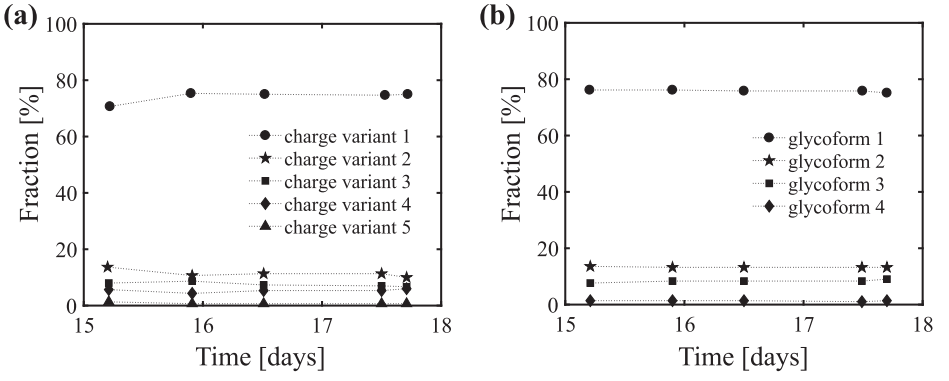

Figure 1.13 (a) Charge variant and (b) glycoform distributions at the outlet of the integrated process reported in Figure 1.12. Adapted from [129] by permission of John Wiley & Sons, Inc.

locations of the process are presented in Figure 1.12. It is seen that no differences in composition are observed between the reactor content and the harvested cell culture fluid, indicating that no undesired retention in the cell filtration device occurred. Then, it is observed that the protein capture step allowed reducing the concentration of both HCP and DNA by approximately 4 orders of magnitude, while achieving an increase in the mAb concentration by a factor of 12. The subsequent polishing steps further reduced the concentration of residual HCP and DNA as well as the concentration of affinity ligands leaching from the mAb capture step. This also resulted in a decrease in the mAb concentration by a factor of around 2. Overall, the polishing steps allowed depleting the content of HCP and DNA by a factor of 5 approximately with respect to the mAb. Figure 1.13 shows the charge variant and glycoform distributions during the last 3 days of steady state operation at the outlet of the integrated process, showing that a product of constant quality is produced over time.

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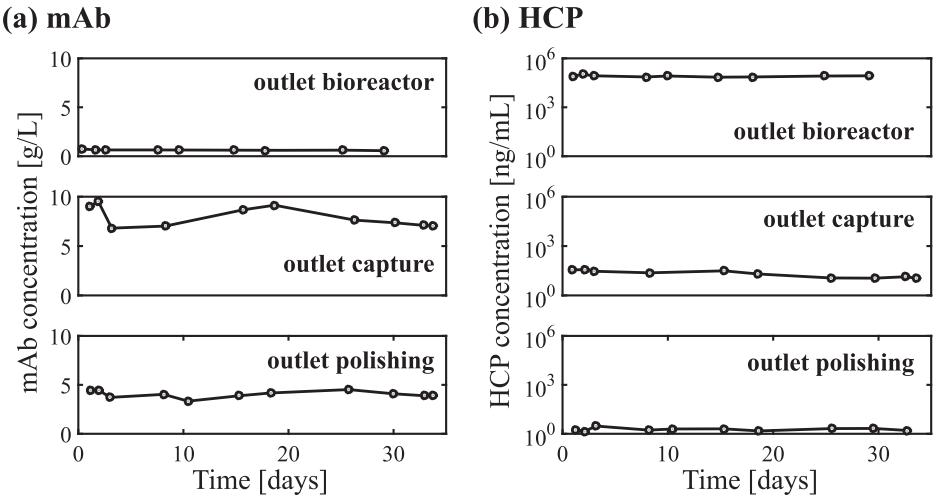

Figure 1.14 Illustrative example of an integrated continuous process for mAb production. (a) Profiles of the mAb concentration at the outlet of the bioreactor, capture and polishing steps during 1 month of operation. (b) Profiles of the HCP concentration at the outlet of the same three steps. Adapted from [128], copyright 2015, with permission from Elsevier.

In the second example [128], the protein capture step was performed with affinity chromatography using a three-column periodic countercurrent chromatographic process, which is abbreviated as 3C-PCC. As in the previous example, the clarified harvested fluid exiting the bioreactor was injected in a surge bag through a sterile filter before being loaded in the first chromatographic unit. The mAb concentration at the outlet of the bioreactor was maintained at 1 g/L during 1 month of operation, as seen in the top panel of Figure 1.14(a). As an example of impurities that need to be removed by the downstream process, the HCP concentration profile at the outlet of the bioreactor is shown in the top panel of Figure 1.14(b). It is observed that the HCP concentration is constant in time at the bioreactor outlet and in the order of 10^5 ng/mL.

The concentration profiles of mAb and HCP after the capture step are shown in the middle panels of Figure 1.14(a) and (b), respectively. It is observed that the capture step allowed increasing the protein concentration by almost an order of magnitude and decreasing the concentration of HCP by four orders of magnitude. The Protein A eluate was then pumped in a stirred vessel with a residence time of 1 h for viral inactivation at pH 3.75. The polishing step was performed with a second 3C-PCC system using a cation exchange chromatographic medium. Importantly, the pH and ionic strength of the solution had to be adjusted in-line before loading the cation exchange column, as in the previous example. The concentration profiles of mAb and HCP after the polishing step are shown in the bottom panels of Figure 1.14. It is seen that the mAb concentration decreased from a bit less than 10 g/L to around 5 g/L and that cation exchange chromatography further reduced the concentration of HCP by one order of magnitude. Overall, the polishing step allowed depleting the content of HCP by a factor of 5 approximately with respect to the mAb.

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These two examples demonstrate the feasibility of integrated continuous bioprocesses. They also highlight some important aspects that need to be accounted for when designing such processes: *(i)* the flow rates and times associated with the different units should be properly adapted to minimize the use of surge tanks and parallel units, which increase the equipment footprint; *(ii)* the buffer solutions should be selected with a view to reducing the use of in-line dilution, which increases the volumes of solution to be treated; and *(iii)* particular care should be taken to avoid contamination during prolongated periods of time. Another important aspect to be considered in the future development of continuous bioprocesses is the implementation of online monitoring strategies and control systems. These are needed to fully exploit the potential of an integrated process, where measurements taken all along the process can be used to adapt the operating conditions of the different units in order to reject disturbances and maintain optimal process performance [130, 131]. Overall, envisioning continuous technologies in the biopharmaceutical industry needs to be part of a general reflection about the integration and the organization of the different unit operations.

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