Therapeutic Proteins and Advanced Therapy Medicinal Products

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Abstract

This chapter is divided in two parts: Therapeutic proteins and Advanced Therapy Medicinal Products (ATMPs).

Therapeutic Proteins.

Therapeutic proteins are large molecules that are different from low molecular weight medicines. They consist of amino acid chains, sometimes decorated with polysaccharides (glycoproteins) that fold in space in secondary and tertiary structures. The first section describes the production process which is divided into upstream and downstream processing. It encompasses cell line development and expression of biopharmaceutical products in Upstream Processing (USP), the purification of the products during Downstream Processing (DSP), as well as the subsequent formulation process (which may be part of DSP or done afterwards).

Therapeutic proteins are sensitive to chemical and physical degradation. The formulation section describes the forces that stabilize a therapeutic protein and potential degradation pathways. It then discusses the challenges a formulator encounters when developing a protein medicinal product: (1) the development of analytical techniques for monitoring critical quality attributes, (2) the selection of the relevant tests to optimize stability and the excipients available, (3) the options for primary packaging materials and (4) the link with the preferred routes of administration. Ultimately, this should lead to achieving the predefined target product formulation profile for a biological.

The predominant routes of administration for therapeutic proteins are the intravenous and subcutaneous (s.c.) route. In contrast to intravenous administration,

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absorption of the protein from the subcutaneous reservoir upon s.c. injection is an extra step, resulting in a longer time to reach the maximum plasma concentration (Cmax) as compared to intravenous administration.

Therapeutic proteins are very important treatment options for a variety of diseases. An adverse event specifically linked to therapeutic proteins is immunogenicity. An immune response against a therapeutic protein can have a major impact on efficacy and/or safety and can be influenced by either patient-, disease-, and product-related factors. Especially, the product-related factors are important to take into consideration during product development.

ATMPs.

The second part of the chapter is devoted to the discussion of Advanced Therapy Medicinal Products (ATMPs). These are medicines made from, or consisting of, cells, genes or tissues. They have introduced a new area of specialism to the pharmacy workforce. The section about ATMPs will explore the role of the pharmacy team in the delivery of ATMPs recognising that operationalising ATMPs requires a collaborative multidisciplinary approach to ensure that the medicines are optimised for patients. This involves ensuring that whilst appropriate pharmacovigilance and pharmaceutical procedures are in place, handling is undertaken by a trained workforce that is competent in the handling of cellular products. The second part describes the legislation applicable to ATMPs and focuses on specific operational considerations including the logistics and product handling requirements related to ATMPs and the implications for pharmacy.

What Is New?

In the previous edition of Practical Pharmaceutics therapeutic proteins and ATMP's were not discussed. Nowadays, a pharmacist is regularly in contact with these classes of medicines. Therefore, this chapter is newly added in the second edition. The chapter is written by several authors, experts in the specific topics of the chapter.

Learning Objectives

After reading this chapter you will understand

- the basics of the upstream and downstream process of the production of therapeutic proteins
- the stability issues typical for therapeutic proteins
- the basic idea of the 'analytical toolbox' used to monitor therapeutic protein quality attributes
- the role excipients play in the formulation process
- the legislation applicable to ATMPs
- the different classes of ATMPs, their characteristics and how they differ from traditional medicines
- the different storage and handling requirements of ATMPs
- the implications of ATMPs for Pharmacy

24.1 Orientation

This newly added chapter deals with therapeutic proteins (biotherapeutics), advanced therapy medicinal products, and -briefly- mRNA vaccines. These new entities of medicinal products were developed over the last decades, and the number of authorized products is rapidly growing. The main differences to small molecule medicines are the production of the active substances for therapeutic proteins by living cells, the specific formulation of the medicinal products, the sophisticated handling, and (usually parenteral) administration of the medicinal products.

Therapeutic (glyco)proteins are developed in living organisms by recombinant biotechnology (rDNA) and monoclonal antibody technologies. Bacteria, yeasts, and mostly mammalian cells are used as host cells. The production of a target protein requires the development and implementation of robust, high-yielding biotechnology processes, including cell line development, fermentation, harvesting, and purification by different filtration and chromatography techniques. In addition, the formulation, manufacturing, storage, and handling of the medicinal products containing therapeutic (glyco)proteins needs special attention. Special efforts were necessary to implement suitable quality controls for the protein products. Various orthogonal physicochemical and biological assays are needed to characterize the protein structure and to cover the critical quality attributes defined for medicinal products. Glycoproteins (including monoclonal antibodies) have intrinsic heterogeneities and they display a large diversity of quality attributes. The compatibility and stability of therapeutic proteins is limited by elevated temperatures, freeze-thawing, exposure to light, pH changes, exposure to oxygen, contact with surfaces, and excessive agitation. Conformational changes and aggregation (higher molecular weight aggregates, as well as submicron-, subvisible- and visible particles) are characteristic for protein products and often the first sign of instability.

The first rDNA biological product brought to the market was recombinant human insulin. Since then, numerous hormones, cytokines, and especially monoclonal antibodies (Mab) were developed and licensed. A large number of Mab products that bind to an increasing number of different targets are meanwhile available for many indications, mainly in autoimmune and malignant diseases. Innovative Mab therapies in oncology indications utilize antibody drug conjugates where the Mabs are linked to cytotoxic or radioactive substances and provide specific delivery of the active substance to the tumour cells.

Next to the originator biotechnological medicines, biosimilars are licensed, when the originator products come out of patent. The first biosimilar guidance was released by the European Medicine Agency (EMA) in 2001. The overarching and product specific guidance documents represent a robust legal framework ensuring the safe use of biosimilars.

Because of their physicochemical properties (glyco)proteins are administered parenterally, mostly s.c. with ready-to-use-syringes or pens or by i.v. infusion. Solution concentrates or powders need to be reconstituted and diluted prior to infusion in most cases. Healthcare personnel need to be familiar with the good reconstitution practice and/or good preparation practice of therapeutic proteins. These proteins are also prepared in stock within the pharmacy for efficiency and safety reasons. Pharmacy preparation must take place within the framework of a pharmaceutical quality management (see Chap. 34) compare [1].

The term "advanced therapy medicinal products" (ATMP) is used to designate gene therapies (GTMP), somatic cell therapies, and tissue engineered products. GTMPs contain genes that lead to a therapeutic, prophylactic or diagnostic effect. They work by inserting 'recombinant' genes into the body, usually to treat a variety of diseases, including genetic disorders or cancer. Rapidly evolving technology in the field of gene therapy brings exciting new opportunities for treating a range of diseases, including many currently considered incurable. Cell-based GTMPs are treatments using cells from the patient or a donor. Because these cells are substantially genetically modified, they are not considered transplants. To ensure safety and benefits of GTMPs and such cell-based therapies, they are regulated in the EU as medicinal products. ATMPs are governed by Regulation 1394/2007 (ATMP Regulation) and must obtain an authorization by the Commission prior to coming to the market. Under certain conditions, member states may permit the use of advanced therapies as so-called "hospital exemption". In any case, the responsible pharmacists need to know the specific regulation, the specialties of product formulation, logistics, storage, preparation practice including occupational and environmental risks as well as proper administration of GTMPs. Pharmacists should also be familiar with the benefits and risks of the treatment and take responsibility for post-authorization pharmacovigilance by collecting, assessing, and preventing adverse events.

Somatic cell therapeutics consist of cells and tissues which were substantially processed so that biological and structural characteristics or physiological functions were modified. These cells or tissues have pharmacological, immunological, or metabolic effects. Tissue-engineered medicinal products contain cells or tissues that have been modified so they can be used to repair, regenerate or replace human tissue. These types of products are also authorized and handled according to the ATMP regulation.

mRNA based vaccines represent the latest development of innovative medicinal products. The innovative vaccines deliver the transcript of interest as mRNA, encoding one or more immunogen(s), into the host cell cytoplasm where expression generates translated protein(s) inducing an immune response [2]. The potential advantages of mRNA technology are rapidity of manufacture in a cell-free and scalable process. Currently, the mRNA technology primarily targets infectious diseases and cancer [3]. Formulation and reconstitution of mRNA based vaccines needs special attention.

The goal of this chapter is to introduce pharmacists to two classes of modern medicines. They should become aware of the special features of biologicals and cell-based therapies, including manufacturing, formulation and Good Preparation Practices.

General information about these products can also be found in EMA documents.

PART 1: Therapeutic Proteins

24.2 Production of Therapeutic Proteins

24.2.1 Introduction

Developing a robust, high-yielding biotechnology process, including cell line development and expression of biopharmaceutical products in Upstream Processing (USP) and the purification and formulation of the products with Downstream Processing (DSP), is a complex and challenging task, see Fig. 24.1 for an example of a USP-DSP train. A good result is based on a combination of sophisticated technologies, high-quality starting materials, and structured process design. A good understanding of the basic process concepts and the underlying biological phenomena is required to succeed, both in USP and DSP [4].

24.2.2 Upstream Processing

An intriguing and challenging characteristic of USP is the fact that living organisms are used, and they do not always behave as predicted. In the past decades, tremendous progress has been made in USP, and modern fed-batch processes can deliver 5–10 g/L of biotherapeutic proteins or more. This is only feasible by an increased understanding of cell cultures, which has led to better cell culture media, more advanced feeding strategies, more robust cell lines, and bioreactor control tailored for specific processes. The basic concepts of USP are cell line development (Sect. 24.2.2.1), cell culture media (Sect. 24.2.2.2), fermentation (Sect. 24.2.2.3) which can be further defined in pre-culture and seed train (Sect. 24.2.2.3.1), bioreactor (Sect. 24.2.2.3.2) and harvesting (Sect. 24.2.2.3.3). Figure 24.2 shows an example of a USP train how to produce product from a vial towards a 2000 L Bioreactor scale [4].

24.2.2.1 Cell Line Development

In this section we will focus on the development of cell lines for production of biotherapeutic proteins, exemplified by

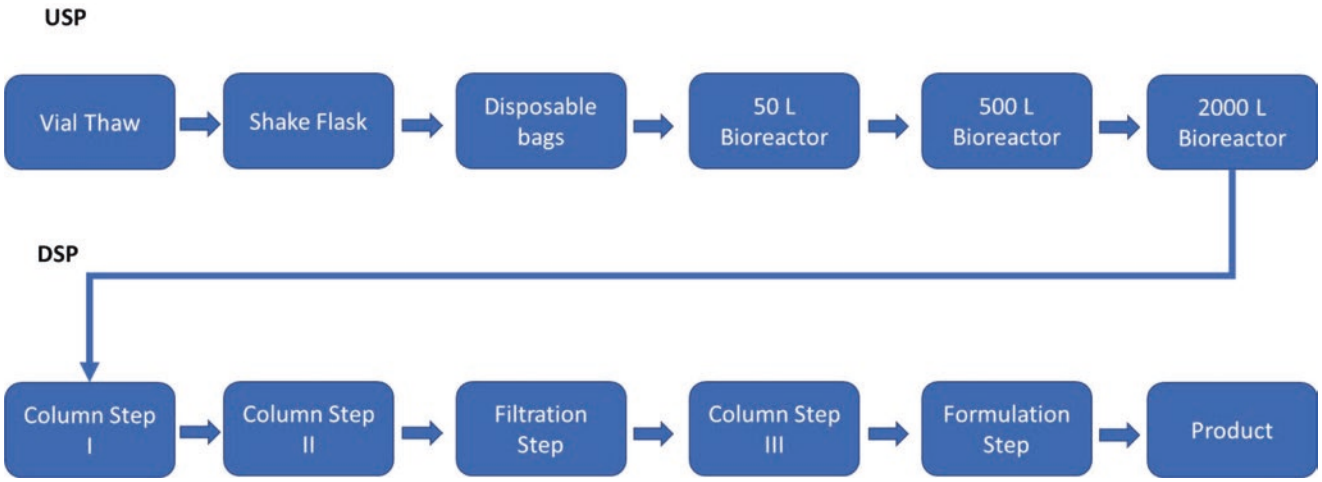


Fig. 24.1 USP-DSP process train for a recombinant protein

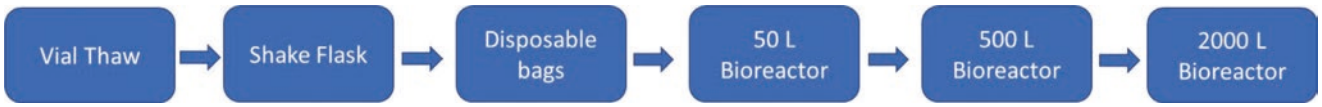


Fig. 24.2 Example of an upstream manufacturing process

Chinese Hamster Ovary (CHO) cells, which is the most widely used cell type for the production of complex biotherapeutic proteins such as monoclonal antibodies. Other typical cell lines are SP2/0, PerC6, NS0 and HEK293. A brief overview will be given on how stable cell lines are generated, requirements for clonality and some insights into the engineering of host cell lines.

Cell line development can be considered the core of the biotechnology processes for developing biotherapeutic proteins. Cell lines suitable for stable production of recombinant proteins must be possible to scale up for commercial Good Manufacturing Practice (GMP) production. The cell line should be:

1. Adapted to serum free media
2. Able to grow in suspension media
3. Able to grow at a high growth rate
4. Able to secrete the biotherapeutic protein

The cell lines should also have an efficient cell metabolism without excess excretion of by-products and a well-tuned protein secretion and glycosylation machinery, which is indispensable for the development of biotherapeutic proteins. Furthermore, cell lines should be stable with respect to product quality and productivity over time (e.g. >70 generations), and the timelines and population doubling from gene transfection to production cell line should be as short as possible. Other critical points are the monitoring of each raw material that is supplemented into the culture medium at any step during cell line development and thus interacting with final production cells. Since the mad cow disease crisis in 1990s, each culture medium component must be certified animal component-free (ACF) to ensure maximum safety profiles.

The process of cell line development starts with the introduction of plasmid DeoxyiboNucleic Acid (DNA) encoding the gene of interest, gene regulatory elements and a selectable marker into the production of host cell lines also known as transfection. An example of a plasmid expression vector is given below in Fig. 24.3. The elements outlined in the figure are the coding sequences for the gene product of interest, the antibiotic selection marker, promotors, signal peptides, and sequences related to the production of the vector in *E. coli*. The vector elements are usually produced in *E.coli* and bacterial plasmids. Normally high copy numbers are used to increase the yield of DNA from bacterial cultures. After bacterial culture, the plasmid DNA is prepared and purified. The construction of the recombinant DNA vector is achieved through modification of the DNA with purified restriction enzymes, e.g. nucleases, ligases and polymerases. After stable transfectants (pools) have been selected, single cell cloning (e.g. by FACS, limiting dilution) is performed to generate a monoclonal cell line which is derived from an individual cell. This is achieved by depositing hundreds of different single cells individually into 384- or 96-well microplates, followed by clonal expansion until enough cells can be cryopreserved. During clone development cells are assessed for cellular productivity and growth performance to select the optimal clones. Eventually, the best clones are characterized in detail using representative scale-down cultivation models

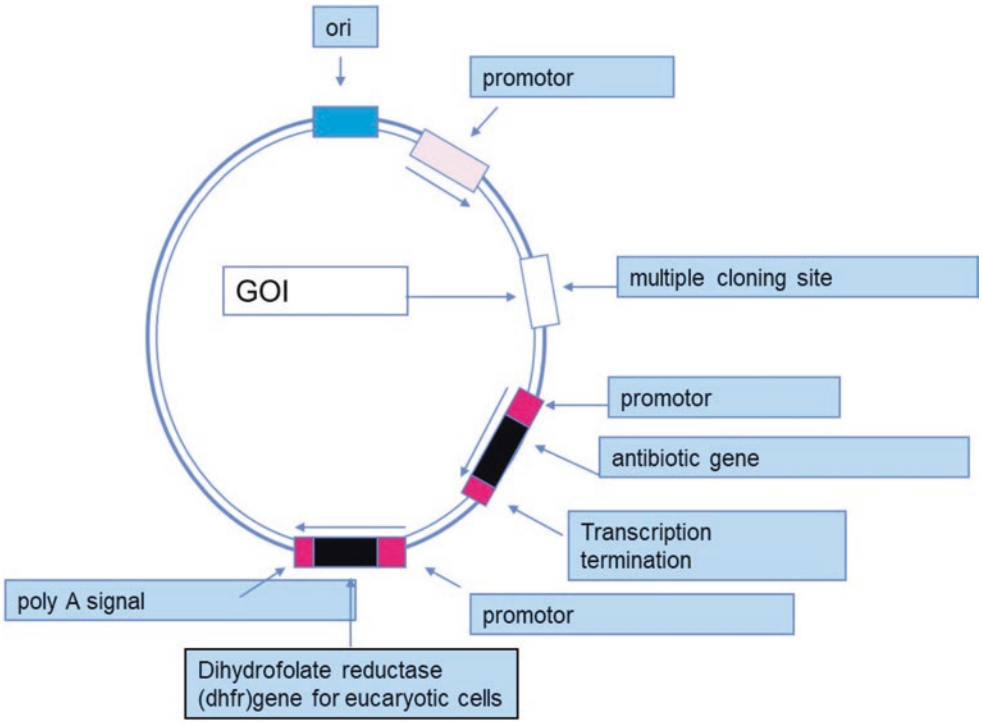


Fig. 24.3 Plasmid expression vector example (*GOI* gene of interest, *Ori* start of the replication)

for bioprocess performance in fed-batch or perfusion cultivation and product quality before the final production clones are selected.

CHO cell engineering can be optimized by host cell optimization using genetic engineering. The following possibilities are available: (A) Beneficial genes which improve bioprocess performance can be introduced into the host cell genome; (B) Genes which negatively affect bioprocess relevant phenotypes can be knocked-out by genome editing technologies such as TALEN or CRISPR/Cas9 in order to prevent their activity [5–7]; (C) Innovative approach to regulate mammalian cell phenotypes without adding a translational burden to the host cell represents the overexpression of advantageous or repression of disadvantageous microRNAs (miRNAs) [8].

24.2.2.2 Cell Culture Media

Basic components of cell culture media include an array of nutrients and factor components the nature and ratio of which vary from cell line to cell line. Serum-dependent media formulations are developed for the express purpose of providing a source of nutrition for mammalian cells being grown in vitro. Most culture media used today are cell line specialized serum free, chemically defined and animal protein free. Moreover, these media are well protected by the suppliers when patented or not remains the most secret part in the development of biotherapeutic proteins. These media consist mostly of pH buffers and indicators, inorganic salts, amino acids, carbohydrates, fatty acids and other lipids, vitamins, trace elements and iron transporters [9].

There are different classes of media:

1. Classic media: media supplemented with serum supporting a wide spectra of cell types and applications. These media contain components for pH, osmotic and shear control (e.g. salts, phosphate, detergents), energy sources (e.g. glucose, glutamine), trace elements and vitamins (e.g. Ca, Cu, Fe, K, Mg, Mn, Se), proteins, lipid and nucleic acid precursors (e.g. amino acids, fatty acids, sterols).
2. Serum Free media: Excluding serum they contain components that provide all the functions of serum without the need for further supplementation.
3. Protein Free media: Media that do not contain protein and use hydrolysates (e.g. peptides), recombinant peptides, synthetic molecules and organic salts.
4. Chemically Defined media: These media consist of materials that are either molecular homogeneous or mixtures of characterized and quantified ingredients.
5. Animal Component Free media: The media do not contain animal derived components and are mostly synthetically derived or plant-based or microbial-based.

There are many other types of media, but these are more specific for certain eukaryotic strains.

Current cell culture media for biomanufacturing are the result of decades of development and refinement. The media support many biopharmaceutical operational critical process parameters and entity critical quality attributes. The development of new media formulations has evolved into a science-

based discipline supported by advanced monitoring and high-throughput techniques. The elimination of serum- and animal-derived components and the establishment of chemically defined formulations has fulfilled many new quality and regulatory requirements.

24.2.2.3 Fermentation

Process development of the fermentation part can be developed in two parts. In the early stage development, a bioprocess quickly produces materials for toxicological studies and clinical phases I/II, whereas for the late stage the focus is on scale-up, productivity improvements, process robustness, and cGMP readiness. There are different alternatives to how an upstream manufacturing process can be designed, but in principle there are a few modes of operations: batch, fed-batch and perfusion (Fig. 24.4). Each of them has his (dis) advantages with fed-batch as the most dominant in mammalian cell culture biomanufacturing and perfusion, which was the alternative if fed-batch did not work out.

Fermentation mode of operations (Fig. 24.4):

1. Batch Culture: Batch fermentation is performed in a system where all necessary medium components and inoculum are added at the beginning of the culture. Batch operations were the method of choice in the early days of biotechnology, due to its simplicity. In these processes a small portion of the production culture is left in the production vessel at harvest, to serve as an inoculum to the next production culture.
2. Fed-Batch Culture: Like a batch culture it starts with essential medium components and inoculum at the start of a culture. During fermentation gradual feed of highly concentrated, fresh nutrients during the culture is added to prolong the growth phase and improve productivity. The feed solution can consist of a single nutrient (e.g. glucose, amino acid) or multiple nutrients, and a fed-batch process can include simultaneous addition of more than one feed.
3. Continuous perfusion Culture: A fermentation that runs at constant volume with a continuous feed of fresh culture medium into the bioreactor at the same time as spent medium is removed at an equal rate. A cell retention device (e.g. Alternating Tangential Flow (ATF)) keeps the cells in the reactor, whereas low molecular components will be removed together with a spent medium. Consequently, the product is continuously harvested, which in practice means that a certain volume of spent medium is collected and then taken to downstream purification.

Before a production culture can start, there is a need to grow enough cells to inoculate the production reactor, this process is called the seed-train. The seed-train starts with thawing cells from a working cell bank (WCB), which are stored at −150 °C, then scaling up in small scale vessels (e.g. shake

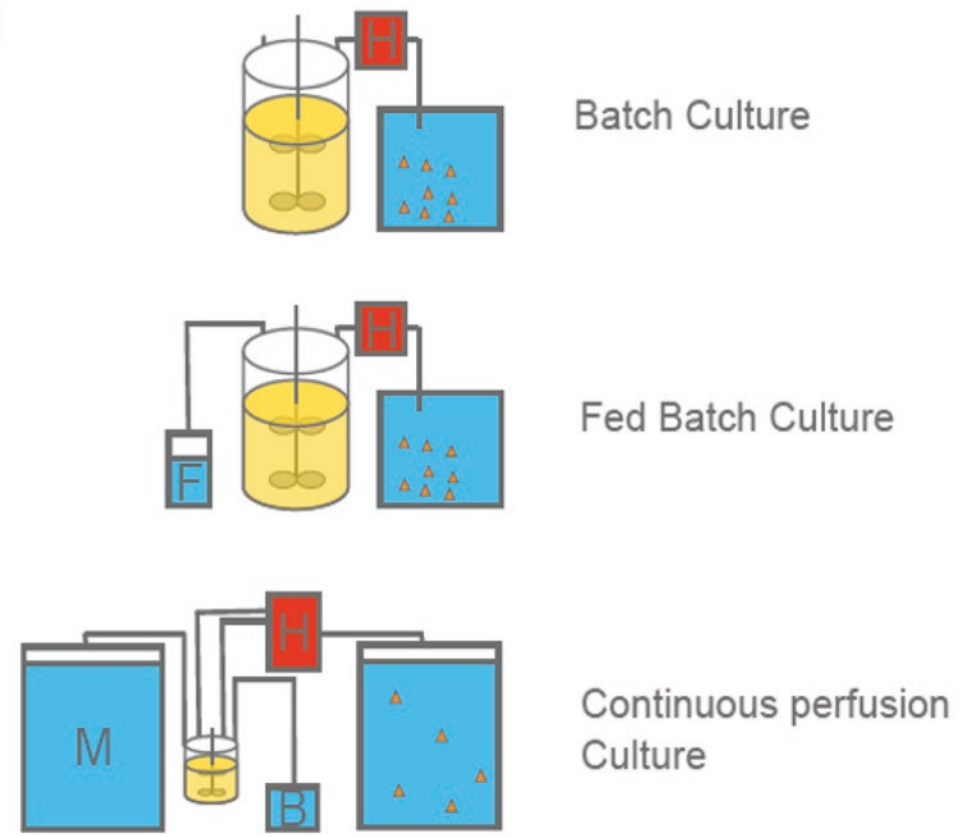


Fig. 24.4 Different fermentation operations: Top figure: Batch Culture; Middle figure: Fed Batch Culture; Bottom figure: Continuous perfusion Culture; M = Media; B = Bleed; F = Feed; H = Harvesting (Filtration unit keeping the cells in the bioreactor) [10]

flasks), followed by continued scale-up in wave bags in the range of 10–50 L and then in bioreactors of increasingly larger size such as 50–1000 L, until the production vessel (single use approx. 2000 L and for stainless steel approx. 25.000 L) is inoculated.

24.2.2.3.1 Preculture and Seed Train

Before a cell culture can be used for the manufacturing of therapeutic proteins a certain cell density is required. This is achieved through a so-called preculture process. This preliminary train of cell cultures is necessary to reach the required minimal cell culture density in the subsequent bioreactor step(s) (see Fig. 24.5). The preculture starts with a small quantity of cells, often called a Working Cell Bank (WCB). The WCB is a deep-frozen vial or bag with cell culture media and genetically modified cells for the manufacturing of the protein of interest. The vial or bag is taken from a nitrogen freezer (−180 °C) and slowly thawed by use of a water bath. Once just a small sliver of ice is still visible in the container the operator takes it into a biosafety cabinet (BSC) or laminar airflow (LAF) hood and transfers the cells by pipetting into a bigger container. This container is often a shaker flask, cell culture flask or bag containing growth stimulating cell culture media. With the cells transferred, the container is placed in a temperature-controlled box on e.g., an orbital shaker. By gentle shaking the cells in the cell culture media, aeration, and gas exchange with the available air in the container is ensured to accommodate an initial cell growth. After 2–4 days the shaker flask is taken from the temperature-controlled environment and a sample is taken to perform a viable cell count. This is done either manually under the microscope or by an automatic cell counter. Knowing the viable cell count is important for the next step in the preculture process, as the cells are further propagated in a new container by inoculating this container with a known cell concentration from the previous. This is called a preculture passage. In this second growth container, the manufacturer may choose to again use a shaker flask with a bigger size. Depending on the growth rate and required cell density the manufacturer may also decide to use a Wave-mixed bioreactor system. These systems mix cell cultures with their surrounding air in a temperature-controlled, aerated bag, creating waves by moving up and down like a see-saw on a playground. This movement ensures gentle mixing of the air with the cell culture, again stimulating cell growth without too much shear stress to the vulnerable mammalian cells. Wave-mixed bioreactor systems may range in working volumes of as low as 100 mL to up to 100 L. Some manufacturers may choose to install a new wave bag for each preculture passage, while others may decide to refresh the cell culture media in the current bag by draining most of the culture volume and refilling with new, until the preferred starting cell concentration of the next passage is reached. By starting a new larger cell culture from the previous over multiple iterations of 2–4 days, the manufacturer propagates their cells until the wanted viable cell concentration and the required volume for the next step in their manufacturing process is reached. The total preculture process can take up to 3 or 4 weeks before these requirements are fulfilled. At the end of the preculture process the manufacturer may choose to use the obtained cell material for the inoculation of a seed bioreactor. This may be a smaller sized version of the final intended production bioreactor and can be operated under various bioreactor modes, such as batch, fed-batch or perfusion. See the next chapter for further reading on bioreactors and bioreactor modes.

24.2.2.3.2 Bioreactor

Once the preculture and seed train process have reached the required number of viable cells, the manufacturer starts their production bioreactor. Cells from the last passage in the seed train are transferred to the bioreactor by pumping. Often through tubing attached with a sterile connection from the wave-mixed bioreactor bag or the seed bioreactor to the production bioreactor. There exists a large variety of bioreactor systems, but each of them will be equipped with at least the

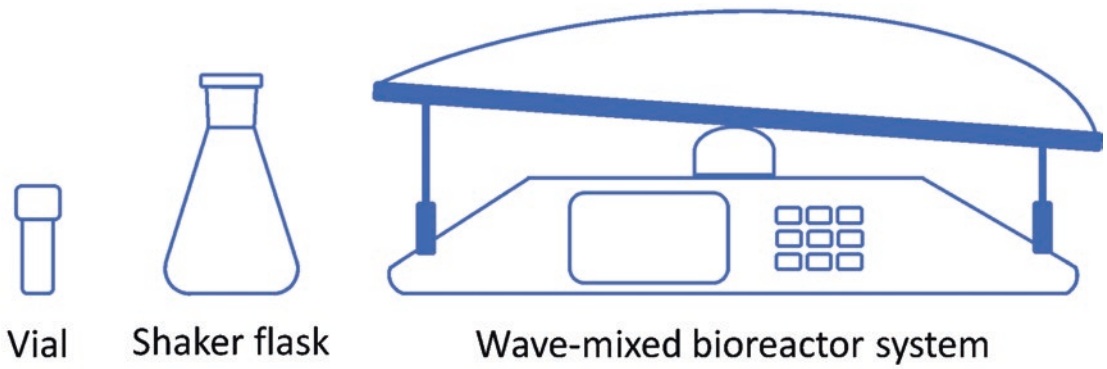


Fig. 24.5 Schematic drawing of preculture train from vial to wave-mixed bioreactor

following properties: temperature control, pH control, mixing and means of sampling and feeding the bioreactor. Bioreactors come in a broad variety of sizes as well. From as small as 1 L of up to 20,000 L or greater. When manufacturers chose a certain bioreactor size, they may take the following into consideration:

- the anticipated production volumes required to supply the market;
- single-use or fixed piping systems;
- the footprint of the bioreactor system in the facility;
- speed of manufacturing and change-over needs to other products;
- the scale of their downstream process equipment;
- their preferred type of bioreactor processing mode;

In general, there are four different bioreactor operation modes: batch, fed-batch, perfusion and continuous (see Fig. 24.4 and –simplified – Fig. 24.6). The latter, continuous, is a bioreactor mode where the inflow of materials is equal to the outflow once a cell culture has reached their steady state. This mode is rarely used for mammalian cell cultures and will not be further addressed in this chapter. As for the remaining three, batch, fed-batch and perfusion, they can all be used for mammalian cell cultures and they are explained here. The simplest bioreactor operation mode is batch. In batch mode all ingredients for the cell culture are added to the bioreactor at the start. The bioreactor is often mixed and oxygen supply, temperature and pH can be controlled. Occasionally antifoaming agents may be added in small quantities to stop or prevent foaming. The separate passages of the preculture process are good examples of batch operated bioreactors. A batch-controlled bioreactor process is ready as soon as the required cell density is reached or when the ingredients the cells need to grow are depleted and thus the cell culture's viability starts to decline.

In fed-batch mode the bioreactor is inoculated with a minimal culture volume and cell culture media, food for the cells, is provided by addition at a certain rate. The rate at which media is added is determined by measuring the concentration of certain ingredients. This can, for example, be done by daily sampling the bioreactor and testing for glucose concentration. Based on the measured glucose concentration and the measured cell concentration the manufacturer can calculate how much media to add to accommodate production for the next 24 h. Once the manufacturer learns more about the behavior of their cells they may feed their fed-batch operated bioreactor according to a pre-established schedule, eliminating the need for daily sampling. Nowadays also more and more manufacturers work with online probes which can control the rate and time points of media addition without the need for offline sampling. A fed-batch operated bioreactor ends as soon as the required volume is reached, or the bioreactor has reached its maximum volume. All produced protein of interest is in the bioreactor until the end of the run and is harvested at once.

Perfusion bioreactors are equipped with a cell retention system. This system ensures that cells stay in the bioreactor, while the protein of interest excreted by the cell culture can be harvested. Different cell retention systems exist, such as,

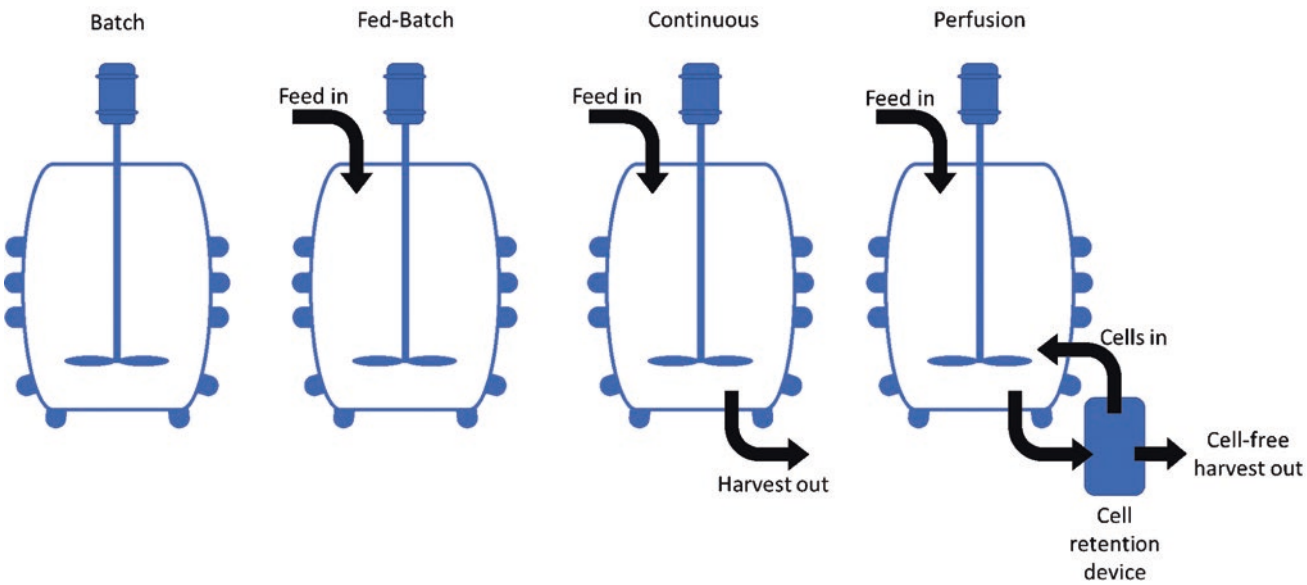


Fig. 24.6 Schematic drawing of different modes of bioreactor processing

internal to the bioreactor, spinfilters or external alternating tangential flow (ATF) filters. Typically, the bioreactor is inoculated until its maximum volume and operated in batch mode for a few days until the cell culture has reached the anticipated cell density and secretes the optimum amount of protein of interest. Once this happens the cell retention system is turned on, and the cell culture is supplied with fresh cell culture media at the same rate at which the bioreactor is harvested. In other words, the volume in the bioreactor is kept stable, because inflow of media and outflow of harvest are equal. It is possible that the cell culture is still increasing in density which is an unwanted situation for the manufacturer. Therefore, the perfusion bioreactor is also equipped with a biomass pump. This pump is used to remove excessive amounts of cells to keep the cell culture at a fixed concentration and optimize the production of the protein of interest. A perfusion bioreactor system can be operated for much longer than batch and fed-batch bioreactors. The length of a run is dependent on the genetic stability of the cell culture and the allowed maximum number of generations that a cell culture may go through. This means that mammalian cell perfusion bioreactors can be operated for up to a few months.

The three bioreactor modes described above can also be used intertwined. A manufacturer may for example choose to start with a fed-batch mode process and switch to perfusion mode as soon as the bioreactor has reached its maximum volume.

24.2.2.3.3 Harvesting

Once a biotherapeutic protein has been produced by a chosen production process, separation and recovery from the host system need to be performed. Cell harvest and recovery serve this function by removing or isolating host cells so that the product can be recovered from the host system and product stream can be clarified prior to the purification process. Harvesting is the link between the expression/production and purification of the biotherapeutic protein. This part is critical to yield the product in its native form from the production system and requires careful optimization. Focus will be given on harvesting systems used for the clarification of CHO cells [11].

Currently, there are three different clarification methods including centrifugation, crossflow filtration and depth filtration (Table 24.1). The primary clarification will remove the cells, fine particles, colloids and soluble impurities prior to the purification steps. A rule of thumb is that clarification of <2000 L scale depth filtration and cross-flow filtration might be the best options and for >2000 L scale centrifugation is the best option. The Table below shows the clarification systems:

Selection of the primary clarification step can significantly impact subsequent filtration and purification operations downstream. To ensure robust and effective clarification, an understanding of the CHO cell culture feed material (or from another eukaryotic cell line) is highly desirable to achieve high product yield, impurity removal and solid separation. Variability is commonly observed between batches of the same CHO cell line and product. Factors that may impact product recovery are (1) solid loads, (2) cell density, (3) cell viability, (4) particle size distribution, (5) Host Cell Proteins/DNA content.

Table 24.1 Different harvesting mode of operations

Technology	Method	Pro's	Con's
Depth filtration	Diatomaceous earth in stacked disks	Good yields, disposable, low maintenance	Low throughput
	Polymer based stacked disks	Low product binding	Higher price
		Improved capacity	Low throughput
Crossflow filtration	Cross flow filtration with 0.6–1.0 μm pore size	High yield	Low throughput
		0.2 μm quality filtrate	Fouling
		Low throughput	Long process time
			Cell density limits
Centrifugation	Disk stack centrifuge	High throughput	Cleaning
		High yield	Energy costs
		Rapid process	Investment costs

24.2.2.4 USP from Lab Scale to Pilot Scale

Most manufacturing processes for therapeutic proteins are not developed at the same scale at which they will eventually need to be for commercial size production. This means that a production process needs to be scaled up at various moments in the process development timeline. For this chapter, we describe USP as the set of process steps in which the protein of interest is manufactured up until the moment that the protein of interest is harvested from the bulk of cellular material and further purification steps in Downstream Processing (DSP) can be performed.

Independent of the final manufacturing scale and volume, all USP processes start with a small quantity of cells: the Working Cell Bank (WCB). Early in development the WCB may also be called Development Cell Bank (DCB) and the bioreactor working volume may be small enough to use this cell bank directly in the bioreactor without the need for a preculture and seed train. This means that the manufacturer can directly produce small quantities of the protein of interest to use, after a set of simple DSP steps, during process-, or analytical-, developmental studies. This is immediately where the first challenge comes to light, because the way cells behave in these small bioreactors without seed train may be

quite different from larger scales. For scale-up, a manufacturer benefits from knowing the chosen cell line well and consequently using the same, preferably completely chemically defined, cell culture media independent of the protein of interest. However, this is not always the case. The cell line producing the protein of interest may have been acquired from a third party and using a different cell culture media. This gives the manufacturer the choice to either first try to fit the new product in their existing platform, or to take the process as it was transferred and try to scale this process to the desired manufacturing size.

Which choice to make depends on various factors, such as:

- Is there enough time for development? The required speed to market for the new product may be too short to spend precious time on trying to fit the new product in the existing platform.
- How does the new cell line perform in the existing manufacturing process? The transferred process may perform better than the manufacturer's platform and the manufacturer may decide to continue with this.
- What is the cost of the new cell culture media? Some cell culture media ingredients may very well promote cell growth and protein production but are so expensive that they become unaffordable when scaling up.

Once the process is fully developed at a small scale and the manufacturer is happy with the process yield, it is time to transfer to a larger scale. This may include the introduction of the preculture and seed, where through a series of batch processes the viable cell quantities are multiplied enough to start a larger scale bioreactor process. When scaling up it is important to mimic the original process scale as well as possible to ensure that the cells produce the protein of interest with the same desired quality attributes and to not further complicate downstream processing. For example, it could happen that a larger scale bioreactor puts a higher shear stress on the cells due to inadequately scaled mixing. The consequence may be a lower viable cell density and higher levels of host cell protein (HCP) being released into the harvest which all need to be removed in DSP. Mixing, cell culture media feed regimens (in case of fed-batch mode), antifoaming agent additions and culture refresh rates (for perfusion mode) are examples of parameters that need to be carefully considered when scaling up the bioreactor process. As it is costly to perform large scale bioreactor processes, manufacturers also benefit from investing in a small-scale bioreactor setup that accurately models and predicts performance of their large-scale bioreactor which can be used for quicker problem solving in commercial production.

Scaling up the harvest process is most challenging for batch and fed-batch bioreactor modes, as the complete volume of the bioreactors needs to be processed at once. Timing is critical here: the longer the harvesting process takes, the more cells may break and thereby release inconvenient host cell proteins and the bigger the risk of losing valuable protein of interest due to protein aggregation or breakdown by proteolysis. A depth filtration may be a fitting solution at small scale but may be impossible to scale-up at large scale due to immense volumes of buffers and processing time. Some manufacturers may therefore choose to use an industrial scale centrifuge for harvesting at large scale while small scale is done with depth filters. Combinations of depth filters with centrifugation steps are also possible.

24.2.3 Downstream Processing

Downstream Processing (DSP) is the purification of biotherapeutic proteins obtained during the Upstream Processing (USP) part. The main focus is to remove product related impurities (e.g. aggregates, modified versions), process related impurities (e.g. host cell proteins, DNA, additives added during USP and DSP processing) and viruses (e.g. minute virus of the mice (MVM), murine leukemia virus (MuLV)) which can be present in eukaryotic cell lines such as in CHO cells. The DSP consists of a couple of unit operations that are able to filtrate the biotherapeutic proteins such as depth filtration (removal of particles) or tangential flow filtration (buffer exchange, product concentration) and chromatographic separations using resins with modified surfaces (e.g. affinity, hydrophobic, ion-exchange) to purify the biotherapeutic proteins to very high purity (Fig. 24.7) [4].

24.2.3.1 Filtration

Filtration is a process in which components of a fluid mixture are separated based on their size during transfer through a porous material. If liquid on one side of a porous material is exposed to pressure it will start to move through the porous material as long as the pressure on the opposite side is lower.

Two modes of operation are the most commonly used in bioprocess filtration processes: (1) Normal Flow Filtration (NFF), also called dead-end filtration, (2) Cross-Flow Filtration (CFF) also called tangential flow filtration (TFF), see Fig. 24.8a. In the case of NFF all solution is passed directly through the filter and material filtered out (retained) is accumulated on the membrane surface or inside the membrane's pore structure. In the case of CFF, the majority of the solution is directed across, or tangential to, the filter membrane (see Fig. 24.8b). This flow is called cross-flow. A frac-

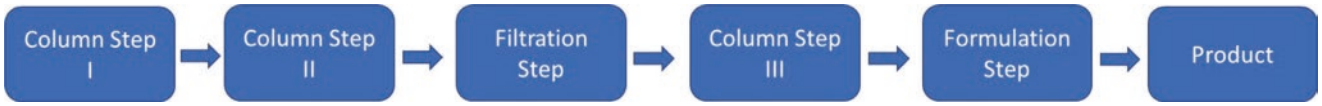
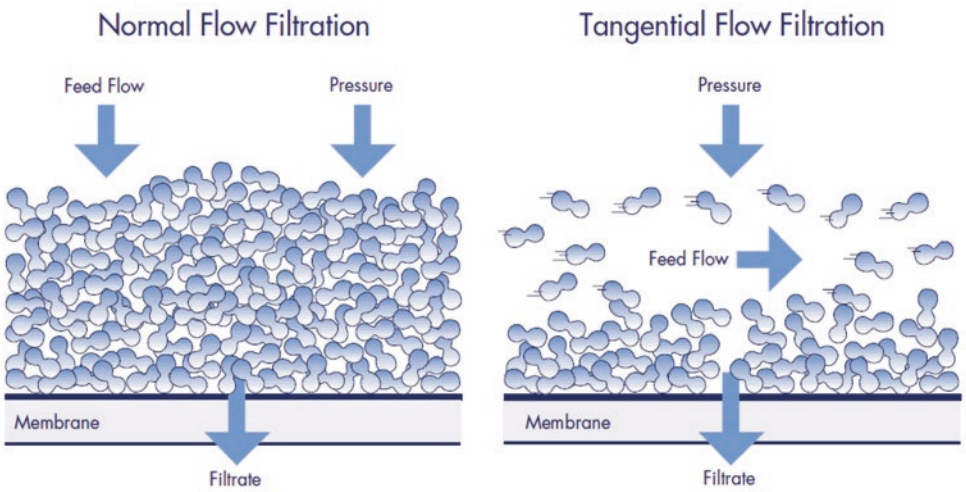


Fig. 24.7 Example of a downstream purification process consisting of different purification steps

Fig. 24.8a Normal flow filtration (depth filtration) and tangential flow filtration (cross-flow filtration)



tion of the cross-flow that is permeating through the membrane is called the permeate flow. The sweeping action of the cross-flow prevents build-up of foulants on the membrane surface. The cross-flow is also called a retentate flow.

Different types of filters are used in the biopharmaceutical industry and two categories can be determined, those made with membranes and those based on other porous media. Membrane based filters are used in a broad range of applications whereby the choice of the membrane polymer decides the surface properties, the most common filters are polyethersulfone (PES), polyvinylfluorodyne (PVDF), Cellulose acetate (CA), polytetrafluoroethylene (PTFE) or Nylon. The choice of material depends on the process requirements as each material will give the filters different characteristics, such as pore size and porosity, mechanical strength, or a certain chemical stability.

Depending on the filtration application, different materials are preferred. For instance, filtration membranes intended for separation of aqueous streams are based on hydrophilic materials, as a hydrophobic membrane which rejects water would require a higher pressure, or the addition of a wetting agent, in order to force water passage through the membrane.

Different pore-size ranges are available ranging from nanofiltration up to microfiltration covering the range from 0.1 nanom – 100 microm. Most common filter ranges are between 5–10 nanom (TFF) and 0.2–10 microm (NFF).

24.2.3.2 Chromatographic Separations

Purification of biotherapeutic proteins occurs by two different phases, whereby a differentiation can be made for a stationary phase and a mobile phase. For separation of proteins the mobile phase is a liquid and the stationary phase a solid (beads which are also known as "resin") and the biotherapeutic proteins will be separated from the liquid or solid phase.

Below in Fig. 24.9 a resin bead is presented with different pores indicating that proteins do not only bind to the surface but are also able to penetrate inside the pores, the pores greatly increase the surface area for protein binding.

The adsorption of proteins to the beads compared to liquid (c) is dependent on the strengths of the interactions, in this way weak and strong adsorption can be observed (Fig. 24.10). This is due to the partitioning between the solid and liquid phase. The binding capacity of the biotherapeutic protein can be low by weak adsorption or very strong by strong adsorption. Mostly, the best way for this chromatographic separation is that the proteins have a good binding to the resin bead, but not too strong otherwise it is very difficult to elute the proteins from the beads and then you might need harsh conditions that would destroy the proteins, such as alkaline conditions (e.g. 0.1 or 0.5 M NaOH).

Two dissolved biomolecules can be separated if one has a better adsorption to a solid phase (resin bead) compared to the mobile phase (liquid) tuned by the pH or salt strength in the mobile phase. For example, Fig. 24.11 shows a chromatogram whereby one biomolecule (protein 1) has less affinity for the resin bead and elutes earlier from the column compared to the other biomolecule (protein 2) so that protein 1 has a shorter

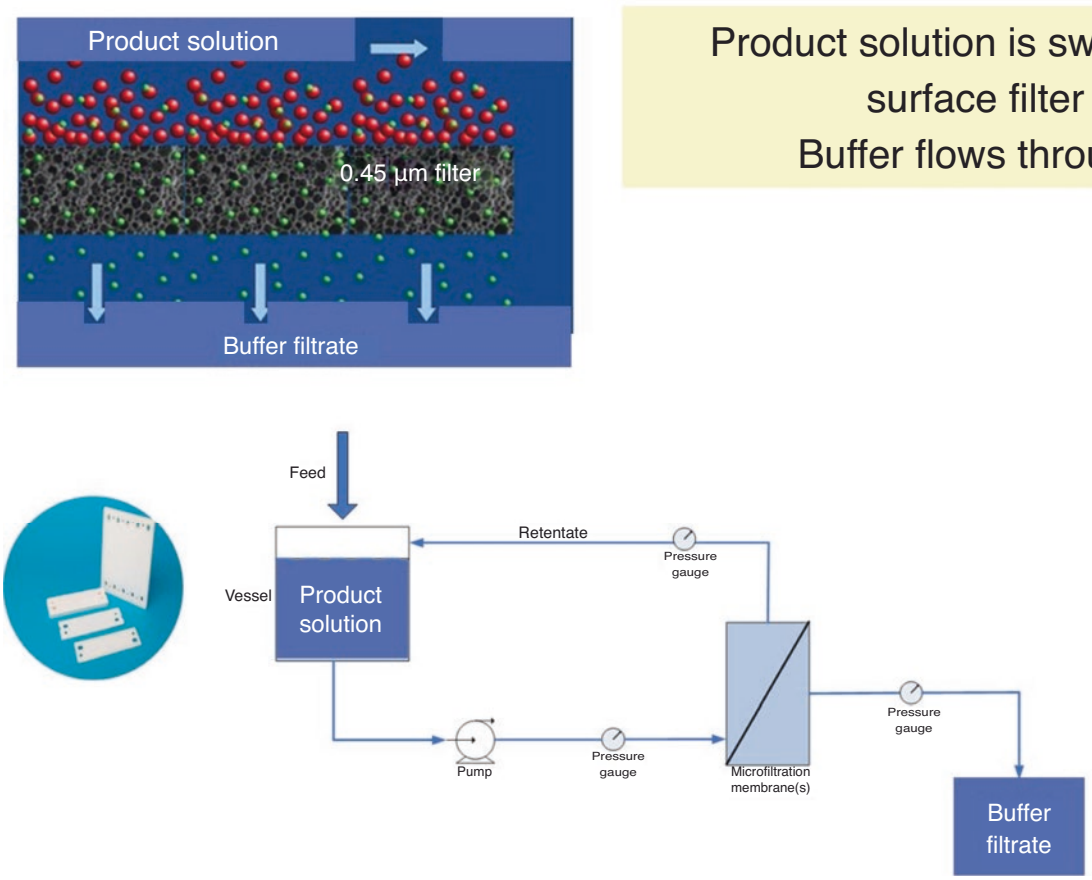
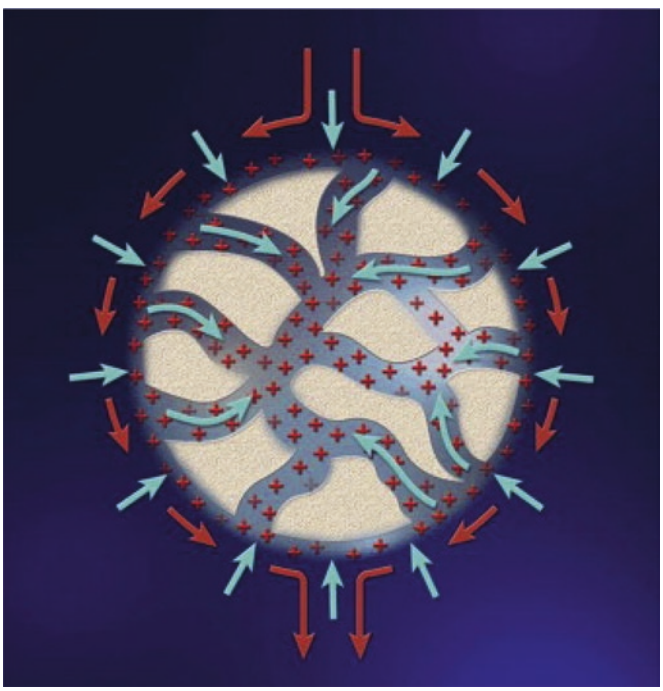


Fig. 24.8b Example tangential flow filtration (cross-flow filtration)

Fig. 24.9 Schematic drawing of a resin bead with pores



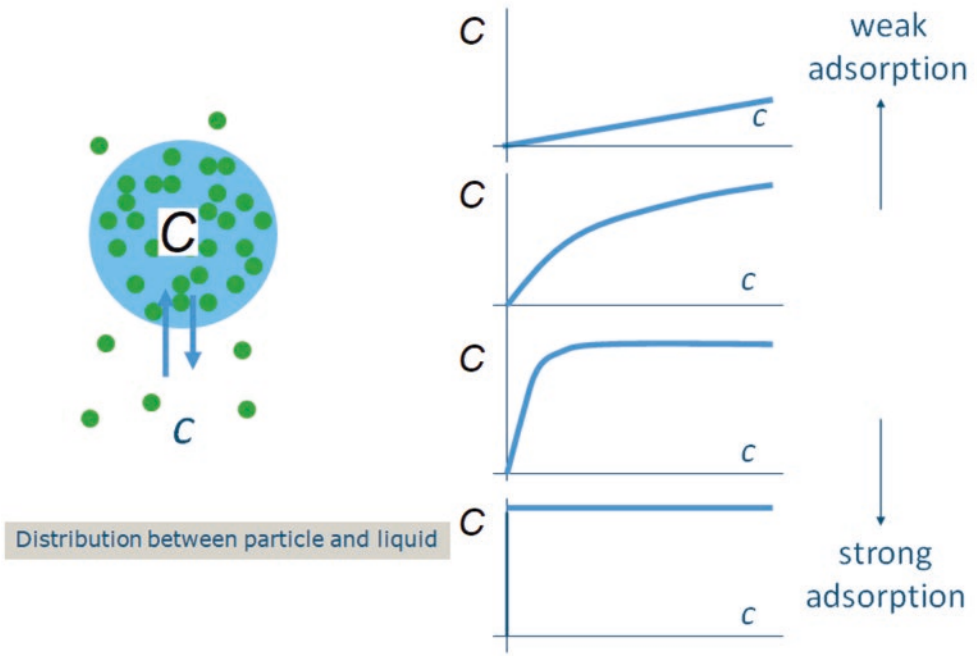


Fig. 24.10 Partitioning of proteins between the bead (C) and liquid (c) in mg/mL

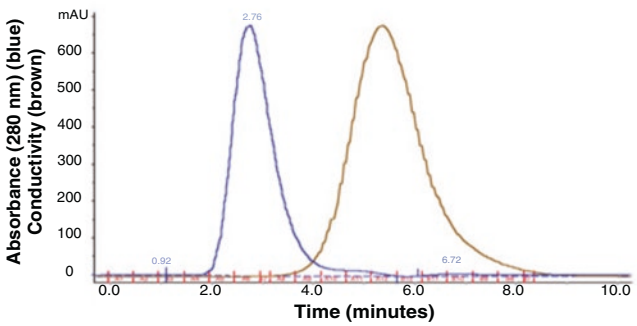


Fig. 24.11 Separation of two proteins with LSC (blue = protein 1; brown = protein 2)

retention time compared to protein 2 as indicated by the $UV_{280\,nm}$ at the end of the column whereby two peaks are being measured and shown in the chromatogram [11].

Next to that adsorptive and non-adsorptive forces play a role in the separation of proteins (Table 24.2). For adsorptive separations electrostatic-, hydrophobic-, van der Waals- forces or combinations such as ionic exchange-, hydrophobic interaction-, reverse phase- or affinity chromatography play a role. For non-adsorptive forces separation is based on size so that small molecules have a longer distance to travel in a column with beads than larger molecules such as with gelfiltration chromatography.

Below in Fig. 24.12 (left figure) a schematic drawing of the separation of a protein from other proteins is presented with column chromatography. In the right figure the chromatogram is displayed whereby different stages (flowthrough, wash, elution, regeneration) can be determined. Proteins that will not bind to the column are in the Flowthrough, the protein of interest should bind to the column. During Washing the weak bounded and rest of the flowthrough will be removed, during Elution the selected protein will be eluted in a concentrated form mostly by changing salt or pH conditions and further processed. During Regeneration resin beads will be regenerated and equilibrated in the right buffer solutions to get them back in the adsorption modus.

Table 24.2 Properties (non) adsorbance

Property	Technique	Adsorption
Charge	Ionic exchange (IEC)	Yes
Size	Gelfiltration (GPC)	No
Hydrophobicity	Hydrophobic interaction (HIC)	Yes
Hydrophobicity	Reverse phase (RPC)	Yes
Biorecognition	Affinity (AC)	Yes

Processes in the Chromatography Column

The efficacy of column chromatography (see Fig. 24.12 for a lab scale and industrial column) depends on different parameters:

- Mobile phase (e.g. buffer composition)
- Stationary phase (e.g. which activated resin bead)
- Sample of the feed (composition of the feed solution)
- Volume (the amount of volume used)
- Retention (elution of the protein or proteins)
- Retention time (time of elution of the protein)
- Retention volume (volume needed for elution of the protein)
- Retention factor (how strong is the retardation on the column)
- Column length (height of the column)
- Partition coefficient (separation of the protein over the solid and liquid phase)

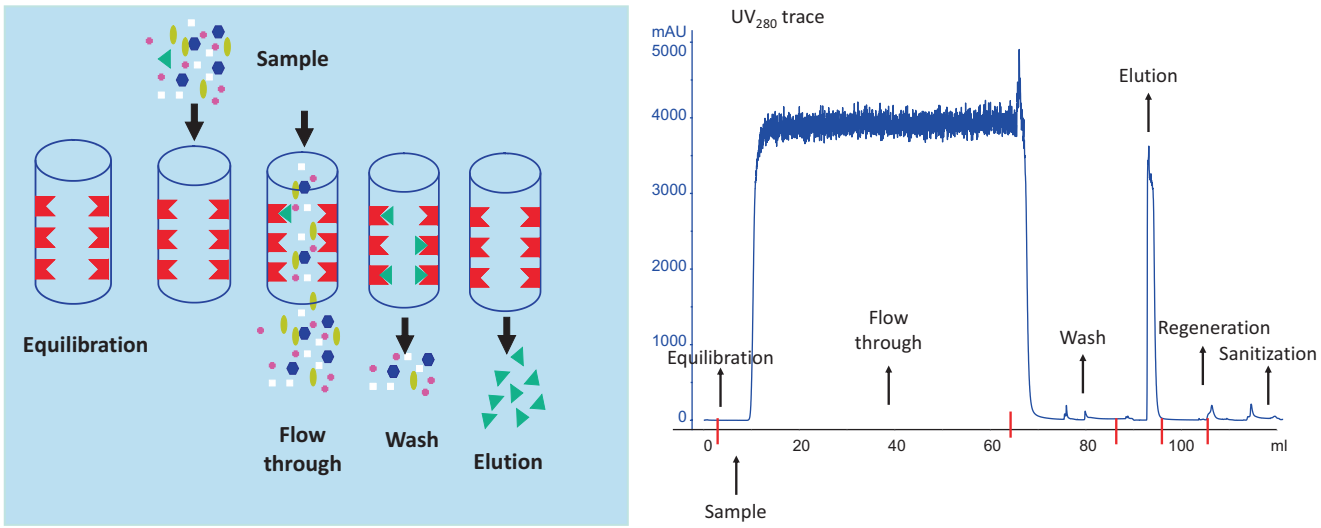


Fig. 24.12 Schematic separation of a protein from other proteins with column chromatography

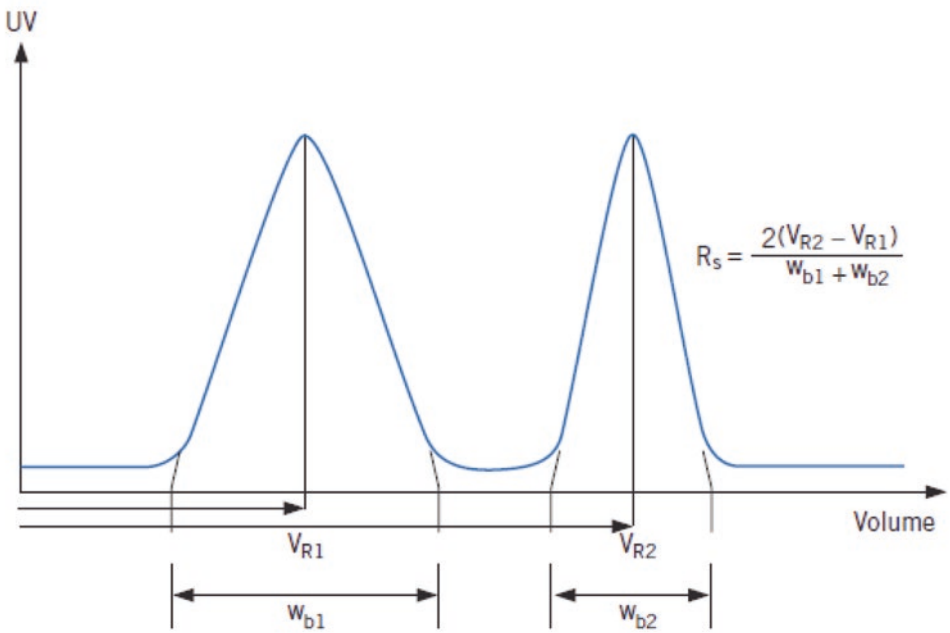


Fig. 24.13 Determination resolution factor (R_s) of two protein peaks

For a good separation between protein components the resolution of the column is very important, in Fig. 24.13 a chromatogram is presented with two different protein components completely separated from each other. This indicates that the column has for these proteins a good resolution.

With the resolution factor R_s the separation factor or the separation efficacy can be determined. V_{R1} = retention time component 1; V_{R2} = retention time component 2; W_{b1} = width of component 1; W_{b2} = width of component 2. Using the formula in Fig. 24.13 the resolution factor R_s can be calculated.

Important for the column resolution is the "Van Deemter" equation, as represented below [4, 12]:

- The Van Deemter equation relates the resolution (H_{ETP}: Height Equivalent to a Theoretical Plate) of a chromatographic column to the liquid stream and kinetic parameters that causes the peak width as shown below:
- H_{ETP} = Height Equivalent to a Theoretical Plate (plate number) is a measure for the column resolution [m], it will give the ratio between the practical and theoretical value
- A = Eddy-diffusion (Fig. 24.15a) parameter is related to the channel formation for a non-ideal packed column [m] so the ration between practical and theoretical values
- B = Diffusion coefficient of the eluting molecules in the longitudinal (= length) direction, resulting in dispersion [$m^2\ s^{-1}$]
- C = Resistance to mass transfer coefficient (Fig. 24.15b) of the component between the mobile and stationary

phase [s]. The path length for a small bead is much smaller as for a larger bead so that peak broadening will occur.
- u = Linear Velocity [m s^{-1}] of the solution through the column

Figure 24.14 shows the influence of the different terms on the separation whereby liquid velocity (V) is set out against H_{ETP} (dish number). The lowest point of the black resultant line shows the lowest column height which meets the design criteria, so the most economical one.

Scale-Up

Scale-up of the chromatographic process from small to large scale, such as presented in Fig. 24.16, normally occurs by broadening the column diameter (Fig. 24.15a), whereby the column diameter of 1 cm is scale-up to a column of even 200 cm, as illustrated in Fig. 24.15b. Most important is that the chromatogram of the 1 cm column should be the same as the chromatogram of the 200 cm column (Fig. 24.15c), showing that scale-up is succeeded.

Development of a Complete Chromatographic Process

The next phase in the process design is the development of a complete purification process for the proteins to be purified with a few chromatographic steps connected to each other using different (non) adsorptive techniques. Figure 24.16 shows a schematic drawing of a few common steps in a chromatographic process. At first the harvest or extract should be a clear solution prepared after centrifugation or filtration, with which this extract the different column steps can be performed. The "Capture step", capturing all proteins of interest and other non-wanted host cell proteins will flow through, in this way the volume can be reduced. Speed and capacity are the most important parameters to catch all the proteins of interest for further purification. In the "Intermediate Purification" step the isolated proteins will be purified by the removal of most impurities (e.g. proteins, aggregates), both capacity and resolution are important. In the last step "Polishing step" both resolution and recovery will result in a pure protein product. When connecting the different purification steps, it is important that the column steps and the buffer conditions (e.g. pH, conductivity) are in-line with each other. As an example the first IEX (ion exchange) step is performed so that the product elutes in high salt, in the next step with HIC (hydrophobic interaction chromatography) the protein solution is loaded on the column and eluted in low salt. In the final step during gelfiltration (GPC) the protein solution is eluted in the right buffer conditions and mostly an additional buffer exchange step with Tangential Flow Filtration is performed to concentrate the final active substance.

The purity of a product is dependent on a few factors:
- What is the product used for (nutrition, medicinal product, diagnostic)
- Regulatory (safety, aseptic, immunogenicity)
- Functional purity (half live, stability)
- Chemical purity (homogeneous, impurities, additives)

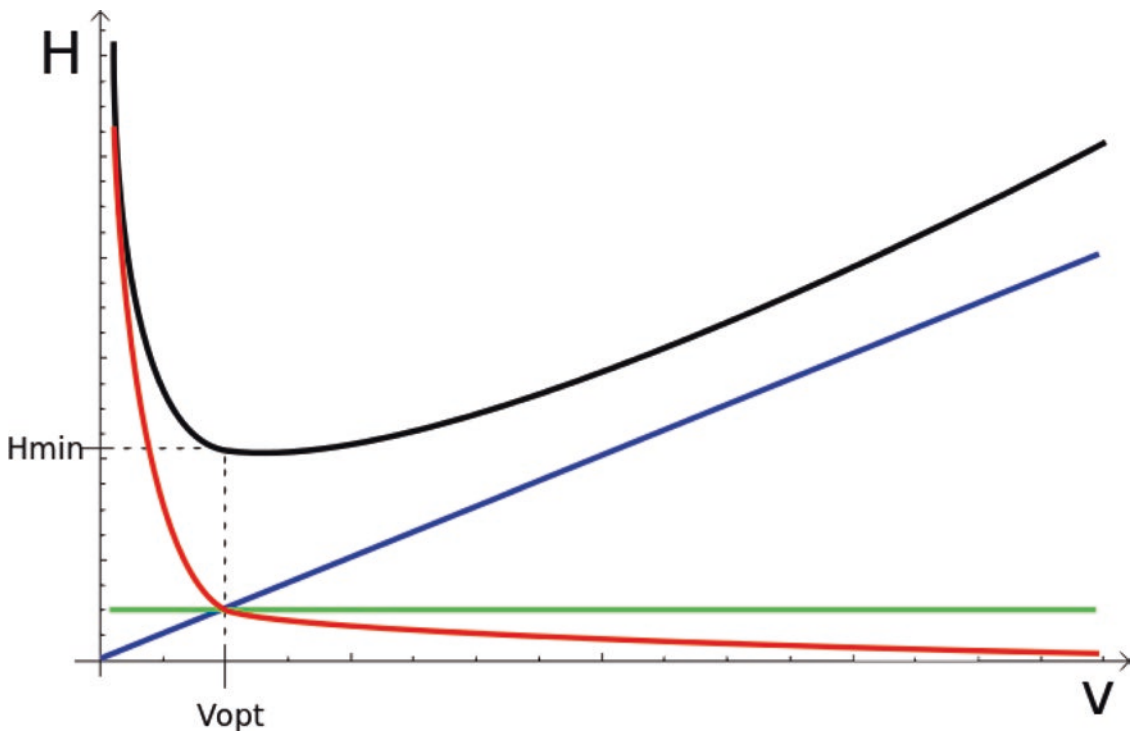


Fig. 24.14 Influence of different terms on the separation of biomolecules in the "van Deemter" equation. Green is the A-Term (channeling), Red is the B-Term (dispersion), Blue is the C-Term (path length) and Black is the sum of the different terms

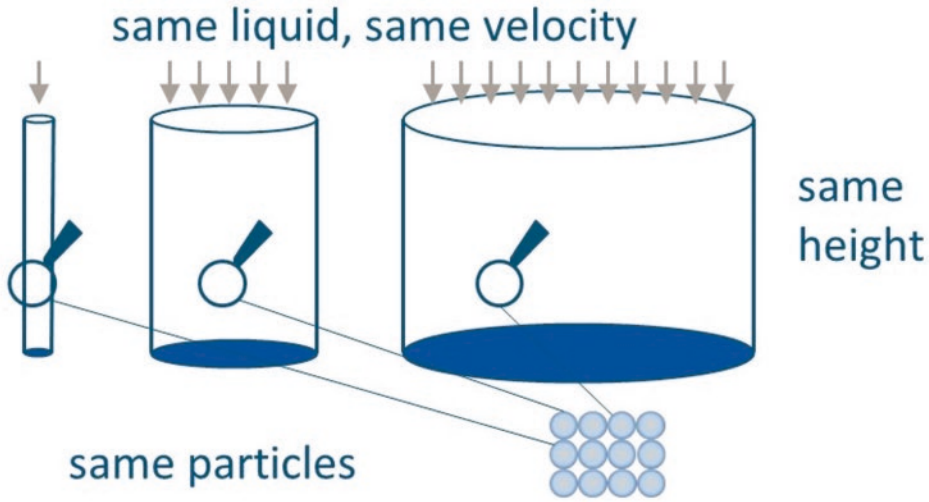


Fig. 24.15a Scale-up in diameter

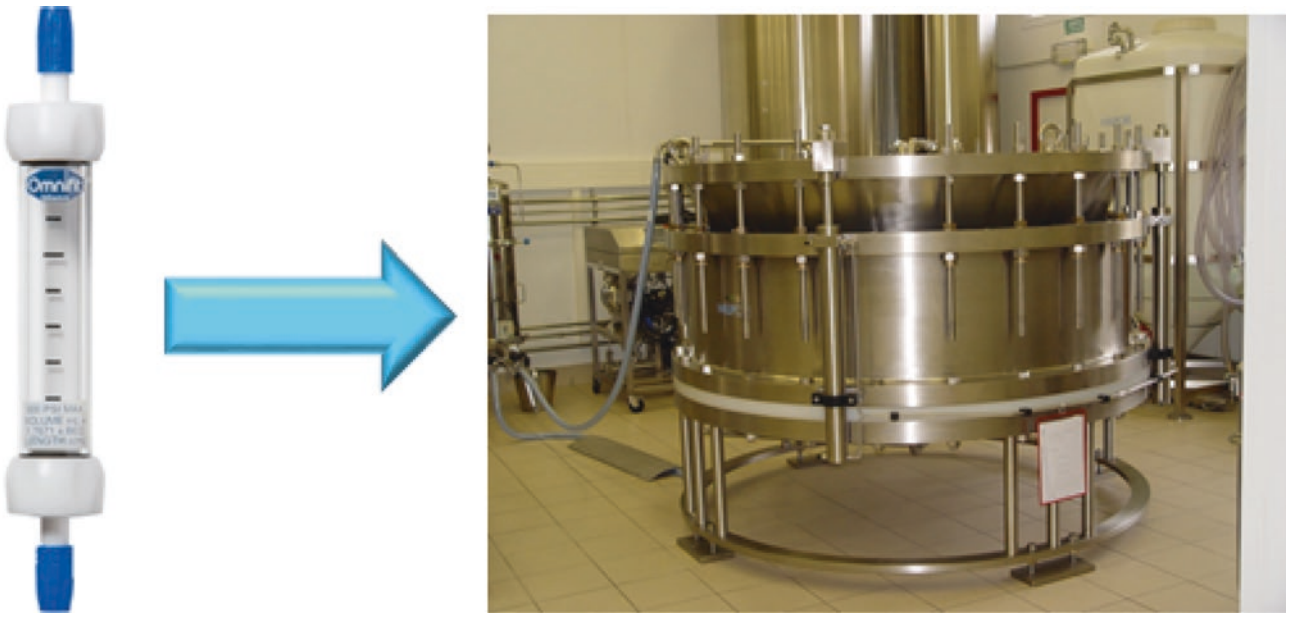


Fig. 24.15b Scale-up in diameter: lab scale (1 cm diameter) to production scale (200 cm diameter)

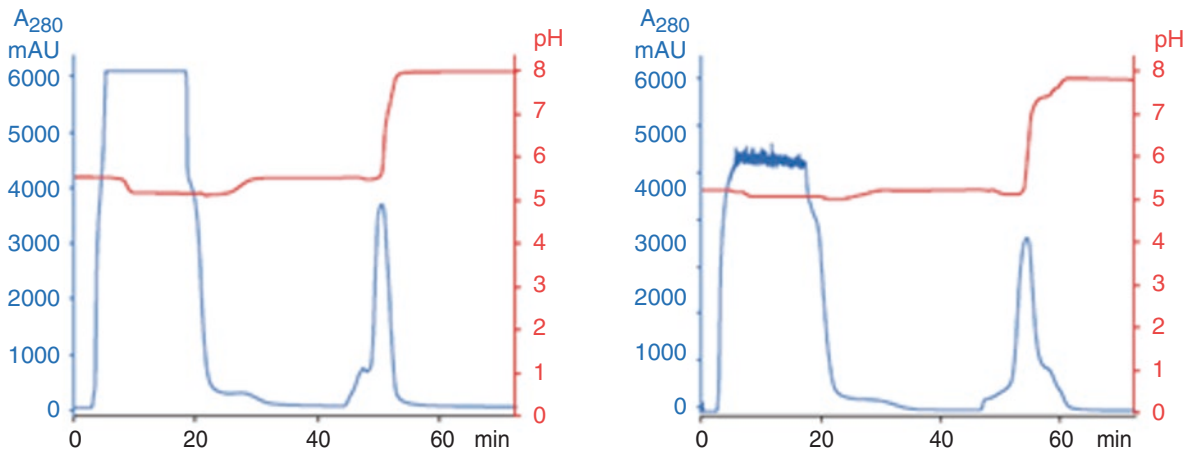


Fig. 24.15c Chromatogram: labscale (left) to production scale (right)

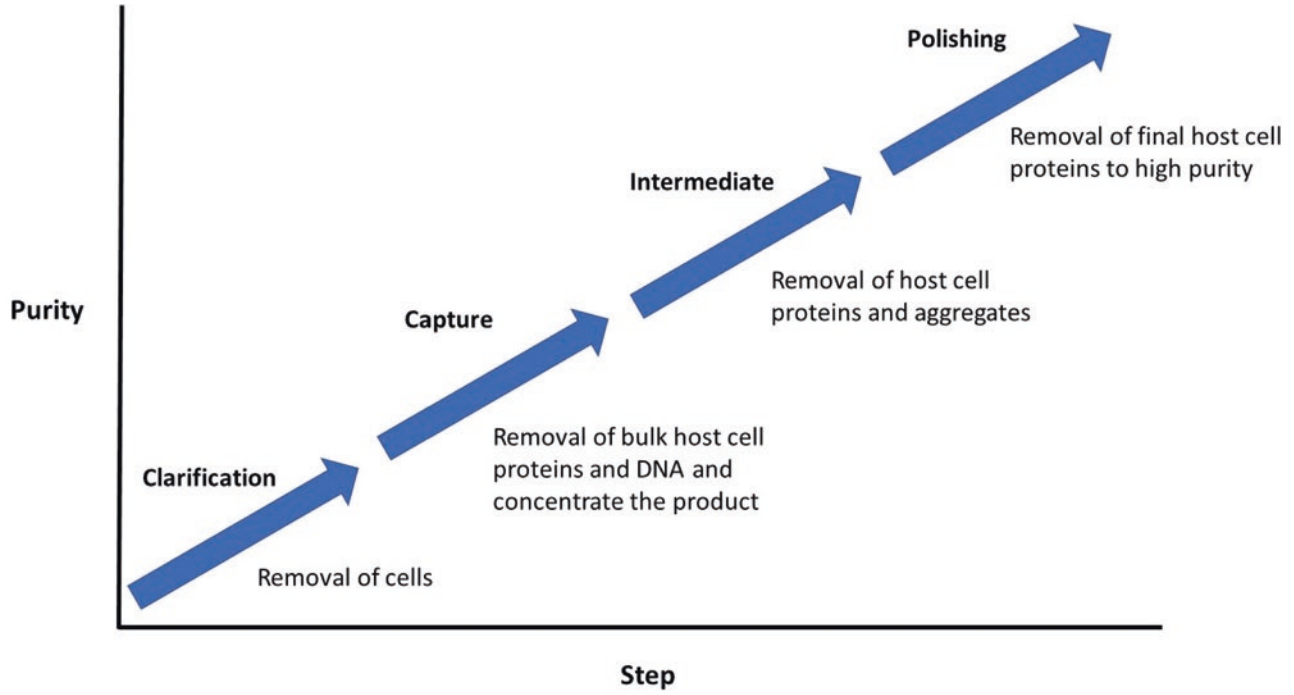


Fig. 24.16 Schematic drawing of a purification process

24.2.3.3 DSP from Lab Scale to Pilot Scale

In the scale-up of Downstream Processing (DSP) the manufacturer needs to constantly balance inevitable product yield losses with speed of manufacturing and purification needs.

During development of the manufacturing process for the protein of interest, the requirements between lab-scale and pilot or commercial scale manufacturing may differ significantly. For example, at lab-scale it might still be sufficient to have a less purified protein of interest than is required for later clinical or commercial use. Therefore, extensive development work is required prior to bringing a lab-scale product to pilot or commercial scale.

In general, most DSP processes for biopharmaceutical proteins consist of combinations of the following steps:

- Purification of the protein of interest by chromatography. Examples are affinity chromatography by protein A for antibodies, (an)ion exchange (AEX or IEX), hydrophobic interaction (HIC) and size-exclusion chromatography (SEC).
- Viral Clearance. FDA and EMA guidelines make it mandatory for manufacturers to include manufacturing steps that remove or inactivate any potentially present viral contaminants. Possible steps are solvent/detergent treatment, viral filtration, or low-pH (acidic) viral inactivation. Low-pH viral inactivation may prove very effective to viral clearance but could negatively impact the structure protein of interest as well

Ultrafiltration/Diafiltration (UF/DF) or Tangential Flow Filtration (TFF). This buffer exchange step serves two purposes: it brings the protein of interest to its targeted concentration and into the right buffer for formulation into the end product. With the optimal combination of flow and transmembrane pressure, the protein of interest should experience the least amount of shear stress while still processing at a convenient speed.

24.2.4 Manufacturing of a Therapeutic Protein Medicinal Product

After active pharmaceutical ingredient (API) manufacturing the protein of interest will be further processed into its final product, the medicinal product (MP). Some manufacturers may choose to split the manufacturing of API and MP at different facilities, because the expertise required for MP is different from API and because of efficiency reasons, e.g. it makes more sense to use the expensive MP manufacturing fill lines for the manufacturing of more than just one API. Medicinal products may be filled in vials and pre-filled syringes (PFS). In some cases, the MP process includes a lyophilization (freeze-drying) step to prepare solid, instead of liquid formulations.

Another topic which was not discussed in the previous chapters is Quality Control (QC). All therapeutic proteins are subjected to a range of stringent assays and tests under GMP conditions prior to release of the product to the market. This quality control is in place to ensure that the product meets its release specifications, that no bacterial or viral contaminants

are present and to check for process robustness over time. Quality Control is a result of a detailed end to end assessment that the manufacturer must perform on the protein of interest, the related manufacturing processes, and raw materials prior to approval by health authorities. This is called a Criticality Analysis and it results in the definition of [13]:

- Critical quality attributes (CQAs). Define which characteristics of the product are critical for its safe and effective performance in the user.
- Critical process parameters (CPPs). Define which characteristics of the process are critical for manufacturing a product that meets its CQAs.
- Critical material attributes (CMAs). Define which characteristics of the materials used in the manufacturing process are critical for a product that meets its CQAs.
- A control strategy. Defines how each of the CQAs, CPPs and CMAs are assessed and controlled. The Quality Control strategy, either by sampling or online measurements, is described in the control strategy.

Once a protein of interest is ready for commercialization after successfully passing the required stages of development, clinical testing and process validation, the manufacturer will write a comprehensive overview of the development and manufacturing process which is submitted to the Health Authorities of the countries in which the manufacturer wishes to sell its product. For example, this is called Marketing Authorisation Application (MAA) for the European Medicines Agency (EMA) or Biologics License Applications (BLA) for the Food and Drug Administration (FDA) in the United States. After approval by the authorities, the applicant must always manufacture their product according to the agreed license. If the manufacturer wishes to make any changes to the manufacturing process this must be done through the proper regulatory variation procedures which may vary per country in which the product is on the market. This can be a time consuming and costly endeavor and manufacturers should therefore carefully consider their production process before the initial filing process. Regular audits by the Health Authorities at the manufacturer check that the filed process is correctly followed.

24.3 Formulating a Therapeutic Protein

24.3.1 Introduction

Formulating a therapeutic protein is a multi-step process where an active pharmaceutical ingredient, API, is converted into a medicinal product that can be administered to the patient. This formulation process starts in the early stage of medicine development and evolves over time into the final product that is approved by the regulatory authorities. Table 24.3 lists points to consider for the formulation team. During this formulation development ‘journey’ major strategic changes may occur. For example, the route of administration, or the dosage form may be changed. In this section several of the considerations mentioned in this list will be discussed. For more detailed information the reader is referred to text books on the formulation of biologicals [14, 15].

Table 24.3 Points for consideration in the formulation process of pharmaceutical proteins

Factor	Description/attributes/examples
API or drug substance	Type of protein, physico-chemical properties, e.g. molecular weight, pI, hydrophobicity, solubility, post-translational modifications, pegylation, physical and chemical stability and concentration, available amount, purity
Clinical factors	Patient population (e.g. age and concomitant medication), self-administration versus administration by professional, compatibility with infusion solution, indication (e.g. one-time application or chronical application)
Route of administration	Subcutaneous, intravenous injection or infusion, intramuscular, intravitreal, intra-articular, intradermal, pulmonal
Dosage form – dosing policy	Single- or multi-dose, vial, prefilled syringe, dual chamber cartridge, pen cartridge; liquid, lyophilizate, frozen liquid, API concentration, injection volume, injection rate, controlled delivery/release, auto-injectors, needle-free
Primary packaging related contact materials	Glass, polymers, rubber, silicone oil, metals, leachables (anti-oxidants, plasticizers, etc.)
Excipients	Pharmaceutical quality, safety record (for intended administration route and dose), manufacturer, tested for critical impurities, stability
Analytical methods	Characterization of API and excipients, stability-indicating assays, quality control assays

Adapted from Pharmaceutical Biotechnology, chapter 5 [16] should read [14]

24.3.2 Protein Structure and Protein Stability

The primary structure of proteins consists of one or a number of chains of amino acids. These amino acid chains may be linked to non-amino acid units, such as polysaccharides (glycoproteins), phosphate or sulphate groups. This chain-like primary structure must fold and form a three-dimensional structure to exert the desired pharmacological effects. Parts of the protein will locally form discrete secondary structures such as alfa-helices or beta-sheets. At the tertiary structure level of the protein different, locally structured subunits i.e., secondary structures, are positioned relative to each other. Some proteins, such as hemoglobin, form quaternary structures. Here individual protein molecules arrange in larger, well defined structures.

Polysaccharides may make up a substantial part of a glycoprotein. E.g., in darbepoetin polysaccharides form 40% of the molecular weight of the molecule. Proper glycosylation

may be essential for the performance of a therapeutic protein. Not only for binding to the target sites, but also to provide the desired pharmacokinetic profile as circulation half-lives increase. Modifying proteins by synthetic chemistry may also improve their performance. Examples are the attachment of polyethylene glycol to interferon alfa as in peginterferon-alfa, or to a TNF-alpha receptor binding fragment as in certolizumab pegol.

The driving forces for the formation and stability of the secondary, tertiary and quaternary structures are relatively weak physical interactions (e.g. van der Waals forces, hydrogen bonding, electrostatic interactions, and hydrophobic interactions). Cysteine bridges are an example of covalent-bonds that contribute to preservation of the secondary and tertiary structure. Hydrophobic structural forces result from apolar amino acids inside the protein structure; they 'try to turn away' from the aqueous phase. Taken together, the folding structure of proteins is easily disturbed, leading to loss of therapeutic activity and increased chances of immunological reactions (See also Sect. 24.4).

Therapeutic proteins are rather unstable, large molecules with hydrophilic surfaces. Cell membrane passage is a major obstacle unless specific transporters are available. Oral availability, for instance, is negligible because of these characteristics: proteins are readily metabolized in the GI tract and no penetration of intact molecules through the gut endothelium occurs. This is true for all other routes of administration except the parenteral route. Thus, for protein therapeutics we have 'to stick to the needle' (See also Sect. 24.4).

24.3.3 Physical and Chemical Stability

24.3.3.1 Chemical Stability

The molecular mass of pharmaceutical proteins varies over a wide range. E.g., insulin and insulin-derived proteins are in the 5000 Da (in total 51 amino acids, two chains) range whereas monoclonal antibodies used in therapy typically are members of the IgG family, which have a molecular mass of around 150,000 Da. They consist of 1350+ amino acids in four protein chains plus polysaccharide units. Degradation reactions are coupled to specific amino acids. Table 24.4 lists 'Common protein modifications and corresponding methods of analysis [16]. This source also gives details about

Table 24.4 Common protein modifications and corresponding methods of analysis

Protein modification	Typical causes and important factors	Physical property affected	Method of analysis
Oxidation	Light, metal ions, peroxides	Hydrophobicity	RP-HPLC, HIC and mass spectrometry
Cys		Hydrophobicity	
Disulfide			
Intrachain			
Interchain			
Met, Trp, Tyr			
Fragmentation	pH, sequence (nearest AA neighbor)	Size	Size-exclusion chromatography, SDS-PAGE
N to O migration		Hydrophobicity	RP-HPLC inactive in Edman reaction
Ser, Thr		Chemistry	
α-carboxy to β-carboxy migration		Hydrophobicity	RP-HPLC inactive in Edman reaction
Asp, Asn		Chemistry	
Deamidation	pH, sequence (nearest AA neighbor), HOS	Charge	Ion-exchange chromatography
Asn, Gln			
Acylation		Charge	Ion-exchange chromatography Mass spectrometry
α-amino group, ε-amino group			
Esterification/carboxylation		Charge	Ion-exchange chromatography Mass spectrometry
Glu, Asp, C-terminal			
Secondary structure changes		Hydrophobicity	RP-HPLC
		Size	Size-exclusion chromatography
		Sec/tert structure	CD
Aggregation		Sec/tert structure	FTIR
		Sec/tert structure	Fluorescence
			Light scattering
			Analytical ultracentrifugation, AF4

From Koulov 2018 [16]

RP-HPLC reverse phase high performance chromatography, *HIC* hydrophobic interaction chromatography, *SDS-PAGE* sodium dodecyl sulfate polyacrylamide gel electrophoresis, *AA* amino acid, *CD* circular dichroism, *FTIR* Fourier transform infrared spectroscopy, *AF4* asymmetric flow field flow fractionation

reaction mechanisms. The conditions under which degradation is favored depend on the type of reaction. For instance, oxidation reactions can be minimized by excluding oxygen, light exposure and (masking) the presence of metal ions during production and storage, e.g. by chelating agents (ethylenediaminetetraacetic acid, EDTA). The deamidation of asparagine to aspartic acid is pH dependent. Low temperatures slow down chemical degradation reactions in general.

Small therapeutic molecules such as paracetamol/acetaminophen are 98% + pure. For relatively small, non-glycosylated proteins, such as insulin-derivatives, active ingredient purity levels are still high i.e., in the 95% + range. However, monoclonal antibody products are mixtures of closely related protein molecules, called isoforms [17].

For example, in one batch heavy chain Asp at positions 388 and 393 are partly deamidated and the C-terminal lysine composition and glycosylation profile vary (e.g., the cation-exchange HPLC tracing in Fig. 24.17). All pharmacological testing in the development phase of the API has been done with these various modifications present, so they are inherent to the product. This heterogeneity can be reproduced from batch to batch only if well-controlled manufacturing protocols are in place.

Analytical techniques to monitor protein stability will be touched upon in the section 'the analytical toolbox'. The effect of chemical degradation reactions on the performance of therapeutic proteins is difficult to predict and has to be established experimentally in the preclinical and clinical stages of development of the protein therapeutic.

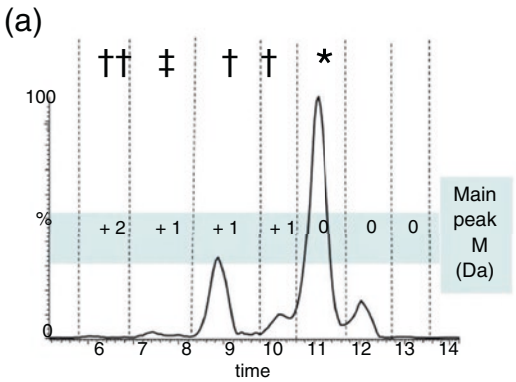
24.3.3.2 Physical Stability

Therapeutic proteins have a natural tendency to adsorb to interfaces, being the water-air (bubble) interface or the water-glass wall interface. During this interaction hydrophobic amino acids from the inside of the protein may be exposed to this interface and remain in that position leading to aggregation when they meet other partially denatured proteins. Figure 24.18 depicts different steps in this protein adsorption and aggregation process. The water-air interface increases dramatically when shaking the container. (In fact the interface is constantly renewed and that therefore, the exchange from the molecule at the interface is fostered). This is the reason why reconstitution protocols for freeze-dried cakes may explicitly stipulate to swirl the vial and its contents instead of shaking it in the reconstitution process as this may lead to aggregate formation [19]. Aggregation has been linked to increased immunogenicity of the protein and is therefore a major point of attention when analyzing protein product characteristics and developing handling protocols, see also Sect. 24.4.

In the formulation process of therapeutic proteins ample attention is paid to stability of the formulation under stress conditions. Table 24.5 gives an example of the panel of stress conditions used. It is clear that one needs to have a validated analytical test panel set up by the times these stress tests are run.

24.3.4 Analytical Toolbox

How does one ensure product quality of pharmaceutical proteins? No one single test will give the answer. A panel of physicochemical and biological assays is needed to shed light on the protein structure and to cover the critical quality attributes defined for the medicinal product (Tables 24.4 and 24.6).



Isoform Modification		M (Da)
††	2 x Deamidation LC-N30	+ 2
‡	IsoAspartate HC-N55	+ 1
†	Deamidation LC-N30	+ 1
†	Deamidation LC-N30 IsoAspartate HC-D102	+ 1
*	Relatively unmodified	0
	IsoAspartate HC-D102	0
	2 x IsoAspartate HC-D102	0

Fig. 24.17 Weak cation exchange (WCX) chromatography results coupled directly to Orbitrap MS for trastuzumab. Assignment of isoforms. LC is light chain, HC is heavy chain. From Bailey et al. 2018 [18]

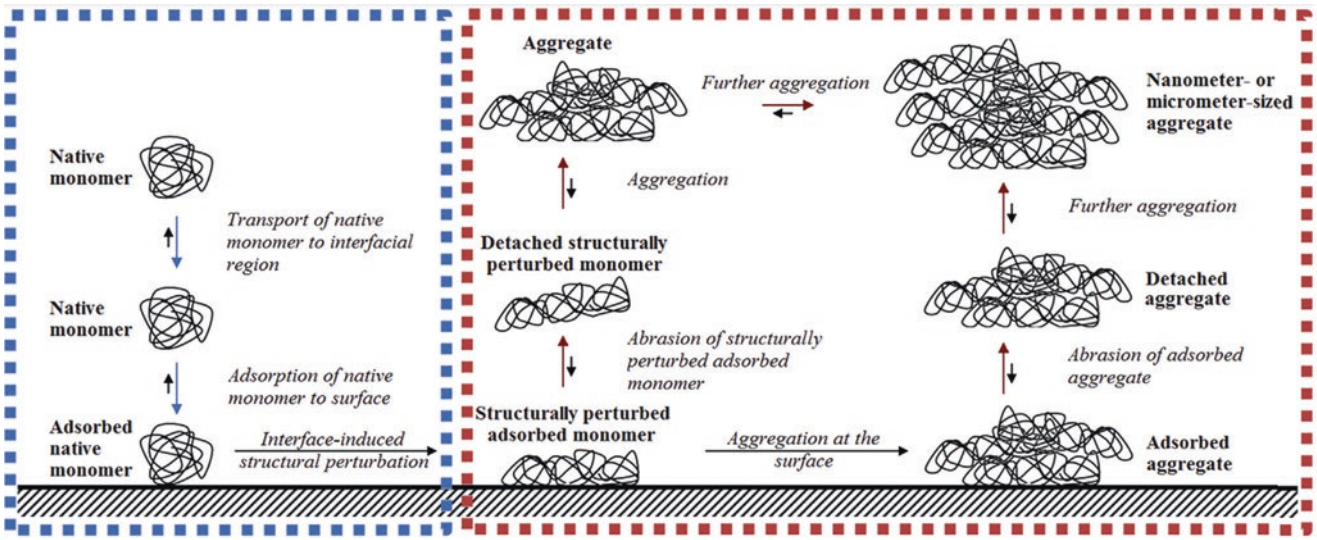


Fig. 24.18 Schematic representation of the suggested mechanism of stirring-induced protein aggregation. The left part (framed in blue) depicts the process of protein adsorption onto solid surfaces with potential perturbation of the native structure of the protein on adsorption. This process is followed by aggregation at the surface and in the bulk (framed in red). Contact sliding results in abrasion of the adsorbed protein layer, leading to renewal of the surface for adsorption of a fresh protein layer. Addition of surfactants, such as polysorbate 20, and avoidance of contact stirring will inhibit the steps shown as blue and red arrows, respectively. (Adapted from Sediq et al. 2016 [19]. Reproduced from Crommelin et al. 2018 [14])

Table 24.5 Examples of real time, accelerated stability and forced degradation studies

Type of stress	Examples of stress conditions	Anticipated instability types
Temperature	Real time (2–8 °C; up to several years)	Aggregation, conformational changes, chemical changes
	Accelerated (e.g. 25 °C, 40 °C, up to several months)	
Mechanical	Shaking (50–500 rpm, hours-days) stirring, 50–500 rpm, hours-days)	Aggregation, adsorption, conformational changes
	Shipment studies	
Freeze-thawing	Freeze-thawing, (1–5 cycles, from 25 °C to −20 °C or − 80 °C)	Aggregation, conformational changes
Light	1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours/square meter (ICH Q1B)	Chemical changes, aggregation, conformational changes
pH	Exposure to low pH (<3) or high pH (>10)	Aggregation, conformational changes, chemical changes
Oxidation	H_2O_2 (0.01–3%, 1–7 days, 2–8° to 40 °C depending on the molecules)	Chemical changes, aggregation, conformational changes
Humidity[a]	0–100% relative humidity	Aggregation, conformational changes, chemical changes

Adapted from Crommelin et al. (2018) [14]
[a]Specifically for lyophilized products

In the medicine development phase establishing the primary structure is the first step to define the protein: a combination of chromatographic steps and mass spectrometric analyses of enzymatically degraded proteins in a digest by trypsin or pepsin provides mass fingerprints of the resulting peptides and their amino acid sequence. The peptide-sequences can also be unraveled by using (an automated) Edman degradation protocol. Sugar groups are typically first enzymatically removed from the protein and then analyzed by chromatography or mass spectrometry or a combination of both techniques.

Table 24.4 lists techniques to assess and monitor possible changes of the secondary and tertiary structure of the protein under: 'secondary structure changes'. Here a set of chromatographic and spectroscopic techniques provides information on the stability of the folding pattern.

Table 24.6 gives a real-life example of the analytical toolbox being used for establishing biosimilarity for a monoclonal antibody. Apart from the (physico) chemical techniques, a number of cell-based and ELISA tests complete the panel.

Table 24.6 Summarized attributes with analytical methods and assessment results to assess biosimilarity for adalimumab

Category	Product quality attributes	Analytical methods	Assessment
Physicochemical characterization			
Primary structure	Molecular weight	Intact mass analysis for glycosylated and deglycoylated under reducing/non-reducing conditions	Highly similar to the reference product
	Amino acid sequence	Peptide mapping by LC-ESI-MS/MS using a combination of digestion enzymes	Identical to the reference product
	Methionine oxidation deamidation non-glycosylation C-terminal and N-terminal variants	Peptide mapping by LC-ESI-MS/MS	Highly similar to the reference product
	Disulfide linkage mapping	Peptide mapping under non-reducing condition	Highly similar to the reference product
	Free sulfhydryl group	Fluorescence detection kit	Slightly higher in free sulfhydryl group but not clinically meaningful
Higher order structure	Protein secondary and tertiary structure	Far- and near-UV CD, FTIR spectroscopy, intrinsic and extrinsic fluorescence spectroscopy H/DX-MS, antibody conformational array	Highly similar to the reference product
	Thermodynamic stability	DSC	Highly similar to the reference product
	Extinction coefficient determination	Amino acid analysis, SEC/UV/MALLS/RI	Highly similar to the reference product
Carbohydrate structure and composition	N-linked glycosylation site determination	LC-ESI-MS/MS	Highly similar to the reference product
	N-glycan identification	Procainamide labelling and LC-ESI-MS/MS	Minor difference was observed but not clinically meaningful
	N-glycan profile analysis	2-AB labelling and HILIC-UPLC	Similar in terms of %Afucose+%HM, %Charged glycans of S85 is slightly higher, but not clinically meaningful
Size heterogeneity	High molecular weight	SE-HPLC, SEC/MALLS, SV-AUC	Highly similar to the reference product
	Low molecular weight	CE-SDS (non-reducing/reducing)	Highly similar to the reference product
Particulates		DLS and MFI	Highly similar to the reference product
Charge heterogeneity	Acidic and basic variants	CEX-HPLC, idEF	Slightly higher acidic and lower basic level compared to the reference product, but not clinically meaningful
Quantity	Protein content	UV/VIS at A280	Highly similar to the reference product
Biological characterization			
Fab-related biological activity	TNF neutralization activity	TNF neutralization assay by NF-kB reporter gene assay	Highly similar to the reference product
	TNF binding activity	FRET	Highly similar to the reference product
	Apoptosis activity	Cell-based assay	Highly similar to the reference product
Fc-related biological activity	Transmembrane TNF-α binding assay	FACS	Highly similar to the reference product
	FcRn binding	AlphaScreen*	Highly similar to the reference product
	FcyRIlla (V/V type) binding	SPR	Highly similar to the reference product
	ADCC using healthy donor PBMC	Cell-based assay	Highly similar to the reference product
	CDC	Cell-based assay	Highly similar to the reference product
	C1q binding	ELISA	Highly similar to the reference product

These methods and results are representative examples of 55 test items were used for similarity assessment
From Lee et al. (2019) [20]. For abbreviations, see original article

Nucleotide Based Vaccines: A Case Study

Biological medicinal products discussed so far are all based on glycosylated or non-glycosylated amino acid polymers. However, there are other basic structures in the category of biologicals. One such family of polymer structures is based on nucleic acids and examples can be found among vaccines and gene therapeutics. These nucleic acids are hydrophilic, negatively charged

(continued)

polymers with as building blocks nucleotides: a nucleobase linked to a ribose/deoxyribose sugar and a phosphate group forming RNA and DNA molecules, respectively. The polymers can vary in length and structure. Some siRNA (small interfering RNA) medicines such as patiseran are based on less than 50 nucleotides in double stranded conformation. On the other side of the size spectrum are viruses used in gene therapeutics with a genome size of >50 kb (kilobases), i.e. a molecular weight of >10 million (g/mol). Nucleotide polymers can form secondary and tertiary structures, just like proteins. The double helix of chromosomal double stranded DNA is a well-known example.

Typical examples of nucleotide medicinal products are the mRNA COVID-19 vaccines. Upon intramuscular injection and cell entrance the single stranded mRNA molecules with around 4000 nucleotide units are translated into the spike protein of the SARS-CoV-2 virus and an immune response against this antigen is triggered.

There are two subjects that need to be discussed related to the action of these mRNA vaccines. (1) the essential role delivery systems play to make these vaccines successful and (2) stability issues related to these mRNA vaccines [21].

Re 1: Lipid nanoparticles: LNP and mRNA vaccines

mRNA is a large, hydrophilic and highly negatively charged polymer that cannot enter a cell on its own. It needs a carrier system. The carrier systems that are being used in the COVID-19 vaccines are lipid nanoparticles (LNP) consisting of (phospho)lipids (Fig. 24.19. Typically, these particles have diameters in the 100 nm range. LNP forms a complex with the mRNA. Complex formation is based on the interaction between the ionizable cationic lipids of the LNP and the negatively charged mRNA polymers. They form the core of these particles and are surrounded by neutral helper-lipids and PEGylated lipids (Fig. 24.19). The PEGylated lipids are present to prevent aggregation. Apart from intracellular delivery through endocytosis the LNPs act as an adjuvant by causing a transient inflammation; this attracts antigen presenting cells to the injection site and that stimulates the desired immune response.

Re 2: Stability issues related to these mRNA vaccines

DNA based products are rather stable during manufacturing, storage, transport and in use. However, mRNA products, with as only difference a ribose sugar instead of a deoxyribose sugar, are highly sensitive to enzymatic and non-enzymatic attacks resulting in hydrolysis of the strand. In therapeutic proteins minor -batch independent- differences in structure are accepted, as they contribute to the therapeutic effect (see Fig. 24.17). However, each break in the mRNA strand causes failure of mRNA translation into the protein antigen. Degradation kinetics are dependent e.g., on the pH (typically pH 7–8 is chosen). Secondary and tertiary structure formation of the mRNA strand also slows down hydrolysis. Formulation in LNP helps in stabilizing the mRNA molecule. However, long-term stability (> months) can only be achieved by storing the product at subzero temperatures. Upon thawing the mRNA-LNP degrades and loses activity dependent on the chosen conditions. Therefore, the healthcare professional should follow the rules about storage, expiry and use as outlined in the company's information leaflets. Real world data indicates that mechanical stress causes aggregate formation and by that precludes vaccine injection.

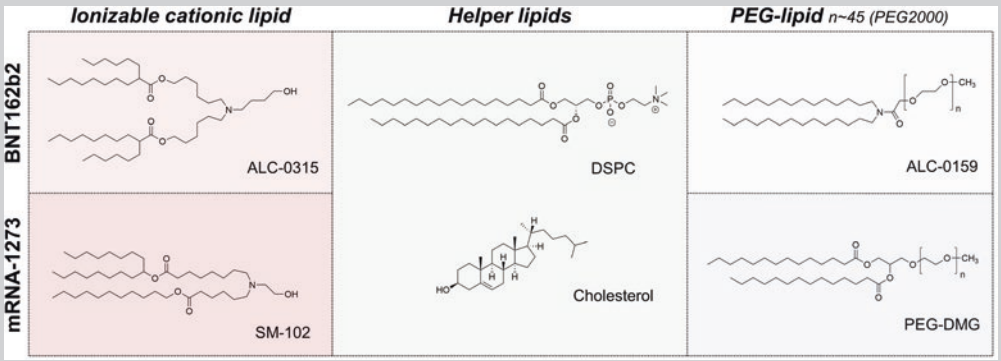


Fig. 24.19 Lipids used in the mRNA-LNP COVID-19 vaccines BNT162b2 (tozinameran) and mRNA-1273 (elasomeran). (From Schoenmaker et al. 2021 [21])

24.3.5 Primary Packaging

The choice of the primary package type and material for biologicals depends on the route of administration i.e., the parenteral routes of administration. The formulator can choose from different types of primary packaging, such as vials, syringes, or cartridges, and materials. Using the subcutaneous route allows the patient to self-administer the biological. This is convenient when long-term dosing schemes are used. However, there are restrictions. For instance, the maximum subcutaneous injection volume has typically been 1–2 ml per injection site. However, over time, approaches to increase the volume e.g., by s.c. infusion or co-administration with hyaluronidase have established a foothold in the practice of administration. Prefilled syringes and pen injectors, but not vials, are candidate packaging devices for subcutaneous or intramuscular injections. When frequent injections of variable doses are required as with insulins, cartridge-based pen injectors with reservoirs are selected for multidose administration. Vials are regularly used for products for intravenous injection or when freeze dried – to be reconstituted on site- products are prescribed (see also Chap. 8 Containers).

24.3.5.1 The Needle Diameter

The thinner the needle (the higher the gauge number (G)), the less pain is sensed at the injection site by the patient. However, that comes at a cost: a higher pressure is needed for a smaller diameter which causes additional shear stress for the active substance.

24.3.5.2 Glass or Polymer?

Prefilled syringes and vials are typically made of glass. However, nowadays, fully polymer-based syringes e.g., made of COP or COC (cyclic olefin (co)polymer) offer an alternative. These polymer-based syringes and glass are transparent. This offers the possibility of visual inspection of solutions to exclude the presence of aggregates or other particulate material. A point of concern is that the wall of glass syringes is coated with silicon oil as lubricant for a smooth, low-friction push of the plunger. However, some silicon oil may be released and form microscopic emulsion droplets in the aqueous phase offering an oil/water interface that leads to aggregate formation of the biological. Non-ionic surfactants such as polysorbate 80 or 20 and poloxamer 188 can mitigate this, as they preferentially adsorb to this interface. Another contaminant may be tungsten or tungsten oxides residues which are remaining in the bore area of the glass syringes when the bore is created with tungsten pins. Tungsten residues are reduced by specific washing procedures or avoided by using ceramic pins.

24.3.5.3 Leachables

The quality of the container material deserves attention. Material may leach out: metal ions from the glass walls and organic material from polymer material, plungers and barrels. Vendors and formulation conditions (e.g. pH) should be carefully selected. Glass delamination may also be mentioned as a cause for instability of the packaging material; this is most relevant for alkaline solutions.

24.3.6 Formulation Development

The therapeutic protein (API) needs excipients to ensure a safe and stable product. Table 24.7 lists the common excipients and their function.

24.3.6.1 Buffer Selection

Regularly used buffers are histidine, phosphate, citrate, acetate or succinate. Typically, protein formulations have a pH between 4 and 8. The pH of a solution can have a major impact on the stability of the protein active substance. Asp deamidation is one of the degradation reactions that is pH dependent. The pH also controls protein solubility. Furthermore, the pH influences protein-protein interactions and thereby aggregation and viscosity (at higher protein concentrations in particular). Protein solubility is minimal at its iso-electric point (i.e.p.), where the net charge of the protein

Table 24.7 Excipients used in therapeutic protein medicinal products, Crommelin et al. 2018 [14]

Excipient class	Function	Examples
Buffers	pH control, tonicity	Histidine, phosphate, acetate, citrate, succinate
Salts	Tonicity, stabilization, viscosity reduction	Sodium chloride
Sugars[a], polyols	Tonicity, stabilization, cryoprotection, lyoprotection[b], bulking agent[b], reconstitution improvement[b]	Sucrose, trehalose, mannitol, sorbitol
Surfactants	Adsorption prevention, solubilization, stabilization, reconstitution improvement[b]	Polysorbate 20, polysorbate 80, poloxamer 188
Amino acids	Stabilization, viscosity reduction, tonicity, pH control, bulking agent[b]	Arginine, glycine, histidine, lysine, proline
Anti-oxidants	Oxidation prevention	Methionine, sodium edetate
Preservatives[c]	Bacterial growth prevention	m-cresol, benzyl alcohol, phenol

[a]Only non-reducing sugars
[b]For freeze-dried products
[c]Multi-dose containers

is zero. Preferably, the pH for the protein solution should be chosen 'far away' from the i.e.p., but other considerations may limit the options e.g., the preferred pH range for optimum protein shelf life and restrictions related to the pH range where pain sensation is acceptable for the patients; for subcutaneous administration that is the pH 4–8 range. Buffers may not always be needed. Highly concentrated protein solutions (≥100 mg antibody/mL) as encountered with a number of monoclonal antibody products have sufficient self-buffering capacity and no extra buffers are necessary.

24.3.6.2 Salts

Sodium chloride may be added for tonicity purposes. However, salts may also be used to physically stabilize the protein or to reduce viscosity in highly concentrated monoclonal antibody formulations (up to 300 mg/mL) that are required because of the subcutaneous injection volume restrictions (1–2 mL).

24.3.6.3 Sugars/Polyols

Sugars and the polyols sorbitol and mannitol are used for tonicity reasons. They have other beneficial properties as they may act as cryoprotectants i.e., they protect proteins from structural changes when the product is accidentally or intentionally exposed to freezing temperatures and goes through a freeze-thaw process. In freeze dried products the sugars sucrose and trehalose act as protectants of the protein structure; they are called lyoprotectants. The mechanisms by which they provide this protection are discussed elsewhere [14]. See also Chap. 6 'Physical chemistry'. For lyophilized products, mannitol can be used as a cryoprotectant and bulking agent. Sorbitol is a cryoprotectant as well e.g., for frozen liquid formulations, but not suitable for lyophilization due to the low glass transition temperature (Tg) which will result in collapsed formulations. Among the sugars, only non-reducing sugars can be used, usually trehalose or sucrose; with reducing sugars the Maillard reaction with primary amines in the protein chain may lead to (colored) reaction products.

24.3.6.4 Surfactants

The foremost reason to use low concentrations of non-ionic surfactants such as polysorbate 20 and 80, as well as poloxamer188 is to cover interfaces (air/water, silicon oil/water, siliconized glass/water, plunger/water, infusion line/water). This prevents adsorption of proteins to these interfaces which may lead to aggregate formation (see above and Fig. 24.18).

24.3.6.5 Amino Acids

Amino acids such as arginine, glycine, lysine and histidine can regularly be found in protein formulations. Their main function is solubility enhancement, except for histidine which also has buffering capacity in the relevant pH range, pH 5–7. Table 24.7 mentioned other functions as well. Viscosity reduction when highly concentrated monoclonal antibody formulations are required may be another reason to add arginine, proline and lysine. The exact mechanisms underlying these actions are still poorly understood.

24.3.6.6 Anti-oxidants

Methionine, cysteine, tryptophan, tyrosine, and histidine are sensitive to oxidation. These amino acids occur in all proteins. The position of these amino acids in the protein structure determines their reactivity as oxidative agents need to gain access. Oxidative stress can be reduced by minimizing the headspace in vials and using inert gasses instead of air (e.g., argon).

Moreover, one may consider the addition of antioxidants, such as methionine. However, one has to be careful, as trace concentrations of heavy metal ions (as catalysts) may turn antioxidants into oxidants. That may happen with ascorbic acid. The addition of the metal chelator EDTA (ethylenediaminetetraacetic acid) blocks this oxidative route.

24.3.6.7 Preservatives

After taking the first dose from a multi-dose container the opened, probably contaminated, container has to be stored under proper conditions and the residual doses should be administered within a prescribed period of time. Preservatives can be added to the formulation to extend shelf life of the opened vial. Chlorobutanol, benzyl alcohol, phenol and meta-cresol are used as preservatives. They may interact with the protein API and that may lead to protein and/or preservative inactivation. But, it can also work out differently. Insulin forms stable complexes -on storage- with zinc-ions and with phenol or m-cresol that rapidly dissociate upon injection [22].

24.3.6.8 Freeze-Drying

Some pharmaceutical proteins are too unstable in solution to achieve acceptable shelf-life conditions, i.e., minimally 2 years of storage time under refrigerator conditions. Then, freeze-drying should be considered. The freeze-drying process protocols are basically similar to freeze drying protocols for non-biologicals. However, a few special features apply when proteins are freeze dried. Lyoprotectants such as sucrose and trehalose are required to stabilize the delicate protein structure. When reconstituting the cake, swirling is preferred over shaking to avoid bubble (air-water interface) formation. See also Chap. 6 Physical chemistry.

24.4 Biopharmaceutics and Use of Therapeutic Proteins

24.4.1 Introduction

During formulation development it is important to design a robust formulation that ensures protein stability also during handling and final administration to the patient in the clinic or at home. The medicine will be subjected to a number of

handling steps, for example, reconstitution for lyophilized products, dilution with vehicle infusion solutions e.g., with 0.9% NaCl, 5% glucose, Ringer-lactate, Ringer-acetate infusion solution, contact with and storage in different primary containers (infusion bags made of different materials, syringes), transportation within the hospital e.g., shear stress through pneumatic tube transport and finally infusion to the patient with contact to tubing and in-line filters.

Some biologicals applied in oncology patients fall under the category of hazardous medicines mainly based on their reproductive toxicity. In the United States, according to the National Institute for Occupational Health and Safety (NIOSH) and USP <800> classifications, it is required to use closed system transfer devices (CSTDs) during handling as part of personal protective equipment even if a biological may not be hazardous [23]. There are different types of CSTDs. They all come with varying and large dead volumes leading to loss of product and additional contact materials because of the complexity of the CSTDs involving numerous parts and adaptors.

All these aspects mentioned are covered during so called Pharmacy Manual studies, clinical in-use stability or compatibility studies as part of the medicinal product development process. The aim of the studies is to confirm that the protein in its formulation remains stable during usage and that the patient receives the correct dose. Furthermore, these studies are the basis for defining in-use stability given in the summary of product characteristics.

As there are numerous types of vendors, brands and materials used in clinical practice, also with regional differences between countries and continents, it is difficult to mimic and cover all aspects. The manufacturer needs to make a careful selection to include materials e.g., for infusion bags made of polypropylene (PP) or polyvinylchloride (PVC), etc. and equipment e.g., type of pumps, relevant for the medicine of interest and planned route of application.

The analytical methods used in this stage can be based on the methods used during formulation development. Adjustments may be required, for instance, for protein content determination at low concentrations in highly diluted infusions. In addition, dilution of the protein with a concentration in the high mg/mL range to a few µg/mL increases the risk for adsorption loss to contact surfaces. Then, the final dose reaching the patient may be too low. In this case, it may be relevant to develop a dedicated diluent containing a surfactant, instead of using off-the-shelf vehicle solutions.

In case the clinical in-use study detects incompatibilities of a protein with certain conditions e.g., leading to a ban of certain vehicle solutions or handling practices, this information can be added to the package leaflet. Examples are statements like “do not shake”, or “do not use 0.9% NaCl for dilution”, or “do not use 5% glucose for dilution”, which e.g. all are applicable for trastuzumab containing originator product and biosimilars.

24.4.2 Pharmacokinetics

In contrast to intravenous administration, subcutaneous administration needs absorption from the subcutaneous reservoir, generally resulting in a longer time to reach the Cmax as compared to intravenous administration. This can be illustrated for the monoclonal antibody trastuzumab which can be administered by both routes. After subcutaneous administration of trastuzumab the median time to reach Cmax is 3 days and the Cmax was about 40% higher in the intravenous treated group, which can be explained by the process of absorption from the subcutaneous reservoir [24]. In contrast, the median observed trough level at the end of cycle 7 was higher in the subcutaneous group. In spite of the differences in pharmacokinetics, the clinical trial showed no difference in efficacy between the intravenously and subcutaneously treated patients. To improve absorption after subcutaneous administration the formulation of trastuzumab has been amended. Recombinant human hyaluronidase was added to temporarily degrade hyaluronan at the injection site resulting in improved absorption [25]. In contrast to the intravenous route, antibodies administered via the subcutaneous route generally pass through the lymphatic system. This may result in a bioavailability of less than 100% [26].

Pharmacokinetic profiles of pharmaceutical proteins vary widely. The relatively small interleukins and insulins, for example, have short half-lives upon injection as they are filtered by the glomeruli, while larger therapeutic proteins, like monoclonal antibodies, have half-lives of several weeks and are usually degraded by proteolysis. One simple solution to prolong the activity is to use continuous intravenous infusion. Alternatively, delivery systems for sustained release may be chosen. Biodegradable microspheres have been highly successful for the prolonged (dosing interval of months) delivery of peptides, e.g. the LHRH agonists. They are now used for GLP-1 extended release systems, i.e. exenatide, for a once per week injection. Finally, several strategies have been developed to increase plasma half-life by chemically modifying the protein. For insulins the protein molecule is modified with a lipid chain so that it links up with the long circulating albumin in blood and is slowly released, insulin detemir. PEGylation of proteins such as interferons dramatically increases plasma half-life as the glomerular filtration rate is reduced. Changes in the production process which might influence, among others, the glycosylation of therapeutic proteins can also have an impact on the therapeutic half-life [27].

The pharmacodynamic characteristics are generally related to the pharmacokinetic profiles, e.g. a longer half-life

generally results in a longer pharmacodynamic effect. For some therapeutic proteins, however, the pharmacodynamic effect is much longer as compared to the pharmacokinetic profile. Rituximab, for example, is a monoclonal antibody which depletes B-cells and has a therapeutic half-life of approximately 3 weeks. However, it might take up to 12 months after administration of the last dose before the B-cells might reach the pre-treatment level. This is not only relevant for the efficacy of rituximab but also for the safety as adverse events might develop months after treatment with rituximab is stopped.

24.4.3 Therapeutic Use

Therapeutic proteins are important treatment options for a variety of diseases. One of the first therapeutic proteins produced by recombinant DNA technology was insulin, which is a hormone used to treat diabetes. Other hormones currently in use have a similar function as insulin: suppletion of a missing endogenous hormone or the presence of an endogenous hormone with limited availability. Monoclonal antibodies are an important part of the therapeutic arsenal and are mainly used in oncology and a variety of auto-immune disorders like rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis. The pharmacological action and the safety can often be related to the specific interference in the process of the disease.

This can for example be illustrated by the Tumor Necrosis Factor alpha (TNF-α) inhibitor, infliximab. TNF-α plays an important role in several auto-immune diseases including rheumatoid arthritis and by (partly) blocking the activity of TNF-α, infliximab directly interferes in the process of the disease.

After marketing approval and wide spread use of infliximab in clinical practice cases of patients developing tuberculosis were reported. After additional research this could be explained by TNF-α playing an important role in the defense against *Mycobacterium tuberculosis*, the bacterium responsible for causing tuberculosis. These cases of tuberculosis could be traced back to patients with latent tuberculosis and further research showed that administration of TNF-α inhibitors diminishes the natural defense against *Mycobacterium tuberculosis* resulting in tuberculosis. This knowledge has resulted in the need for screening for latent tuberculosis before treatment with a TNF-α inhibitor is initiated. If the patient has latent tuberculosis this should be eradicated before treatment with a TNF-α inhibitor is started [28]. For other therapeutic proteins the relation between a specific adverse event and the pharmacological action is less clear but, in general, it is known that infections occur in patients treated with therapeutic proteins with an immunosuppressive mode of action. Another example is the use of high doses of epoetin resulting in a haemoglobin level above the reference values resulting in blood with enhanced viscosity and, consequently, in thromboembolic events.

24.4.3.1 Immunogenicity

An adverse event which is specific for therapeutic proteins is immunogenicity. Immunogenicity consists of an immune response against an administered therapeutic protein and relates to the administration of a foreign protein which might be recognized as such by the human immune system. It is important to realise that this immune response is specifically needed after the administration of vaccines. However, for therapeutic proteins the goal is generally to minimize the risk for immunogenicity. The immune response against an administered therapeutic protein can have several consequences. There are cases of the existence of transient anti-drug antibodies (ADA) without any clinical consequence. ADAs can, however, also have an impact on the efficacy of a therapeutic protein in those cases where the patient develops neutralizing ADAs. These ADAs neutralize the effect of the therapeutic protein with a direct negative impact on efficacy. The presence of ADAs can also have a negative effect on safety. For some therapeutic proteins, it is known that patients with ADAs have a higher risk of developing unwanted reactions during administration. These infusion- and injection related reactions will be discussed in more detail in the section on administration. Very severe safety problems might occur in case neutralizing ADAs cross react with endogenous available protein [29]. This can be illustrated by a landmark example in patients treated with epoetin α, which is administered to patients on dialysis who are not able to produce enough endogenous epoetin. After a change in formulation of the epoetin α some patients developed antibodies which cross reacted with the endogenous epoetin resulting in patients becoming epoetin deplete and developing the very serious reaction, pure red cell aplasia [30]. Another example, are clearing antibodies which break down the therapeutic protein and, therefore, have an impact on the pharmacokinetics of the therapeutic protein generally resulting in shorter half-lives.

An immune response against a therapeutic protein can be influenced by either patient- and disease-related factors and/or product related factors. Patient-related factors include genetic factors which might influence the immune system and age-related factors. Regarding disease-related factors, it is known that patients with allergies and infections have a higher risk for an unwanted immune response. Concomitant use of other immunosuppressive medicines might lower the risk for an unwanted immune response as the immune system is suppressed. Immunogenicity might also be influenced by the route of administration with intravenously administered therapeutic proteins, in general, having a lower level of immunogenicity as compared to the subcutaneously administered proteins. Product related factors can either be related to the active substance, the medicinal product (active substance including formulation and packaging) or a combination of both. The active substance can either relate to the protein structure and/or post-translational modifications which can either have a negative or a positive impact on the immunogenic profile. Suppletion of an endogenous protein may, for example, trigger an immune response if the amino acid sequence differs between the exogenous therapeutic protein and the endogenous protein. Glycosylation of the therapeutic protein can also increase immunogenicity, as well as pegylation of therapeutic proteins. Pre-existing anti-PEG-antibodies have shown to cross-react with the PEG-part of pegylated therapeutic proteins. On the other hand, pegylation and glycosylation might also limit the immunogenic potential of the therapeutic protein by shielding immunogenic epitopes. Formulation and packaging is an important aspect to take into consideration as these might impact immunogenicity [29]. The previously described case of PRCA with epoetin α was related to a change in formulation of the product probably resulting in the formation of aggregates of epoetin α which triggered the immune system. Impurities, e.g. contaminants of the manufacturing process, in either the medicinal product or active substance might trigger an unwanted immune response but also the formation of aggregates as a result of improper storage of the therapeutic protein might have a negative impact on the immunogenic profile [29].

In summary, an immune response against a therapeutic protein can have a relevant impact on efficacy and/or safety and can be influenced by either patient-, disease-, and product-related factors. Especially, the product-related factors are important to take into consideration during product development.

24.4.4 Administration

Most therapeutic proteins are administered either via the subcutaneous or intravenous route. The subcutaneous route generally provides patients the possibility to administer the product themselves in the home setting. A well-known example of a subcutaneously administered therapeutic protein is insulin. Preferably, a subcutaneously administered therapeutic protein is available in a pre-filled pen or syringe which can easily be used by the patient. This prerequisite directly relates to the characteristics of the therapeutic protein and the availability of a formulation in which the therapeutic protein is stable in a solution. Administration of therapeutic proteins by inhalation or nasal administration has been explored for specific proteins, e.g. human insulin In addition, certain therapeutic proteins are administered for local effects, which is, for example, the case with therapeutic proteins administered via the intravitreal route resulting in local effects in the eye.

Requirements regarding the formulation are described in more detail in Sect. 24.3. The formulation, e.g. pH, and the use of certain excipients, e.g. use of citric acid, might influence the frequency and severity of injection-related reactions which are frequently reported in relation to subcutaneous administration of therapeutic proteins. In addition, packaging, e.g. presence of latex, thickness of the needle, of the medicinal product can also have an impact.

Therapeutic proteins administered via the intravenous route generally need handling and preparation at the pharmacy to make the product ready for administration and intravenous administration requires a nurse to administer the protein. In most cases, intravenously administered therapeutic proteins are diluted in an infusion bag before administration. The stability of the therapeutic protein after dilution in an infusion bag might be limited resulting in products with a relatively short period until expiration.

Due to the parenteral route of administration therapeutic proteins are often related to injection-, and infusion-related reactions. Reactions can either be very mild, like redness at the injection site, or very serious resulting in life-threatening reactions. Infusion-related reactions are common with monoclonal antibodies and timely related to administration and have been reported as anaphylaxis (IgE-mediated), anaphylactoid reactions and cytokine release syndrome (rapid release of proinflammatory cytokines) [31]. Symptoms might be the same and include, among others, severe hypotension, bronchospasm, laryngeal or pharyngeal oedema, wheezing and/or urticaria. Treatment encompasses among others, corticosteroids, epinephrine, and oxygen. Suspected or proven allergic reaction is a contra-indication for further exposure to the therapeutic protein [31]. The symptoms for injection- and infusion related reactions can also be very general, like fever and headache, which makes them difficult to relate to the administered therapeutic protein.

Non-allergic, infusion-related reactions generally occur during the first administration of therapeutic proteins. The risk for these reactions is often mitigated by the administration of co-medication, e.g. antihistamines and/or corticosteroids. Another measure to mitigate the risk for these non-allergic immune reactions includes administration of the protein at a low speed which is gradually increased in case the patient does not develop an immune reaction during administration. For the monoclonal antibody bevacizumab, for example, the advised time for administration is 90 min for the first administration which can be reduced to 60 min for the second administration and 30 min to subsequent administration unless the patient develops reactions. Also the recommended time for observation of the patient after the first administration is for some therapeutic proteins longer as compared to following administrations in case the patient did not develop a reaction at the previous administration [29].

24.4.5 Logistics

Increased immunogenicity of a therapeutic protein might occur due to, for example, the formation of aggregates after improper storage and/or transportation. This can be illustrated by reported cases of pure red cell aplasia in patients treated with epoetin α in Thailand, which could be traced back to improper storage conditions by unauthorized vendors and increased formation of aggregates [32]. Storage conditions should, therefore, be carefully controlled during storage and transportation of therapeutic proteins. Therapeutic proteins often need to be stored and transported between 2 °C and 8 °C. If the conditions during storage and transportation become either too low or too high this can negatively impact the quality characteristics, as illustrated by the previous cases of pure red cell aplasia in Thailand. Besides the need for controlled temperature, some hospitals use pneumatic tube transportation for syringes and infusion bags with therapeutic proteins. It has been shown that use of pneumatic tube transportation can increase the number of particles in the solution, which might have a negative impact on the characteristics of the therapeutic proteins [33].

Some therapeutic proteins are, however, stored and administered by the patient themselves. This often results in improper storage conditions during transportation from the pharmacy to the home of the patient. It has been shown that frequently used cool boxes to transport therapeutic proteins from the pharmacy to the home of the patient are often not compliant with the recommended storage conditions. In addition, a study among 255 patients showed that most patients do not store their therapeutic proteins according to the recommended temperature range [34]. Although the therapeutic proteins were stored in the refrigerator, the temperature of the refrigerator was often outside the specifications and the place of storage also had an impact, e.g. storage in the door of the refrigerator often resulted in higher mean temperatures. Improper transportation and storage of therapeutic proteins can impact the formation of aggregates. It has been shown that low temperatures (−20 °C) and multiple freeze-thaw cycles in consumer refrigerators can increase the number of subvisible particles as a result of the formation of aggregates [35]. The clinical relevance of these aggregates are currently not known but these findings in relation to the cases of pure red cell aplasia in Thailand supports careful storage and transportation of therapeutic proteins.

PART 2: Advanced Therapy Medicinal Products

24.5 Advanced Therapy Medicinal Products

24.5.1 Definitions and Legislation

24.5.1.1 The "ATMP Regulation"

Cell and tissue based ATMPs have evolved as a result of scientific advances in the stem cell transplantation field. As the development in this area resulted in increased complexity of processing cellular products, a European regulation was introduced in 2007 (the "ATMP Regulation" 1394/2007) which amended the Medicines Directive 2001/83/EC and defined ATMPs as medicinal products, thereby introducing the requirement for manufacturers to be approved by medicines regulators and for licensed products to hold marketing authorisations. The definition is also applicable to investigational medicinal products used in clinical trials.

An advanced therapy medicinal product is a biological medicinal product that can be classified as either one of or a combination of the following three categories:

Somatic Cell Therapy: a biological medicinal product which contains or consists of cells or tissues that; (1) have been substantially manipulated, or (2) – are not intended to be used for the same essential function(s) in the recipient and the donor.

Tissue Engineered Product (TEP): a biological medicinal product that contains or consists of cells or tissues administered to human beings with a view to regenerating, repairing or replacing human tissue. A tissue engineered product may contain cells or tissues of human or animal origin, or both. The cells or tissues may be viable or non-viable. It may also contain additional substances, such as

cellular products, bio-molecules, bio-materials, chemical substances, scaffolds or matrices.

Gene Therapy Medicinal Product (GTMP): a biological medicinal product which; (1) contains an active substance which contains or consists of a recombinant nucleic acid used in, or administered to human beings, with a view to regulating, repairing, replacing, adding or deleting a genetic sequence, and (2) its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence.

An ATMP can also be classed as a combined product; this is a combination of any of the above with a medical device.

Products may fit into more than one of these categories. Where a product fits the definition of either a somatic cell therapy or a tissue engineered product, and also a gene therapy, the product is classified as a gene therapy medicinal product.

In addition to standard medicines regulation and adherence to the 'ATMP Regulation', additional legislation may be applicable depending on the class of the ATMP as described below.

24.5.1.2 Contained Use of Genetically Modified Micro-organisms

For gene therapies the requirements of the European Directive 2009/41/EC on the contained use of genetically modified micro-organisms must be taken into account. This directive has been enshrined in local legislation according to individual member state requirements but the principles universally require a risk assessment to assess the risks posed to the environment and to human health by introduction of the genetically modified organism, and to ensure that suitable risk mitigation measures are in place. In practice, the risk assessment should consider the arrangements in place in relation to the patient, the product and the waste generated by the product handling and by the patient. Hence the involvement of pharmacy in this assessment is of vital importance to ensure all aspects of handling are included [36].

There are four classes of activities:

Class 1 – activity of no or negligible risk for which containment level 1 is appropriate to protect human health and the environment.

Class 2 – activity of low risk for which containment level 2 is appropriate to protect human health and the environment.

Class 3 – activity of moderate risk for which containment level 3 is appropriate to protect human health and the environment.

Class 4 – activity of high risk for which containment level 4 is appropriate to protect human health and the environment.

Containment measures should be proportionate to the risks and introduced on a case by case basis but guidance is given in the European Directive [37] and this has been carried forward into the requirements for the member states.

Where a Gene Therapy is classed as an investigational medicinal product and contains a genetically modified organism a risk assessment, as described, is mandatory. Where the product has been granted a marketing authorisation then the required handling is detailed in the summary of the medicinal product characteristics and the risk assessment is not mandated. Pharmacists are recommended, however, to consider the value of using the same approval process, including a risk assessment as part of the governance process when introducing a marketed gene therapy. The expertise is beneficial for licensed medicines and non GMO investigational GTMPs, as traditional access to new medicines' systems (e.g. medicines management committees) and Research and Development committees may not include specific GTMP handling expertise and therefore will benefit from having expert input. In this way it can be assured that the local governance process will always ensure that suitable handling arrangements are in place for the product, including, for example, what to do in the event of a spillage of a replicating gene therapy; and for the patient, including, for example, whether patient waste can be discharged to the sewerage system or should be collected and incinerated in the event of the use of a shedding gene therapy.

The GMO risk assessment should be divided into the three separate, but overlapping, pathways: **The product pathway** considers:

- the properties of the GTMP
- receipt and storage of the GTMP
- preparation of the GTMP for administration
- transport and containment of the GTMP
- GTMP tracking system – from receipt through to destruction

The patient pathway considers:

- administering the GTMP
- patient handling and emergency procedures
- sampling and monitoring of shedding (if required)
- criteria for patient monitoring and discharge post treatment
- interactions with other patients and staff, visitors and family

The waste pathway considers:

- stages at which contaminated waste is generated
- transport and containment of waste
- inactivation and disposal

24.5.1.3 Cell and Tissue Directive

For ATMPs that contain or consist of cells or tissues, some requirements of Directive 2004/23/EC, also referred to as the European Tissues and Cells Directive, must be taken into account. The Directive covers all steps in cell processing from donation or procurement, testing, processing, cryopreservation, storage to distribution and is applied in the early stages of the ATMP manufacturing process. The Tissue and Cells Directive applies to all stages of ATMP starting material handling up until the point of the start of the medicine manufacturing process, at which point the ATMP Regulation takes effect.

24.5.1.4 Regulation in Different Member States

ATMPs are legally classed as medicinal products, therefore manufacture of the product is regulated by the medicines regulator and QP certification is required.

In Europe ATMPs are centrally regulated by the European Medicines Agency (EMA) in a process that aligns closely with EMA processes for other biological medicines. In the UK a parallel process is undertaken by the Medicines and Healthcare Regulatory Agency (MHRA). Hence the process for gaining a marketing authorisation is clearly set out. There is similar clarity surrounding the requirements for the manufacture of investigational ATMPs for use in clinical trials.

On occasion, the manufacture of unlicensed ATMPs is permissible. The ATMP Regulation states that an ATMP which is prepared on a non-routine basis according to specific quality standards, and used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner, in order to comply with an individual medical prescription for a custom-made product for an individual patient, can be exempted from the requirement for the manufacturer to hold a manufacturer's authorisation.

There is no definition of "non-routine basis" and the requirements imposed by the competent authority for individual member states differ and should be confirmed by the pharmacy team as part of the organisational governance arrangements.

When introducing the use of an ATMP into any healthcare organisation pharmacists should be central to the process ensuring appropriate organisational governance, operational handling and clinical suitability (see section on Considerations for Pharmacy). Governance considerations must ensure that regulatory compliance is in place.

It is recommended that pharmacists understand the regulations appropriate to their environment and document local requirements for governance in organisational policies such as an ATMP policy, and/or an unlicensed medicines policy [38].

24.5.2 Different Classes of ATMPs and Their Manufacture

Pharmacists, as healthcare professionals concerned with optimising medicines use for patient benefit, are key stakeholders in the adoption and implementation of ATMPs into routine clinical practice.

ATMPs however pose challenges for Pharmacy professionals. One of the challenges is that the concept of cell and tissue based "living medicines" are unfamiliar to pharmacy. Another is that the 3 categories of ATMP are uniquely different and require different approaches to adoption into routine practice.

As with traditional medicines, a range of techniques are used during the manufacture of ATMPs and the final product comes in a range of dosage forms, depending on the properties of the product. As ATMPs are biologic medicines based on tissue, cells or genes, the manufacture process is different to that of traditional medicines and the final dosage formulation must be suitable to maintain the integrity and, where applicable, the viability of the biologic product.

24.5.2.1 Gene Therapy

Gene therapies are designed to introduce genetic material into cells to compensate for abnormal genes or to make a beneficial protein which then multiplies and exerts a positive effect. Another mode of action is in place where a mutated gene causes a necessary protein to be faulty or missing, the GTMP may be able to introduce a normal copy of the gene to restore the function of the protein.

The manufacture of GTMPs is complex as a carrier called a vector is required to deliver the gene to the cell. Modified viruses are often used as vectors because they can deliver the new gene by infecting the cell. The viral vectors are genetically modified to ensure that they are non-pathogenic and do not cause disease when used in people. Upon delivery, the viral vectors infect dividing and non dividing cells and deliver a sustained expression of the gene under controlled manufacturing conditions. The viral vectors may be integrating or non-integrating. An example of an integrating viral vector is lentivirus. Lentiviruses enable the integration of the therapeutic gene into the cell's genome and the therapeutic gene is then retained as the cells divide and multiply. The most common non-integrating viral vector used for gene delivery purposes is adeno associated viruses (AAV). Adenoviruses are considered safer as they do not integrate the host cell genome but introduce their DNA into the nucleus of the cell. Another advantage is that AAV strains can preferentially infect certain cell types which enables targeting but due to a limited transgene capacity the AAV will be lost from dividing cells. Other commonly used vectors include retrovirus and herpes sim-

plex virus. Throughout manufacture care must be taken when handling the viral vectors and containment requirements as laid out in European Directive 2009/41/EC on the contained use of genetically modified microorganisms must be taken into account. There is currently a lot of research into the development of safer and more efficient vectors.

Gene therapies are further divided into two categories; in-vivo gene therapy and ex-vivo gene therapies, based on the process of genetic modification (see Fig. 24.20). If genetic modification occurs inside the body, it is called an in-vivo gene therapy whereas genetic modification which occurs outside of the human body is called an ex-vivo (cell based) gene therapy.

24.5.2.1.1 In-Vivo Gene Therapy

In-vivo gene therapies do not contain living human cells or tissues and the genetic modification of cells occurs directly within the patient's body. Routinely a vector is used to deliver the gene to the cells inside the patient as the medicine is injected directly into a specific tissue in the body or is administered intravenously where it then successfully infects the individual cells. In-vivo gene therapies have a formulation most similar to traditional intravenous medications. In-vivo gene therapies are usually formulated in vials which require aseptic preparation in the pharmacy prior to infusion. In-vivo gene therapies often require storage at ultra-low temperatures (e.g. −20 or −80 °C).

An example of in-vivo gene therapy is Voretigene neparvovec. Voretigene neparvovec is a gene transfer vector that employs an adeno-associated viral vector and is used to treat vision loss due to inherited retinal dystrophy caused by RPE65 mutations. RPE65 is a gene that codes for a protein needed for vision. RPE65 gene mutations cause vision to deteriorate and may progress to complete blindness. Voretigene neparvovec is administered via surgical injection beneath the retina of each eye and provides a working RPE65 gene to act in place of a mutated RPE65 gene which then works to restore the visual cycle (see SmPC Luxturna).

24.5.2.1.2 Ex-Vivo Gene Therapy

Ex-vivo gene therapies are a form of individualised medicine that contain living cells and where the genetic modification of cells occurs whilst outside the body. Ex-vivo gene therapy requires cells as a starting material to manufacture the product.

Cells are taken from a donor, usually the patient, to be used as the starting material for the medicinal product. The cells are then transferred to a manufacturing unit where a vector is used to introduce the gene to the donor cells. The donor cells undergo genetic modification and expansion in cell culture to form the medicinal product. The modified cells, now classed as a medicine, are shipped back to the hospital to be administered to the patient. Where the starting material originates from the patient's own cells, this is called an "autologous" therapy. Occasionally, the starting material may originate from another donor and this is termed "allogeneic" therapy.

Different viral vectors have been used during the manufacture of ex-vivo gene therapy, but in general the majority of final products received back in the hospital are class 1 GMOs and therefore pose negligible risk.

In addition to GMO considerations, principles of cell handling must apply to ex-vivo gene therapies. All cell based products are patient specific and require traceability from

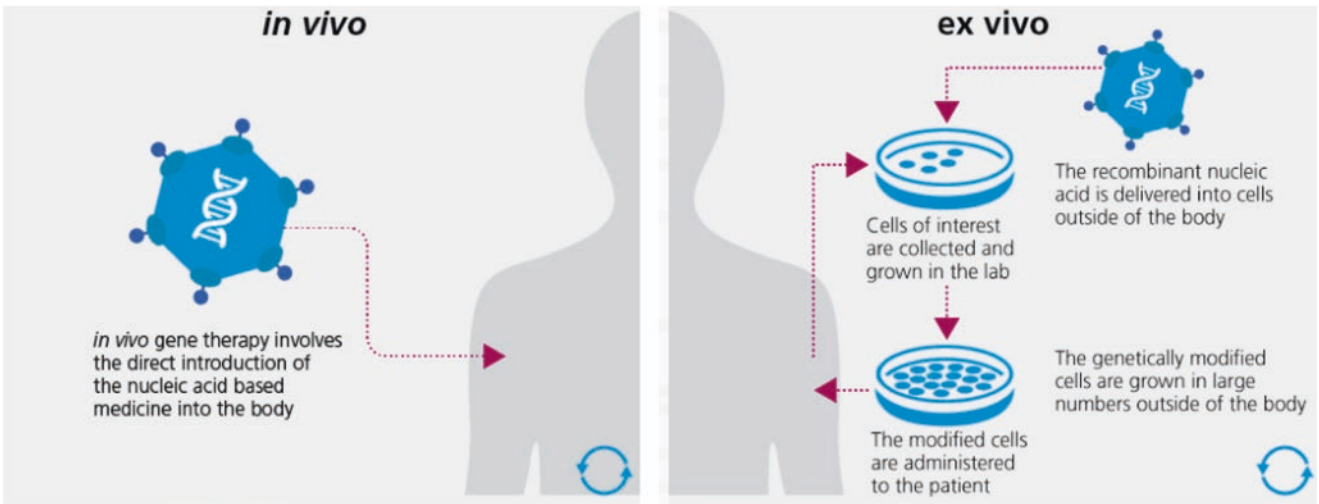


Fig. 24.20 Difference between in-vivo and ex-vivo gene therapy [39]. (REF: HEE elfh Hub (e-lfh.org.uk))

'vein-to-vein' as per the cells and tissue directive. This is most important when a product is autologous as there are potentially disastrous consequences if the medicine is administered to a patient for whom it is not intended.

Ex-vivo gene therapies contain cells so the final product formulation must ensure cell viability is maintained. The majority of cell products are cryopreserved, although some may be fresh. Cryopreservation involves storage at ultra low temperatures below −120 °C. Cryopreservation freezes cells and maintains them in a viable state. The medicines are therefore contained with dosage forms that are able to withstand these temperatures and are presented in cryobags or cryovials.

Occasionally cell based ATMPs will come 'fresh'. In this instance they will still be presented in bags or vials, but do not require the specialist material to withstand cryopreservation temperatures. 'fresh' cells should be kept refrigerated.

24.5.2.2 Somatic Cell Therapy

Somatic cell therapies contain or consist of cells or tissues that have been subject to substantial manipulation. As with ex-vivo (cell-based) gene therapy, somatic cell therapy is a type of cellular therapy in which viable cells are administered to a patient to elicit a therapeutic effect.

The manufacture and formulation of somatic cell therapy is similar to that of ex-vivo gene therapy and the principles of cell handling apply. The key difference is that the substantial manipulation undergone by the cells during manufacture of somatic cell medicinal products can consist of a variety of processes, but not genetic modification.

Cells or tissues are collected from a patient or donor to provide the source material for manufacturing the somatic cell therapy. The cells undergo substantial manipulation in the GMP manufacturing facility to form the medicinal product. Examples of substantial manipulation include cell

An example of an ex-vivo gene therapy is Chimeric Antigen Receptor T cells (known as CAR-T cell therapy; see Fig. 24.21). CAR-T cell therapy is a type of immunotherapy currently licensed to treat certain blood cancers. CAR-T cells enable T cells to recognise cancer cells and destroy them. T cells are collected from the patient and genetically modified in a GMP manufacturing facility to contain a chimeric antigen receptor (CAR) which is designed to recognise and target a specific protein on the cancer cells. Once administered back to the patient the CAR-T cells are now able to recognise and kill the cancer cells.

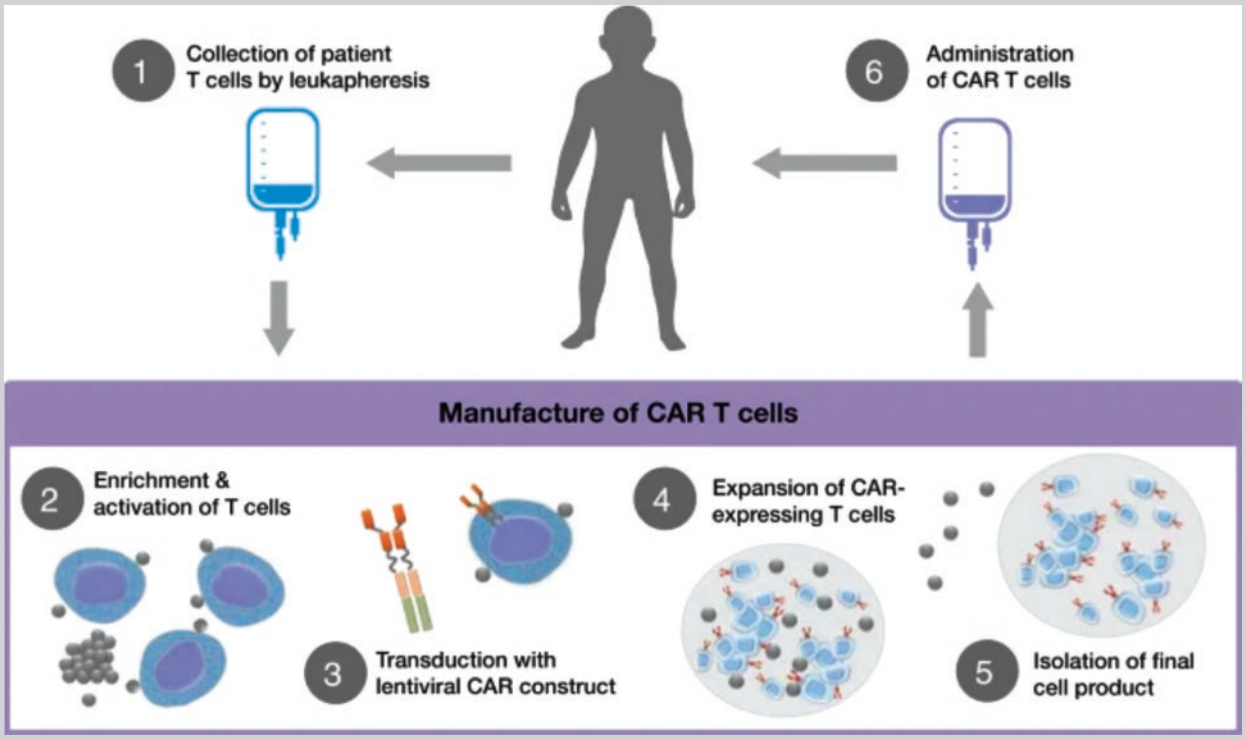


Fig. 24.21 Process of CAR-T cell manufacture [40]

expansion/culture, enzymatic digestion of tissue and differentiation or activation with growth factors. If the manipulation involves genetic modification of the cells, the medicine would be classed as an ex-vivo gene therapy as opposed to somatic cell therapy. The manipulated cells, now classed as a medicine, are shipped back to the hospital and administered to the patient.

Somatic cell therapies can be autologous (patient derived) or allogenic (donor derived). The principles of cell handling apply and traceability from 'vein to vein' is essential. Where a product is autologous there are potentially disastrous consequences if the medicine is administered to a patient for whom it is not intended. There is a role for pharmacy in ensuring that the risks, particularly around tracking and traceability, are minimised.

An example of somatic cell therapy currently being evaluated in clinical trials, is autologous tumour infiltrating lymphocytes (TILs) to treat melanoma and other solid tumours. Naturally occurring TILs are identified and isolated from the patient's tumour, expanded and purified ex vivo, and infused back into the patient where they kill the cancer cells but not healthy cells.

24.5.2.3 Tissue Engineered Products

A TEP consists of or contains engineered cells or tissues and is used in or administered to human beings with a view to regenerating, repairing, or replacing a human tissue. Where a product is autologous (uses a patient's cells or tissues as starting materials) there are potentially disastrous consequences if the medicine is administered to a patient for whom it was not intended. The starting material harvest and the administration are often part of a surgical procedure. There is a key role for pharmacists in ensuring that the risks, particularly around tracking and traceability, are minimised.

TEPs are usually 'fresh' and require storage at refrigerated temperatures. As with cell therapies, extreme care must be taken with product handling to ensure product viability is maintained. In addition, TEP often have very short expiry times (hours to days) which pose operational challenges.

An example of a TEP is autologous cultured chondrocytes indicated for the repair of symptomatic cartilage defects. It comprises a scaffold formed of collagen on which autologous chondrocytes are seeded. At implantation, the scaffold is trimmed to the size and shape of the cartilage defect. The collagen structure is held in place in the lesion with fibrin glue [41].

24.5.2.4 Manufacturing Challenges

ATMP s must be manufactured according to Good Manufacturing practice as described in Part IV EudraLex Volume 4 (Good Manufacturing Practice specific to ATMPs). Compliance brings several challenges for manufacturers including the following:

Raw materials: Sourcing donor starting materials carries extra regulatory requirements. There is a need to understand the consent arrangements and provide proof of the infectious disease markers prior to using the material. Labelling must ensure traceability and the logistics of distribution must ensure robust tracking.

Sterility assurance must ensure adequate viable and particulate contamination control to enable the manufacture of a sterile product. It is not possible to sterilize cellular medicines by heat, by irradiation or by filtration, therefore assurance of sterility must be built into the manufacturing process.

Quality Control is complex. Each cell or tissue based product must be functionally characterized during the validation phase. The release specification must take into account a potency assay as well as cell number, viability and impurity limits. This is in addition to microbiological tests. On occasion where the shelf life of the ATMP is very short, a two stage release process may be required.

Uniquely for cellular ATMPs where the product is personalized for an individual patient, if the product does not meet its release specification, it can under exceptional circumstances be requested for use by the treating physician where they feel it would be in the best interests of their patient to receive the out of specification product.

The **Shelf-life** of an ATMP should be maximized and should be determined by a stability study. This is often dependent on the starting material and the shelf life of ATMPs vary from hours (fresh product only) to years (Cryopreserved product).

24.5.3 Product Handling

24.5.3.1 Specialist Handling and Storage Requirements Within the Clinical Setting

As a result of the unique formulations, ATMPs have specialist storage and handling requirements which can be complex and disruptive to traditional pharmacy processes. As with all medicines, ATMPs require secure storage within a temperature controlled environment. Hospitals and ATMP treatment centres must have validated facilities and appropriately trained staff to safely handle these products in line with their unique storage requirements.

For gene therapies the storage facility must be suitable for the containment level of product. For cell and tissue based

therapies, facilities must support the required temperature ranges.

The handling and storage requirements depend on the formulation on the product. This section explores the key considerations for different formulations.

24.5.3.1.1 Cryopreserved Products

Cell based ATMPs (somatic cell therapy and ex-vivo gene therapy) are often cryopreserved. Cryopreserved ATMPs must be stored in cryopreservation environments until the point of thaw which is usually immediately prior to infusion. It also requires excipients (cryopreservatives) to protect the cells. An example is DMSO which protects cells during freezing. However, during thawing DMSO needs to be removed again by washing to prevent cell damage in that stage.

Cryopreservation requires temperatures below −120 °C. This is maintained by using vapour phase liquid nitrogen (VPN). Pharmacies do not routinely have VPN storage tanks and so collaborative agreements between the pharmacy and a cell storage facility need to be in place. This may be a stem cell laboratory (or equivalent) within the same hospital, or may be outsourced to a different physical and legal entity. Regardless of the storage location, the pharmacy must maintain oversight of the ATMPs and a technical agreement, clearly defining roles and responsibilities is recommended.

Cryopreserved ATMPs are usually delivered in dry shippers before being transferred to a larger storage tank. Staff involved in the handling of these products must be trained and competent as there is risk to both the handler and product if not handled correctly. VPN poses risk to human health and must have appropriate safety training. Pharmacy guidance is available [42].

Cryopreserved products must remain frozen up until the point of administration, where they are thawed immediately prior to administration. Operational pathways must be in place to ensure a robust pathway is in place to maintain these conditions.

24.5.3.1.2 'Fresh' Cells and Tissues

Some cell based ATMPs and tissue engineered products are delivered 'fresh'. Fresh cells need to be stored at 2–8 °C in a designated fridge for cellular products. A standard medicines fridge is not suitable for cells. Fresh cells have short expiries and routinely need to be used within 72 h from the end of collection, prior to manufacture. Due to the short expiry, these products may be delivered directly to the clinical area for immediate administration and may never enter pharmacy. Pathways must be in place to ensure appropriate product handling, by trained staff and pharmacy must maintain oversight of the entire pathway.

24.5.3.1.3 Ultra Low Freeze Products

In-vivo gene therapies often need storage at −20 or −80 °C. These products can therefore be safely stored in the pharmacy upon purchase of the appropriate freezer and training of staff in management of ultra low freezers. In-vivo gene therapies are usually delivered on 'dry ice' to maintain temperature during transport. Pharmacy staff must be trained in the safe handling of dry ice shippers and ultralow freezers as harm can be done if not handled correctly. The product is extremely delicate and brittle so extreme care must be taken to not damage the product.

24.5.3.2 Good Preparation Practice

It is essential that, where applicable, ATMPs are handled by healthcare professionals with demonstrated competency in manipulating cells and tissues. These skills are currently rare within pharmacy teams and therefore, with the exception of in-vivo gene therapies and cell and tissue based ATMPs which are provided in a ready to administer presentation, it is routinely recommended that pharmacies work in close collaboration with expert colleagues in their healthcare organisation. Suitable expertise can be found in stem cell laboratories.

Part IV EudraLex Volume 4 (Good Manufacturing Practice specific to ATMPs) specifies that activities required after batch release prior to administration of an ATMP, which are not considered to be a manufacturing steps can be performed outside of a licensed GMP environment at the administration site, for example in aseptic preparation suites or clinical areas.

Where there are any reconstitution steps as defined in Part IV EudraLex Volume 4 which include thawing of cryopreserved products in a ready to administer presentation, pharmacists should provide oversight of the process. This recommendation recognises that ATMPs are medicines, and that Chief Pharmacists are routinely appointed by their hospital board to be the named individual responsible for the safe and secure handling of all medicines within their organisation [43].

Pharmacists should therefore be involved in the process of producing/approving the procedure and ensure that it is performed in line with the SmPC or clinical trial protocol. The pharmacist's presence for the activity or process to be carried out each time by appropriately qualified and competent members of staff is not necessarily required, providing the appropriate training has been provided and a pharmacy approved SOP is in place. For example, if a stem cell lab staff member has been trained and is competent to carry out the activity, it may be good practice for the pharmacist to be present the first time the activity is performed, and to monitor the activity at future intervals to ensure continued compliance, but it is not necessary for the pharmacist to be present each time the staff member performs the preparation activity.

For in-vivo gene therapies stored at ultra-low temperatures, the ATMP will need to be thawed before being prepared and administered to the patient. The area of preparation depends on the risk and containment level of the product, and the post preparation expiry time. For in-vivo gene therapies which are non viable, pharmacy aseptic facilities provide an optimal location for preparation and thawing. Preparation may be undertaken within a pharmacy unit where in use stability data allows sufficient time for transport to the clinical area, but thaw may need to be undertaken in the clinical area or at the patient's bedside if this is not feasible. Good preparation practice should be utilised for any such reconstitution activity [44].

24.5.3.3 Administration of ATMPs

Once prepared ATMPs usually have a very short expiry time and special care must be taken when handling and administering ATMPs to maintain product viability.

ATMPs are most commonly given intravenously although some ATMPs may be injected directly to the site of action (e.g. intra-tumoral).

Specific equipment and facilities are required for the administration of certain ATMPs. For example, gloves/masks and other personal protective equipment may be needed for handling GMO products class 2 and above. For cell infusions it is essential to ensure a non-leukodepleting filter is used to ensure the active cellular product is not filtered out.

Another consideration during administration is dosage. Traditional medicines are prescribed at a fixed dose. For living treatments containing cells, this is not possible, therefore it is standard to prescribe the treatment at a cell dose range.

Some ATMPs have novel toxicity profiles and specific monitoring of the patient is required after administration. Some may require access to an intensive care unit. For example, CAR-T therapy carries a high risk of cytokine release syndrome and it is a requirement to have specific treatment readily available. Always follow the latest product recommendations and ensure the pharmacy has developed guidelines for the toxicity management of these patients.

24.5.4 Considerations for Pharmacy

The principles of managing the adoption of any new medicine recognise that optimal patient benefit requires collaboration with patient and carers, between healthcare professionals and that support may be required at different times across the patient journey and this lies at the heart of providing an optimal pharmacy service. This is in line with the International Pharmaceutical Federation objectives for Hospital Pharmacists, which promote integrating pharmacy services through communication and collaboration [45].

These principles align and resonate with the requirements for ATMPs. Regardless of the setting, be it as a licensed medicine holding a marketing authorization, as an investigational medicinal product in a clinical trial or as an unlicensed medicine for an individual patient with a special clinical need, a collaborative approach is required to ensure that any advanced therapy medicine is safely introduced into use. In particular, pharmacists recognize that handling and manipulation of cellular medicines is a specialist competency which is currently outside of the pharmacy workforce's curriculum, however ensuring that processes and procedures are in place to enable this to occur and to enable good clinical practice or compliance with the SMPC and/or pharmacovigilance responsibilities requires a multidisciplinary collaboration including pharmacists as key stakeholders to ensure that the use of the medicine is compliant and that safety is optimised.

24.5.4.1 Medicines Governance Role

Medicines governance is required at various levels encompassing both national and local requirements. As ATMPs are innovative products often associated with challenges and service disruption; all requests to use ATMPs require scrutiny from the appropriate organisational multidisciplinary committee. This is in addition to approval via routine Research and Development routes where the ATMP is for use in a clinical trial.

The governance process should confirm the suitability and readiness of sites to deliver treatment with these medicines. For CAR-T products the marketing authorisation also required audits to be undertaken by the MA holders, and national governance requires audits of prospective sites by the Joint Accreditation Committee of ISCT (Europe) and EBMT. Pharmacists are encouraged to document local requirements and to play a role in each of these stages which encompass clinical, operational and local medicines management. The principles outlined for the role of the pharmacist will apply in any country adopting ATMP therapies.

As ATMPs may be (very) high cost medicines, local pharmacy medicines management is also required to ensure compliance with relevant requirements at an individual patient level which will support financial reporting and ensure that agreed payments are received by the healthcare establishment.

(continued)

Due to the unique nature of cell/tissue-based medicines, there are occasions (often but not always due to inherent biological variation of starting materials) when the manufactured medicines are not in full compliance with their release specification. It is recognised that due to the specialised nature of the medicines and depending on the nature and degree of non-compliance it may be that the administration of an out-of-specification (OOS) ATMP remains in the best interest of the patient and that administration is the correct course of action. Although EMA guidance clarifies the role of the manufacturer, pharmacists are recommended to provide a clear organisational governance route in this situation to ensure, in collaboration with the treating physician, that administration is in the best interests of the patient and is approved locally from both a clinical and financial perspective [46].

24.5.4.2 Operational and Clinical Role

The operational role and responsibilities of the pharmacist can be best explained by using the example of marketed CAR-T cell therapies. Fig. 24.22 demonstrates the complexities of delivering autologous cellular medicines.

The journey of the patient shown in blue in Fig. 24.22 begins with a patient who fulfills the eligibility criteria for CAR-T consenting to the treatment. The patient then visits the apheresis center and after a period of weeks during which some bridging chemotherapy may be required to stabilize their disease, continues with the administration of a lymphodepleting regimen followed by the CAR-T therapy. Toxicities may ensue which require careful clinical management and the availability of an intensive care bed.

The corresponding product journey is shown in red.

The starting material is procured in the apheresis center and is processed if required in the stem cell laboratory. It is subsequently packaged and shipped to the manufacturer where the regulatory controls shift from the local Tissues and Cells Directive regulator to the local medicines competent authority. The medicinal product is cryopreserved and shipped to the stem cell laboratory where it is verified that it is of suitable quality and then stored under vapour phase nitrogen until required. It is then thawed in the clinical area under pharmacy oversight before being administered to the patient.

Pharmacists are advised to have oversight of all of the processes involved.

In order to ensure consistency and minimize variation in service provision, it is recommended to implement checklists for each stage identified above requiring pharmacy input, which detail key points to ensure are covered by local systems. This allows the pharmacy to consistently discharge their oversight of medicines responsibility whilst ensuring that the products are handled by staff with the appropriate skills and competencies. Exemplary templates for such checklists are provided by [47]. This has introduced a concept of Considerations for Pharmacy Implementation (see Sect. 24.5.5).

Given the complexity of these novel treatments there is an essential role of the clinical pharmacist to ensure that these

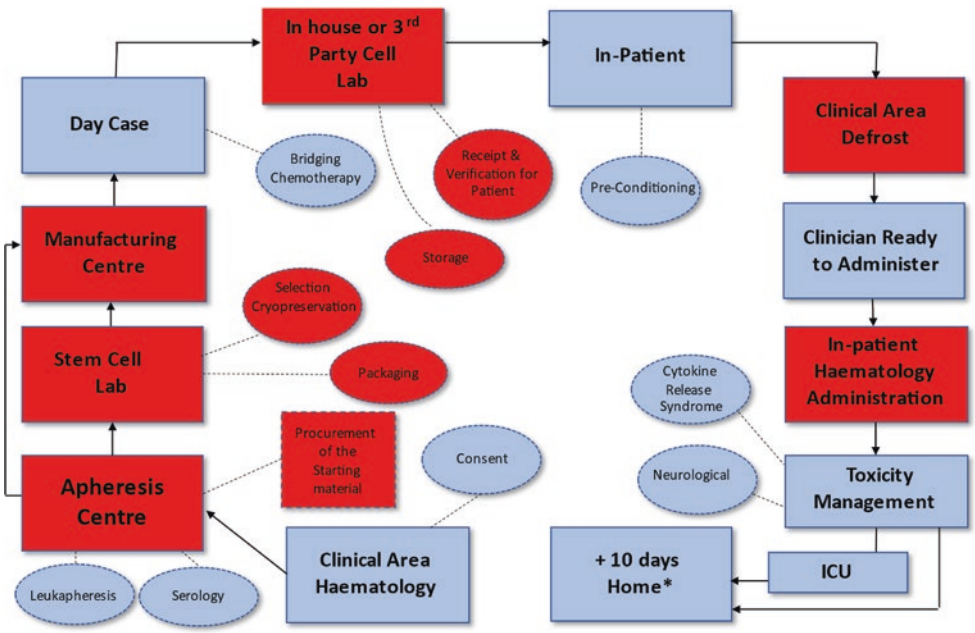


Fig. 24.22 Processes involved in the provision of CAR-T therapy, showing where the product and patient hourneys interact (Product Journey in RED, Patient Journey in BLUE).

treatments are optimised. The clinical pharmacist should be involved throughout the entire patient pathway, including the management of any toxicities.

24.5.5 Considerations for Pharmacy Implementation

There is a benefit to pharmacy understanding and having a consistent approach to their governance, operational and clinical roles.

The concept of pharmacy institutional readiness recognises that systems for delivery need to be set up and tested in advance of being able to routinely offer treatment with an ATMP. Systems and procedures will differ by locality but the principles being addressed should be similar regardless of geography.

In order to be ready to implement a new ATMP, Pharmacy Institutional Readiness Guidance suggests that a generic pathway can be followed for implementation of an ATMP type in a somatic cell therapy, a tissue engineered product, an in-vivo and an ex-vivo Gene Therapy.

It gives overarching principles for pharmacy consideration at each stage of the pathway and has checklists supplied either for implementation or to use to check that local systems have considered all items recommended.

Collaborative Groups throughout Europe have developed Pharmacy Readiness Guidance for all ATMP categories. Pharmacists are recommended to use these as a basis to set up local systems when wishing to implement an ATMP service. EBMT, EHA and JACIE provide additional guidance for cellular therapies.

Pharmacy professionals work in many sectors and in many clinical specialisms. Regardless of which sector or specialism it is clear that the potential offered by ATMPs is exciting though can also be challenging. It is essential therefore that pharmacy teams look forward and ensure that our workforce is suitably informed to meet and overcome the barriers encountered to delivery of these complex therapies as we embrace these medicines which have the potential to be life changing for so many of our patients.

Questions

1. A monoclonal antibody formulation has the following compositions.
   Solutions
   IV injection
   20 mg/mL
   sucrose 50 mg/mL
   polysorbate 80 (0.2 mg/mL)
   sodium phosphate 0.015 M, pH 6.5
   Subcutaneous injection
   180 mg/mL
   arginine HCl 21 mg/mL
   methionine 4.4 mg/mL
   polysorbate 80 (0.2 mg/mL)
   histidine 1.7 mg/mL
   histidine HCl 1.9 mg/mL (0.02 M), pH 6.0
   Why are the concentrations API different?
   What are the excipients and why are these excipients added?
2. What is immunogenicity. What are the main parameters that can cause immunogenicity
3. Which of the following are not classed as ATMPS?
   (a) Somatic cellular therapies
   (b) Gene therapies
   (c) Monoclonal antibodies
   (d) Tissue engineered product
4. Which of the following pieces of legislation apply to in-vivo gene therapy?
   (a) Regulation (EC) 1394/2007 "The ATMP Regulation"
   (b) The Tissues and Cells Directive 2004/23/EC
   (c) European Directive 2009/41/EC on the contained use of genetically modified micro-organisms
5. If an ATMP requires cryopreservation, what is the optimal storage temperature

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