Advances in Biochemical Engineering/Biotechnology 171
Series Editor: T. Scheper

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Current Applications of Pharmaceutical Biotechnology

171
Advances in Biochemical Engineering/Biotechnology

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Aims and Scope

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Volumes are organized topically and provide a comprehensive discussion of developments in the field over the past 3–5 years. The series also discusses new discoveries and applications. Special volumes are dedicated to selected topics which focus on new biotechnological products and new processes for their synthesis and purification.

In general, volumes are edited by well-known guest editors. The series editor and publisher will, however, always be pleased to receive suggestions and supplementary information. Manuscripts are accepted in English.

In references, Advances in Biochemical Engineering/Biotechnology is abbreviated as *Adv. Biochem. Engin./Biotechnol.* and cited as a journal.

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Current Applications of Pharmaceutical Biotechnology

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Preface

Biopharmaceuticals have been showing high therapeutic potential, especially for medical conditions associated with unmet medical needs. Examples of these conditions include cancer and autoimmune, metabolic, dermatological, neurological, and genetic diseases. Thereby, current clinical relevance of biopharmaceuticals is unquestionable. Since the 1980s, several biological medicines have been approved and, due to patents expiration, many biosimilars have been marketed.

Manufacture and formulation of biopharmaceuticals comprising therapeutic proteins generated by DNA recombination is challenging. Depending on the protein's molecular weight and structure complexity, industrial manufacturing processes have employed different host cells. Furthermore, biopharmaceuticals formulation is fundamental to achieve an effective medicine, being necessary to stabilize the protein conformation, enabling its efficacy while avoiding immunogenicity and other safety problems. Commercial biopharmaceuticals are typically for parenteral administration, although several efforts have been made to find alternative administration routes and/or new delivery systems enabling improved pharmacokinetic properties and better patient compliance.

Therapeutic applications of biopharmaceuticals are highly diverse and include active substances such as monoclonal antibodies, cytokines, growth factors, hormones, blood products, therapeutic enzymes, as well as vaccines, cellular therapies and products of gene therapy. In respect of biopharmaceuticals (including biosimilars and orphans), the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) have approved so far 89 cytokines, 78 hormones (insulin, glucagon, growth hormone, gonadotropins, and thyroid-stimulating hormone and parathyroid hormone), 49 blood products (blood factors, anticoagulants, and thrombolytic agents), and 22 therapeutic enzymes. Current research has been also focused on identifying new clinical applications for existing therapeutic proteins.

A number of different advances in the development, manufacturing, and characterization of vaccines have enabled the prevention of infections and promoted human health worldwide. Approved recombinant vaccines include Shingrix, Ebola

(Ad5-EBOV), meningococcal subgroup B, human papilloma virus (HPV), dengue, enterovirus 71 (EV71), and hepatitis B (HBV), while vaccines against rVSV-ZEBOV Ebola, respiratory syncytial virus (RSV), and malaria are under clinical evaluation.

In addition to biopharmaceuticals and vaccines, advanced therapy medicinal products based on the use of cells, tissues, or genes offer new therapeutic possibilities. For example, cell-based therapies have been used in regenerative medicine, disease modelling, and drug screening studies. This has also constituted an opportunity to engineer new manufacturing concepts as 3D bioprinting technology, which have succeeded in generating living tissues and organs from a combination of cells, biomaterial bioinks, and growth factors. Examples of bioprinted tissues include nerve, skin, heart, bone, cartilage, skeletal muscle and soft tissues such as liver, cornea, intestine, and bladder.

In the last years, gene therapy medicines, such as DNA-based therapies, antisense oligonucleotides, small interfering RNA, and T-cell-based therapies, have shown promising results to such a point that they have started to change the treatment paradigm, as in cancer. Furthermore, the use of gene-editing approaches, such as the CRISPR (clustered regularly interspaced short palindromic repeats), has started to be assessed at the clinical level. Besides cancer, the clinical usefulness of gene therapy is also being assessed in monogenic infections, inflammatory, cardiovascular, and neurodegenerative diseases.

Within the scope of the different therapeutic tools herein addressed, pharmacogenomics assumes a relevant role on their performance, in terms of both efficacy and safety. In fact, the use of a medicine adapted to the genetic variants of each patient, reducing adverse effects and improving treatment outcomes, supports pharmacogenomics as a relevant component of personalized medicine.

According to the wide range of pharmaceutical biotechnology applications, this book aims at providing the most relevant up-to-date information for healthcare professionals, students, and researchers.

We would like to thank all the authors of this book for contributing with high quality chapters. We are also very grateful to all the reviewers, who had the kindness of critically evaluating the chapters. In addition, we would like to thank to the Series Editor, Professor Thomas Scheper and to the Associate Editors, Dr. Sofia Costa and Dr. Tanja Weyandt, for giving us the opportunity of organizing this volume, and to the publishing team (Ms. Alamelu Damodharan and Ms. S. Dhivya Geno) for their kind help.

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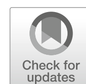
Industrial Challenges of Recombinant Proteins



Scott R. Rudge and Michael R. Ladisch

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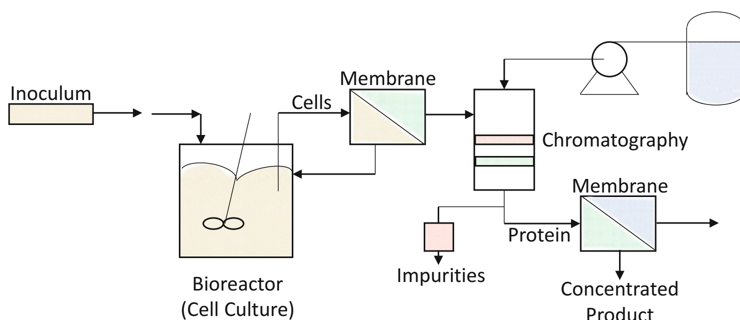
Abstract The use of recombinant DNA methods to produce large quantities of protein for therapeutic uses has revolutionized medicine. Industrial challenges for manufacture of biotherapeutic proteins are related to the characteristics of these proteins and the increasing quantities required to address needs of patients, worldwide. A brief overview of therapies in which proteins are employed helps to frame some of the challenges facing their industrial production. The number of molecules and their applications have significantly expanded over the last 15–20 years, together with the quantities used to address specific indications. Challenges addressed include achieving cell density, protein expression, separations of cells and protein, protein purification, and segmentation of protein production into smaller quantities with the evolution of personalized medicine and products designed for increasingly small patient populations. This chapter highlights some of the current challenges.

Graphical Abstract



Keywords Biologics, Bioprocessing, Cell culture, Cell separation, Chromatograhpy, Expression systems, Fermentation, Filtration, mAbs, Recombinant proteins, Separations

1 Introduction

Since the early commercialization of recombinant human insulin, recombinant human growth hormone, and recombinant bovine growth hormone, the field has expanded to include over 174 US-approved therapeutic proteins, including 98 antibodies (including biosimilars) [1, 2]. While insulin was among the first recombinant products, its lower molecular weight (~6 kD), long history of use, and molecular

characterization positioned it for scaling up [3]. Subsequent early products such as erythropoietin and tissue plasminogen activator, with their higher molecular weights and complex structures, required production through mammalian cell culture and the many attendant scaling and regulatory factors that needed to be addressed. Along the way, the development and laboratory culture of hybridoma cells enabled the production of monoclonal antibodies, proteins with molecular weights in the 120–150 kD range, and for which initial applications were limited to research tools. It required about 20 years of further molecular engineering as biopharmaceuticals to develop this class of proteins. Further advances have been made in yeast, specifically *Pichia pastoris* (reclassified as *Komagataella pastoris*), to enable the production of large and small proteins including antibodies and proinsulins. Gene therapy has now emerged with potential to not only treat diseases but perhaps provide cures. With the emergence of gene therapy has come the need for vectors consisting of nanoparticulate viral capsids, i.e., structured protein assemblies, for delivery of nucleic acid payloads. Manufacturing approaches have employed similar unit operations throughout the development of biotherapeutic products since their introduction about 40 years ago. What has changed are cells and culture methods and the separations media utilized during the manufacturing processes.

2 Classes of Products (Agonists and Antagonists)

Therapeutic recombinant proteins generally fall within two classes, those meant for replacement therapy (agonists) and those meant for antagonist therapy.

2.1 Agonists

Replacement therapies are those in which the intended patient has lost the ability to produce a protein, and that lack of ability results in disease. Type I diabetes is an excellent example of such a disease, in that the patient has lost the ability to produce insulin due to an autoimmune destruction of pancreatic islet cells. In such patients exogenous insulin is required for the patient's body to properly regulate glucose in the blood stream. These patients were treated with insulin purified from porcine pancreas before the introduction of recombinant protein production [2]. The injection of insulin at intervals related to meal times and times of fasting (overnight, for example) allows these patients to regain some control, albeit imperfect, of their blood glucose levels. In this case, the ability to inject a recombinant form of human insulin *replaces* the patient's ability to produce their own insulin. In general, low doses of proteins in this category are required, as the protein being replaced physiologically has a specific function. These proteins are also called "agonists," as their specific function is to make something happen. Other examples of agonists

are erythropoietin, granulocyte-colony stimulating factor, growth hormone, and factor VIII.

New agonist therapies are not common, as most diseases requiring a replacement protein are well-known and fatal and are/were being treated with a purified protein from an animal, plasma fractionation, or cadavers. Additionally, these diseases are usually in small patient numbers, as a rare genetic defect may be involved or an autoimmunity. The search for new agonists continues, as these treatments are almost certain to be clinically successful with few side effects. Progress in understanding the function of the brain and the neurological system may be the last frontier for agonist therapies.

2.2 Antagonists

The other class of recombinant proteins is called antagonists. These proteins are used to block some physiological action. Antagonists bind to receptors or other proteins, or other molecules in the blood stream, and prevent them from having their normal physiological function. An excellent example of an antagonist therapy is the chemotherapeutic antibody Avastin or antihuman vascular endothelial growth factor (VEGF) immunoglobulin G1. VEGF is a protein that stimulates the growth of new blood vessels, which are produced by tumors growing in the body, which require a source of nutrition from the blood stream. Avastin blocks the action of VEGF, thereby depriving tumors of the source of nutrition they need to grow [4].

Antagonist therapies typically require higher doses than agonists, as the antagonist molecule has to battle stoichiometry and biology to bind and neutralize all the target molecules in the patient. As the target molecules are neutralized by the antagonist, the body tends to make more as the biological feedback loop indicates that the desired effect is not being achieved. Additionally, the complete elimination of a physiological mechanism is usually not desirable. For example, patients taking Avastin will have difficulty growing new healthy tissue as well as tumor mass.

2.3 Humanized Antibodies

The development of humanized antibodies to treat human disease has been a second major milestone in the history of recombinant protein therapies. Early attempts to use antibodies to seek out and bind targets in humans were set back by immune responses to the nonhuman epitopes in the antibody structure. Methods for “humanizing” antibodies, including critically in the “complement defining region” or CDR, have made the construction of antibodies specific for distinct biological targets possible [5].

Over the last 20 years, antibody antagonists have largely displaced recombinant agonists as the top selling protein pharmaceuticals, as shown in Table 1 (compare [6] to [7]) with the pipeline of therapeutic mAbs now at 560 in various stages of clinical trials [8, 37].

Table 1 Top selling biopharmaceuticals from 1997 and 2011

	1997 [6]	2011 [7]
1	Erythropoietin (agonist)	Anti-TNF monoclonal antibody (antagonist)
2	Granulocyte-colony stimulating factor (agonist)	Anti-TNF receptor monoclonal antibody (antagonist)
3	Insulin (agonist)	Anti-VEGF monoclonal antibody (antagonist)
4	α-Interferon (agonist)	Anti-CD20 monoclonal antibody (antagonist)
5	Hepatitis B vaccine (agonist)	Anti-HER2 receptor monoclonal antibody (antagonist)
6	Glucocerebrosidase (agonist)	Insulin (agonist)
7	Tissue plasminogen activator (agonist)	PEGylated granulocyte-colony stimulating factor (agonist)
8	Growth hormone (agonist)	Erythropoietin (agonist)
9	GPIIb/IIIa antibody (antagonist)	Anti-VEGF monoclonal antibody fragment (antagonist)
10	Interferon β-1a (agonist)	Interferon β-1a

Today, the top ten selling recombinant protein pharmaceuticals are mostly monoclonal antibodies. Antibody products accounted for about $114.6 billion in sales in 2018, while all biopharmaceuticals were about $237 billion. Total pharmaceutical sales were $1,200 billion for reference. Interestingly, all of these products received their marketing approval within the last 15 years, which demonstrates the rapid growth of the product category and may indicate the large potential still remaining.

However, as with most technologies, the targets for antibodies and other antagonists are becoming harder to identify, and the targets that are being pursued are in higher concentrations, requiring higher doses of antagonists [9]. Doses as high as one or more grams per day are becoming more common. High doses can be problematic because (1) they require greater purity, (2) they are harder to administer to the patient, and (3) they require lower cost of goods. The third constraint is easily understood, the treatment of disease is an economic proposition, and the market, or society, will only bare so much cost. The cost of treating versus not treating must be carefully balanced, and in cases where other therapies exist, a price is already set. The market cares little about the mass of protein required to produce the desired effect, and the cost of goods for an active antibody can vary between $100 and $1,000/g, excluding testing, dosage form manufacturing, and other expenses [10].

Higher doses also require higher purity. This is best illustrated with the impurity endotoxin, which is produced in very large quantities when *Escherichia coli* is used to produce the recombinant protein. Endotoxin is part of the cell wall of gram-negative bacteria and causes an immune response and inflammation when present in the blood stream of humans. This activity of endotoxin, called pyrogenicity, is measured in endotoxin units (EU) by a few standardized biological assays. The total amount of endotoxin that a patient can be exposed to per 1 h administration is limited by medical standards to 5 EU/kg (the kg refers to the weight of the patient). A 50 kg patient may be exposed to a total of 250 EU. If the dose of the protein is 10 mg, the protein must be purified to at most 25 EU/mg, but if the protein dose is 2 g/day, then the most endotoxin the product can contain is 0.125 EU/mg.

2.4 Biosimilars

With a regulatory pathway now open for biosimilar products, there will be a focus on cost of goods for biologics like never before. When patent exclusivity expires, the cost of making and purifying the protein will become a more significant part of the selling price. The cost for making a purified therapeutic protein ranges from about $100 to $1,000/g, with cell culture-derived proteins requiring higher expenditures. Therefore, reduction of cost of goods at higher purity requirements will be a major challenge for biopharmaceutical manufacturing in the immediate future.

2.5 Pharmacoeconomics

Finally, the political climate is one in which justification of high reimbursement for pharmaceuticals is increasingly difficult. Pharmacoeconomics may not be sufficient to justify reimbursement in all cases. Therefore, due to high doses, biologics intended for treating diseases where other treatments exist, pressure from biosimilars, and price restraints, the cost of manufacturing a therapeutic protein is under pressure. This chapter will review some current efforts to decrease the cost of manufacturing.

3 Upstream Manufacturing

Upstream manufacturing includes fermentation, cell culture, or another means of expressing the therapeutic protein at high titer and reasonable purity. Upstream manufacturing typically also includes the removal of the production cells or biomass. This may be done with a centrifuge, a filter, by flocculation and settling, or another unit operation.

3.1 Expression Systems

The expression of the protein is the first and primary step in producing the product in its desired form. Expression systems consist of a host cell and the foreign DNA that encodes the product. The most commonly used host cells are *E. coli* and Chinese hamster ovary (CHO) cells. *E. coli* is used when the protein requires little posttranslational modifications, while CHO cells are used when posttranslational modifications are required for the structure or function of the protein. Antibodies require fairly extensive posttranslational modifications. For example, immunoglobulin Gs (the most common therapeutic antibody and the simplest) require posttranslational

modifications in the form of the assembly of two heavy chains with two light chains to form a hetero-tetramer, and glycosylation of an asparagine located on the heavy chain constant region, so typically antibodies are made in CHO. Insulin only requires enzymatic activation, which can be done in manufacturing with the correct protease, so typically, insulin is made in *E. coli*.

Protein expression is complicated, and there are several steps that have to be optimized to get the maximum expression of the desired protein. There are several considerations:

- The codons in the gene for the desired protein must be optimized for the host cell.
- The gene must possess flanking regions, such as a poly-adenosine tail and a ribosome binding site.
- The gene must be under the control of a promoter that will cause its expression, either constitutively or in response to an inducer or derepressor.
- If it is desired to export the product to another compartment of the cell, or to secrete the product, a leader sequence must be attached to direct the protein to that cellular compartment.
- The gene must have the proper reading frame to ensure the appropriate sequence is transcribed and translated.

There are numerous expression systems available, both proprietary and in the public domain. The expression of every recombinant protein is different and unpredictable, but the expression of antibodies tends to be fairly consistent from antibody to antibody. A titer of 3 g/L can be expected for an antibody expressed from nonproprietary expression constructs in CHO cells [11]. Titers as high as 15–25 g/L have been reported in vendor marketing data; however the corresponding product quality is unknown.

3.2 Promoters

A common promoter for protein expression in CHO cells is the cytomegalovirus (CMV) promoter [12]. This promoter is constitutive, meaning it is always activated and does not require induction. The titer is also dependent on the cell density – the more cells, the more product. A typical cell density for CHO cells is 20×10^6 cells/mL. However, high cell density cultures with up to 100×10^6 cells/mL are now being developed which can increase the titer a commensurate fivefold. These cell densities are a relatively new development, and their impact on downstream processing is still being determined. Cell culture medium costs about $100/L on average, so the media alone can contribute $10–$33/g to the cost of the product, depending on the titer. Media used to achieve high cell density is more expensive, but not five times more expensive, so the investment in high cell density is justified.

Product expression in *E. coli* is typically greater than 5 g/L and can be as high as 15 g/L. At very high expression levels in *E. coli*, the product is usually found in inclusion bodies, small precipitate particles that have to be solubilized and renatured

to make them usable. A common expression system for *E. coli* is the T7 promoter, which is induced with iso-propyl thio glycerol (IPTG) [13]. IPTG derepresses the T7 promoter, allowing transcription of the gene and subsequent translation. An alternate promoter system is the xyz switch where amino acid content in the media is used to induce insulin expression [3]. In most bacterial systems, the expression of the foreign protein is toxic to the cells, and so inducible rather than constitutive promoters are used. Inducer is added to the fermentation after high cell density is reached, at which point the cells do not grow significantly. The final wet weight of solids in the fermentation is typically 12–16% (120–160 g/L) of which the expressed protein is about 10% (12–16 g/L as previously stated). On a dry weight basis, the proportion of expressed protein is closer to 20–25% by weight. *E. coli* media is much cheaper to prepare, usually \$5–\$10/L. If the expression level is reasonably high, the cost of the media will only be \$1–\$2/g.

Efforts to increase protein expression are ongoing and remain important. When the protein expression is increased, the purification is easier. However, additional impurities are typically generated with highly expressed proteins as the protein synthesis machinery is overtaken by the higher level of expression. One common impurity is the mis-incorporation of norleucine for methionine residues [14]. Covalent protein aggregates are also prevalent in highly expressed proteins. Some attention to the likely downstream yield purity must be paid when designing high expression systems.

3.3 High Cell Density Reactors

Another way to increase product throughput is to grow cells to very high cell density. In mammalian cell culture, this means densities on the order of 100×10^6 cells/mL, and for bacterial and yeast fermentations, this means ODs greater than 100 and approaching 300 or 400, and wet cell weights around 50%. These higher cell densities are made possible by new media formulations that are rich in key nutrients, as well as advances in oxygen transfer into and heat removal from the reactor vessel.

Increased cell density gives productivity gains linearly in proportion to the increase of numbers of cells. CHO cultures are routinely run at 20×10^6 cells/mL, so the ability to achieve 100×10^6 cells/mL offers an opportunity to increase the productive time in the bioreactor given that protein expression and cell growth occur concurrently, unlike *E. coli* where expression follows accumulation of cells and subsequent induction. Since mammalian cell culture expression is typically constitutive, the culture is producing product throughout, again in proportion to cell density. The area under the cell density vs. time curve gives the basis for the product expression. As shown in Fig. 1, 43% of the productivity of a 25-day culture can be accounted for in the 6 days the culture achieves $>80 \times 10^6$ cells/mL.

Bacterial expression systems are typically induced, and when they are induced, the growth of the cells slows or stops, as metabolism is taken over by the protein synthesis machinery of the cell. The fermentation may only last some hours after

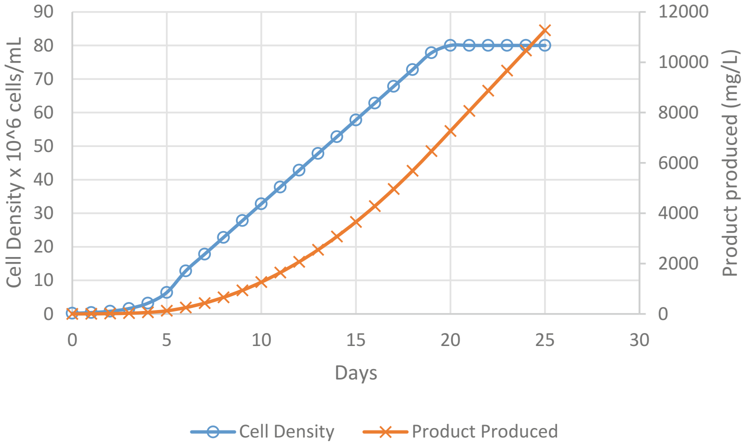


Fig. 1 Typical cell density vs. time curve and protein production, assuming a doubling time of 24 h and specific productivity of 1 pg/cell/day

induction of the expression system; 10–20 h is typical. In this case, the specific productivity is unchanged as the productivity gains are directly proportional to the cell density at induction.

Both high cell density techniques are limited in the further advantage that they can bring to protein production. At 50% wet weight, another doubling in bacterial fermentation productivity by this method is not possible. Extending the length of time such high cell densities can be maintained with cells in maximum productivity will be the most practical way to increase production in the future.

High cell density cell culture poses challenges in cell separation. These will be discussed in a subsequent section.

4 Single Use Manufacturing

Many new products are designed for increasingly small patient populations. These products are known as orphan products, and they are highly reimbursed. Gene and cell therapies are also beginning to emerge in this space. Efficient expression systems have made it possible to produce a worldwide supply of these drugs in a few batches per year. Since it is not practical to dedicate an entire clean facility to produce a few batches of a product per year, multi-product facilities have become more common, and contract manufacturing organizations are being tasked for production on a more frequent basis.

4.1 Single-Use Bioreactors

Keeping different products segregated from one another is a critical requirement of multi-product facilities, which means extensive and detailed cleaning of the equipment during product change-over. Another solution is to make inexpensive, disposable equipment. Disposable bioreactors have been in use at commercial scale for about 10 years for mammalian cell culture. Disposable systems increase the flexibility of multi-product manufacturing facilities by removing the requirement for post-use cleaning of the bioreactor. These bioreactors are typically made of plastic and come pre-sterilized by gamma irradiation. While larger bioreactors have been made, the most common large single-use bioreactor has a 2,000 L working volume, a version of which is available from at least three different manufacturers. These are accompanied by other single-use unit operations, such as filtration pods, chromatography systems, and vessels. A fully "disposable factory" may still be cost prohibitive, but it could become less expensive in the future.

While flexibility is promoted as a feature of disposable systems, particularly bioreactors, such systems are not as flexible as might be imagined [15]. The bioreactors require a superstructure made of steel to support them. The single-use bioreactors are very heavy and require a crane to lift them into the support structure and then to lift them out again. Shipping is expensive and can result in pinholes and creases in the plastic, which lead to bioreactor rupture or sterility failure. And while some early economic analysis made the case that single-use plastic was more economical and environmentally friendly than cleaning fixed stainless-steel reactors, these analyses [16] ignored shipping, gamma irradiation, the clean room required to fabricate the single-use reactors, and the effect of the plastic load on the biosphere.

Single-use systems are not readily available for large-scale microbial fermentation. As bacteria grow many times faster than mammalian cells, their oxygen transfer and heat transfer requirements are much larger. Plastic bioreactors are not able to hold significant pressure which facilitates oxygen transfer, and the magnetic drive that is used to agitate plastic bioreactors cannot deliver the torque required for mixing and heat and oxygen transfer.

5 Perfusion Reactors and Continuous Processing

One method for increasing bioreactor productivity is to increase the time the fermentation or bioreaction remains at high cell density and maximum productivity. One obvious way to do this is to continuously supply fresh nutrient medium while removing spent medium and product (if secreted) while retaining cells. There are two challenges in this endeavor, one is to continuously provide sterile medium, and the other is to remove spent medium while retaining cells and maintaining sterility.

The nutrients required for mammalian cell culture are complex and not stable to autoclaving. Therefore, the introduction of fresh medium is typically via filtration.

Mammalian cultures are also susceptible to viruses, and so viral reduction devices such as "high-temperature-short-time" and UVC irradiators are being designed to continuously introduce sterile, viral-free media into the bioreactor [17]. This challenge appears to have been substantially met.

Sterile cell retention is a different challenge. The bioreactor contains cells, along with dead cells, cell fragments, lipids, and other debris. The cell separation device must operate efficiently in this environment. Several methods that are currently in use are:

- Cross-flow filtration
- Centrifugation and sedimentation
- Acoustic separation

It is important to separate the cells without lysing them. When lysed, cells release proteases that can degrade the product and additional impurities that must be subsequently removed by downstream purification processes. Ideally, the retention device would retain only cells and allow removal of debris and unproductive solids. Otherwise, the bioreaction will be choked off by accumulation of the unproductive solids.

5.1 Cell Retention by Cross-Flow Filtration

The alternating tangential flow filtration device may be the most widely used device for perfusion reactors today. It works by drawing culture fluid across a micro-filter in tangential flow fashion. The culture fluid accumulates in an expanding bulb downstream of the filter. Cells are retained, and spent culture fluid with secreted product crosses into the filtrate. Then the cycle is reversed, the bulb is collapsed, and the retained cells are returned to the bioreactor. Since bioreactors typically operate under slight positive pressure, the driving force for filtration is vacuum on the filtrate side of the membrane. An example is shown in Fig. 2 [18].

The alternating tangential flow filter allows high throughput and essentially total retention of cells. Perfusion flow rates of two volumes/reactor volume/day are readily achievable. The technology is easy to scale up as the principals of tangential flow filtration are well-known. The stream is already filtered and so already clarified, so no further filtration is needed. When a filter clogs, which will happen ultimately, it must be replaced without stopping operations. This can be done with tube welding technology, or steam in place valves, although it is inconvenient and requires the purchase of a second device.

The alternating tangential flow filter retains all solids, including nonproductive solids, which can accumulate and choke the cell culture if not removed. Additionally, as the perfusion rate increases, the alternating flow filter must operate faster, increasing shear on the cells and creating additional debris. This is typically addressed by adding a cell syphon stream where cells, debris, and medium are all removed. This is an imperfect solution, however, and eventually the cell debris will overtake the cell culture process.

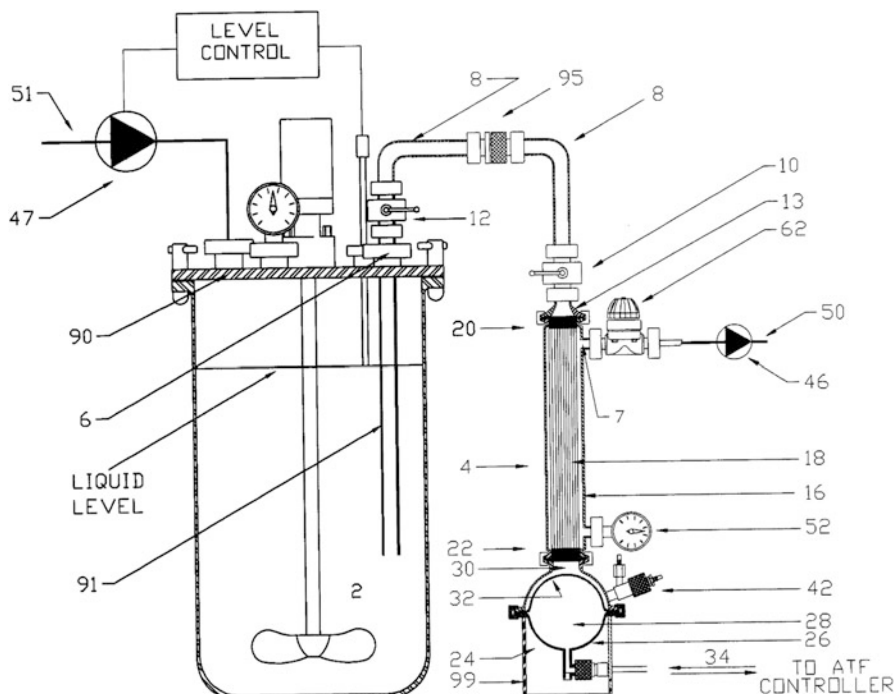


Fig. 2 An alternating tangential flow filtration device [18]

5.2 Cell Retention by Centrifugation or Sedimentation

Centrifugation is also used as a cell retention mechanism. These centrifuge systems use a modest centrifugal force (approximately 100–300× g) to sediment cells and other solids and to enable withdrawal of a clear fluid stream. Care must be taken not to damage the cells and not to pack them too tightly so that they can freely flow back to the bioreactor. The moving parts of a centrifuge also make maintaining sterility difficult. Because of these challenges, sedimentation has also been used as a cell retention mechanism.

Inclined settling uses an inclined plane to settle and return live cells to a bioreactor, while allowing product-containing medium to be removed from the overflow. This method also allows dead cells and debris to be removed with the product stream. A recent improvement has been made to this system by allowing the flow over stacked cones, much like in a disk-stack centrifuge but without the added centrifugal force [19, 20]. An example is shown in Fig. 3.

The downside to inclined settlers is the slow settling, which results in fairly large volumes required to achieve the appropriate residence time. The large volume/long residence time results in the settling device required being nearly as large as the bioreactor itself, meaning cells spend significant time in an environment where pH

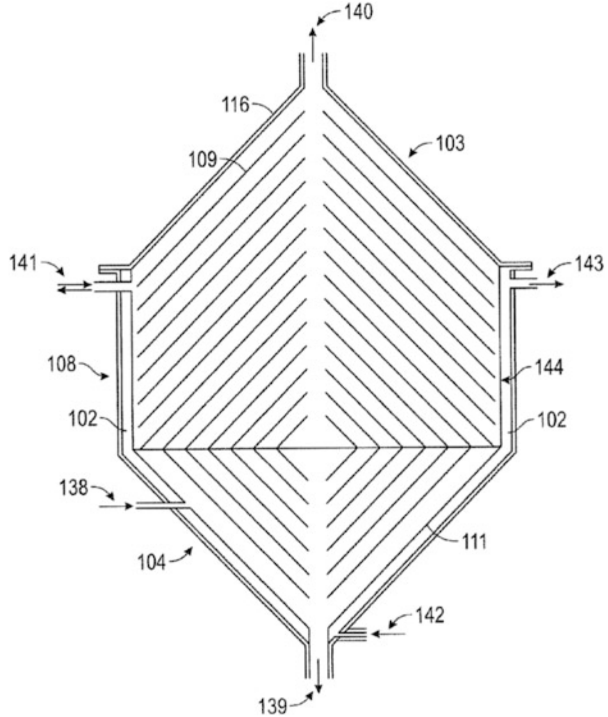


Fig. 3 Compact settler diagram [20]. Media containing cells are fed through port 142. Product and nonproductive solids may be withdrawn through port 140, and live cells can be returned to the bioreactor through port 139

and dissolved oxygen are not being actively controlled. Attaining high flow rates has also been difficult in inclined settling systems, meaning high rates of turnover of bioreactor contents are not achievable. However, as a solid/liquid separating device with no moving parts that can be made of inexpensive, light weight disposable materials, compact settlers have some advantages that cannot be ignored.

5.3 Cell Retention by Acoustic Separation

A final method for cell retention is the application of an ultrasound field [21]. The ultrasound is able to retain particles of a certain size against a small flow field. This method is also difficult to scale up, since the dimensions increase to provide a small enough linear velocity for the flow field, the device becomes large relative to the bioreactor, and the acoustic field heats the fluid throughout, while heat can only be removed from the periphery of the device. This method for cell retention is rarely used outside very small-scale lab experiments.

5.4 Perfusion Reaction Control

Continuous processing is being explored as a method for faster and less expensive manufacturing of biologics such as therapeutic proteins and antibodies [1]. The advantage of continuous processing is a relatively high productivity at a smaller overall volume. Continuous processing may improve overall quality by reducing hold times between batch steps and increase flexibility by allowing for longer production times at higher volumetric productivities [22].

Continuous processing requires that a steady state be achieved. In typical steady-state reactions, reactants are fed to a reactor to produce product at a constant rate, which can then be removed and purified. In order to maintain the steady state, feedback control is used to account for small fluctuations in conditions that can cause variability in the rate of production, such as the concentration of reactants, temperature and pressure of feed streams, and fouling of surfaces, for example. The state of development of feedback control for bioreactors (and other bioprocessing steps) is in its infancy, and much more work is needed in order to achieve true continuous processing in biopharmaceuticals. To further develop feedback control, more information will be needed on the effects of media, temperature, metabolite concentrations, debris and unproductive solids, oxygen transfer, pH and osmolarity on product quality, and production over time. This information is likely to be complex and process specific and may need to be studied on the genetic level, where different genes in a cell are upregulated and downregulated in response to various environmental factors. Without models for the performance of the cells, the time of a batch can be extended perhaps by a factor of 2–3, but without adequate feedback control, true continuous bioprocessing is still a concept rather than a reality [23].

6 Cell Separation

Once the bioreaction phase is over, it is typical to remove cells from the spent or conditioned medium in order to begin purification of the product. In mammalian and yeast culture, the product is typically secreted from the cell, so the cell is considered part of the waste stream and need not be recovered. In bacterial culture, the product may ultimately reside in an intracellular compartment or an extracellular compartment depending on how the recombinant gene is constructed. If the product is secreted, it is desirable to have a cell separation method that does not lyse the cells. If the cells are lysed, proteases are released that could degrade the product, and the released DNA, RNA, lipids, and carbohydrates increase the purification challenge downstream. Disk stack centrifugation has been the state of the art for this step for more than 30 years but has recently become limited by the high cell densities now achieved with mammalian cell culture and yeast cell culture. Depth filtration is supplanting centrifugation as a gentler separation methodology.

6.1 Centrifugation

Centrifugation is limited by shear force and solids concentration. A disk stack centrifuge, which can intermittently or continuously discharge a concentrated solids fraction, is only able to achieve a solids concentration of about 50% wet weight. Above this solids concentration, the viscosity of the solids fraction increases, and the solids cannot be discharged through the nozzles used to regulate the flow. Yeast culture can easily achieve 50% wet weight, so these cultures must be diluted prior to use of a disk stack centrifuge. If dilution is used, yield is still a consideration, as the upper limit of 50% wet weight will not be overcome. For example, if a 50% wet weight harvest broth is diluted to 16% by adding 2.125 kg of water for every kg of harvest broth, the maximum product that can be recovered is 81%, presuming recovery of 2.125 kg of liquid phase for every 1 kg of solid phase and assuming that the 1 kg of solid phase includes 500 g of solids and 500 g of liquid. Unless in-line dilution is used, a tank that can contain 6,250 kg of diluted harvest broth and another one that can collect 4,250 kg of liquid phase from the centrifuge will be needed. The need for large tanks reduces the flexibility that is considered one of the advantages of single-use technology.

Shear force is another consideration. In a typical disk stack centrifuge, the disks spin at 3,000–10,000 rpm [24], producing high rates of shear at the fluid inlets and outlets. Filtration shear rates are at least an order of magnitude lower, which matters for mammalian cells that are fragile enough to be lysed by bubbles [25]. This limits the applicability of centrifugation to bacteria and yeast primarily, since cell lysis is a factor for mammalian cells.

6.2 Depth Filtration

Depth filtration has been used as a method for collecting cells and other solids. Depth filters are designed for high solids loads but do not provide an absolute barrier to solids of a certain size. Rather, filtration is due to adsorption or entrapment of cells in tortuous channels. It is useful to think of depth filters as resembling cotton or glass wool, through which a solution will be poured. Solid is captured by the random threads of the material, and the deeper the bed of random threads, the more likely the solid is to be captured. However, solids do eventually break through this type of filter. Some depth filters are supplemented with filter aids, such as diatomaceous earth, which provide more solids retention. Eventually the filters clog as a solids cake begins to form at the surface of the filter medium, and the filtration has to be terminated, or the filters changed out for new ones. It is not uncommon for depth filters used as the primary means of cell separation to process only 10–100 L/m^2. Once loaded with solids, these filters are discarded, as they cannot be regenerated. Although the filters themselves are relatively inexpensive, the waste stream is considered biohazardous and is expensive to dispose of. As the cell density in the bioreactor increases, more area is needed for depth filtration, increasing cost.

6.3 Cross-Flow Filtration

Given the limitations of the two traditional means of providing cell separation at harvest, other approaches are being tested. Not unlike the cell retention devices described above, cross-flow filtration and sedimentation are both being employed, and both suffer from limitations as described above. Cross-flow filtration has high shear, and the membranes used are subject to fouling. Cross-flow filtration also has a maximum solids limitation and requires dilution for recovery of cells, although the system in Fig. 2 overcomes some of the plugging by reversing flow every 30 s to 1 min.

6.4 Sedimentation

Sedimentation is slow and requires too much volume. In order to speed up sedimentation, flocculants can be added to the harvest broth, such as polyethyleneimine or diatomaceous earth. The ideal flocculant would have high capacity for cell solids with low affinity for the product, a density much higher than water, no extractable compounds, and minimal toxicity in humans. Acid can also be used to aggregate some cells but would pose a degradation risk for some products. Significant opportunity for solid/liquid separation improvements exists for therapeutic protein production.

7 Downstream Processing

Downstream processing is often portrayed as a bottleneck in biomanufacturing [26]. Chromatography and ultrafiltration have been the work horses in downstream processing since they displaced differential precipitation as a means of fine purification for biomolecules. Chromatography is slow, with low capacity, and is typically not optimized for throughput. Chromatography has also been difficult to adapt to continuous operations. Ultrafiltration is usually not used for purification but rather for the change of a buffer salt and pH and is not as rapid and inexpensive as the alternatives of dilution and pH adjustment with acid and base, and therefore it is avoided in general. However, a founding principal in preparative biochemical purification is that removing water is expensive and the balance between dilution and operating at higher concentrations is not always appreciated. Challenges, principles, and design criteria are the subject of several books and are briefly outlined below [24, 27, 28].

7.1 Chromatography and Adsorption

Chromatography for biotherapeutic molecules bears more resemblance to a traditional adsorption /desorption step than to analytical chromatography, although counterflow for product recovery is rarely employed. The objective is to purify a single component from a multicomponent mixture, rather than to separate each component from each other. For this reason, the preparative chromatography feed stream should be thought of as having three components: (1) the target product molecule, (2) those molecules that bind more tightly to the resin, and (3) those molecules that bind less tightly or not at all. This ternary mixture is the most complicated to purify since the desired molecule has properties in between the two others [29].

There are two operating modes that are a special case of this general one: affinity adsorption and flow-through operation. In affinity adsorption, a ligand with high selectivity and avidity for the product is bound to the chromatography resin, and in principal, there should be no molecules that bind more tightly. There may, however, be "product-related impurities" such as dimers or oxidation products that bind with the same selectivity and avidity as the product itself.

Alternatively, some adsorption separations are made in "flow-through" mode, in which the product does not bind to the resin at all, in which case, there is no fraction of molecules that bind less well to the resin. Using the resin to capture only contaminants is an excellent strategy, as contaminants such as RNA and DNA are typically in very small amounts relative to the product (parts per million to parts per billion) so a small amount of adsorbent can be used relative to the product. This separation mode also does not typically separate product-related impurities from product.

When the most general condition exists, that the separation is intended to purify the product from molecules binding more and less strongly, the separation must be carefully thought out and tested. The most efficient separation is likely to occur when the resin binding capacity is saturated with product although there are trade-offs in product purity when this occurs. Consequently, multiple sequential steps may be used based on "orthogonal" binding chemistries, i.e., ion exchange, reversed phase, or size exclusion, where protein retention is based on different principles.

Examples of binding isotherms are shown in Fig. 4. Proteins and DNA typically have a Langmuir isotherm form, with a very high slope at low concentrations. At high concentrations, the resin is saturated and the isotherm flattens horizontally. Under these conditions, the less strongly binding components will largely be displaced from the resin. To get the most binding from the resin, a length of unused bed (LUB) approach may be used [24]. To minimize the LUB, dispersion must be minimized in the column, and a condition where a sharp breakthrough curve would be obtained is favored. A sharp breakthrough curve can be generated by operating the unit operation at high protein concentration as above, away from the linear portion of the binding isotherm. If gradients are utilized, it is possible to encounter a number of nonideal phenomena, and these must be accounted for in the selection of column operating conditions (i.e., gradient slope, column load, mobile phase composition, protein concentration, media chemistry, and particle size) [24, 27, 30].

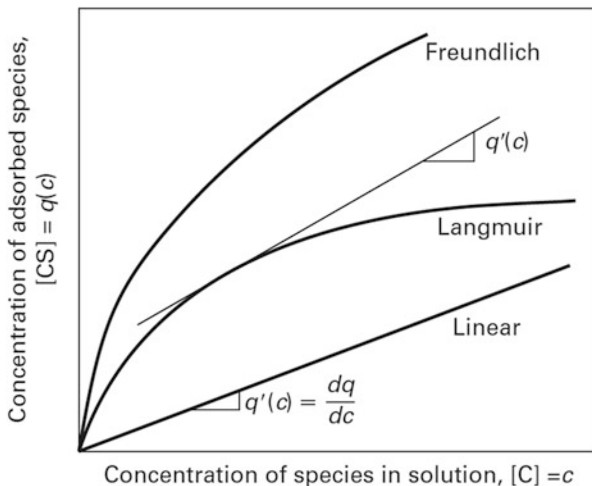


Fig. 4 Different binding isotherms. Proteins and biotechnology products often have a Langmuir shape with a very high slope at low concentration [24]

Dilution of the product prior to loading decreases the saturation of the resin. As the concentration of the product goes from right to left on the *x*-axis in Fig. 4, the concentration bound to the resin decreases. This moves the operation down the binding isotherm and will lead to a more diffuse breakthrough profile and a longer LUB. Operating at high concentration will offset dispersion caused by mechanical and mass transfer effects that are hard to overcome in protein purification [28]. Minimized dispersion requires good packing and good mechanical column design with even flow distribution. It also requires the smallest resin particle size practical for the application and a flow rate that allows sufficient mass transfer [31]. Unfortunately, small particles are difficult to handle and pack in a manufacturing environment, and the linear velocity that matches to the rate of inter-particle diffusion for a protein is on the order of 1×10^{-8} cm/s, which is not very practical. Operating at high protein concentration is the most beneficial and easiest parameter to control for efficient adsorption behavior.

7.2 Intensified Chromatography

While operating at high protein concentration could improve the efficiency of adsorption significantly, decreasing mass transfer path lengths could also increase the efficiency of adsorption and the throughput of downstream processing. There have been at least three variations on this theme with varied success, macroporous resins, membrane adsorption, and ordered media.

Macroporous resins have been synthesized that have an outer diameter on the order of 100 μm but have flow paths within the particles on the order of 1 μm [32]. A type of this resin is sold under the brand name POROS and is very popular for the separation of antibodies and other therapeutic proteins. In principal, these resins should allow some amount of convection within the pores of the particle, so mass

transfer is not limited by pore diffusion within the particle. The diffusion length would be closer to 0.5 μm, characteristic of a 1 μm particle, rather than the existing 50 μm diffusion path length that accompanies a 100 μm particle. This inter-particle convection is possible, but the pressure drop across one particle diameter is the pressure required to move fluid through a bed of 100 μm particles, so not sufficient to drive much convection through 1 μm pores. Additionally, since the particles are macroporous, they have lower ligand densities and consequently lower binding capacities on a volumetric basis. This might mean that more resin is required to process the same amount of therapeutic protein compared to a more conventional resin. Nevertheless, there is some improvement in LUB in these resins, leading to their popularity.

Membrane chromatography was initially conceived of in the form of parallel hollow fiber membranes with adsorbing ligands within the membrane material [33]. The concept provided a low Reynolds number methodology for decreasing the diameter of the flow path to increase the rate of mass transfer. The thickness of the hollow fiber wall was ideally as small as possible, making the largest resistance to mass transfer the transfer of a molecule through the liquid phase to the interface with the ligand-bearing solid phase. This methodology was reduced to practice and showed promise. However, much like macroporous adsorption, the ligand density is low. This technology is also difficult to scale, as the hollow fibers have to be held tightly at each end and have a monodispersed inner diameter; otherwise the widest fibers will have a higher flow rate at constant pressure (according to Poiseuille's law, flow increases as diameter to the fourth power at constant pressure), and dispersion will be introduced that way.

The concept morphed to membrane chromatography, in which existing filtration membranes are derivatized with ligands and the feed stream is forced through the membrane's tortuous flow paths [34]. This idea is categorically different from the original idea which sought to keep the flow path straight and narrow in order to get maximum mass transfer at minimum momentum transfer. In today's membrane chromatography, the pressure drop is high, and the feed stream must be completely clear of particulates or the membrane will become clogged. Furthermore, membrane manufacturing processes are well adapted to make membranes wider, but not deeper, so volumetric binding capacity is problematic. Because of this, membrane chromatography is almost always operated in flow-through mode, where small levels of impurity are scavenged from a concentrated product stream. This is a very efficient method for removing DNA, endotoxin, and RNA from a protein product, for example. This methodology allows for an inexpensive single-use format as well.

Finally, the original idea for hollow fiber membrane chromatography was extended into a more manageable form by utilizing a separations media consisting of wound woven cloth [35, 36]. In this idea, a derivatized fabric is tightly rolled on an axis and packed into a column. The directions of the fibers in the cloth are both parallel and perpendicular to the direction of flow, but not random as in a packed bed. Fibers on the order of 1 μm can be used to minimize the mass transfer path length, but because the cloth is an "ordered medium," the pressure drop is a small fraction of the pressure drop generated in a random medium. Furthermore, cloths can

be made and derivatized inexpensively and enclosed in thermoplastic, making this configuration a reasonable candidate for a single-use application.

8 Single Use Downstream Processing

There is significant penetration of single-use technologies in downstream processing. Plastic bags in portable superstructures are commonly used to hold buffers and in-process intermediates. When working at high concentration, smaller columns and buffer volumes can be used. However, a true single-use adsorption system has not yet been developed, primarily due to the high cost of the resins. Resin costs can be up to $10,000/L for affinity resins, so their reuse is mandatory in an economical process. Prepacked columns are available that are advertised as single use, but this practice is only advised for very price-insensitive molecules, which will be very few as biosimilar molecules become more conventional. Some of the configurations mentioned above may be more amenable to single-use modalities if their lower cost can be proven.

9 Continuous Downstream Processing

Continuous downstream processing is a field of active research. Simulated moving bed chromatography has been adapted to biomolecule separations and may be feasible for some extended batch size. However, feedback control is not yet developed for this operation, so true continuous processing will not be possible in the short term. Large gains in volumetric efficiency and cost reduction have been reported using simulated moving bed chromatography.

10 Conclusions

There are many challenges remaining for producing therapeutic biomolecules at large scale for reasonable prices. Just a few have been reviewed in this chapter, including:

- Cell cultures with higher cell densities and protein production levels
- Cell retention methodologies that allow the discharge of dead cells and cell debris, while retaining viable cells
- Solid/liquid separations with high yield and no cell lysis
- High concentration/high intensity downstream processing
- Adsorbent development with improved mass transfer properties

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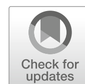
Insights on the Formulation of Recombinant Proteins



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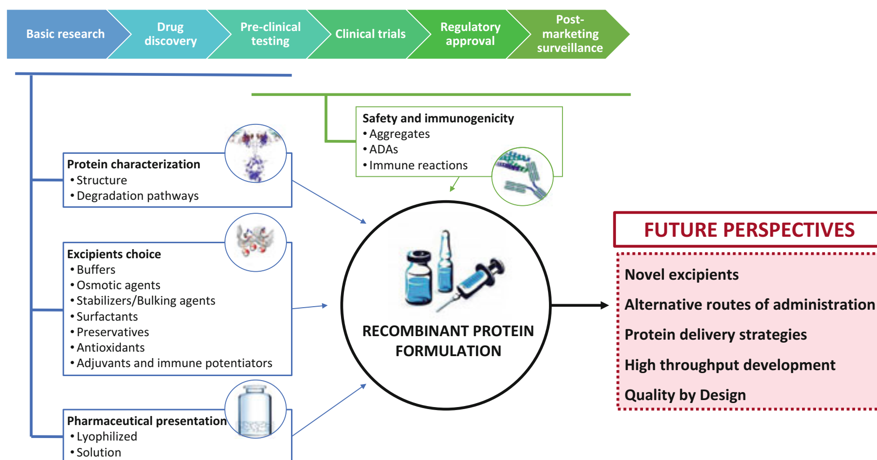
Abstract Recombinant proteins are large and complex molecules, whose therapeutic activity highly depends on their structure. Formulation of biopharmaceuticals aims at stabilizing protein conformation, promoting its efficacy, and preventing safety concerns, such as immunogenicity. Currently, the rational design of formulations is possible due to the availability of several techniques for molecule characterization and an array of both well-known and new excipients. Also, high-throughput technologies and Quality by Design approaches are trending and have been contributing to the advancement of the field. Still, there is a search for alternatives that ensure quality of the medicines through its life cycle, particularly for highly concentrated formulations, such as monoclonal antibodies. There is also a demand for strategies that improve protein delivery and more comfortable administration to the patients, especially with the arising of recombinant proteins in the treatment of chronic diseases, such as autoimmune conditions or heart diseases. In this chapter, current and future advancements regarding recombinant protein formulation and its impact in drug development and approval will be addressed.

Graphical Abstract



Keywords Formulation, Immunogenicity, Recombinant proteins, Stability

1 Introduction

Recombinant proteins represent an increasing fraction of the therapeutic arsenal available nowadays. Relative to small molecular weight drugs, recombinant proteins are larger and more complex active pharmaceutical ingredients, whose efficacy and safety highly depend on the ability to recapitulate their structure in the different stages of the protein lifetime. Additionally, challenges arise from the development of formulations with high concentrations of protein, as in the case of monoclonal antibodies, as well as for low-volume formulations (as demanded for self-administration devices). The pursuit of a formulation that can stabilize the active protein, from manufacture to administration, in an economically effective way, is then a crucial aspect to consider in the development of biological medicines. Herein, the formulation of recombinant proteins will be discussed, addressing general concepts, current trends, and future perspectives.

2 Protein Structure

2.1 Molecular Structure

Proteins are large molecules composed of one or more chains of amino acid residues, displayed in an order that is determined by a DNA sequence. The linear polypeptide

form of the protein corresponds to the protein's primary structure. Intermolecular interactions promote the arrangement of these chains into secondary (alpha-helices and beta-sheets) and tertiary structures. The latter can be assembled into quaternary structures, when two or more subunits interact. As different amino acids have different side chains with unique chemical properties, intra- and intermolecular interactions vary in strength, from weak forces (hydrophobic, electrostatic, hydrogen, van der Waals) to strong covalent bonds (disulfide bridges), adding to the complexity of the final structure [1].

If chemical and/or physical degradation occurs, protein may unfold, and hydrophobic residues that would be hidden in the native state may become exposed. Consequently, the molecule can remain unfolded or misfolded in a thermodynamically non-stable state, interacting with other proteins and causing aggregation and precipitation [2]. The presence of aggregates in biological medicines has been associated with immunogenicity, loss of biological activity, and renal failure [3]. Further in this chapter, mechanisms of protein degradation will be addressed, as well as approaches to minimize aggregates formation and assure the efficacy and safety of the final medicine.

Protein degradation can take place at different stages of the product's life cycle, from manufacturing and processing to storage, administration, and delivery [4]. Because recombinant proteins are produced in cell-based systems, purification and recovery steps are needed to isolate the product of interest from destabilizing impurities. Long-term shelf-life (about 18–24 months for a protein-based therapeutic) could promote degradation pathways (e.g., hydrolysis or oxidation), even when the adequate storage conditions are maintained [5]. Different delivery systems are also to consider, since the materials and processes used for their preparation and final presentation may impact protein stability [6].

2.2 *Chemical Degradation*

Chemical degradation involves modifications of covalent bonds of the protein, such as deamidation, isomerization, hydrolysis, oxidation, and disulfide bond shuffling [7]. It affects the primary sequence of the protein, which may lead to significant changes in the final three-dimensional structure [3].

Deamidation is one of the most common forms of chemical degradation. It consists in the hydrolysis of asparagine (Asn) to aspartic (Asp) and iso-aspartic acid, or that of glutamine (Gln) to glutamic acid (Glu), via a succinimide intermediate. Subsequently, a neutral amino acid residue is converted into a negatively charged polar one [8]. The reaction is favored by neutral and basic pH [9] and temperature above 25°C [10], as well as by the steric effect of adjacent residues (mainly in the C-terminal) and their conformation. In this respect, the higher the size and branching of the side chain, the lower the rate of deamidation, with glycine (Xxx-Asn-Gly-Xxx) resulting in the highest extent of deamidation [11].

Hydrolysis of a peptide bond can take place either under acidic or alkaline conditions, or it can be enzymatically mediated. As a result, the polypeptide chain breaks, leading to protein fragmentation. Some bonds are more susceptible to hydrolysis, like Asn-tyrosine or Asn-proline [12].

Oxidation can be a consequence of several external factors, including exposure to light, oxygen, transition metal ions (iron and copper [13]), or formulation degradation products (e.g., hydrogen peroxide from polysorbate autoxidation [14]). Amino acid residues such as tryptophan (Try) and histidine (His) are more susceptible to oxidation due to their aromatic rings, while methionine (Met) and cysteine (Cys) are vulnerable to oxidation as the result of their highly reactive sulfur atoms [15]. The steric exposure of the reactive residue and pH of the solution also influence the oxidation rate [16].

Cross-linking is an additional chemical modification involving the formation of disulfide bonds, either from the formation of new Cys-Cys bonds or exchange of pre-existing ones. This modification is favored with increasing pH values, as thiolate ions are the major reactive species involved [17].

2.3 Physical Degradation

Physical degradation implies changes in the secondary, tertiary, or quaternary structure of the protein, leading to aggregation, precipitation, or adsorption to surfaces. Mechanisms of physical degradation usually involve unfolded or semi-unfolded intermediates and are triggered by stress factors, such as mechanical stress (stirring, pumping, filtration, and filling during manufacturing and shaking during administration [18]), high temperatures (during shipping and storage), and freeze/thaw cycles. High temperatures, i.e., temperatures close to the protein's melting point (value at which half of the protein population is unfolded and the other half is folded [19]), lead to the formation of aggregates via non-covalent interactions between the unfolded proteins [20]. Freeze/thaw cycles may cause partitioning of the protein to ice-water interface, cryo-concentration, and change in pH due to nonuniform buffer recrystallization, affecting protein stability [21]. Cold denaturation is not very common, as it takes place only below the freezing point of water, when water molecules are ordered in a layer around the protein, changing its thermodynamics and leading to destabilization [22]. These low temperatures (usually below $-20°C$) are not achieved under regular manufacturing, transport, or storage conditions. However, upon freeze-drying for lyophilization purposes, the glass transition temperature usually goes below $-20°C$, which increases the chances of cold denaturation.

Aggregation is one the most common forms of protein physical degradation. The trigger of aggregation and the characteristics of aggregates depend on protein's environment: pH, temperature, ionic strength, concentration, and presence of co-solutes and excipients [23]. This form of degradation is particularly significant when dealing with monoclonal antibodies, as they are usually formulated at high

concentrations [24]. Moreover, large, multidomain proteins are more prone to aggregate, because gentle conditions, such as exposure to temperatures below their melting temperature (T_m) [25], can be sufficient to break the non-covalent interactions that usually maintain the several domains properly organized, unfold the protein, and induce aggregation. On the other hand, small, single-domain proteins only have their intramolecular forces disrupted under more extreme conditions, such as pH below 3 or above 10, or temperatures above 40°C [26]. Nowadays, it is possible to predict the extent and rate of aggregation based on the physicochemical properties of the protein, upon experimentally testing the effect of certain residues' mutations [27, 28], complementing with in silico prediction tools [29]. Recent developments on statistical modeling and machine-learning enable the assessment of aggregation potential of proteins using a high-throughput screening, and thus the selection of a lead with the lowest risk of aggregation, in an early stage of development [30, 31].

2.4 Characterization of Proteins

The complex structure of recombinant proteins demands a thorough characterization in early development to better control the impact on their stability and biological activity. Several techniques of biophysical and biochemical analysis have to be performed in order to determine, in a rational way, the best conditions for drug stabilization through its whole life cycle. Information derived from this early characterization will be further used to establish the specifications to which the active substance, excipients, and final product must conform to be considered acceptable by manufacturers and regulatory authorities. In this respect, the International Conference on Harmonization (ICH) Q6B guideline unifies a set of international analytical procedures and respective acceptance criteria regarding biotechnological products, based on their molecular and biological characteristics [32].

These methodologies are based in separation (chromatographic, SDS-PAGE) and spectroscopic methods, complemented by calorimetric and light scattering techniques. They assess protein size, conformation, hydrophobicity, and hydrodynamic features [33, 34]. The detection of aggregates requires imaging techniques, such as microscopy and light scattering that provide information to be used in computer modeling predictive tools, giving insight in possible degradation, aggregation, and immunogenicity. A review of computational tools used to assess protein aggregation can be found elsewhere [29], while the most commonly performed assays and respective measured properties are summarized in Table 1.

Finally, it is very important to do a functional characterization of the active protein, according to its mechanism of action, in order to evaluate its performance. Blotting, electrophoresis, chromatography, immunoassays (e.g., ELISA), and in vivo bioassays are examples of such methodologies [33].

Table 1 Methodologies and properties assessed for protein characterization (Adapted from Angkawinitwong [35])

Method	Technique	Measurement	Property assessed			
			Primary structure	Secondary structure	Tertiary structure	Aggregates
Chromatographic	Ion exchange chromatography	Charge variants	X			X
	Size exclusion chromatography	Hydrodynamic size				X
	High-pressure liquid chromatography (HPLC)	Degraded product	X			
Spectroscopic	UV absorbance	Protein concentration		X	X	X
	Raman spectroscopy	Conformation		X	X	
	Fourier-transform infrared spectroscopy (FTIR)	β-sheet, α-helix		X		X
	Near-/far-UV circular dichroism	Thermal stability		X	X	
	Fluorescence	Folding/unfolding, hydrophobic surface			X	X
	Optical density (OD)	Turbidity				X
Calorimetric	Differential scanning calorimetry (DSC)	Melting temperature (T_m), thermal unfolding/aggregation		X	X	X
Light scattering	Static/dynamic light scattering	Diameter and monodispersity of monomers				X
Microscopic	Transmission electron microscopy (TEM)	Particles				X
	Fluorescence microscopy	Particles				X
	Atomic force microscopy (AFM)	Particles				X
Mass spectrometry		Size, peptide mapping, degradation products	X			
SDS-PAGE		Molecular weight				X
Analytical ultracentrifugation		Molecular weight/shape				X

3 Formulation of Proteins

Efficacy and safety of recombinant proteins is highly dependent on their unique structure, which is why it is critical to develop a formulation with an optimum qualitative and quantitative composition for each therapeutic. The choice of excipients will depend on several factors, aiming at stabilizing the active protein through the life cycle of the product, up to administration to the patient. Excipients can promote protein stabilization by increasing thermodynamic stability, either through strengthening protein-stabilizing forces or destabilizing the unfolded state, forcing the refolding to the native conformation [36]. This is possible because of their direct binding to the protein (e.g., stabilizers) or their exclusion from protein surface and replacement by water molecules, i.e., protein hydration (e.g., sugars) [37].

Aside from the intrinsic physical and chemical properties of the active protein and excipients, some other aspects have to be considered when choosing the most appropriate excipients. First, special attention to chemical and physical stability issues during manufacturing is demanded. This requires a good understanding of the critical attributes and processes that impact quality, from early development [38]. The formulation approach intended is important, since liquid or lyophilized formats require different processing [39]. The final concentration of protein is another significant aspect to consider. As mentioned before, formulations with highly concentrated protein (50–150 mg/mL) [40], as monoclonal antibodies, are more susceptible to aggregation. It is also important to consider possible non-specific adsorption to surfaces, as to the primary packaging or to the administration device, especially for hydrophobic proteins [41]. The route of administration must be regarded too, as adjustments in formulation may be necessary to keep the required tonicity or osmolarity and pH for parenteral administration [39].

All the components to be used in a formulation must be characterized, and their compatibility with each other and with the therapeutic protein must be evaluated. Depending on the concentration that is being used, the same excipient may have different roles in different formulations, making it difficult, sometimes, to categorize them. Herein a classification of the most common excipients used in the formulation of recombinant proteins is presented (Table 2).

3.1 Buffers

At a pH value at the protein isoelectric point, the net neutral charge of the protein limits electrostatic interactions, thus favoring protein-protein interactions and subsequent aggregation. Buffers are, therefore, an important component of protein formulation in order to enable a pH of the protein solution below or above of the corresponding isolectric point [42]. As therapeutic proteins are preferably administered parenterally, on the choice of the most appropriate pH, the physiological range (pH = 7.35–7.45) [43] necessary to avoid irritation or pain during administration

Table 2 Classification, role, and examples of commonly used excipients in the formulation of recombinant proteins

Excipient class	Role in formulation	Examples
Buffers	pH stabilization	Amino acid (histidine, methionine, glycine, arginine), non-amino acid (phosphate, acetate, citrate), and salts (sodium hydroxide, hydrochloric acid, phosphoric acid)
Osmotic agents	Isotonicity maintenance	Sodium chloride, potassium
Stabilizers/ bulking agents	Protein stabilization and formulation support	Amino acids (histidine, glycine, arginine, glutamate) and sugars (mannitol, sucrose, trehalose)
Surfactants	Solubility enhancement and anti-aggregation	Sodium dodecyl sulfate and polysorbate 20, polysorbate 80
Preservatives	Minimization of bacterial contamination	Benzyl alcohol, phenol, metacresol
Antioxidants	Minimization of oxidative stress degradation	Ascorbic acid, acetylcysteine, glutathione
Adjuvants and immune potentiators	Modulation of immune response	Aluminum salts, squalene-based oil-in-water emulsions, monophosphoryl lipid A

must be also considered. When balancing these aspects, pH ranging between 3 and 11 may be considered acceptable [44]. Substances like amino acid, as histidine, methionine, glycine or arginine, or non-amino acid, phosphate, acetate or citrate, are examples of buffers used in protein formulation. Acids and bases can also be used as buffers, either as pH modifiers or to form salts in combination with other components of the buffer (e.g., sodium hydroxide, hydrochloric acid, phosphoric acid). Along formulation development, the presence of other excipients or the influence of certain processes that may promote precipitation or crystallization of the buffer, altering the pH, should be carefully assessed. This is the case of acetic acid that being volatile, its use in lyophilized formulations is not indicated [39]. Interestingly, formulations with high concentrations of protein may even dismiss the need of buffer, namely, when they have buffering amino acids in their composition, such as aspartate, glutamate, and histidine [45].

3.2 *Osmotic Agents*

In medicines for parenteral administration, the maintenance of the isotonicity of the formulation in a range between 280 and 300 mOsm/L is required, to avoid damage of the tissues (phlebitis or infiltration) or pain at the site of injection. This is particularly difficult for formulations with a high concentration of protein. The most common tonicifiers are sodium chloride and potassium chloride [39]. Careful choice of the

agent is required as, for example, sodium chloride and water react to form an eutectic mixture at −21°C. In this case, water crystallizes during freeze-drying, and the concentration of sodium chloride increases until it precipitates. For this reason, sodium chloride is added to the diluent for reconstitution instead to the lyophilized product [46].

3.3 Stabilizers/Bulking Agents

The solvent surrounding the protein and the solutes interacting with it are very important in maintaining the correct folded structure. The pharmaceutical presentation of the protein-based dosage form, either in solution or lyophilized, influences the nature of those interactions and impacts the choice of stabilizers and bulking agents.

The most common stabilizers are amino acids (histidine, glycine, arginine, glutamate) and sugars (mannitol, sucrose, trehalose). They stabilize the proteins by direct interactions or solute exclusion (stabilizer amino acids are repulsed from the protein surface, increasing the free energy of the unfolded state and favoring folding in the native form) [47], but their specific mechanism is sometimes difficult to assess due to the diversity and complexity of their side chains. Mixtures of different amino acids (e.g., arginine and glutamic acid) [48] or combinations of amino acids and sugars (e.g., histidine and sucrose or mannitol) might enable a synergistic effect in the protein stabilization [49].

Bulking agents are incorporated in lyophilized formulations to increase product mass (thus preventing its collapse), to aid in rehydration (facilitating dissolution) and to improve product appearance (by providing mechanical support) [50].

Due to their amorphous or crystalline physical state, sugars may act as stabilizers or bulking agents, respectively. For example, sucrose maintains an amorphous state after lyophilization, acting as a stabilizer, while mannitol is capable of crystallizing and supporting the lyophilized cake, thus acting as a bulking agent. Another case shows that, in a formulation of a human growth hormone, when mannitol and glycine were used separately, both crystallized during freeze-drying. However, when combined in the same formulation, mannitol crystallized, while glycine remained amorphous. This partially amorphous system offered the best protein stabilization [51]. Sugar recrystallization should be avoided, since the conversion between amorphous and crystalline states can compromise protein stability [52].

3.4 Surfactants

Surfactants are molecules whose mechanism of action will depend on their nature (either ionic or nonionic), acting as solubility enhancers, anti-adsorption or anti-aggregation agents. Upon competitive binding to interfaces like air-water or

container-water, surfactants prevent the non-specific adsorption of proteins through binding to their hydrophobic residues. They also compete with other proteins for the binding to protein surface, preventing aggregation [53]. Their activity has a significant impact in highly concentrated formulations, which are more prone to form aggregates.

Ionic surfactants are rarely used in protein formulations, due to their tendency to denature proteins. Nonionic surfactants, such as sodium dodecyl sulfate and polysorbate 20 or polysorbate 80, are efficient, less toxic, and less sensitive to the presence of electrolytes [12]. Polysorbates are the more common surfactants found in protein formulations, but their concentration must be selected carefully, since they can undergo autoxidation and give origin to hydrogen peroxide, which will not only compromise protein stability but also the safety of the product [14].

3.5 Preservatives

Preservatives are present in formulations to minimize microbial contamination. They are particularly important in products with multiple doses, due to their likelihood to contaminate in repeated usages. Examples of preservatives include metacresol, phenol, and benzyl alcohol. The latter was even shown to stabilize the partial unfolded state of a protein, inhibiting its aggregation [54]. In the case of volatile and reactive preservatives, incorporation in lyophilized products is rather performed in the solvent for reconstitution, than in the lyophilized cake [39]. The addition of preservatives to a formulation may, however, impact protein stability in a negative way. In this respect, it is proposed that preservatives interact with the active proteins, destabilizing their weakest links (so-called hot-spots), thus decreasing conformational stability and increasing propension to aggregation [55, 56]. This issue may be minimized upon deepening the understanding of the effect of preservatives on proteins and by applying strategies enabling protein stability. Besides, a case of binding of preservatives to a model peptide decreased their antimicrobial effect, thus raising possible safety concerns [57].

3.6 Antioxidants

Proteins rich in methionine, cysteine, tryptophan, tyrosine, and histidine residues are more susceptible to degradation due to oxidative stress. In these cases, formulation with antioxidants is a requirement. Ascorbic acid and acetylcysteine are examples of antioxidants often used in the formulation of proteins. Glutathione also behaves as reducing agent, upon creating disulfide bonds with cysteine residues of the protein. Replacement of oxygen by an inert gas in the vial is a complementary measure to reduce oxidation [58]. Other mechanism of oxidation prevention is the chelation of

metal ions. In this regard, ethylenediaminetetraacetic acid and calcium chloride have been used as antioxidants [59].

3.7 Adjuvants and Immune Potentiators

Recombinant peptide or protein-based vaccines have the ability to induce immune responses against a specific antigen. Their formulation often includes adjuvants that help to modulate or even increase immune responses. Aluminum salts are the most commonly used adjuvants. They act in the site of injection, adsorbing the antigen, enabling its stability and uptake by antigen-presenting cells, and inducing local pro-inflammatory reactions [60]. Other examples are MF59 and AS03, squalene-based oil-in-water emulsions, that consist of oil nanoparticles suspended in an aqueous phase via a surfactant. They have shown to be more effective than aluminum in the avian H5 pandemic flu vaccines [61].

Currently, the objective is to go beyond antibody-mediated immunity and accomplish long-lasting cellular responses, like those mediated by T helper and T cytotoxic lymphocytes. This can be achieved by the activation of Toll-like receptors (TLR) or NOD-like receptors (NLR) by immune potentiators, like pattern-recognition receptors (PRR) agonists [62]. Monophosphoryl lipid A (MPL) is a natural molecule that targets TLR4 and has been also used as an adjuvant. Synthetic analogues have been studied, namely, in combination with aluminum or emulsions [63].

A relevant formulation aspect relies on the assessment of the mechanism of action of the antigen and of the adjuvants, as well as the interaction of these components with each other, as this may impact both the efficacy and the safety of the drug [64]. For example, aluminum salts can adsorb the antigen (with a strength that is antigen-specific), destabilizing it and promoting protein aggregation [65]. As for emulsions, they can lead to protein denaturation or desorption, structural rearrangements, and inter-protein interactions, with subsequent aggregation [66]. To minimize these effects, particle-based formulations for stabilization and delivery of the adjuvant are under development, such as nanotechnology-based delivery systems, virus-like particles, and polymeric systems [67].

3.8 Trends in Formulation Approaches

An overview of recombinant protein medicines currently approved by European Medicines Agency (EMA) and Food and Drug Administration (FDA) gives an idea of the formulation approaches that are preferentially being followed. In Europe, monoclonal antibodies comprise the major class of therapeutic proteins, which, as mentioned earlier, implies the use of excipients appropriate for highly concentrated products. Therefore, upon briefly running through the major classes of excipients, buffers, surfactants, preservatives and tonicifiers arise as the major components of

current protein formulations [39]. In the United States, the scenario is similar. Antibody therapeutics are also the highest selling class of biopharmaceuticals, delivered by parental routes of administration and stored either in solution or powder formats. Among these, the most commonly used excipients fall into the buffers, bulking agents, and surfactants categories [68]. For a more detailed and practical understanding of the formulations currently used in commercialized drugs, the European Public Assessment Reports (EPAR) [69] of the 10 top-selling biopharmaceutical products in 2017 [70] were analyzed, and their excipient composition and pharmaceutical presentation were compiled in Table 3.

Table 3 10 top-selling biopharmaceuticals' formulations

Product	Active substance	Excipients	Pharmaceutical presentation
Humira®	Adalimumab	Mannitol, polysorbate 80, water for injection	Solution for injection
Enbrel®	Etanercept	Mannitol, sucrose, trometamol	Lyophilizate for reconstitution and injection
Rituxan® MabThera®	Rituximab	Sodium citrate, polysorbate 80, sodium chloride, sodium hydroxide, hydrochloric acid, water for injection	Concentrated solution, for dilution and infusion
Remicade®	Infliximab	Sucrose, polysorbate 80, monobasic sodium phosphate, dibasic sodium phosphate	Lyophilizate for reconstitution, dilution, and infusion
Herceptin®	Trastuzumab	L-histidine hydrochloride monohydrate, L-histidine, polysorbate 20, α,α-trehalose dihydrate	Lyophilizate for reconstitution, dilution, and infusion
Avastin®	Bevacizumab	Trehalose dihydrate, sodium phosphate, polysorbate 20, water for injection	Concentrated solution for dilution and infusion
Lantus®	Insulin	Zinc chloride, metacresol, glycerol, hydrochloric acid, sodium hydroxide, water for injections, polysorbate 20 (when stored in vial)	Solution for injection
Eylea®	Aflibercept	Polysorbate 20, sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate heptahydrate, sodium chloride, sucrose, water for injection	Solution for injection
Opdivo®	Nivolumab	Sodium citrate dihydrate, sodium chloride, mannitol, pentetic acid, polysorbate 80, sodium hydroxide, hydrochloric acid, water for injection	Concentrated solution for dilution and infusion
Neulasta®	Pegfilgrastim	Sodium acetate, sorbitol, polysorbate 20, water for injection	Solution for injection

3.9 Formulation of Biosimilars and Biobetters

A biosimilar is a biopharmaceutical that is highly similar to an already approved biologic (reference medicine) in terms of quality, biological activity, safety, and efficacy. The complexity of proteins associated with their different levels of structural organization and high molecular weight determines a demonstration of similarity between a biosimilar and the reference medicine, based on the assessment of toxicity and efficacy in a clinical setting. This implies not only a structural similarity between the active protein in the biosimilar and reference medicine, but also the same posology and route of administration. Some deviations from the reference product may be accepted, such as pharmaceutical dosage form, formulation, excipients, or presentation, provided that they are properly justified [71].

In fact, both EMA and FDA allow for advancements in formulation science to be incorporated in the biosimilar presentation, admitting excipients to differ from those of the reference. In this respect, any relevant effects of the revised formulation on the stability, physiochemical, and functional characteristics of biosimilars must be assessed [72]. However, as stated before, it is hard to collect information on the safety and immunogenicity of biopharmaceuticals based only in the characterization of the active molecule and nonclinical data. The same is applied to their biosimilars. Therefore, clinical trials and post-marketing vigilance are required by the regulatory authorities to ensure at least the same efficacy and safety from the innovator medicine [73, 74].

Biobetters are the new players in biopharmaceutical development. They act against the same target as an already marketed medicine and include changes in the molecular structure or formulation. However, unlike biosimilars, these alterations aim to improve pharmacokinetic or pharmacodynamic properties, to decrease toxicity or immunogenicity, or to make administration more comfortable or less frequent, i.e., biobetters are clinically superior than the respective references either in efficacy, safety, or compliance, or both [75]. Biobetters are not regulated yet, so they are treated as new drugs and can only enter the market after a full application submission, which is both expensive and time-consuming. Nonetheless, they have been used for biopharmaceutical companies that want to extend the life cycle of drugs whose patent is expiring, as a way to manage competition from biosimilars [76]. For example, in 2013, Roche obtained marketing authorization for a subcutaneous formulation of Herceptin®, since intravenous trastuzumab patent would expire in 2014. The new formulation presented efficacy and safety profiles similar to the intravenous one and further enabled administration of higher volumes. Along with the advantage of shorter duration of treatment, saving time and resources for healthcare professionals, and improving patient compliance [77], it became a concern for biosimilars of intravenous trastuzumab developers. This case illustrates the importance of formulation development beyond the drug's marketing authorization.

4 Pharmaceutical Presentation

Even with an appropriate choice of excipients supporting the stability of the active protein, the dosage form and drug packaging can have an impact in maintaining the medicine's quality throughout its life cycle. Therefore, pharmaceutical presentation must be regarded during formulation development [78].

4.1 Lyophilized Therapeutic Proteins

Most biopharmaceutical products are stored in the form of aqueous-based solutions or lyophilized powders [39]. As mentioned before, protein solubility depends on several aspects, including pH, ionic strength, and associated excipients. If stability and solubility can be achieved, a solution is preferred over a suspension, since the latter is harder to process and handle. Suspensions have increased chemical stability, but not necessarily higher physical stability, due to the propensity to form agglomerates when exposed to mechanical stress [79].

Often, the liquid formulation does not provide the required long-term stability of the protein and quality of the product, as the presence of water may promote physical or chemical degradation. The dried state is thus the preferred form, and the protein must be lyophilized.

Lyophilization is a process that comprises three sequential phases: freezing, primary drying, and secondary drying. During the freezing step, temperature is reduced below the eutectic temperature (T_e) of the mixture (i.e., a temperature that is lower than the melting point of each component of the formulation). Then, during primary drying, crystallized water and water that is not bound to the protein or excipients are removed by sublimation, with temperature still below the T_e, and under reduced pressure. Secondary drying allows the removal of water that is bound to the protein and excipients, by slowly increasing the temperature, although still below T_e, and gradually rising the pressure [80]. The result is a dried powder that contains the protein in a glassy phase, amorphous excipients, and some residual water. It must have a uniform and elegant appearance [81] and be rapidly dissolved in the reconstitution solvent.

This process of water removal from frozen solutions under reduced pressure may damage the active protein. The incorporation of sugars and polyols (e.g., sucrose, trehalose, sorbitol) will mostly replace the hydrogen bonds between the protein and the surrounding water molecules by hydrogen bonds created with the excipients, stabilizing it thermodynamically and thus acting as lyoprotectants. From a kinetic perspective, it is proposed that these sugars stabilize the active molecule by forming an amorphous glass matrix, so they should not recrystallize during storage [82].

Due to the crystallization of different solutes in the mixture at different stages during freeze-drying, a differential precipitation of excipients may occur. Upon precipitation of buffer components, a shift in the pH of the mixture may take

place, with negative consequences for protein stability. This is hardly detected as, upon reconstitution, buffers will redissolve and the pH measured will be the targeted one. Salts are used to minimize changes in pH and tonicity, but one must be conscious in their choice, since pure solvent freezes first than the salts, leading to an increase of their concentration and altering the ionic strength and compromising the protein. Sugars like lactose and mannitol can also crystallize and separate from the solution. They should be replaced by carbohydrates (e.g., sucrose) or amino acids (e.g., histidine or glycine) that present amorphous characteristics [39]. During freeze-drying carbohydrates can also react with amino groups of the protein, promoting the Maillard reaction and originating a yellow-brown cake. To avoid this, nonreducing sugars like sucrose or trehalose may be used [34].

When considering lyophilized biopharmaceuticals, the reconstitution step is of major importance. Reconstitution solvent can interfere with protein stability. Therefore, besides using sterile water as diluent, excipients like stabilizers and preservatives may be added to maintain the quality of the drug until administration. Reconstitution times may have an impact in protein stability, especially for high-concentration therapeutics that are formulated with higher concentrations of stabilizer excipients. This increases reconstitution time and protein propensity for degradation. The incorporation of wetting agents, like surfactants, in the reconstitution solvent is a possible solution [83].

4.2 Containers and Administration Devices

Packaging of biopharmaceuticals must accompany the chosen form of pharmaceutical presentation in the maintenance of drugs' stability during shelf-life and administration, while providing ease of handling and improving patient compliance. Biologics are stored in vials, pre-filled syringes or cartridges, ampoules, or bags, according to the physicochemical properties of the formulation, product volume, materials of the containers, and their intended functionality [84]. There is a high potential of interactions between containers and components of the formulation, especially due to phenomena of leaching (migration of unwanted particles from the container to the medicine) and sorption (diffusion of an ingredient to the container material, through mechanisms of adsorption, absorption or permeation) [85], thus requiring a rigorous compatibility assessment.

Pre-filled delivery devices are increasing in popularity, as they allow for the patients to self-administer the drug in ambulatory, without the help of a healthcare professional, improving compliance and reducing time and cost expenses with specialized practitioners. This is relevant in a time of increasing development of biopharmaceuticals to treat chronic diseases (diabetes, rheumatoid arthritis). They also reduce the risk of contamination and waste-associated costs, since there is no need to overfill the container [58]. When choosing the excipients and packaging material for such devices, some aspects must be considered. For example, to be administered in a syringe, liquid solutions must have low viscosity [40]. Also, during

transportation or handling, agitation may lead to the incorporation of air, which could potentially originate new liquid-gas interfaces, and subsequently protein unfolding.

5 Safety and Immunogenicity of Biopharmaceuticals

5.1 Quality Framework

Safety is a major concern regarding the use of biopharmaceuticals, and it can be influenced by the quality of the medicine. In fact, unlike small drugs, recombinant proteins are produced in living systems and are subjected to downstream steps of processing and purification. It has been shown that the complexity of their manufacturing may result in contaminants or impurities that affect the medicine's safety [86, 87]. Moreover, the physical and chemical features of therapeutic proteins are hard to fully characterize, as well as their potential for aggregation and precipitation. To add to the active protein's intrinsic features, the corresponding formulation can have a significant impact in the drug's safety profile. In fact, excipients are perceived as pharmacologically inactive substances, but this concept is quite simplified, as the safety of a biopharmaceutical also involves the quality and toxicity of its excipients [88]. For known excipients, it is only required to prove quality to ensure safety, since they are well-characterized and associated with market approval. For novel excipients, however, regulatory authorities request preclinical studies to demonstrate the desired safety of the compound on its intended formulation, which can be very expensive and time-consuming [89]. This justifies a strict regulatory framework on the quality control and toxicological data of the active protein and excipients [89].

The ICH provides guidance on the quality and safety of medicines. The ICH Q5A-Q5E and the S6(R1) guidelines provide information on contaminants evaluation, stability testing, and preclinical safety testing within the scope of biotechnological products. The World Health Organization (WHO) also presents recommendations for both manufacturers and regulatory agencies on the quality, safety, and efficacy of biotherapeutic products, giving guidance on manufacture and quality control of recombinant protein therapeutics, and on nonclinical and clinical evaluation [90]. The *Good Manufacturing Practice Guide for bulk Pharmaceutical Excipients* is harmonized across the different regulatory agencies and helps manufacturers to evaluate the quality of raw materials, in order to comply with *Good Manufacturing Practices* [91]. The European and the United States pharmacopoeias also have a natural impact in formulation development and excipients quality control. These documents support pharmaceutical companies on the fulfillment of requirements set by the EMA's regulatory guidance on excipients [92] and the FDA's Code of Federal Regulations (Title 21, Chapter I, Subchapter C).

Despite the effort to assess all possible safety issues during development and manufacturing, adverse events may occur when the final medicine is administered to

patients. Biologics adverse reactions have been classified based on the already existing classification for small drugs adverse reactions (type A to E), having into account mechanisms of action rather than clinical symptoms [93]. Thus, to distinguish this classification from the one existing for chemical drugs, adverse events can be categorized from type α to type ε, as immune stimulation, immunogenicity (against therapeutic protein), immune deviation (immunosuppression or autoimmunity), cross-reactivity (with similar molecule), and non-immunological.

5.2 *Mechanisms of Immunogenicity*

Immunogenicity is a major issue in recombinant proteins' safety and efficacy, because of their high potential to elicit anti-drug responses [94]. The immunogenic potential of biopharmaceuticals depends on a variety of factors, such as structural features of the active molecule or excipients, presence of impurities, route of administration, dose and duration of treatment, and patient features [95].

Reactions against biologics can be a consequence of either breakdown of immune tolerance to autoantigens (human homologous) or activation of the host's immune system to neoantigens [96]. The first mechanism is not yet well understood, but it seems that repeated administration of therapeutic proteins that are similar to endogenous ones, depending on their dose, may induce immune responses and clinical adverse effects. This is exemplified by the development of thrombocytopenia in patients who were administered with a pegylated recombinant megakaryocyte growth and development factor. This therapeutic protein shares the first 163 amino acids of endogenous thrombopoietin, which led to the development of antibodies that cross-reacted and neutralized the endogenous promoter of platelet production [97].

The second proposed mechanism of immunogenicity is related to the degree of non-self of the active protein, glycosylation, and protein aggregates, all of which can be examples of neoantigens (not previously recognized by the immune system). The degree of non-self, i.e., of structures foreigner to one's organism, is related with the source of the protein: animal (e.g., bovine insulin is more immunogenic than porcine insulin, which is more immunogenic than the human recombinant protein [98]), bacterial, or plant origin (absence of posttranslation modification mechanisms [99]). Glycosylation is one important characteristic that influences protein three-dimensional conformation. When a glycan composition is not recognized by the immune system, or affects protein structure, decreasing its stability and solubility, immune reactions against the drug may be triggered. However, in some cases, a glycosylation pattern different from the native protein's one can be beneficial, and it is even a modification proposed to decrease protein immunogenicity, as it can improve solubility through protein-solvent interaction or upon hiding antigenic sites [100].

5.3 Protein Aggregation

Protein aggregation has been considered the most important structural change related to immunogenicity [101]. It is hypothesized that the immune system was developed to recognize repeated patterns of proteins, sugars, or lipids present at the surface of invading microorganisms and develop a humoral response upon generating antibodies against the active site of the molecule. In the case of therapeutic proteins, the generated antibodies are named anti-drug antibodies (ADAs). This is likely the reason why multimerization of proteins is a key factor in the development of an anti-drug immune response, along with protein aggregates, regardless the size of the monomeric or aggregated form of the protein [102]. Aggregates can be classified according to their size (submicron soluble aggregates, insoluble particles up to 100 μm, and visible particles – above 100 μm), binding type (non-covalent or covalent) [3], or format (aggregates of proteins in the native structure; denatured structure, aggregated in an irreversible way; and covalently bound native and denatured proteins) [103]. There are no regulatory limits regarding the size of aggregates in biopharmaceutical products, but the United States [104] and the European [105, 106] pharmacopoeias determine the recommended limits for injectables and methods to assess them.

5.4 Anti-drug Antibodies

When ADAs are produced in a conformation-dependent manner (instead of the linear form of the epitope), they will alter the pharmacokinetics and pharmacodynamics of the protein, therefore neutralizing its activity and therapeutic efficacy [107]. Non-neutralizing antibodies did not used to be considered clinically significant, as they do not neutralize the activity of the protein. However, they can bind to it and form immune complexes that are rapidly eliminated, thus altering the pharmacokinetics of the drug and affecting its bioavailability [94]. Consequences of ADAs can range from clinically non-relevant to severe responses, such as hypersensitivity, anaphylactic reactions [103], or deficiency syndromes (thrombocytopenia or pure red cell aplasia [108]). Thus, it is important to use ADA determination as a predictive marker of immunogenicity during drug development or as an assessment tool after drug's approval [109]. Nonclinical studies have a low predictive value. Despite the efforts, there is still the need to develop more accurate in silico models that can predict protein-immune system interaction, as well as animal models that can overcome the differences between human and animal immune systems and their inevitable immune reactions against recombinant human proteins [110]. This supports the requirement from the regulatory authorities to generate data on ADA detection in clinical trials, as well as the incorporation of ADA detection methods in the drug's risk management plan [111]. EMA and FDA generated documents that

provide guidance and harmonization on the analytical methods to be used for ADA detection [112–114].

6 Current Trends in Formulation and Future Perspectives

The biotech industry has had a huge development, with great scientific and technical improvements, that increased the efficacy, safety, and quality of biopharmaceutical products. Nonetheless, challenges associated with the efforts to better understand the structure-function relationship, rational design of suitable formulations, engineering novel protein formats, avoidance of aggregates formation and immunogenicity, or development of less invasive administration routes, still remain.

6.1 Novel Excipients

The research on novel excipients is a significant aspect to consider in the development of novel biotechnology-based drugs and delivery devices. Excipients are considered novel when they are associated for the first time with a drug or route of administration. Therefore, both new substances and existing compounds may fit in this definition [92].

Novel excipients can bring numerous advantages: they can be used in smaller concentrations even in highly concentrated solutions; they can decrease viscosity and increase protein stability; they can improve manufacturing processes or allow to differentiate a medicine. Therefore, they can have a central role in increasing drug's efficacy and safety and contribute to patient's compliance [115]. Some novel substances have been approved or are under development. Recombinant human hyaluronidase has been recently used in products for subcutaneous administration. It enhances protein bioavailability by creating an interstitial matrix that promotes dispersion of the therapeutic protein [116]. Surfactants based on trehalose fatty acid esters are being tested as stabilizers in shake-induced stress conditions, reducing aggregate formation and adsorption to container surfaces. These properties make them a plausible alternative to poly(ethylene glycol)-derived surfactants that induce protein oxidation during static storage [117]. With the increasing development of high-concentration antibody solutions, excipients that reduce viscosity are needed. Combined use of arginine and glutamic acid seems to reduce intermolecular attractions, thus decreasing aggregation [48]. Hydrophobic salts, which compete with hydrophobic protein residues in protein-protein interactions, are also helpful in avoiding aggregation [118]. Cyclodextrins are cyclic oligosaccharides that have been used as excipients before, but not in parenteral protein formulations [119]. Their interest in recombinant protein formulations has been rising since they form inclusion complexes of the drug, increasing solubility and stability, and reducing aggregation [120].

Despite the awareness on the importance of novel excipients and the efforts made to develop them, they are not frequently found in approved products. This is because regulatory authorities have very strict requirements about the safety of these new substances, making the evaluation of the application process very complex, time-consuming, and expensive [121]. In fact, the experience associated with human use of excipients approved by regulatory authorities determines that any or only little additional toxicological data is needed. For novel excipients, stricter requirements are demanded [121], such as extensive preclinical evaluations, as well as data on the pharmacokinetics of the new excipient on its intended formulation. In Europe, the dossier that needs to be submitted for the application of a new excipient is similar to the one of an active substance. It requires information on manufacturing, characterization, in process controls and safety. It must be submitted together with the application for marketing authorization, in accordance with the EMA *Guideline on Excipients in the Dossier for Application for Marketing Authorization of a Medicinal Product* [92]. The FDA elaborated a guidance on *Nonclinical Studies for the Safety Evaluation of Pharmaceutical Excipients* that includes recommendations on how to obtain a safety profile for these new entities [122].

One last concern regarding the regulatory requirements for new excipients is that their development generates proprietary data that demands protection. As a way of promoting the development of novel compounds by the pharmaceutical companies, manufacturers of the United States of America and Japan follow an *Excipient Master File*, which contains both open and confidential information on quality and toxicity of the new excipients. By using this information, new excipients can be approved apart from a marketing drug application, a situation that is different at the present moment in Europe [121].

6.2 Alternative Routes of Administration

Therapeutic proteins have been formulated for parental administration, to allow for maximum availability and minimum pre-systemic degradation. Intravenous formulations are administered either in a single bolus dose or in a continuous infusion. Subsequently, maximum bioavailability is rapidly achieved. However, this pharmacokinetic profile may not be the desired one for all therapeutics. In fact, for proteins with short blood half-lives, an absorption of the drug through a prolonged period may be beneficial. One way to obtain the desired concentration-time profile is to change the administration route from intravenous to subcutaneous or intramuscular, which delay the absorption of the drug into the blood circulation [123].

Nowadays, other routes of administration are being studied for biopharmaceuticals, in search for a better convenience of administration (noninvasive) for the patient, improving therapeutic adhesion and self-administration in ambulatory [124]. This is especially relevant in a time where more biopharmaceuticals to treat chronic diseases are available. The oral administration provides the previously mentioned advantages. However, bioavailability achieved by this route is very

low, not only because of the nature of the proteins themselves (polarity and high molecular weight) but also due to protein degradation in the gastrointestinal tract and in first-pass hepatic metabolism. Strategies to overcome these difficulties are being studied and include the co-administration of absorption enhancers, which can act by temporarily disrupting the intestinal barrier or serving as a carrier (e.g., calcium chelators, surfactants, and bile salts), or protease inhibitors, which prevent enzymatic degradation of the drug (e.g., aprotinin and puromycin) [125].

Inhalation and intranasal administration routes can be advantageous due to the convenience of administration, the highly vascularized large surface area for absorption and avoidance of first-pass metabolism. Exubera® was a recombinant human insulin that, when inhaled, was absorbed more rapidly and had a shorter half-life than subcutaneous insulin. This profile was actually similar to the one of endogenous insulin after a meal [126]. However, the product was withdrawn from market in 2008 due to the low bioavailability and local side effects. Intranasal administration has the benefit of a direct delivery of the drug from the nasal cavity to the brain, without being retained by the blood-brain barrier. This is particularly interesting in therapeutics for central nervous system diseases, as exemplified for some neurotrophins, neuropeptides, and cytokines in in vivo studies [127]. On the other hand, intranasal administration is associated with more variability in absorption, due to the physiological mucociliary clearance mechanism and uncertain mucus secretion in cases of cold, allergies, or hay fever.

Transdermal administration also offers the advantage of bypassing hepatic and gastrointestinal degradation and allows for a prolonged drug release. However, bioavailability is limited due to proteins' high molecular weight and hydrophilic nature. Penetration enhancers like cell-penetrating peptides, ethanol, propylene glycol, dimethyl sulfoxide or surfactants can be incorporated in the formulation to change the permeability of the skin, facilitating drug absorption [128]. However, these passive strategies of transdermal delivery are limited to peptides and small molecular weight proteins. In this regard, permeation techniques like sonophoration (application of low-frequency ultrasound), iontophoresis (use of low-level electric current), and polymeric microneedles (either coated, dissolvable, degradable, or bioresponsive, according to the type of drug-polymer system desired) [129] have been used to allow transdermal delivery of proteins like insulin, hormone-releasing factor, calcitonin, and parathyroid hormone [130].

Based on the advantages associated with alternative routes of administration stated above, a new route of administration may be an interesting strategy to extend the life cycle of a drug already marketed in an injectable formulation. In this case, new nonclinical and clinical data would be required, to ensure comparability of efficacy, safety, and tolerability between the two formulations [131].

6.3 Other Strategies to Improve Protein Delivery

Manipulating the choice and concentration of excipients may not be enough to achieve the desired pharmacokinetics of certain proteins. Therefore, other strategies

to improve drug delivery have been proposed. A controlled delivery system enables the maintenance of drug therapeutic levels for an extended period, decreasing the need of frequent administrations and thus improving patient comfort and compliance. The components of the system must be nontoxic, non-immunogenic, biodegradable, and/or biocompatible [132]. Options available today include mechanical pumps (that can deliver the drug at a constant or pulsar delivery rate), subcutaneous osmotic mini-pumps (mechanism based on a difference of osmotic pressure between the interior of the pump and the outside), and regulated approaches (like biosensor-pump combinations or even self-regulating systems) [133]. Other strategies include the delivery of the therapeutic drug encapsulated in matrix-type delivery systems. Examples of such approach are nanoparticles and microparticles that prevent the contact of the protein with proteases and other enzymes and, consequently, its degradation before reaching the target site [134], or immobilization in hydrogel networks (beneficial in maintaining protein three-dimensional structure) [135].

6.4 High-Throughput Characterization and Formulation Screening

Due to the complexity of recombinant proteins and the diversity of excipients now available, drug characterization and formulation development are difficult and time-consuming. The use of high-throughput screening for protein characterization has been increasing, with the improvement of equipment such as high-density microplates, microfluidics and more sensitive detectors [136], and automatization of conventional technologies like spectroscopy, light scattering, or imaging [137]. When applied to formulation development, high-throughput assays give a large amount of information about protein stability, in a short period of time, and with a reduced amount of sample, which is critical in an early development stage [37].

Such assays should be preceded by pre-formulation studies, in order to increase knowledge on the physical and chemical properties of the protein and optimize the choice of experimental conditions to perform. These studies can go from the aforementioned characterization methods to mathematical and computer modeling tools. Mathematical models are an interesting source on the prediction of the protein's conformational dynamics and aggregation propensity [138, 139]. To assess protein structure, in silico characterization software is available. It is mainly based on the knowledge provided from protein sequence and signature databases (e.g., UniProt, Protein Data Bank). They combine data from amino acid sequences and three-dimensional data, to give an insight on protein structure, hydrophobicity, or even function [140]. For subsequent steps, several tools and online services have been developed that allow a fast and efficient analysis of the protein's active conformation, protein-ligand interactions, and affinity data [141].

Forced degradation characterization must be also included in pre-formulation studies, in order to detect and quantify possible degradation products [142]. It is

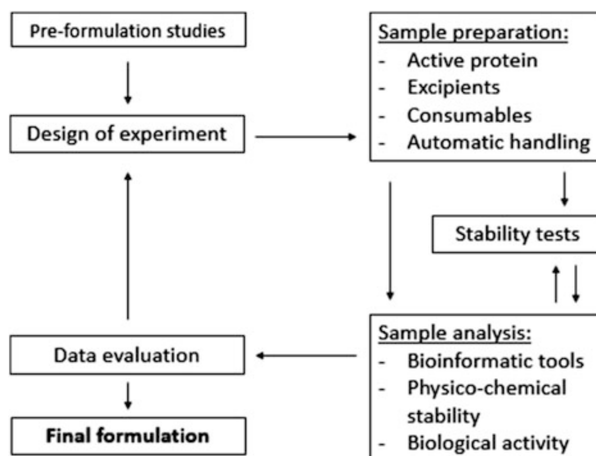


Fig. 1 Flow diagram of the high-throughput formulation development concept (Adapted from Martinus et al. [145])

assumed that when exposed to stress conditions for a short period of time, such as elevated temperatures, freeze-thawing, extreme pH, mechanical stress, or oxidizing environments, the degradation profile of the drug will correlate with degradation during the medicine lifetime [143]. This will enable the determination of the best stabilizing conditions, selection of stability parameters for quality monitoring and comparability, and prediction of degradation upon accidental exposure to unrecommended conditions. Most common forced degradation studies and analytical methods to assess degradation products have been reviewed by Hawe et al. [144]. After data collection from pre-formulation studies, an experiment can be designed, and a high-throughput platform (Fig. 1) can be put in place, either for characterization assays or for selection of appropriate formulation. There is a first step of sample preparation, using automated handling systems that place the samples in microplates (96–384 wells), allowing for the screening of multiple parameters simultaneously (concentration, pH, buffers, dosage form, and storage temperature). This is followed by sample analysis, usually with the help of specific software [145]. Several methodologies have been performed in high-throughput platforms. Noninvasive methods are the ones that allow for a recovery of the formulation, thus reducing waste. They include techniques such as UV-Vis absorbance, fluorescence spectroscopy, and light scattering. Invasive procedures should be executed after the noninvasive ones, to make use of the same sample. Examples of such methodologies are calorimetry, capillary electrophoresis, and mass spectrometry.

An example of a high-throughput platform for protein characterization that has been successfully developed is that of an automated workstation for large-scale protein identification, using reverse liquid chromatography and mass spectrometry, followed by analysis using SEQUEST software [146]. Regarding formulation development, Zhao et al. engineered an automated system that can test up to 500 different samples through a variety of analytical techniques, allowing for the screening of more formulations in a very short time, with the same reliability than classical approaches [147].

6.5 Developability Assessment and Quality by Design

Developability assessment is the likelihood of a drug candidate to be successfully developed into a commercial product [148]. It comprises a set of approaches that, when implemented in early stages of development, enable a more rational choice of lead candidates to be developed into optimal commercial products. These strategies include pre-formulation studies, stability evaluation, interaction between discovery and formulation design teams, and manufacturability assessment [149]. Therefore, developability offers the chance to re-engineer the lead molecule or to adapt the development process according to potential undesired features discovered in early development. This decreases the risk of failure in more resource-consuming and costly stages of drug development [150].

Developability assessment correlates with the implementation of Quality by Design (QbD), another trend in biopharmaceutical development and manufacture [151]. ICH guidelines Q8 and Q8(R2) define QbD as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" [152]. With a great understanding of the active protein and excipients from pre-formulation studies and developability assessment, it is possible to define, monitor, and adjust the parameters that contribute the most to consistently deliver products with a defined quality. Within the QbD, the parameters that are proven to impact protein stability, named *Critical Quality Attributes* (CQAs), should be monitored throughout the manufacturing process, such as type of excipients, concentration, or grade. *Design of Experiments* (DoE) is another important element of QbD, defined as a tool that incorporates the possibility to change the formulation attributes within a defined *design space*, whose limit is justified based on solid scientific data. This qualitative and quantitative flexibility allows for a rapid screen and optimization of a formulation during manufacturing [153]. Any deviations in formulation composition made within the defined design space are not considered changes in manufacturing, which is very attractive from the regulatory point of view. Risk analysis can help defining CQA's upon, for example, using tools of prediction of aggregates formation and immunogenicity propensity, which may impact the efficacy and safety of the medicine [100]. In respect to the establishment of the range enabling the maintenance of the protein quality (in terms of physical stability and activity), a risk management plan must be developed to minimize the impact of any damage that may occur, which is contemplated in the ICH Q9 *Quality Risk Management* [154]. This plan must be defined by a multidisciplinary team that can correctly assess the major technical and logistic aspects that can impact the drug (from physical and chemical stability problems, to operation errors, or issues associated with raw material suppliers) [155].

7 Conclusion

Protein-based therapeutics are complex products, whose stability from manufacturing to patient administration is hard to achieve. From formulation optimization to the development of delivery systems, several strategies have been studied to try to overcome biopharmaceuticals stability limitations and safety concerns or to improve product quality and patient compliance. Despite these efforts, many challenges remain unsolved, like immunogenicity and safety concerns, stability issues, and exploration of alternative routes of administration, giving space for further research for different solutions and technical advances in this field.

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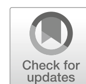
Therapeutic Antibody Engineering and Selection Strategies



Joana Ministro, Ana Margarida Manuel, and Joao Goncalves

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Abstract Antibody drugs became an increasingly important element of the therapeutic landscape. Their accomplishment has been driven by many unique properties, in particular by their very high specificity and selectivity, in contrast to the off-target liabilities of small molecules (SMs). Antibodies can bring additional functionality to the table with their ability to interact with the immune system, and this can be further manipulated with advances in antibody engineering.

The expansion of strategies related to discovery technologies of monoclonal antibodies (mAbs) (phage display, yeast display, ribosome display, bacterial display, mammalian cell surface display, mRNA display, DNA display, transgenic animal,

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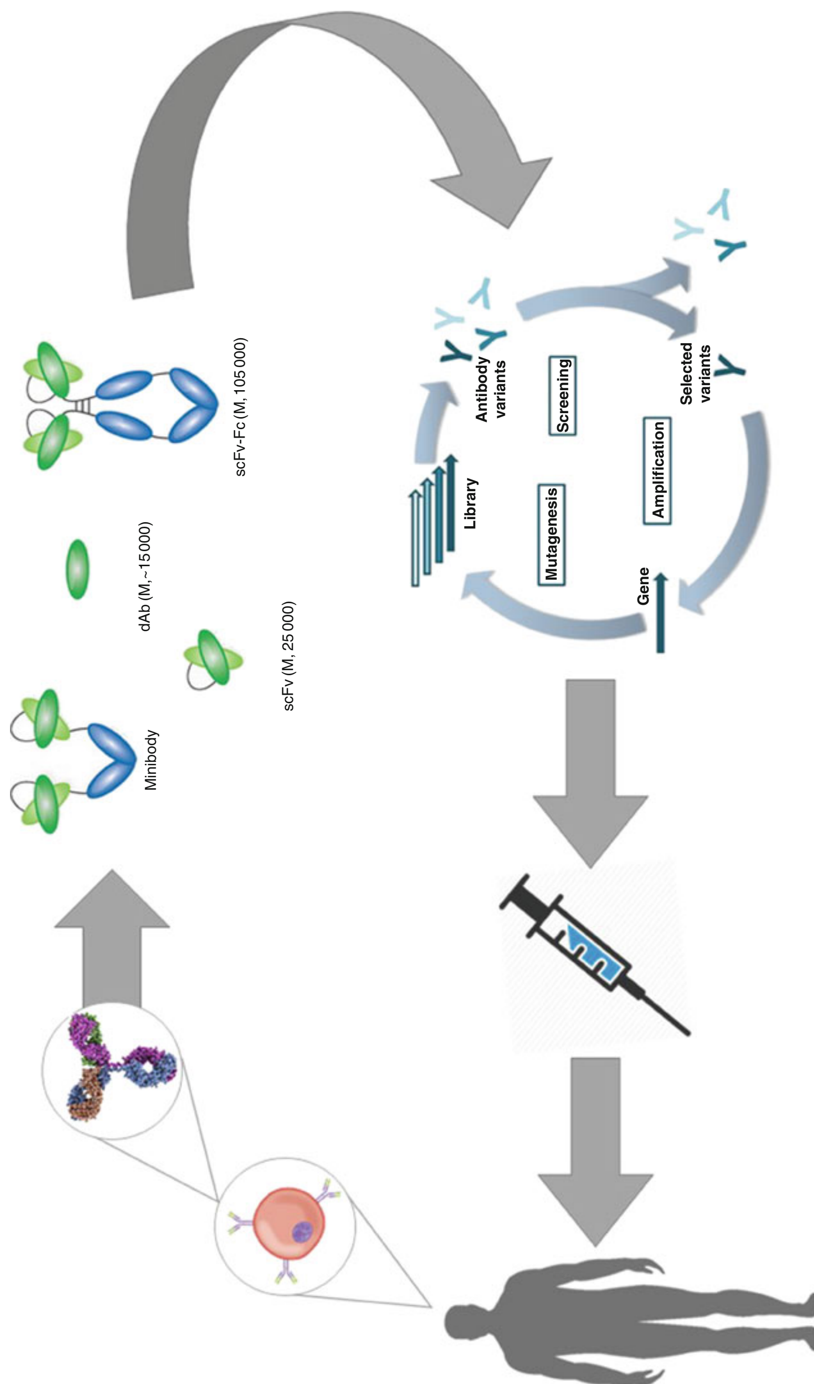
and human B cell derived) opened perspectives for the screening and the selection of therapeutic antibodies for, theoretically, any target from any kind of organism. Moreover, antibody engineering technologies were developed and explored to obtain chosen characteristics of selected leading candidates such as high affinity, low immunogenicity, improved functionality, improved protein production, improved stability, and others. This chapter contains an overview of discovery technologies, mainly display methods and antibody humanization methods for the selection of therapeutic humanized and human mAbs that appeared along the development of these technologies and thereafter. The increasing applications of these technologies will be highlighted in the antibody engineering area (affinity maturation, guided selection to obtain human antibodies) giving promising perspectives for the development of future therapeutics.

Graphical Abstract



Keywords Anti-drug antibodies, Biosimilars, European market, Immunogenicity, Product information, Recombinant drugs, Summary of product characteristics, Therapeutic biologics

1 Antibody Overview

1.1 Antibody Discovery

When in the 1700s the first perceptions of the immunology field were being explored through vaccination experiments, no one could imagine the impact of antibodies in today's society. In fact, immunity acquisition against a previous encountered disease has been documented for many centuries [1]. The first reference to antibodies appeared in 1890 from Emil von Behring and Shibasaburo Kitasato. In a breakthrough experiment, they treated diphtheria-infected animals with serum from immunized animals [2]. The potential of this therapy was immediately foreseen for human application, and Behring was later awarded with the Nobel Prize.

The term Antikörper (antibodies) was introduced around 1900 by Paul Ehrlich, who performed several studies about toxicity and immunology. He is considered one of the fathers of modern immunology for his insights into the antibody mechanics and for the suggested theory that side chains of the antibodies react with antigens and bind them and subsequently travel around the body in the blood [3]. In the 1940s, Linus Pauling showed that the interaction between antibodies and antigens was more dependent on their shape than on their chemical composition, confirming the lock-and-key theory proposed by Ehrlich [4].

The modern era of antibody research started in 1975 with the development of the hybridoma technology, the first mechanism to produce monoclonal antibodies in large quantities [5]. Since then, antibodies have become crucial in biotech and pharma industry, either for research purposes or for high-profit therapies. The first monoclonal antibody for therapy was approved in 1986 [6], and by December 2017, the FDA had already approved 79 therapeutic antibodies, with much more currently under evaluation in various phases of clinical trials [7, 8].

1.2 Characteristics and Structure of Antibodies

Today it is well-known that the recognition of foreign agents by immunoglobulins (Igs) is a core function of the adaptive immune response in mammals. Igs are glycoproteins produced by all vertebrates and can be localized on the surface of B lymphocytes, known as B cell receptors (BCR), or free in the blood or lymph, known as antibodies (Ab). If produced by the same cell, these two molecules are identical

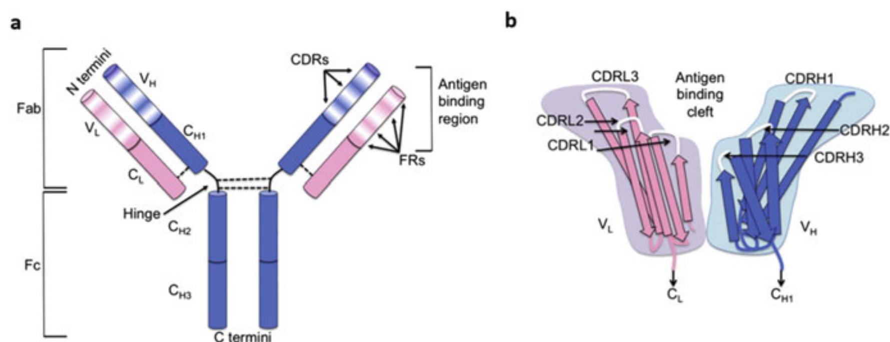


Fig. 1 General structure of antibodies. (**a**) A typical IgG molecule is composed of two heavy chains (blue) and two light chains (pink), linked by disulfide bonds. C_H and C_L are constant domains of heavy and light chain, respectively. V_H and V_L are variable domains of heavy and light chain, respectively. CDRs stand for complementary determining regions and are responsible for antigen binding. FRs are conserved regions that confer structure to the CDRs. The two antigen-binding fragments (Fab) are linked to the crystallizable fragment (Fc) by the hinge region. (**b**) An IgG has two antigen-binding regions, containing the V_H and V_L domains. Each variable region has three CDRs, flanked by the FRs regions. Adapted from O'Kennedy et al. [20]

except for a small portion that allows membrane anchoring in BCRs and secretion in Abs [9]. Since antibodies are soluble and can be secreted in large quantities, they are easily obtainable and studied. They are Y-shaped molecules consisting of two heavy and two light polypeptide chains, linked by disulfide bonds, and divided into constant and variable domains (Fig. 1a). They can be also divided in three equal-sized portions, two antibody fragment (Fab) arms, containing the variable domains at both ends, and the crystallizable fragment (Fc) domain. All domains are connected by a flexible amino acid chain, called the hinge region [10, 11]. The Fab and the Fc domains perform the two main functions of antibodies; while the Fab recognizes a foreign element (or an antigen), through the variable domains, the Fc induces an immune response by activating the complement system and/or the antibody-dependent cellular cytotoxicity [12].

All antibodies are assembled in the same way. However, two classes of constant light chains (κ and λ) and five classes of constant heavy chains (μ, δ, γ, α, and ε) can be distinguished. Immunoglobulins (IgM, IgD, IgG, IgA, and IgE) are named by their class of constant domains of the heavy chain. Also called isotypes, these different constant regions determine the functional properties of an antibody. Moreover, IgA has two sub-isotypes (IgA1 and IgA2), and IgG has four sub-isotypes (IgG1, IgG2, IgG3, and IgG4). IgM is the largest antibody and is mainly expressed in immature B cells. IgD is expressed in naïve B cells and activates basophils and mast cells upon exposure to an antigen. IgE is involved in allergic responses and IgA in mucosal immunity. IgG is the most frequent isotype, accounting for 70–85% of total immunoglobulins in serum. Its stability, long half-life and generally high affinity make IgG the most used isotype for therapeutic use [13–15].

1.3 Antibody Paratope

For an antibody to exert its biological function, it must bind to a specific target. The variable domains provide the contact to the antigens and determine the specificity of each antibody. Each variable domain forms an immunoglobulin fold that consists of a pair of β-sheets, linked by a disulfide bond, and three hypervariable loops lying at the end of the structure, known as complementary determining regions (CDRs). There are three CDRs in each variable domain, and their hypervariable characteristic allows diversification and the recognition of an almost unlimited number of antigens. Variation of the amino acid sequences inside these loops provides the major mechanism for the generation of the vastly diverse set of antibodies and T cell receptors expressed by the immune system. While CDR1 and CDR2 domains vary somewhat between different immunoglobulins, CDR 3 differs very dramatically. This happens because contrary to CDR1 and CDR2, CDR3 suffers imprecise rearrangements during the process of V(D)J recombination (Sect. 1.4.1) [16]. The three CDRs of each variable domain are distributed between four structural and less variable regions, known as framework regions (FR). The FR regions function as a scaffold to hold the CDRs in position to contact the antigen. Even though sequence diversity is concentrated in the CDRs, the framework regions are also diverse to some extent, which may be advantageous in antibody function and stability to accommodate highly diverse CDRs [17].

The variable regions of both chains interact to form a molecular site (the paratope) that binds strongly to a part of the antigen (the epitope), and the strength of this binding is called antigen-binding affinity (Fig. 1b). While identification of paratopes is often done through identification of CDRs, not all the residues within the CDRs bind the antigen. In fact, 3D analysis of the antibody structure suggested that only 20–33% of the residues within the CDRs participate in antigen binding. Nonetheless the residues that are directly involved in the interaction with the antigen are, in general, the most variable ones. The paratope is ultimately a function of the folding pattern of the association of heavy and light polypeptide chains [18, 19].

1.4 B Cell Development and Activation

The antibody producing cells are called B cells or B lymphocytes. They perform a core function in humoral immunity not only by secreting antibodies but also by presenting antigens (antigen-presenting cells) and by secreting cytokines [21]. In mammals, B cells start maturation in the bone marrow, from hematopoietic stem cells, where they undergo a process called somatic recombination or V(D)J recombination (see Sect. 1.4.1). This process is responsible for the generation of the first level of antibody diversity and comprises the rearrangement of immunoglobulin genes through random recombinations [22]. While developing, B cells undergo both a positive and negative selection, a process that assures that the B cell receptor

(BCR) is binding to a proper ligand and not to a self-ligand. In case this would not occur, the cell would not receive the right signals to proceed and would stop the development. B cells then migrate to peripheral lymphoid organs, such as the spleen, where they become mature B cells expressing both IgM and IgD; these cells are also called naïve B cells before antigen exposure [23, 24].

Mature B cells circulate through the blood to the secondary lymphoid organs where they contact with antigens that circulate in the lymph. The activation of a B cell is triggered when the BCR binds to an antigen. This event induces the cells to migrate to the interface between the follicle and T cell zone, and it is at this stage that the B cells present the antigen to helper T cells. At this point both cells integrate inputs to decide between death and proliferation. B cells can then start to proliferate and form stable connections with helper T cells near the interfollicular zone, where there is a great number of dendritic cells. A few days after antigen exposure, cells integrate more inputs to differentiate into either plasma cells (in order to secrete large amounts of antibodies) or memory cells (responsible for a secondary immune response) or to go to the germinal center for affinity maturation [25]. In the germinal centers, cells divide quickly and hypermutate the BCR V-regions in a process called somatic hypermutation. They also undergo a process called class switch recombination, a mechanism that changes the immunoglobulin isotype (see Sect. 1.4.2). Depending on their BCR affinity for foreign and self-antigens, the cells may be selected again for survival or death [9]. Some antigens can activate B cells directly in the absence of helper T cell. The ability of B cells to respond directly to these antigens provides a rapid response to many important pathogens. However, as these antibodies are not subject to affinity maturation, they are therefore less variable and less functionally versatile than those induced with T cell help [26].

B cell development comprises a series of decision points where many inputs are integrated influencing the cells' fate. Evidence from cell dynamics and integration of signals support the theory that more complex principles of control also exist [27].

1.4.1 V(D)J Recombination

An efficient immune response absolutely requires genetic diversity at the immunoglobulin gene locus. The first level of diversity consists in the rearrangement of multiple immunoglobulin genes, in a process called V(D)J recombination. It occurs in the bone marrow, during maturation, and before antigen encounter. In mammals, immunoglobulin gene segments contain more than 100 variable (V), 30 diversity (D), 6 joining (J), and 9 constant (C) exons. Random choices of these genes encode different proteins, generating in the order of a million different antibodies and matching an enormous panoply of antigens. Rearrangements occur orderly (Fig. 2a). At the heavy chain locus, D–J segments assemble first, followed by V segment gathering, and, after transcription, the heavy variable regions are connected to the constant regions by RNA splicing. At the light chain locus, D segments are absent; thus only V–J exons are paired. CDR 1 and CDR 2 are encoded by the V gene segments, while CDR 3 (the most variable and exposed CDR) is generated as a

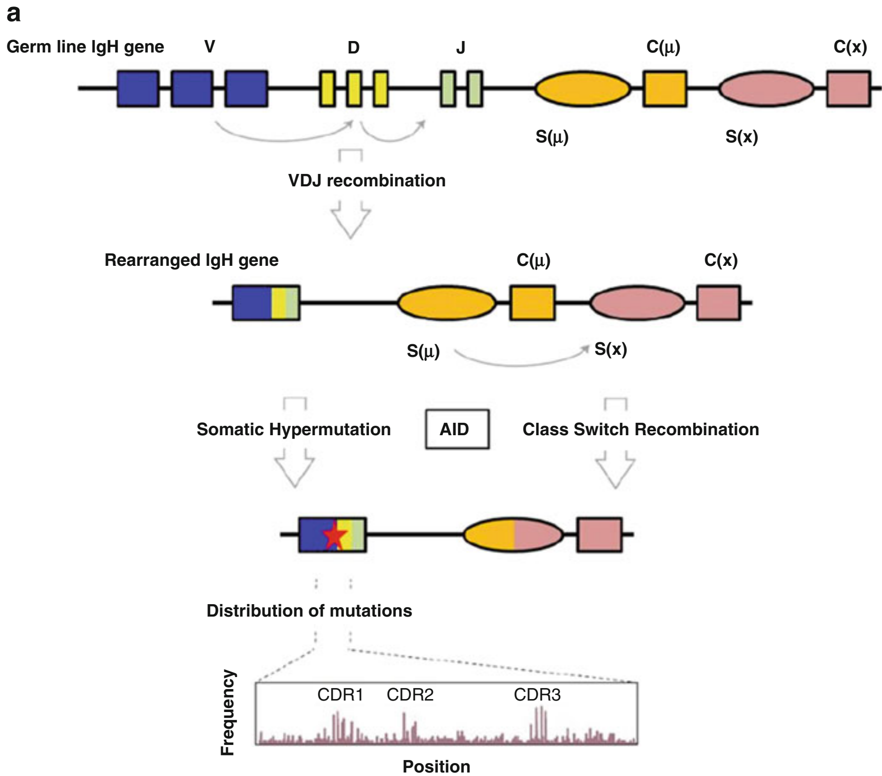


Fig. 2 Immunoglobulin heavy chain diversification processes. (**a**) Random formation of diverse V(D)J heavy combinations generates combinatorial diversity. Curved lines with arrows indicate gene rearrangement events. The regions where somatic hypermutation accumulates are mainly the CDRs; (**b**) schematic representation of protein–DNA complexes in V(D)J recombination. The 12-RSS and 23-RSS are represented as white and black triangles, respectively. Blue and yellow rectangles represent the coding segments to be recombined, and the red rectangle represents the newly generated nucleotide sequence at coding junction. Gray areas represent protein complexes; *AID* activation-induced cytidine deaminase, *C* constant regions, *D* diversity segments, *J* joining segments, *S* switch regions, *TdT* terminal deoxynucleotidyl transferase, *V* variable segments. Adapted from Di Noia and Neuberger [39] and Mansilla-Soto and Cortes [31]

result of the imprecise joining of the VDJ or VJ exon. The antibody diversity is then further increased by the assembly of different heavy and light chains [22, 24, 28].

V(D)J recombination is activated by a V(D)J recombinase that consists of two lymphoid specific proteins, RAG1 and RAG2, that cleave DNA [29]. The recombinase recognizes conserved DNA sequences, as well as the recombination signal sequences (RSS) that flank each V, D, and J segments. In order to control this mechanism, recombination only occurs between RSSs with different spacer lengths (the "12/23 rule"), which prevents joining of two gene segments from the same group [30]. RAG proteins introduce a pair of site-specific double-strand breaks

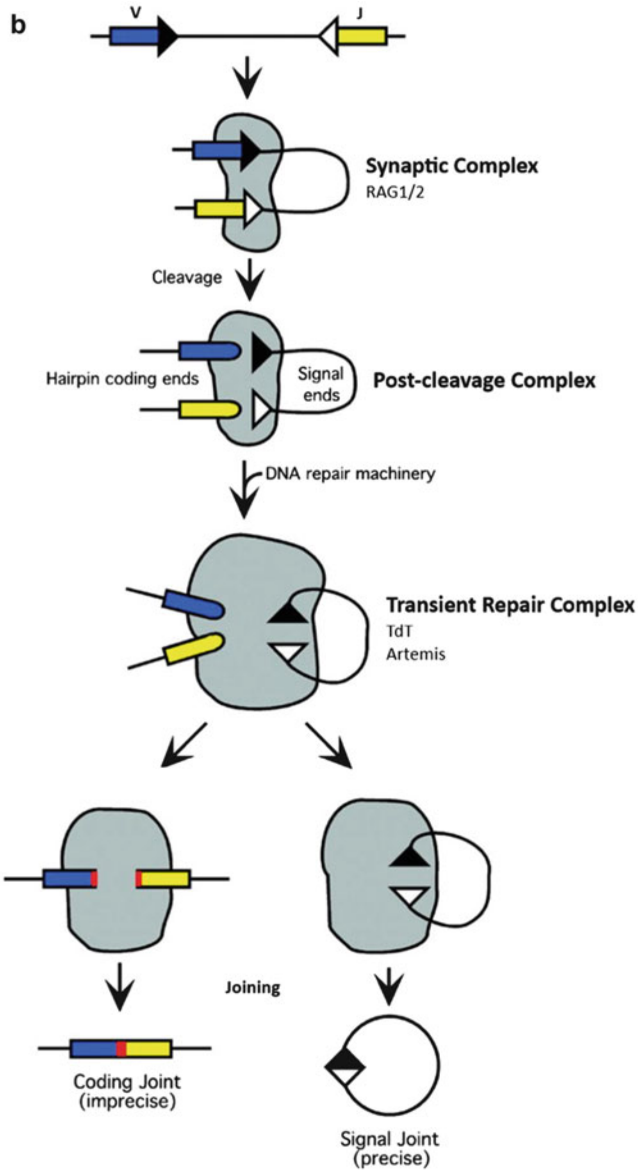


Fig. 2 (continued)

between the coding segments and the RSSs, creating a circular signal joint and a linear coding joint. This cleavage generates hairpins at the extremities, which cannot be directly ligated (Fig. 2b). The random opening of the hairpins by the endonuclease protein Artemis at each extremity generates a combination of different DNA ends that will strive for a mismatch between the two ends, enabling therefore the

formation of junctional diversity [31]. Thus, junctional diversity arises by the imprecise fusion of V, D, and J segments and can result in deletion and/or addition of nucleotides in the joint region. Deletions occur through the activity of an exonuclease that is responsible for trimming the DNA ends before ligation. In fact, 80% of human Ig genes show junctional diversity caused by deletions. A less common process is the addition of template nucleotides (P nucleotides) in the junction site [32]. Another source of junctional diversion is the addition of non-template N nucleotides. These random nucleotide insertions are added to the DNA ends by a lymphoid-specific enzyme, called terminal deoxynucleotidyl transferase (TdT), in a template-independent manner, which can add up to 15 extra residues in the joint, further increasing junctional diversity [33]. Actually, the fact that this rearrangement process provides around 10^5–10^6 different antibody specificities in humans is in a great extend due to TdT activity. An end-joining process strictly restricted to fully complementary ends would be unable to ligate the coding joints. In contrast, a versatile but conservative process, such as nonhomologous end joining (NHEJ), is able to join these DNA ends and generate a highly diverse immune repertoire while protecting against side genomic instability [34, 35]. During the process of V(D)J recombination, the numerous combinations of multiple gene segments and the junctional modifications brought about by their joining result in a large repertoire of antigen receptors, thereby assuring the hallmark diversity of the adaptive immune system [36].

1.4.2 Somatic Hypermutation and Class Switch Recombination

Despite the great diversity achieved by V(D)J recombination, the B cell requirements for antigen targets exceed largely the information presented in the cell genome. Thus, the cell needs to achieve larger repertoires by itself. After encountering an antigen and moving to the germinal centers, immunoglobulin genes suffer both somatic hypermutation (SHM) and class switch recombination (CSR) [37].

In mice and humans, SHM causes mutations in the variable immunoglobulin both in the light and heavy chains locus. The mutation rate is about one million-fold higher than the spontaneous mutation rate in other genes, and it is mainly due to single base substitutions, with occasional insertions and deletions. The mutation activity of SHM starts around 150 nucleotides downstream of the IgV promoter, and it is extended for about 2 kb pairs [38]. All four bases can be mutated, but the targeting of individual bases is, however, not random. Interestingly, certain regions, especially those responsible for antigen binding, are intrinsically more mutable than others. The result of these mutations may alter the specificity or affinity of the encoded antibody, potentially enhancing the response to a specific antigen [39, 40]. In the same B cell, the heavy chain is rearranged down the chromosome through CSR or isotype switching (Fig. 2a). CSR is the process by which C_H domains encoding one isotype are exchanged for another, thereby influencing the effector function of the resulting antibody. In addition, CSR is accompanied by an increase in antigen-binding affinity [41–43].

The mechanisms of SHM and CSR are not completely elucidated, but a key factor of the two processes is well-known: the activation-induced cytidine deaminase (AID) [44]. Upon B cell activation, AID expression is upregulated and is associated with the cell transcription apparatus, gaining access to single-stranded DNA regions. In most of the cases, AID recognizes specific small sequences (hotspots), where it deaminates cytosines and converts them to uracils, creating dU:dG mismatches. This DNA lesion is then processed by mismatch repair (MMR) and base excision repair (BER) pathways, resulting in point mutations [45, 46]. In the CSR process, a protein machinery is recruited after AID deamination process creating double-strand breaks in the switch regions (S) located upstream of the constant regions. The DNA between the S-regions is subsequently deleted from the chromosome, removing unwanted heavy chain constant regions. The free ends of the DNA are rejoined by NHEJ yielding B cells that express IgG, IgE, or IgA [41].

SHM and CSR are usually more evident after a second exposure to an antigen and are responsible for the dramatically strong secondary immune response. These events of affinity maturation work in combination to generate an antibody repertoire estimated to be superior to 10^9 in humans [47].

1.5 Monoclonal Antibodies and Their Recognition as Tools

Due to their high specificity and selectivity, antibodies have the potential to be of great use for biochemical, diagnostic, and therapeutic purposes. Antibodies are categorized into two groups, polyclonal or monoclonal. Polyclonal antibodies (pAbs) are a heterogeneous mixture of antibodies directed against various epitopes on the same antigen. These antibodies are generated by different B cell clones and, as a consequence, are immunochemically different, with different specificities and affinities. While they are inexpensive and easy to produce, pAbs cannot be reliable due to the high variability from batch to batch. In contrast, monoclonal antibodies (mAbs) originate from a single antibody-producing B cell and therefore only bind to one epitope of an antigen. Thus, while more expensive to produce, mAbs have high specificity to a single epitope and present batch-to-batch homogeneity [48].

The development of the hybridoma technology was a big hallmark in the antibody field, allowing the relatively easy production of large quantities of mAbs for diverse applications. These antibodies were generated by fusing antibody-producing mouse cells with myeloma mouse cells, resulting in the formation of immortal cell lines expressing a single antibody with specificity for one particular epitope of an antigen. Once produced, hybridomas can be cultured in vitro indefinitely [5].

Many of the current laboratory procedures use mAbs as tools to answer basic research questions. They allow researchers to identify molecules not seen by the naked eye, enabling conclusions about the target molecule and pathway of interest. Routine techniques such as Western blot, flow cytometry, immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA) all rely on antibody properties. MAbs have also become an important component of many diagnostic techniques

including detection of infections, measurement of biological markers in blood, and recognition of allergies, having increased the speed and accuracy of many tests [49]. Also, radiolabeled antibodies can be used in the imaging diagnostic allowing, for instance, differentiation between cancerous and non-cancerous cells [50].

With the ability to bind an almost unlimited number of targets with high specificity and stability, mAbs were sought for therapeutic applications since the first time they were developed in vitro. Therapeutic mAbs can act through multiple mechanisms, such as blocking of targeted molecule functions or disrupting a signaling pathway [51]. Nevertheless, it soon became clear that antibodies were facing many problems for therapeutic use, mainly due to their murine nature. Developments in molecular biology made possible the creation of recombinant variants of mAbs that led to optimized antibodies and ushered the age of antibody engineering [52, 53].

1.6 Antibody Engineering

1.6.1 Reduce Immunogenicity

The success of the first therapeutic antibody, OKT3 (muromonab), as a treatment for transplant rejection, was not immediately followed by a series of approvals as anticipated. Although very promising, patients treated with antibodies from hybridoma technology often developed an immune response that attenuated the half-life and the efficacy of the antibodies [54]. In order to reduce immunogenicity, numerous strategies have been pursued for the humanization of animal antibodies, replacing constant regions (chimeric antibodies) or all the non-specificity determining residues (humanized antibodies) with corresponding human antibody sequences [55]. Since the late 1990s, with the introduction of chimeric and humanized antibodies, therapeutic antibodies have become one of the fastest-growing classes of therapeutics in the biological drug market [56]. More recently, the generation of transgenic mice expressing a repertoire of human heavy and light chain genes, followed by the generation of human hybridomas, was shown to be an effective way for the generation of human antibodies against many antigens [57–59].

1.6.2 Antibody Formats

Besides increasing safety, antibody engineering allowed the development of several antibody formats for different functions. Immunoglobulins are large proteins (between 150 and 180 kDa) and have difficulty to penetrate tissues or to target antigens in small areas such as enzyme active sites. In drug development a heavy chain may not be desirable or even necessary [60]. Researchers have dismembered antibodies into their component parts, taking advantage of the smaller sizes in order to develop new therapeutics with exciting properties (Fig. 3). These “domain antibodies” come in numerous formats: fragment antigen binding (Fab), single-chain

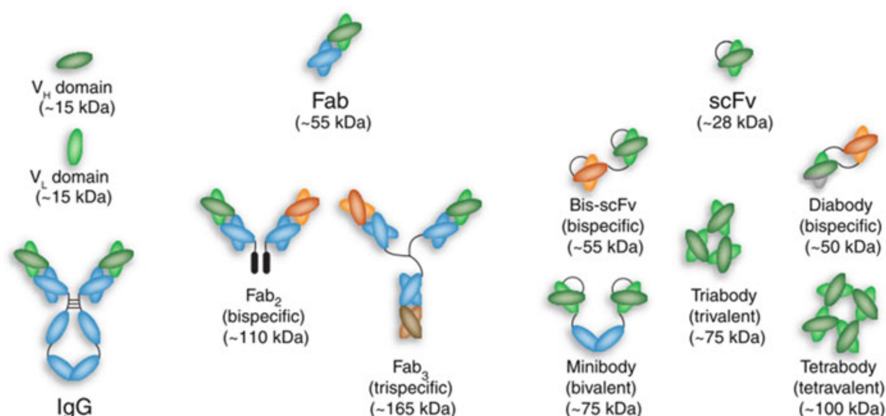


Fig. 3 Examples of antibody formats. A variety of antibody fragments are represented including Fab, scFv, and single domains V_H and V_L. Multimeric formats such as minibodies, bis-scFv, diabodies, triabodies, and tetrabodies are also depicted. Adapted from Holliger and Hudson [61]

variable fragment (scFv), or V_H and V_L domains [61, 62]. Disadvantages of these small domains comprise aggregation, poor solubility, and reduced half-life in circulation. However, genetic engineering also allowed multimerization of antibody fragments, and these formats can help to overcome the decrease of affinity and stability from fragment domains. Moreover, this has also led to the generation of multispecific antibodies, with the ability to recognize different epitopes or different antigens [63–65]. The large panel of antibody formats that has been developed reflects the strong interest for these molecules. While in many cases the manufacturing remains challenging, several antibody formats are currently in clinical trials with some already approved for commercialization [61, 66].

1.6.3 Target Delivery

Recently, mAbs are being exploited for modern drug delivery systems, aiming to direct a therapeutic agent to a specific tissue or cell for enhanced pharmacology. Antibody-based conjugates have opened up a whole new field of clinical possibilities with several platforms emerging on the market, with most promising applications in cancer therapy [60].

Without the toxicity associated with chemotherapeutic agents, antibody therapy held a tremendous promise in the elimination of tumor cells. However, only modest success has been achieved in patients with solid tumors, with cell elimination not being as effective as expected [67]. Antibody-drug conjugates (ADCs) combine the targeting ability of an antibody with the cell-killing activity of a cytotoxic drug [68]. The basic ADC technology is composed of an antibody vehicle, the cytotoxic drug, and a linker, and each ADC is constructed in a customized manner for the specific antibody and drug

combination. By the end of 2018, there were four ADCs already approved for cancer therapy [69, 70].

Therapeutic efficacy can also be increased by coupling an antibody with a nanoparticle conjugate. In the context of drug delivery, nanoparticles are used to encapsulate a drug for enhanced drug release and reduced side effects, similar to ADCs. While fused to nanocarriers, mAbs can be used for targeting cell surface receptors resulting in enhanced intracellular drug accumulation [71, 72].

The understanding that multiple factors might contribute for disease progression led to the development of bispecific antibodies, in a large variety of formats. Bispecific T cell engagers (BiTE®) have stood out from the panoply of bispecifics due to their function in recruiting the host immune system, more specifically T cells cytotoxic activity, to the cancer cells. They consist of two different scFvs, one binding to T cells, usually through the CD3 receptor, and the other to a tumor cell, through a tumor-specific molecule. One example of a BiTE antibody construct is blinatumomab (trade name Blincyto), approved in 2017 for relapsed or refractory B cell precursor acute lymphoblastic leukemia [73–75].

Another innovative approach for antibody use is the chimeric antigen receptors (CARs). CARs are engineered receptors, which combine specificity (conferred by the antibody) with immune effector function of T cells. In cancer therapy, T cells are removed from patients, and the receptors are modified so that they become specific to the patient's cancer. The engineered T cells are then reintroduced into the patient, with the new ability to recognize and kill the cancer cells [76, 77]. A CAR therapy has been recently approved for use against acute lymphoblastic leukemia [78].

The development of creative antibody engineering technologies together with the progress toward deciphering disease pathways has generated robust interest, resources, and investments in antibody discovery.

1.7 Market Importance of Therapeutic Antibodies

Over the past 40 years, therapeutic antibodies have rapidly become the leading product within the biopharmaceutical market. The global market for monoclonal antibodies was valued at 85.4 billion dollars in 2015 and is expected to reach a value of 138.6 billion dollars by 2024 [79]. The growing health concerns and the increasing of diagnostic techniques have boosted the demand for these therapeutics in recent years, particularly for chronic diseases. Nevertheless, the spectrum of diseases potentially treated by antibody therapies is massive, including cardiovascular, respiratory, hematology, kidney, immunology, and oncology diseases. While antibodies are very efficient, their cost-effectiveness has always been discussed due to their high costs, estimated to be more than one billion dollars from preclinical development to market approval. Consequently, therapeutic antibodies are inaccessible to many patients in both developed and developing countries. The development of biosimilar antibodies, which show a similar quality, efficacy, and safety as the

original antibody, may help decrease the associated costs and provide alternative treatment options [80, 81].

2 Antibody Libraries

2.1 Generation of In Vitro Antibody Libraries

As discussed before, the immune system has the incredible ability to create a vast antibody repertoire, combining antibody gene segments recombination and somatic hypermutation. Since the perception of the several applications for antibody molecules, scientists have been trying to mimic the vast diversity achieved inside B cells by constructing antibody libraries in vitro [82].

It was only in 1989 when the antibody repertoire from the B cells of an organism was recombinantly cloned in large combinatorial libraries that mAb technology really exploded [83, 84]. Another hallmark for in vitro antibody discovery occurred roughly 1 year after, with the development of a display technology for the isolation of mAbs from these large collections of recombinant antibody fragments [85]. Display technologies provided a way to quickly select antibodies from libraries on the basis of the antigen-binding behavior of individual clones and allowed to overcome the limitations from toxicity and immune tolerance. Moreover, the continuous development of automation techniques made it possible to identify hundreds of different antibody leads against a single therapeutic target, opening a new field for different kinds of libraries without the need for immunization [86].

The most important parameter for in vitro antibody discovery is the quality of the antibody libraries. Evolution has allowed the development of a wide range of strategies for library generation that can differ in both design and means of construction. They were first focused on affinity and thus on library size and diversity, and, more recently, they are also focused on biophysical properties and reduced immunogenicity. Based on the source of the antibody repertoire, four types of libraries can now be identified: naïve, immune, synthetic, and semisynthetic (Fig. 4) [87, 88].

2.1.1 Naïve Libraries

Naïve libraries are derived from B cells of nonimmunized donors. Although other tissues can be used for B cell extraction, most naïve libraries are originated from peripheral B lymphocytes, where they are not biased from the process of affinity maturation that occurs in secondary lymphoid organs. Moreover, IgM repertoire is often preferred because it closely reflects the diversity following immunoglobulin gene rearrangement, without tolerance or antigen selection [89]. mRNA isolated from B cells is used as template for the amplification of the variable regions of antibody genes using degenerate oligonucleotide primer sets. The amplicon is then

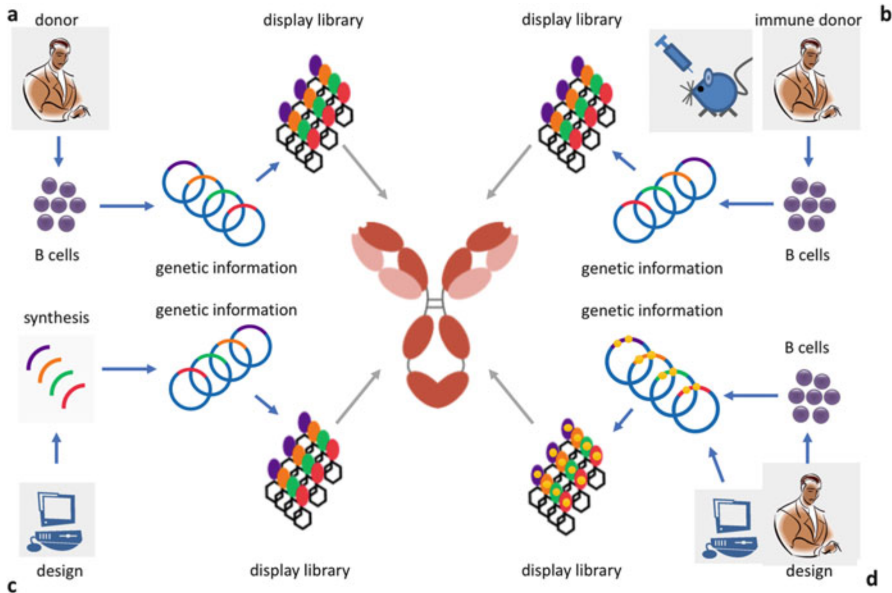


Fig. 4 Different types of antibody libraries. (**a**) Naïve libraries are amplified from nonimmunized donors. (**b**) Immune libraries are derived from immunized or immune donors. (**c**) Synthetic libraries are based on computational in silico design and gene synthesis. (**d**) Semisynthetic libraries comprise a combination of natural sources with in silico design. All libraries can be cloned in suitable vectors to be used in display techniques. Adapted from Ponsel et al. [87]

cloned into a suitable vector for display and selection against a desired target. One of the primary limitations of naïve libraries is the fact that the immune system deletes clones with affinity to self-antigens, in order to prevent the development of autoimmune diseases, which may prevent the isolation of antibodies with affinity for many important human targets. Despite the uncertainty of its content, the main value of these kinds of libraries relies in the large repertoires (of up to 10^{11} antibodies) that can be constructed and in the fact that a single library can be used for the selection of antibodies against several types of antigens, including toxins [90, 91].

2.1.2 Immune Libraries

Immune libraries are derived from B cells of immunized donors or previously in contact with an antigen. The library construction is performed using the same method, but, contrary to naïve, immune libraries are smaller in size (normally 10^6–10^8) and are not well suited for the identification of antibodies against a large panel of antigens. Nevertheless, the pre-exposure of the host to an antigen results in the production of very specific and high-affinity antibodies against the particular antigen, mainly due to affinity maturation, which makes immunization a very suitable

model for antibody discovery. As human libraries can only be generated from B cells obtained from disease-affected patients, several animal models are used for the generation of this kind of libraries, including mice, rabbits, camelids, and sharks [92, 93]. The latter two express antibodies that are only composed of heavy chains. Thus, antigen binding is performed by only one single domain referred to as V_HHs in camelids and vNARs in sharks. Those fragments are naturally highly stable and soluble, and it has been shown that they can generate high-affinity binders [94, 95]. However, there are some important limitations when considering immune libraries, including the time required for animal immunization, the unpredictability of the immune response to the immunized antigen, and the fact that a new library must be generated for each desired antigen [89].

2.1.3 Synthetic Libraries

While naïve and immune libraries are based entirely on naturally occurring sequence diversity and do not require human input during the generation of sequence diversity, synthetic libraries are diversified according to design and need a strategy for the sequence diversification. In this approach the antibody diversity is designed in silico and then synthesized in a controlled manner [96]. A key advantage of synthetic diversification is that the composition of the CDRs and frameworks can be exactly defined and controlled. Synthetic libraries prevent the natural biases and redundancies of in vivo antibody repertoires and allow control over the sequences of variable genes and the introduction of diversity [53]. From simple degenerate oligonucleotide synthesis to physiochemically optimized library design, many different strategies have been applied since the first reports of synthetic antibodies, in 1992 [97]. Most of the constructed synthetic antibody libraries maintain a single framework sequence and have their diversity concentrated in the CDRs, which are generated by random combinations of mono- and trinucleotide units [98–100]. Nonetheless, there are a few synthetic libraries with multiple variable heavy and light chain framework regions, such as the HuCAL libraries and Ylanthia library [101, 102]. Multiple framework sequences can accommodate more diverse CDR structures, but it makes library design and construction more complex, and the clones are not as uniform in their properties as in the case of single framework libraries [103].

The increasing knowledge of sequence, structure, function, and physicochemical properties of antibodies has allowed researchers to design and construct highly functional and sophisticated synthetic antibody libraries. However, it is still a very complex process to assemble synthetic libraries, and intensive study regarding structure and folding needs to be performed in order to guarantee functional antibodies [104].

2.1.4 Semisynthetic Libraries

In semisynthetic libraries there is a combination of naturally derived and synthetically designed parts, and the ratio of both parts may vary in different strategies [97]. They are often created to increase the natural diversity while maintaining a good level of functional diversity. Such libraries have been created, for example, by combining natural CDR regions or by joining naturally rearranged and highly functional CDR 3 sequences with synthetic CDR 1 and CDR 2 diversity [105, 106].

2.2 Technologies for Antibody Selection

Antibody libraries need to be followed by a selection step in order to collect the leads with the required affinity, specificity, and stability. In the immune system, B cells link genotype to phenotype through presentation of the expressed antibody on the surface, where only the positive selected cells will succeed [107]. The most common selection methods also rely on the display at cell surface of each antibody from a desired library. The antibody display can be on the surface of phages, on eukaryotic cells, or even on ribosomes following in vitro transcription. All of these methods consist of antibody incubation with a relevant antigen followed by several rounds of washing and recovery of the best binders. Display systems offer a number of advantages for antibody discovery and optimization as they are significantly fast and can be carried out in a high-throughput mode [108].

2.2.1 Phage Display

Phage display was the first display technology developed for antibody discovery and still remains widely used due to its simplicity to present large libraries. It was first described by George P. Smith, in 1985, when he demonstrated the display of peptides by fusing a peptide of interest to the gene III of a filamentous phage. This technology was further developed and improved for display of antibodies mainly by the groups of Winter and McCafferty, at the Laboratory of Molecular Biology (Cambridge, UK), and by the groups of Lerner and Barbas, at The Scripps Research Institute (La Jolla, USA) [85, 109, 110]. In phage display, the library DNA is usually inserted in fusion with the gene of the phage coat protein pIII or pVIII. After infection of bacteria, a progeny of phages is released, each phage displaying an antibody on its surface while containing the antibody gene inside. By immobilizing a desired target to the surface of a microtiter plate well, it is possible to incubate the phage library, in which the phages displaying antibodies with affinity for the target will remain bound while others are removed by washing. Those that remain can be eluted, and this resultant pool contains a phage mixture that is enriched with relevant antibodies. This mixture is then amplified by infecting bacteria and subjected to another incubation step. This

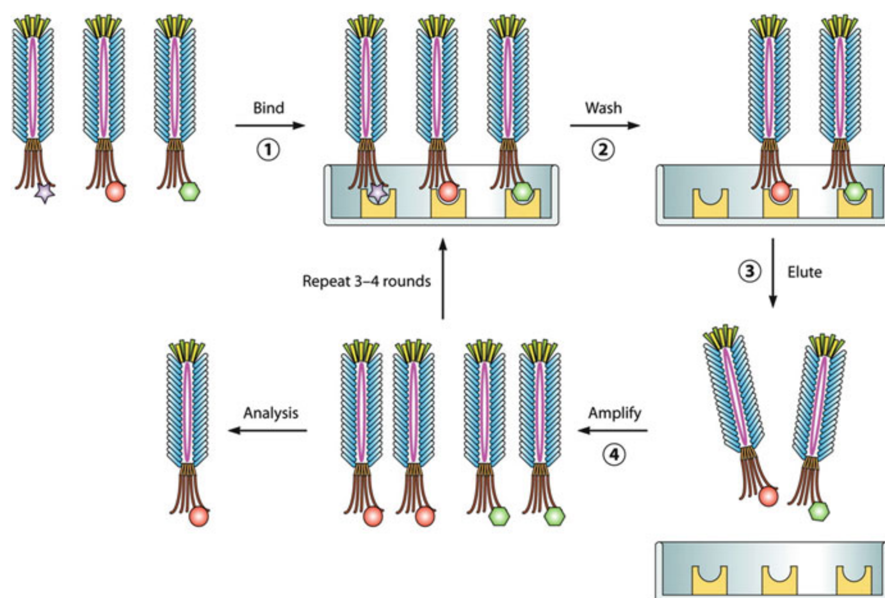


Fig. 5 Sequence of events of phage display technology. Biopanning of a phage display library to select antibody binders to an immobilized target. This cycle is usually repeated 3–4 times and includes binding of the phage library to an antigen-coated surface, washing with the desired stringency, elution of phages containing the antibody genes, amplification, and analysis. Reprinted from Huang et al. [111], with permission from ASM

repeated cycling is referred to as "panning" (Fig. 5), and usually after three or four pannings, the DNA from eluted phages is collected and sequenced [111].

Phage display of antibody libraries has become a powerful method for a fast selection of antibodies for therapy. It was used to select synthetic human libraries, allowing fully human antibodies to be created in vitro [112]. One of the most successful antibodies discovered by phage display was adalimumab (HUMIRA), an antibody with affinity to human TNF-α, the world's first fully human antibody [113]. Nevertheless, phage display also presents some drawbacks, as it cannot accommodate all antibody formats and often requires reformatting to produce soluble and well-expressed antibodies with properties compatible with efficient manufacturing. Also, in most of the cases, antigens are not presented in their native conformations, which decreases the likelihood of selecting successful leads for therapeutic uses [114].

2.2.2 Ribosome and mRNA Display

In ribosome and mRNA display, a DNA library is transcribed and transduced in vitro. Without the need to transform cells for library selection, it is possible to achieve much higher library diversity. While in ribosome display, the translated

protein remains connected to the ribosome and to its encoding mRNA for the selection step, in mRNA display, the mRNA is first translated and then covalently bound to the protein it encodes, using puromycin as an adaptor molecule. Also, a mutagenesis-based PCR can introduce random mutations after each selection round, allowing combination of selection with affinity maturation [115, 116].

2.2.3 Yeast Cell Display

In recent years, several cell-based screening technologies have emerged. They rely on the multi-copy display of antibodies or antibody fragments on a cell surface in a functional form followed by high-throughput screening. Cell-based screening harbors the benefit of single-cell analysis and characterization of individual library candidates [117]. The most common method used for cell-based screening is flow cytometry, which allows the analysis of hundreds of thousands of cells per minute according to their size, granularity, and fluorescence properties. Selection of monoclonal antibodies presented on the cell surface can be performed by antibody expression level, by display strength, or by antigen affinity [118].

The yeast display technique was first described by the group of Wittrup, where a protein of interest was displayed in fusion to a cell wall protein of *Saccharomyces cerevisiae* [119]. Although not suitable for large libraries, due to limitations on yeast transformation which limit the complexity of these libraries [120, 121], yeast display has still the advantageous of linking an eukaryotic secretory pathway, with the potential for the selection of antibodies with improved folding characteristics, to the flow cytometry system, for a high-throughput screening, making it still widely used. It is a very effective method for the isolation of high-affinity antibodies against labeled targets and provides the possibility to discriminate between different affinities of distinct clones [122].

2.2.4 Mammalian Cell Display

Despite the clear advantages, all of the above platforms share the downside of expressing antibodies in a nonnatural environment. As large and complex proteins that possess several functional domains, antibodies can only be efficiently expressed and assembled into functional forms during the mammalian expression and secretory pathway. The display of antibodies on mammalian cell surface often requires the fusion of a transmembrane domain to the C-terminus. The ability to identify antibodies directly from mammalian cells therefore enables selection of antibodies with desirable properties from an early stage, such as high-level expression and stability [123, 124]. Several groups have developed different mammalian display strategies, from directly isolation of B cells specific for an antigen to more complex approaches, as introduction of combinatory libraries through lentivirus infection [125, 126]. Most of these systems, however, are complex and time-consuming, which significantly hamper the application of mammalian display [127].

2.2.5 In Vitro Compartmentalization

A next generation technology, called in vitro compartmentalization, intends to substitute cells by water droplets for antibody production and display. The water droplets are surrounded by an oil phase and contain all components required for protein synthesis, as well as single genes that are competent for transcription and translation. This system allows the co-compartmentalization of DNA and protein, and this cell-like droplets (up to 10^{10} per ml) can therefore be used in flow cytometry-based selections of large libraries, with a high degree of control over selection conditions and stringencies [128, 129].

A multitude of discovery technologies are nowadays available to isolate high-affinity antibody binders to nearly any given target. Promising strategies are also emerging to improve the discovery process (Fig. 6). The biggest challenge remains on how to include other important features into the screening process as, for example, high folding and expression, low tendency to aggregate, and correct glycosylation patterns. Various innovative approaches are expected to appear in next years that will include as many parameters as possible and, certainly, will increase the likelihood of antibody molecules to successfully reach the market [117].

2.3 Maturation Strategies for Directed Evolution

One of the greatest challenges in library generation when compared to in vivo methods is the absence of somatic hypermutation (SHM), which enables nature to create high-affinity antibodies. The in vitro occurrence of spontaneous mutations is generally insufficient to obtain desired gene variants on a practical time scale. Therefore, several genetic diversification techniques can be used to direct and evolve antibody libraries toward a defined goal, thereby accelerating the identification of desired proteins. This bottleneck process is named directed evolution and can be applied in vitro with the purpose of mimicking the process of natural selection [131, 132]. A single gene or a library is evolved by being subjected to mutagenesis, screening, and amplification. Rounds of these steps are repeated, using the best variants from one round as the templates for the next round, in order to reach the best improvements [133].

Some methods based on introducing a certain degree of diversity into selected, moderate affinity candidates are used and can be generally differentiated between targeted and nontargeted diversification strategies [134]. Nontargeted approaches, such as error-prone PCR and the use of a mutator *E. coli* strain, can be used for random introduction of nucleotides into the whole antibody. Error-prone PCR is based on the use of low-fidelity polymerases as well as on modified reaction conditions, creating a high error rate during amplification [135]. *E. coli* mutator strains are conditional mutants that produce single-base substitutions with higher rates than normal cells [136]. Chain shuffling is another method used for nontargeted

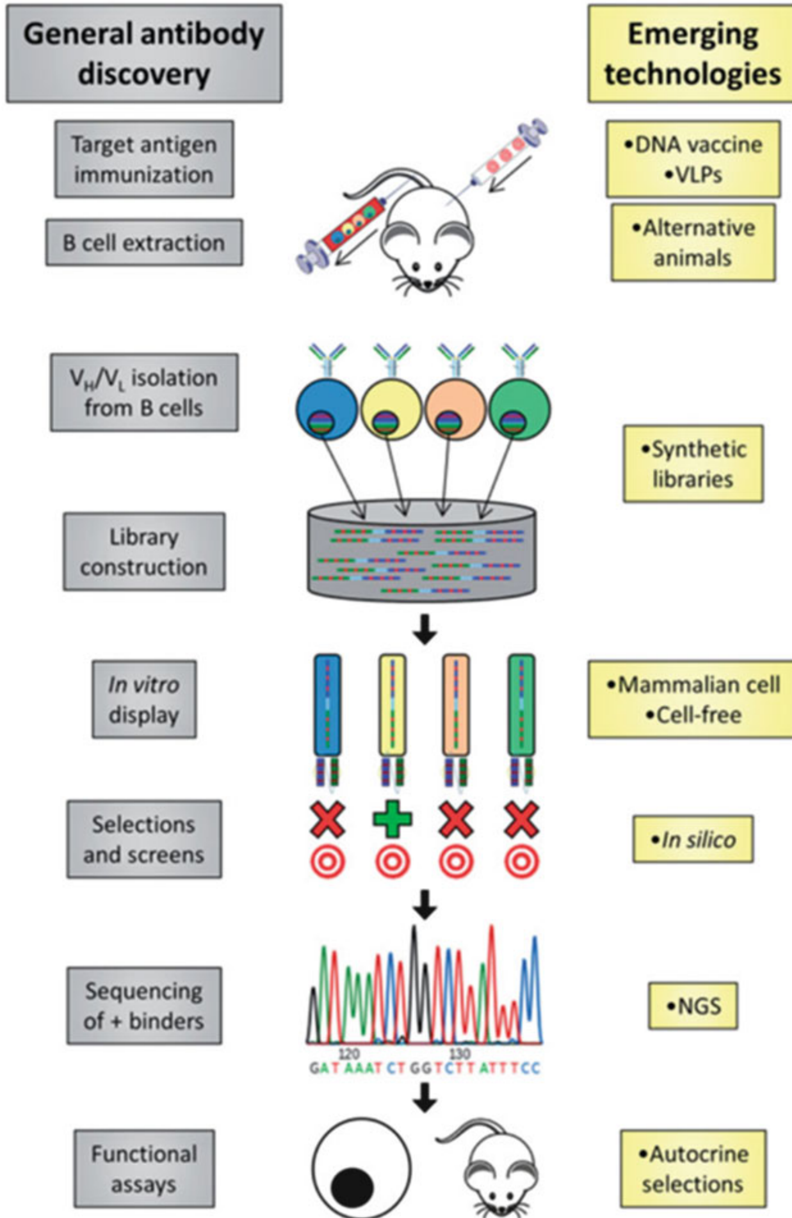


Fig. 6 General antibody discovery process and the emerging technologies. The most common antibody discovery process starts with animal immunization with the target antigen. The variable genes are isolated from the antibody-producing B cells of positive responders, and polyclonal libraries are constructed and packaged into in vitro display technologies. The displayed libraries are then selected and screened for binding to the target antigen. Positive binders are sequenced, recloned, and further characterized in functional assays. Improvements in the discovery process include DNA vaccines and virus-like particles (VLPs) to enhance immunogenicity, alternative

diversification, where one of the two antibody chains is replaced by a repertoire while the other chain is kept constant [137].

In contrast, targeted strategies introduce diversity at predicted positions, mainly at the ones contributing for antigen binding, the CDRs. CDR walking, an approach where diversity is introduced in short amino acid stretches, can provide up to a 420-fold increased affinity [138]. A more recent approach for targeted modifications relies on programmable nucleases, which can be easily customized to cleave DNA at almost any desired site. MAGE, abbreviated from multiplex automated genome engineering, takes advantage of a template repair where different *loci* on the chromosome can be targeted simultaneously, generating a multiplex genomic mutant library. This is achieved by homologous recombination of artificial single-stranded oligonucleotides with flanking homology to the target region. The oligonucleotides hybridize to the complementary exposed strands during the process of replication [139].

Both target and nontargeted strategies are usually followed by stringent selections with targeted strategies being more likely to improve affinity and least likely to create problems with protein stability [87]. Besides, the majority of mutations are usually deleterious, and so mutated libraries often have most of the variants with reduced activity. Therefore, a high-throughput selection and screening is important to find the rare variants with beneficial mutations that improve the desired properties [140]. Another important feature of directed evolution is the genotype-phenotype link. When functional antibodies are isolated, it is necessary that their genes are also collected. In phage display, for example, this is performed by compartmentalization of gene (inside the phage) and protein (phage surface). The isolated gene sequences are then amplified by transforming host bacteria or by PCR. The best pool of sequences are then used for the next round of mutagenesis, and this repeated cycle can generate protein variants adapted to the applied selection pressures (Fig. 7) [141].

In practical terms it is impossible to cover the entire possibilities of mutations in a typical protein. The entire randomization of a decapeptide would yield 10^{13} unique combinations of amino acids, which exceeds the library size of all known library creation methods. Nonetheless, directed evolution represents already a hallmark of protein development and is being practiced for many academic and industrial purposes. The easy manipulation of biological systems makes them very good substrates for engineering and for redesigning new evolutionary approaches [142].

Fig. 6 (continued) animals to increase repertoire diversity, fully synthetic libraries to eliminate the need for immunized animals, mammalian cell display for a native presentation, high-throughput technologies to decrease discovery time, and autocrine-based selection systems to bypass the target-binding process and directly select for antibodies with a defined functional phenotype. Reprinted from Kennedy et al. [130], with permission from Taylor & Francis publisher

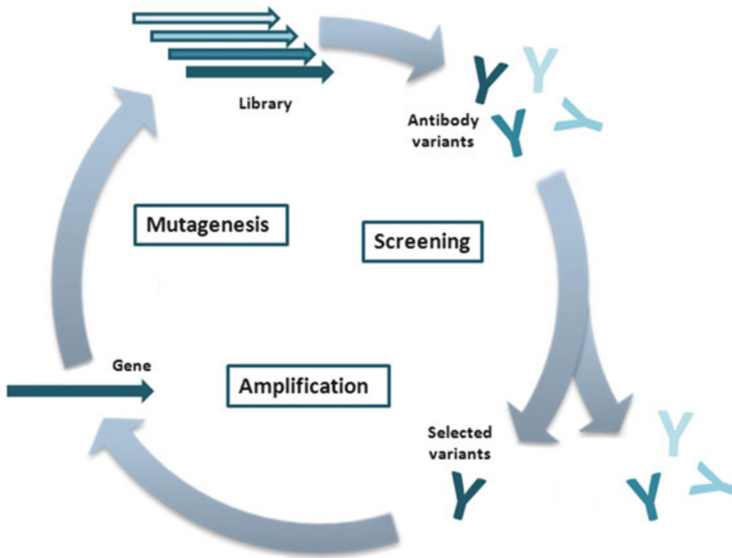


Fig. 7 Key steps in the cycle of directed evolution. A diverse library of genes is translated into a corresponding library of gene products and screened for functional variants in a manner that maintains the correspondence between genotype (gene) and phenotype (protein). These functional genes are replicated and serve as starting points for subsequent rounds of diversification and screening or selection

Over the last 40 years, monoclonal antibodies have stood out as important scientific tools and powerful human therapeutics. Due to their high specificity to the target, these proteins are extremely important for innumerous therapeutic applications and are recognized as a major drug modality in a variety of diseases. Until now, the leading methodologies for therapeutic antibody generation, immunization, and display approaches have been effective for the generation of antibodies against various targets and have led to a significant number of commercialization approvals.

Despite the moderate success, the common technologies for antibody discovery are time-consuming and imply complex methods. Today, there is a need to improve the antibody discovery process, creating more efficient technologies. There is a need, for example, to avoid the cost and time-consuming practice of humanization or de-immunization, which could be surpassed by a system capable of developing human antibodies. Also, it is a main concern to increase the size and quality of antibody libraries. The higher the quality and size of antibody libraries, the higher the likelihood of finding efficient therapeutic leads. Furthermore, the most widely used display technique, phage display, does not allow antibody and antigen molecules

(continued)

to interact in their native conformation. Having the possibility to select different antibody formats in their native conditions would also be a big benefit, increasing the chances of success in further assays. Finally, it would be more efficient if one could select good candidates for high affinity and, at the same time, optimized candidates for large-scale production.

Hybridoma technology and phage display have revolutionized the field of antibody discovery by providing important approaches for the isolation of monoclonal antibodies. However, the low efficiency of the hybridoma isolation process and the limitations of the prokaryotic expression-based technologies have been important technical drawbacks difficult to overcome [143, 144]. Various in vitro approaches have been developed in recent years to display full-length IgG on the surface of eukaryotic cells, with the aim to render discovery of therapeutic monoclonal antibodies more efficient, with favorable biophysical properties [122, 123, 126, 145]. Despite the improvements, these technologies are limited either by the size of the antibody libraries that can be expressed or by the lack of a stable genotype to phenotype link. With the increased number of antibodies reaching the market, there is a need to develop new technologies that stem from the limitations of current discovery platforms.

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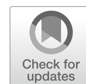
Cytokines and Growth Factors



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Abstract Several cytokines have been used to treat autoimmune diseases, viral infections, and cancer and to regenerate the skin. In particular, interferons (INFs) have been used to treat cancer, hepatitis B and C, and multiple sclerosis, while interleukins (ILs) and tumor necrosis factors (TNFs) have been used in the management of different types of cancer. Concerning the hematopoietic growth factors (HGFs), epoetin has been used for anemia, whereas the colony-stimulating factors (CSFs) have been used for neutropenia. Other growth factors have been extensively explored, although most still need to demonstrate in vivo clinical relevance before reaching the market.

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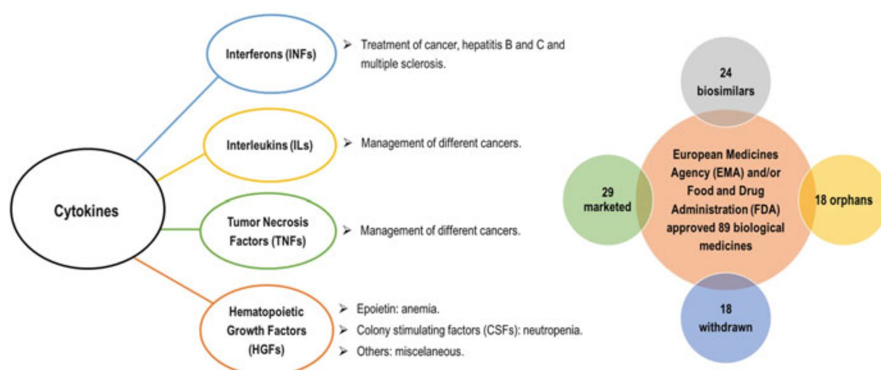
This chapter provides an overview on the therapeutic applications of biological medicines containing recombinant cytokines and growth factors (HGFs and others). From this review, we concluded that the clinical relevance of recombinant cytokines has been increasing. Since the 1980s, the European Medicines Agency (EMA) and/or Food and Drug Administration (FDA) have approved 89 biological medicines containing recombinant cytokines. Among these, 18 were withdrawn, 24 are biosimilars, and 18 are orphans.

So far, considerable progress has been made in discovering new cytokines, additional cytokine functions, and how they interfere with human diseases. Future prospects include the approval of more biological and biosimilar medicines for different therapeutic applications.

Graphical Abstract



Keywords Cytokines, Growth factors, Hematopoietic growth factors, Interferons, Interleukins, Tumor necrosis factors

1 Introduction

Biopharmaceuticals are generally recombinant therapeutic proteins obtained by biotechnological methods, such as genetic engineering techniques (e.g., recombinant DNA technology) that use biological systems (e.g., microorganisms, cells, plants, or animals) to produce proteins similar to those that occur naturally in the body. Sometimes biopharmaceuticals are called biologicals or biological products, although this is a broader concept that includes pharmaceutical substances produced or extracted from living sources. Examples of biological products are vaccines, blood-derived products, allergenics, somatic cells, gene therapy, and recombinant therapeutic proteins [1–3].

The clinical use of recombinant proteins requires the manufacture of the corresponding biological medicine, which is defined by the European Medicines Agency (EMA) as a medicine containing an active substance produced by a living organism [4]. In contrast, the Food and Drug Administration (FDA) includes biological medicines in biological products that may contain sugars, proteins, nucleic acids, or combinations of these molecules, living cells, or tissues and are obtained from natural sources or produced by biotechnology techniques or other innovative technologies [3].

Biological medicines have been used to treat medical conditions that do not have other treatments or when they are the best therapeutic option for the treatment of some diseases. Examples of these conditions include autoimmune, cardiovascular, metabolic, dermatological, neurological diseases, cancer, and eye and respiratory disorders [5]. Furthermore, several biological medicines have been granted with the orphan designation, being used for the treatment of rare life-threatening or chronically debilitating conditions [6].

The advent of biological medicines brought a new era for therapeutics, being the first biological medicine approved in the 1980s (recombinant human insulin). To date, the expiration of some patents led to the development of biosimilar medicines, which are medicines with the same quality, safety, and effectiveness of an approved biological medicine, the reference medicine [7, 8].

Biopharmaceuticals can be divided into different classes, including monoclonal antibodies, cytokines, growth factors, hormones, blood products, enzymes, vaccines, cells and tissues, and products of gene therapy. In this chapter, an overview is given on the therapeutic applications of biological medicines containing cytokines and growth factors (hematopoietic growth factors and others).

2 Cytokines

Cytokines display a crucial role in the mediation of immune response, controlling effector cells activity on the target cells. Immune regulation, disease pathogenesis, and management of immune-mediated diseases are among the activities of cytokines [9, 10]. These molecules are generally proteins or glycoproteins that are secreted by cells in small quantities and bind to other cell type's specific receptors, triggering and modelling the immune responses (Fig. 1). Leukocytes and other cells that interact with hematopoietic cells produce most cytokines. These mediators are essential to regulate immune and inflammatory responses, hematopoiesis, and wound healing. Thereby, the administration of cytokines improves immune responses against some viral infections and cancers and manages skin regeneration [2, 10]. Most of these molecules are hydrophilic, being the formulation of the respective medicines challenging. Notwithstanding, they have high affinity to the binding receptors, usually are administered in small doses and show a narrow therapeutic index. So far, several cytokines have been identified, and some are used in clinics to treat cancer, autoimmune diseases, and viral infections, for example, the colony-stimulating factors to

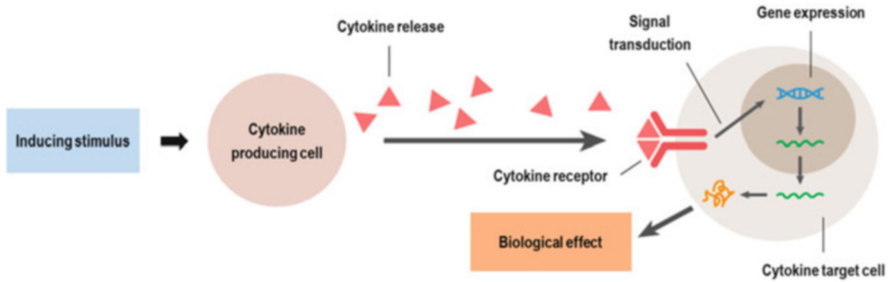


Fig. 1 Schematic representation of the general activity of cytokines

the treatment of neutropenia; the interferons to the treatment of viral infections, neurodegenerative disorders, and cancer; and the interleukins and tumor necrosis factor to the management of different cancers [11, 12]. In contrast, the overexpression of cytokines can induce diseases. Accordingly, cytokine antagonists (e.g., cytokine-binding receptors, such as monoclonal antibodies) have been used in clinic [10], but these products are out of the scope of this chapter.

Table 1 shows examples of the different clinical applications of interferons (INFs), interleukins (ILs), and tumor necrosis factors (TNFs) and corresponding biological medicines.

2.1 Interferons (INFs)

Different cell types, showing diverse biological effects, including cellular antiviral states, regulation of immunological and inflammatory responses, cell growth processes, and apoptosis, produce INFs. Regarding these activities, INFs have been used in clinical practice to promote immune responses against infections and to treat autoimmune disorders and different types of cancer [2, 15].

INF-α, INF-β, and INF-γ comprise the three human classes of INFs, where INF-α and INF-β have similar amino acid sequences and bind to the same cell receptors, originating identical biological activities, particularly, against viral infections and antiproliferation of tumor cells. In contrast, INF-γ has a distinct amino acid sequence and binds to different cell receptors, having an activity related to anti-inflammatory and immunological responses. Owing to their different biological effects, INF-α and INF-β are also named type I interferons, and INF-γ is classified as type II interferon. Type I has 12 subtypes or isoforms of INF-α and 1 subtype of INF-β, while type II is only the INF-γ. Current INFs used in clinical practice are recombinant, being typically produced in *E. coli*, since they do not require posttranslational modifications to display the biological activity, although some INF-β have been produced in CHO (Chinese hamster ovary) cells [2, 15].

Table 1 Examples of interferons (INFs), interleukins (ILs), and tumor necrosis factors (TNFs), therapeutic indications, and marketed biological medicines

Recombinant cytokine	Therapeutic indications	Marketed medicines	References
INF-α	Various cancers; chronic hepatitis B and C	Intron A®/Alfatronol®, Virtron®/Viraferon® [a], Roferon A®, Infergen® [a], Rebetron®, Pegasys®, PegIntron®, Sylatron®, Alferon N®	[2, 11–23]
INF-β	Multiple sclerosis	Betaferon®, Betaseron®, Avonex®, Rebif®, Plegridy®, Extavia®	[2, 11–15, 24–28]
INF-γ	Chronic granulomatous disease; osteopetrosis	Actimmune®	[2, 11, 14, 15, 29]
IL-1 receptor antagonist (anakinra)	Rheumatoid arthritis; Schnitzler's syndrome; Familial Mediterranean fever; mevalonate kinase deficiency; TNF receptor-associated periodic syndrome	Kineret®	[2, 15, 30–32]
IL-2 (aldesleukin)	Renal carcinoma; melanoma	Proleukin®	[11, 12, 15, 33]
IL-2 (denileukin diftitox)	Cutaneous T-cell lymphoma	Ontak® [a]	[11, 15, 34]
IL-3 – diphtheria toxin	Acute myeloid leukemia	Orphan medicine	[35]
IL-7	Progressive multifocal leukoencephalopathy	Orphan medicine	[36]
IL-7 – antibody	Idiopathic CD4 lymphocytopenia	Orphan medicine	[37]
Pegylated IL-10	Pancreatic cancer	Orphan medicine	[38]
IL-11 (oprelvekin)	Prevention of chemotherapy-induced thrombocytopenia; avoidance of platelet transfusions after chemotherapy	Neumega®	[2, 11, 15]
IL-12	Acute radiation syndrome	Orphan medicine	[39]
TNF-α-1a (tasonermin)	Adjunct for surgery tumor removal; palliative care after irresectable soft tissue sarcoma of the limbs	Beromun®	[2, 12, 40]
NGR-human TNF	Malignant mesothelioma	Orphan medicine (Zafiride®)[a]	[41, 42]
	Hepatocellular carcinoma	Orphan medicine	[43]

IL interleukin, *INF-α* interferon alpha, *INF-β* interferon beta, *INF-γ* interferon gamma, *NGR-human TNF* human TNF coupled to cngrcg peptide, *TNF* tumor necrosis factor
[a]Medicine withdrawn

Concerning therapeutic applications of INFs, INF-α subtypes are the most used. For example, INF-α-2a (Roferon A®) has been used to treat hairy cell leukemia, Kaposi's sarcoma, chronic myelogenous leukemia, cutaneous T-cell lymphoma, chronic hepatitis B and C, follicular lymphoma, renal cancer, and malignant melanoma [20]. INF-α-2b (Intron A®/Alfatronol®) has been indicated to the treatment of multiple myeloma, chronic myelogenous leukemia, chronic hepatitis B and C, carcinoid tumor, hairy cell leukemia, follicular lymphoma, malignant melanoma, condylomata acuminate, and Kaposi's sarcoma [16]. IFN-α-n3 (Alferon N®) has been used to treat condylomata acuminate [23]. Regarding other INFs types, INF-β-1b (Betaferon®, Betaseron®, and Extavia®) and INF-β-1a (Avonex®, Rebif®, and Plegridy®) have been used to the management of multiple sclerosis [24–28], while INF-γ-1b (Actimmune®) is indicated to the treatment of chronic granulomatous disease and osteopetrosis [29]. Furthermore, the use of recombinant INF-γ has been suggested to improve atopic dermatitis treatment in patients with predisposition for skin infections [44]. Pegylated INFs (i.e., INFs chemically linked to polyethylene glycol – PEG) have also been marketed (Pegasys®, PegIntron®, and Sylatron®) to increase molecules half-lives, improving administration regimens [2, 11, 15, 18, 21, 22]. Table 1 shows examples of the different clinical applications of INFs and their corresponding marketed biological medicines.

Depending on the dose regimen, the administration of INFs usually induces flu-like symptoms, since they promote the production of endogenous INFs that are also produced during influenza virus infection. These symptoms are mild and can be relieved with paracetamol. Nonetheless, there are reports of more severe adverse effects for INF-α and INF-β [2, 15].

To our knowledge, there are no marketed biosimilar medicines with recombinant INFs, although some are expected in the near future. Recently, Mufarrege et al. used of a multiplexed gene expression system, based on a human monocytic cell line, to characterize the bioactivity of recombinant INF-β. The researchers suggested the application of this system to compare recombinant INF-β medicines (Rebif® and Betaseron®) with the bioactivity of the respective biosimilar candidates [45].

2.2 Interleukins (ILs)

Interleukins (ILs) are a large group of cytokines comprising several subtypes that are produced by different cells, such as macrophages, eosinophils, vascular endothelial cells, fibroblasts, and keratinocytes. The biological activities of ILs are complex and not totally understood and include the regulation of normal and malignant cells growth and differentiation and the management of immunological and inflammatory responses. Similar to INFs, ILs bind to specific receptors on the surface of neighborhood cells, stimulating the production of more ILs. The medical use of ILs is limited, due to the lack on the knowledge of all biological functions. Thereby, only IL-1, IL-2, and IL-11 are approved for clinical use [2, 15]. Nonetheless, the therapeutic potential of other ILs subtypes has been studied. For example, the use of IL-4,

IL-10, and IL-11 to the treatment of psoriasis and rheumatoid arthritis has been tested, but the results of clinical trials were not satisfactory, since patients modestly improved upon administration of the ILs [46]. Steen-Louws et al. proposed the use of IL-4–10 fusion protein as an alternative to the administration of isolated IL4 and IL-10, obtaining a synergistic therapeutic effect [47]. Tang et al. performed a clinical study using a recombinant fusion protein consisting of two IL-22 molecules linked to an immunoglobulin constant region, which promotes tissue repair and suppresses bacterial infection. The results showed that the tested recombinant fusion protein was well tolerated and can be used to treat inflammatory diseases and organ failure, such as alcoholic hepatitis [48, 49].

The efficacy of ILs as adjuvants for cancer immunotherapy has been demonstrated in clinical trials. Naing et al. reported promising results from a phase I trial using pegylated recombinant IL-10 to treat advanced solid tumors, suggesting its potential for cancer immunotherapy [50, 51]. The use of IL-12 to the treatment of melanoma and cutaneous T-cell lymphoma was tested, being observed the occurrence of antitumor activity and undesired toxicity effects [46]. Currently, the National Cancer Institute (United Sates) is carrying out a phase I clinical trial to evaluate the efficacy of a combined therapy with monoclonal antibody (pembrolizumab) and recombinant IL-12 to treat solid tumors. Herein, the therapeutic interest of using recombinant IL-12 is related with its ability to destroy tumors, by obstruction of the blood flow to the tumor and stimulation of the white blood cells that kill tumor cells [52]. Coyne et al. carried out a clinical trial where it was observed that the use of immune checkpoint inhibitors combined with recombinant IL-15 (which promote the production of $CD8^{+}$T-cells, natural killer cells, and inflammatory cytokines) improved the antitumor immune responses [53]. In other study, Miller et al. observed the clinical efficacy of subcutaneous recombinant IL-15 to the treatment of refractory solid tumors in cancer patients [54]. Francois et al. carried out a clinical trial using recombinant IL-7 in oncologic and lymphopenic patients and observed an increase on the $CD4^{+}$ and $CD8^{+}$ lymphocytes levels. From their findings, these researchers proposed the use of recombinant IL-7 for the treatment of sepsis [55].

Table 1 shows examples of the different clinical applications of ILs and corresponding biological medicines. From our research, no commercially available biosimilars containing recombinant ILs were found.

The two subtypes of IL-1 (IL-1α and IL-1β) bind to the same receptors and induce similar biological activities. Among these, the main function is the pro-inflammatory activity that originates the production of inflammatory mediators. Furthermore, it has been described that IL-1 plays a role in the activation of T-lymphocytes and hematopoietic cells differentiation and growth. Besides, together with IL-6, IL-1 induces hepatocytes acute-phase proteins. The extension of these effects depends on the amount of IL-1 produced, being local for low quantities and systemic for high concentrations. Clinical investigations related with the potential of using IL-1 for treating several cancers and chemotherapy-induced bone marrow suppression have been carried out, but the results were not satisfactory, since no significant antitumor activity and toxic side effects were observed. However, regarding the ability to

mediate acute and chronic inflammation, the modulation of IL-1 levels has been showing interesting clinical results, in particular using IL-1 receptor antagonists. In this field, there is one marketed medicine, anakinra (Kineret®), a non-glycosylated recombinant IL-1 receptor produced in *E. coli.*, which was first approved to treat rheumatoid arthritis [2, 15]. Later, this medicine was approved to treat periodic fevers and auto-inflammatory disorders at all ages, being the first-line treatment for Schnitzler's syndrome and second-line treatment for Familial Mediterranean fever, mevalonate kinase deficiency, and TNF receptor-associated periodic syndrome [30]. Moreover, anakinra is used in combination with tocilizumab to the treatment of adult-onset Still's disease refractory to second-line therapy [31]. Thomas et al. are conducting a clinical trial to assess whether the use of anakinra in combination with corticosteroids improves outcomes in patients with acute severe ulcerative colitis [56]. Recently, Zhu et al. performed in vivo experiments where it was observed that the use of recombinant IL-1 provides a protective role against myocardial ischemia-reperfusion injury in rats, suggesting its potential for the treatment of this disorder [57].

IL-2 is the most studied type of ILs, being the first identified T-growth factor and having a crucial role in immune response activation, tolerance, and memory induction. IL-2 is produced by T-lymphocytes and stimulates natural killer cells and antibody production by B-lymphocytes. The clinical relevance of IL-2 is related with its immunostimulatory activity that promotes body antitumor responses [2, 15]. Two medicines based in IL-2 have been approved, despite only one is currently marketed. Aldesleukin (Proleukin®) is a recombinant IL-2 approved to treat renal carcinoma and melanoma [33]; denileukin diftitox (Ontak®) is a recombinant engineered fusion protein composed by diphtheria toxin fragments linked to IL-2, which targets T cells and was used to the treatment of cutaneous T-cell lymphoma, being discontinued by FDA in 2014 [11, 15, 34].

The use of high IL-2 concentrations originates cardiovascular, hepatic, and pulmonary adverse effects, which limits the duration of treatments. Besides, some adverse effects can be induced indirectly, promoting the synthesis of other cytokines [2, 58]. Some recent works referred the potential of using IL-2 to the management of other disorders. For example, Humrich and Riemekasten showed that a low-dose of IL-2 is well tolerated and influences positively the clinical course of patients with active systemic lupus erythematosus. Nonetheless, phase II clinical trials are in progress to confirm these preliminary results [58]. In other study, Zhang et al. proposed the use of recombinant IL-2 as an adjunctive immunotherapeutic agent to treat tuberculosis. The results of the performed experiments suggested that the use of IL-2 increases the proliferation of $CD4^+$ and natural killer cells and improves sputum culture and smear conversion in patients with pulmonary tuberculosis. Nonetheless, the real clinical efficacy of this use needs to be demonstrated [59].

When activated by IL-1, bone marrow stromal cells and fibroblasts produce IL-11. The latter acts as a hematopoietic growth factor (Sect. 3.1), stimulating thrombopoiesis (i.e., platelet production) and the growth and differentiation of bone marrow cells. Recombinant IL-11, called oprelvekin (Neumega®), is produced in *E.coli* and indicated to the prevention of severe thrombocytopenia and avoidance

of platelet transfusions after chemotherapy. Despite IL-11 is well tolerated, some adverse effects have been reported, including edema, tachycardia/palpitations, dyspnea, and oral moniliasis [2, 11, 15]. Kuo-Ming et al. demonstrated that the use of pegylated recombinant IL-11 did not originate additional toxicities in primates, when compared to the recombinant IL-11 alone. Therefore, these researchers proposed the use of pegylated recombinant IL-11 as a safe long-acting treatment, originating less fluid retention, which is most common adverse effect [60].

EMA approved the market of some recombinant ILs as orphan drugs, for example, pegylated IL-10 (in 2017) for pancreatic cancer [38]; IL-12 (in 2016) for acute radiation syndrome [39]; IL-7 (in 2014) for progressive multifocal leukoencephalopathy [36]; IL-7 fused with antibody (in 2017) for idiopathic CD4 lymphocytopenia [37]; and IL-3 fused with diphtheria toxin (in 2015) for acute myeloid leukemia [35].

2.3 Tumor Necrosis Factors (TNFs)

Tumor necrosis factors (TNFs) comprise the subtypes α and β that induce similar biological effects. Among these, the most studied is the TNF-α, also named TNF, cachectin, macrophage cytotoxic factor, macrophage cytotoxin, or necrosin. This cytokine is synthetized by activated macrophages, although several cell types can produce it, for example, natural killer cells, eosinophils, Kupffer cells, glomerular mesangial cells, fibroblasts, B- and T-lymphocytes, polymorphonuclear leukocytes, astrocytes, Langerhans cells, and brain microglial cells. The biological effects of TNFs include immunological activation in response to the presence of microorganisms (e.g., Gram-negative bacteria), regulation of inflammation, necrosis of some tumor cells types, and mediation of several disorders (e.g., septic shock, cachexia, and anorexia) [2, 61].

Table 1 shows examples of the different clinical applications of TNFs and corresponding biological medicines. From our research, no commercially available biosimilars containing recombinant TNFs were found. The first clinical attractiveness of TNF-α was for cancer therapy. Nonetheless, it has been observed that some tumors are not susceptible to TNF, due to a reduced necrosis activity and occurrence of adverse effects upon the administration of therapeutic doses. In contrast, some clinical studies focused in the neutralization of the negative outcomes originated by the overexpression of TNF. Examples of these adverse effects are induced cachexia and tumor growth stimulation in cancer patients; tissue necrosis and vascular leakage in patients with septic shock; inflammation in patients with rheumatoid arthritis; and diabetes by induction of insulin resistance after pancreatic cells death. The administration of anti-TNF monoclonal antibodies or anti-TNF receptor reduces the severity of these effects. Regarding the direct use of TNF in therapy, only one product (tasonermin – Beromun®) is approved [2]. This medicine contains recombinant TNF-α-1a that has been produced in *E. coli* and is indicated as adjunct for surgery tumor removal, to prevent or delay amputation, or in a palliative situation,

for irresectable soft tissue sarcoma of the limbs in combination with melphalan [2, 12, 40]. In 2008, EMA granted the orphan designation to NGR-human TNF (human TNF coupled to cngrcg peptide) for the treatment of malignant mesothelioma [42], being the respective medicine (Zafiride®) withdrawn in 2017 [41]. Latter, in 2009, EMA approved NGR-human TNF as an orphan medicine to the treatment of hepatocellular carcinoma [43].

Li et al. reported the results of a retrospective trial where it was observed that the intrapleural instillation of recombinant TNF-α controlled the malignant pleural effusion and minimized invasive intervention in a cohort group of lung cancer patients. However, more trials are required to define the optimal therapeutic dose and confirm clinical efficacy [62].

3 Growth Factors

3.1 Hematopoietic Growth Factors (HGFs)

Growth factors are cytokines that stimulate cell proliferation, differentiation, and/or activation. Among these, hematopoietic growth factors (HGFs), which are glycoproteins that regulate the production and maturation of blood cells (i.e., hematopoiesis), have been showing high therapeutic potential [2, 63, 64].

HGFs with clinical relevance have been produced by DNA recombinant techniques and include IL-11 (Sect. 2.2), recombinant erythropoietin or epoetin and darbepoetin alfa, and the white cells factors: granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) [2, 63, 65].

Erythropoietin is an uncharacteristic cytokine that acts as an endocrine hormone and is produced by the kidneys, although the liver synthetizes a small amount. Its main function is the stimulation and regulation of the production of red blood cells (i.e., erythropoiesis), by a mechanism that increases the number of cells able to differentiate in erythrocytes and promotes the migration of mature red blood cells from the bone marrow to the peripheral circulation. In addition, anemia-related tissue hypoxia induces erythropoietin production by the kidneys. Besides, erythropoietin improves body resistance to exercise and well-being and reduces the need of blood transfusions in anemic patients. This hormone is present in low concentrations in the urine of anemic patients, being initially isolated from there for clinical use. Nonetheless, due to the small amount available by this method, the erythropoietin currently used in therapy is produced by DNA recombinant technique, in mammalian cells (e.g., CHO), being called epoetin [2, 63].

The white cell factors, granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF), play a major role in the differentiation of neutrophils from hematopoietic stem cells. G-CSF is a glycoprotein synthetized by different cells (bone marrow stromal cells, macrophages, and fibroblasts) and has a biological activity related to the proliferation of neutrophils and further activation of mature cells (i.e., gain of specific functions). Moreover,

G-CSF promotes the proliferation and migration of endothelial cells and, when associated with other HGFs, has a synergic effect on the differentiation of various hematopoietic cells. In contrast, GM-CSF is a glycosylated polypeptide produced by several cells (macrophages, T-lymphocytes, fibroblasts, and endothelial cells), which induces the proliferation of neutrophils, macrophages, eosinophils, erythrocytes, and megakaryocytes [2]. G-CSF and GM-CSF have been used to treat neutropenia. Furthermore, they show therapeutic effect against infections and some cancers and in the management of bone marrow transplants. The approved G-CSF and GM-CSF medicines are marketed under several trade names and are usually produced in *E. coli*. Recombinant G-CSF include filgrastim, pegfilgrastim (filgrastim linked to a PEG molecule), and lenograstim, while recombinant GM-CSF comprise molgramostim and sargramostim [2, 63].

Table 2 shows examples of the different clinical applications of HGFs and corresponding biological and biosimilar medicines.

FDA approved the first recombinant erythropoietin in 1989 (Epogen®/Procrit® – epoetin alfa) to treat anemia in patients with chronic renal failure that are unable to produce this hormone due to the loss of renal function. Later, the clinical use of epoetin was extended to treat or avoid anemia caused by other disorders, for example, to improve the production of red blood cells in patients undergoing myelosuppressive chemotherapy and bone marrow transplantation and those undertaking therapy for viral infections, such as human immunodeficiency virus (HIV) and hepatitis C. However, some adverse effects have been associated with the use of Epogen®/Procrit®, including cardiovascular events, hypertension, seizures, stroke, thrombosis or thromboembolism, tumor recurrence or progression, and severe anemia [2, 63, 65, 69]. Later, several subtypes of epoetin were approved for similar therapeutic indications. For example, epoetin beta (NeoRecormon® and Mircera®) [70–72] and epoetin theta (Eporatio® and Biopin®) [73, 74] were approved to treat symptomatic anemia originated by chronic renal failure or post-chemotherapy nonmyeloid malignancies. Two medicines containing darbepoetin alfa (Aranesp®, Nespo®) were approved to treat anemia originated by renal failure or myelosuppressive chemotherapy, although Nespo® was withdrawn in 2009 [78–80].

With the expiration of epoetin patents, some biosimilar medicines have been developed and more are expected for the next years. Current approved epoetin biosimilars are based in the reference medicine Epogen®/Procrit® and include alfa epoetin (Alfa Hexal®, Abseamed®, and Binocrit®) and zeta epoetin (Retacrit® and Silapo®). Among these, Retacrit® is the unique approved by FDA, being all approved by EMA. Despite the cost reduction of using biosimilar epoetin, some clinicians have been showing skepticism regarding its similar therapeutic efficacy and still prescribe the reference medicine [8, 111].

The most common indications of epoetin biosimilar medicines include the treatment of anemia caused by several disorders, such as chronic renal failure or kidney problems, chemotherapy treatments (reducing the need of allogenic blood transfusions), and defective production of blood cells. In addition, epoetin biosimilars are used to regulate normal blood levels before surgery in patients that undergo autologous blood transfusion and to reduce the need of allogenic blood transfusions in

Table 2 Examples of hematopoietic growth factors (HGFs), therapeutic indications, and marketed biological and biosimilar medicines

Recombinant HGFs	Therapeutic indications	Marketed medicines	References
Epoetin alfa	Anemia caused by several disorders	Epogen®/Procrit®, Eprex®/Erypo®, Epoetin Alfa Hexal®[a], Abseamed®[a], Binocrit®[a]	[2, 8, 63, 66–69]
Epoetin beta	Anemia originated by chronic renal failure or post-chemotherapy nonmyeloid malignancies	NeoRecormon®, Mircera®	[2, 63, 70–72]
Epoetin theta		Eporatio®, Biopin®	[73, 74]
Epoetin zeta	Anemia caused by several disorders	Silapo®[a], Retacrit®[a]	[8, 75–77]
Darbepoetin alfa	Anemia originated by renal failure or myelosuppressive chemotherapy	Aranesp®, Nespo®[b]	[2, 63, 78–80]
G-CSF (granulocyte colony-stimulating factor) or filgrastim	Neutropenia and avoidance of febrile neutropenia in patients receiving myelosuppressive chemotherapy, or patients undergoing myeloablative chemotherapy followed by bone marrow transplantation; reduction of severe neutropenia effects in patients with congenital, cyclic, or idiopathic neutropenia	Neupogen®, Tevagrastim®[a], Zarzio®[a], Ratiograstim®[a], Grastofil®[a], Nivestim®[a], Nivestym®[a], Accofil®[a], Filgrastim Hexal®[a], Biograstim®[a,b], Filgrastim Ratiopharm®[a,b]	[2, 63, 81–92]
G-CSF (granulocyte colony-stimulating factor) or filgrastim	Amyotrophic lateral sclerosis	Orphan medicine	[93]
	Spinal cord injury		[94]
G-CSF (granulocyte colony-stimulating factor) or pegfilgrastim	Neutropenia and avoidance of febrile neutropenia in patients receiving myelosuppressive chemotherapy, or patients undergoing myeloablative chemotherapy followed by bone marrow transplantation; reduction of severe neutropenia effects in patients with congenital, cyclic, or idiopathic neutropenia; increased drug residence time in the body	Neulasta®, Lonquex®, Granix®, Ziextenzo®[a], Pelgraz®[a], Pelmeg®[a], Udenyca®[a], Fulphila®[a], Ristempa®[a,b], Neupopeg®[a,b], Efgratin®[a,b], Cavoley®[a,b]	[2, 63, 95–108]
GM-CSF (granulocyte macrophage colony-stimulating factor) or molgramostim	Acute respiratory distress syndrome	Orphan medicine	[63, 109]

(continued)

Table 2 (continued)

Recombinant HGFs	Therapeutic indications	Marketed medicines	References
GM-CSF (granulocyte macrophage colony-stimulating factor) or sargramostim	Hematopoietic syndrome of acute radiation syndrome	Leukine®	[2, 63, 110]

[a]Biosimilar medicine
[b]Medicine withdrawn

patients with moderate anemia that undergo major surgery (e.g., hip or knee surgery) [66, 75, 76]. Epoetin Alfa Hexal® and Silapo® have also been used in patients with risk of developing acute myeloid leukemia that show low levels of erythropoietin [66, 75]. Regarding Retacrit®, it has been used for the same medical indications of the other epoetin biosimilars, and FDA also approved its use for the treatment of anemia in HIV patients that have been treated with the antiviral zidovudine [76, 77].

Filgrastim (Neupogen®) was the first recombinant G-CSF approved by FDA, in 1991, to reduce neutropenia and avoid febrile neutropenia in patients receiving myelosuppressive chemotherapy, except the ones with chronic myeloid leukemia and myelodysplastic syndrome. This medicine has also been used to reduce neutropenia period in patients undergoing myeloablative chemotherapy followed by bone marrow transplantation, which show high risk of prolonged severe neutropenia. Furthermore, filgrastim reduces the incidence of severe neutropenia effects (e.g., fever, infections, and oropharyngeal ulcers) in patients with congenital, cyclic, or idiopathic neutropenia. Later, in 2015, Neupogen® was indicated to treat patients acutely exposed to myelosuppressive doses of radiation, designated by hematopoietic syndrome of acute radiation syndrome, promoting the recovery of neutrophils production by bone marrow cells and improving body immunity. Nonetheless, the use of filgrastim has been associated with the occurrence of some adverse effects, such as fever, pain, rash, headache, cough, breath difficulty, and nose bleeding. In 2015, FDA authorized Zarxio® to the same indications of Neupogen® [90, 91]. More recently, FDA approved sargramostim (Leukine®), which is the recombinant form of GM-CSF, to increase the survival rate of patients showing hematopoietic syndrome of acute radiation syndrome [110].

EMA approved the use of pegfilgrastim since 2002, by means of Neulasta® (pegfilgrastim) and Lonquex® (lipegfilgrastim), to the same therapeutic indications of filgrastim. The PEG linkage to filgrastim molecule increases the body circulation time of the drug, avoiding fast elimination and, therefore, reducing the number of required administrations [106, 107].

Two orphan designations have been attributed to filgrastim: treatment of amyotrophic lateral sclerosis, protecting nerve cells from damages [93], and reducing the effects of spinal cord injury, due to the capacity for protecting spinal cord cells from death [94]. Molgramostim, a recombinant form of GM-CSF, is approved

to treat acute respiratory distress syndrome, due to the ability to repair cells and eliminate microbes from the lungs, improving the oxygen flow into the blood [109].

To date, there are eight approved filgrastim biosimilars from the reference Neupogen® [112]: Filgrastim Hexal® [88], Accofil® [87], Nivestim® [86], Nivestym® [92], Grastofil® [85], Ratiograstim® [84], Zarzio® [83], and Tevagrastim® [82]. Concerning pegfilgrastim, there are five approved biosimilars to the same indications of the reference Neulasta® [106]: Fulphila® [95], Udenyca® [97], Ziextenzo® [99], Pelgraz® [100], and Pelmed® [101]. Furthermore, some biosimilars have been withdrawn: Biograstim® and Filgrastim Ratiopharm® for filgrastim [81, 89] and Ristempa®, Neupopeg®, Efgratin®, and Cavoley® for pegfilgrastim [102–105].

The clinical use of G-CSF has been explored in the management of several disorders. For example, Affentranger et al. reviewed the clinical trials that investigated the synergic therapeutic effect of using G-CSF combined with erythropoietin in anemic patients with lower risk of myelodysplastic syndromes and concluded that an improved efficacy can be achieved [113]. In other revision, Hamel et al. suggested that the use of G-CSF can accelerate the white blood cell count in patients with leukopenia related to kidney transplant, despite more data is required to confirm this evidence [114]. The benefits of using G-CFS in infertile women, after embryo implantation, seem to play an important role in the clinical outcome of assisted reproductive technology [115, 116]. Herrmann et al. carried out a pilot study and observed that the use of G-CSF improved bone regeneration in patients with large segmental defect, fracture non-unions, or insufficient vascularization [117].

3.2 Other Growth Factors

Apart from the HGFs, other growth factors have been applied for different therapeutic indications. For example, the recombinant human epidermal growth factor (EGF) is used to treat diabetic foot ulcers (Heberprot-P®, Regen-D® 150, and Easyef®), vascular ulcers and bed sores (Regen-D® 150) [118], and healing burns and donor site skin grafts (Regen-D® 60) [119]. The EGF mechanism of action is related to the improvement of keratinocytes proliferation and increase on the tensile strength of the new skin. Current EGF-based medicines are topical, for cutaneous administration or intralesional injection [120, 121]. Nonetheless, in 2018, EMA released a decision on granting a waiver for all EGF approved indications [122]. Several researches showed that the topical use of EGF in individualized formulations (i.e., magistral formulations) is effective in the management of diverse adult skin lesions, without showing adverse effects. However, more studies are required to establish clinical protocols for this application [123]. Furthermore, some products containing EGF for wound healing are currently under clinical trials, being expected to be marketed soon [124].

The platelet-derived growth factor (PDGF), in particular the isoform BB (PDGF-BB), is used in the management of chronic wounds, regulating the healing process.

This growth factor is released by activated platelets at the site of the damage and is a mitogenic and chemoattractant for mesenchymal stem cells, promoting the initialization of the tissue repair process. It also plays a role in angiogenesis [2, 121]. A single medicine containing human recombinant PDGF-BB was approved for the treatment of chronic diabetic ulcers (Regranex®) but was withdrawn by EMA [125, 126]. FDA approved an injectable containing PDGF-BB (Augment®) for arthrodesis and ankle hindfoot in patients that need supplemental graft material, being an alternative to autografts [127]. In this sense, Sun et al. conducted a meta-analysis to evaluate the efficacy of using recombinant PDGF-BB in comparison to autologous bone grafts, in ankle and foot fusion patients. From their study, the authors concluded that the use of this growth factor avoids the problems associated with the autografts procedures (e.g., pain, scarring, blood loss, and extra surgery time), although more studies are required to confirm these evidences [128]. Human recombinant PDGF-BB is used in dental therapy to treat periodontal defects, by means of an osteoconductive matrix enriched with this growth factor (GEM 21S®) [129].

Similar to PDGF-BB, the fibroblast growth factor 2 (FGF-2) or basic FGF (bFGF) shows ability to improve wound healing, stimulating the proliferation of epithelial and mesenchymal cells and promoting neovascularization. Furthermore, the use of bFGF in the recovery of skin burns is also effective. Fiblast® spray (trafermin) is the first marketed product containing bFGF, which is indicated to the treatment of skin ulcers, including leg and burn ulcers. Latter, this medicine was approved for dental therapy to promote the regeneration of periodontal and bone tissues [121, 130]. EMA attributed the orphan designation to the fibroblast growth factor 19 (FGF-19) for the management of primary biliary cirrhosis, primary sclerosing cholangitis and avoidance of liver damages, since this protein decreases the production of bile acids [131, 132]. The keratinocyte growth factor (KGF), palifermin or FGF-7, belongs to the FGF family. This growth factor stimulates the epithelial cells proliferation, improving tissue formation, and was approved by EMA (Kepivance®) for the treatment of oral mucositis in patients undergoing chemotherapy and radiotherapy, being withdrawn in 2016 [133]. Some researchers suggest the potential of KGF for the management of cutaneous wounds, despite human clinical studies are required to prove this evidence [121].

The vascular endothelial growth factor (VEGF) has been explored to improve wound healing, since it plays a major role in the angiogenesis initiation, improving the proliferation and migration of endothelial cells. Nonetheless, to our knowledge, there are no available products in the market. In this sense, Hanft et al. carried out randomized controlled trials to evaluate the efficacy of using human recombinant VEGF in patients with neuropathic diabetic foot ulcers. These researchers observed that the topical application of VEGF originated an improved wound healing, reached in a smaller period, when compared to the placebo [121, 134]. The orphan designation was granted by EMA to the human recombinant VEFG to treat amyotrophic lateral sclerosis, due to the ability of this growth factor to stimulate the development of blood vessels. This medicine is for direct brain administration, promoting the growth of blood vessels that irrigate nerve cells, increasing their survival [135]. In

contrast, some medicines containing VEGF inhibitors are available and are used to treat age-related macular degeneration and different cancers [136].

The placental growth factor (PlGF) is an angiogenic protein that belongs to the VEGF family, which is highly produced during pregnancy and is fundamental to the growth of placenta blood vessels. Thereby, PlGF is fundamental to normal pregnancy and baby's development and is a useful clinical indicator to predict adverse outcomes. For example, low levels of PlGF are associated with the occurrence of preeclampsia [137, 138]. EMA granted the orphan designation to human recombinant PlGF to restore blood PlGF levels, improving preeclampsia symptoms [139].

Transforming growth factors (TGFs) are a family of mitogenic polypeptides that includes the subtype TGF-α, which has an activity similar to EGF and induces angiogenesis. Some studies suggested that TGF-α promotes the reepithelialization, but more experiments are required to confirm this activity [2, 121]. TGF-β is another subtype that has been showing an important role in the early stages of wound healing, which has three isoforms (β1, β2, and β3). Isoforms β1 and β2 promote fibroblast differentiation, contraction, synthesis of the extracellular matrix, and scarring, while isoform β3 reduces scar formation [121, 140]. So et al. carried out successful phase I and II clinical trials with human recombinant TGF-β3 (avotermin), where observed a significant scarring reduction [141]. However, this product failed phase III trials and is not marketed [121].

Insulin-like growth factors (IGFs) family includes the subtypes I and II, which are structurally similar to proinsulin. Thereby, the administration of IGF decreases the levels of insulin and glucagon and increases the cellular glucose uptake. Diverse activities have been attributed to IGFs, regarding their ability to inhibit apoptosis and regulate cells growth and differentiation [2]. Accordingly, high circulating levels of IGF-I have been associated with the development and progression of different cancers [142], including breast [143], prostate [144], and thyroid [145] cancers. In 2005, FDA approved the use of IGF-I (mecasermin – Increlex®) to treat growth failure in children with severe IGF-I deficiency or with growth hormone insensitivity syndrome (alterations of the growth hormone gene that unable the use of this hormone by the body). However, IGF-I should not be used instead of growth hormone [146]. IGF-I was also approved by FDA to treat amyotrophic lateral sclerosis [147], and EMA granted the orphan designation to IGF-I for different therapeutic indications. For example, the recombinant human IGF-I/insulin-like growth factor-binding protein-3 (IGF-I/IGFBP-3) was approved to treat type A [148] and B [149] extreme resistance insulin syndrome, inherited extreme insulin resistance (or Rabson Mendenhall syndrome) [150], primary growth hormone insensitivity syndrome (or Laron syndrome) [151], and leprechaunism [152]. Nonetheless, all these medicines are withdrawn.

Neurotrophic factors are a large group of molecules that play an important role in the development and survival of nerve cells [2]. Among these, the nerve growth factor (NGF) has been the most studied, since it regulates the development and survival of specific peripheral neurons and basal forebrain cholinergic nuclei. Furthermore, NGF levels are high in several anti-inflammatory and autoimmune disorders (e.g., chronic arthritis, systemic lupus erythematosus, and multiple sclerosis),

and a relation with diabetic pathology was observed. Thereby, NGF constitutes a multifactorial modulator of neuro, immune, and endocrine systems [153].

Regarding its ability to regulate the growth and survival of retina and cornea cells, in 2013 EMA granted the orphan designation to NGF to treat retinitis pigmentosa. This growth factor improves the survival of retina cells, slowing the progression of the disease and preserving vision [154]. Latter, in 2015, EMA also attributed the orphan designation to NGF to treat neurotrophic keratitis. The use of NGF improves the eye normal healing process and repairs the damages to the cornea, which are typical of this disorder [155]. In 2018, FDA approved the medicine Oxervate® containing the recombinant human NGF (cenegermin) to treat neurotrophic keratitis [156]. The use of NGF to improve the recovery of other ocular degenerative diseases has been suggested. For example, Mesentier-Louro et al. observed that the ocular application of NGF reduces the retinal ganglion cell degeneration that occurs after optic nerve crush in adult rats, suggesting the potential of this growth factor to treat optical neuropathies, such as glaucoma [157]. Other researches have investigated the use of NGF for different clinical applications. For example, Sacchetti et al. carried out a phase IIa prospective, open label and multiple-dose clinical trial in 40 patients with dry eye disease, which received eye drops of recombinant NGF during 28 days. The results showed that the use of NGF is safe and effective to treat dry eye disease, although randomized clinical trials are required to confirm these findings [158]. Aloe et al. reviewed the results of the researches related with the implication of NGF in the induction and progression of carcinogenesis that remains open to debate [159]. In contrast, Denk et al. highlighted the clinical relevance of NGF antagonists as effective analgesic drugs for the treatment of several conditions, including osteoarthritis and back pain [160].

Table 3 shows examples of the clinical applications of diverse recombinant growth factors and corresponding biological medicines.

4 Conclusion

Among the most important activities of cytokines are the triggering of immune responses against cancer and viral infections and the ability to regenerate the skin. In this sense, numerous recombinant cytokines have been used in clinical practice. For example, the INFs to the treatment of several cancers, hepatitis B and C and multiple sclerosis, and the ILs and TNFs for the management of different cancers. Concerning the HGFs, epoetin has been used to treat anemia caused by diverse disorders, while colony-stimulating factors have been used for neutropenia. Regarding other growth factors, those used for wound management have been extensively explored, although most still need to demonstrate clinical relevance in vivo before reaching the market.

From this review, we concluded that the clinical relevance of recombinant cytokines has been increasing. Since the 1980s, EMA and/or FDA have approved 89 biological medicines containing recombinant cytokines (INFs, ILs, TNFs, HGFs, and other growth factors). Among these, 18 were withdrawn, 24 are biosimilars, and

Table 3 Examples of recombinant growth factors for different therapeutic indications and marketed biological medicines

Recombinant growth factor	Therapeutic indications	Marketed medicines	References
EGF (epidermal growth factor)	Diabetic foot ulcers	Heberprot-P®, Regen-D® 150, Easyef®	[120, 121]
	Vascular ulcers and bed sores	Regen-D® 150	[118, 121]
	Healing of burns and donor site skin grafts	Regen-D® 60	[119, 121]
bFGF/FGF-2 (basic fibroblast growth factor or fibroblast growth factor 2) or trafermin	Skin ulcers, regeneration of periodontal and bone tissues	Fiblast® spray	[121, 130]
FGF-19 (fibroblast growth factor 19)	Biliary cirrhosis	Orphan medicine	[131]
	Primary sclerosing cholangitis	Orphan medicine	[132]
IGF-I (insulin-like growth factor-I) or mecasermin	Growth hormone insensitivity syndrome	Increlex®	[2, 161]
	Amyotrophic lateral sclerosis	Iplex® [a]	[2, 162]
IGF-I/IGFBP-3 (insulin-like growth factor-I/insulin-like growth factor-binding protein-3)	Type A and B extreme resistance insulin syndrome	Orphan medicine	[148, 149]
	Inherited extreme insulin resistance	Orphan medicine	[150]
	Primary growth hormone insensitivity syndrome	Orphan medicine	[151]
	Leprechaunism	Orphan medicine	[152]
PGF (placental growth factor)	Preeclampsia	Orphan medicine	[139]
PDGF-BB (platelet-derived growth factor-isoform BB)	Ankle arthrodesis and/or hindfoot when supplemental graft is needed	Augment®	[127]
	Periodontally related defects	GEM 21S®	[2, 129]
	Chronic diabetic ulcers	Regranex® [a]	[2, 125, 126]
NGF (nerve growth factor) or cenegermin	Retinitis pigmentosa	Orphan medicine	[154]
	Neurotrophic keratitis	Oxervate®	[155, 156]
KGF (keratinocyte growth factor) or palifermin	Oral mucositis	Kepivance® [a]	[133]
VEGF (vascular endothelial growth factor)	Amyotrophic lateral sclerosis	Orphan medicine	[135]

[a] Medicine withdrawn

18 are orphans. The withdrawal of the medicines from the market has been requested by the producers and is related to economic reasons, occurrence of toxicity or non-efficacy events. Regarding biosimilars, only the HGFs epoetin and filgrastim were approved. This can be explained by their antiquity over other classes of recombinant cytokines, which allowed patent expiration. Thus, the approval of more biosimilars containing recombinant cytokines is expected for the next years. Orphan designation was granted mainly to non-HGFs, for different clinical uses. However, HGFs, ILs, and TNFs also have orphan medicines.

So far, considerable progress has been made in discovering new cytokines, additional cytokine functions, and how they interfere with human diseases. Future prospects include the approval of more biological and biosimilar medicines for different therapeutic applications.

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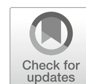
Hormones, Blood Products, and Therapeutic Enzymes



Ana Catarina Silva, Cládia Pina Costa, Hugo Almeida, João Nuno Moreira, and José Manuel Sousa Lobo

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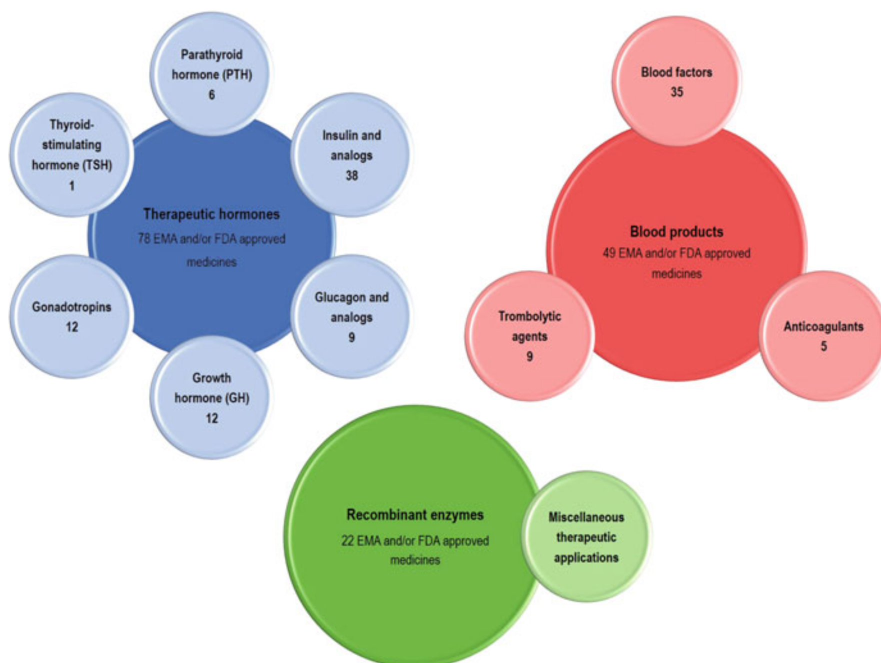
Abstract Therapeutic uses of biological medicines are diverse and include active substances from different classes. This chapter provides an overview on the clinical applications of biological medicines containing hormones, blood products, and therapeutic enzymes. Currently, therapeutic hormones have 78 approved medicines, including insulin and analogs, glucagon and analogs, growth hormone, gonadotropins (follicle-stimulating hormone, luteinizing hormone, and human chorionic gonadotropin), thyroid-stimulating hormone, and parathyroid hormone. In contrast, recombinant blood products, and particularly blood factors, anticoagulants, and thrombolytic agents, incorporate 49 approved biological medicines. Regarding recombinant therapeutic enzymes, there are 22 approved medicines. Among the referred biological medicines, there are six biosimilar hormones, and no biosimilars have been approved for recombinant blood products and therapeutic enzymes, which is unexpected.

Current investigations on recombinant hormones, recombinant blood products, and therapeutic enzymes seem to follow the same directions, searching for alternative non-injectable administration routes, development of new recombinant molecules with improved pharmacokinetic properties and discovering new clinical applications for approved medicines. These approaches are showing positive results and new medicines are expected to reach clinical approval in the coming years. Future prospects also include the approval of more biosimilar medicines.

Graphical Abstract



Keywords Anticoagulants, Blood factors, Glucagon, Gonadotropins, Growth hormone, Insulin, Therapeutic enzymes, Thrombolytic agents

1 Introduction

The approval of recombinant human insulin as the first biological medicine in the 1980s marked a new era for medical therapy that brought together the biotechnological and pharmaceutical industries. Biological medicines are often based on recombinant proteins (also named as biopharmaceuticals) and produced in living organisms, including microorganisms, cells, plants, and animals. However, due to cost-effective manufacturing, some biological medicines contain proteins extracted directly from natural sources. Thus far, the number of biological medicines that gained clinical approval is impressive along with an increased number of biosimilars approved. Current therapeutic applications of these medicines are diverse and include active substances from different classes, such as monoclonal antibodies, cytokines, growth factors, hormones, blood products, enzymes, vaccines, and cellular therapies [1–5]. Almost all of these medicines are of parenteral administration, as other routes pose a number of different biological barriers that are yet to be overcome [6].

This chapter focuses on the clinical applications of biological medicines containing hormones, blood products, and therapeutic enzymes. Therapeutic hormones that were initially extracted from natural sources are currently produced through biotechnological techniques (i.e., recombinant-DNA technology), and include insulin and analogs, glucagon and analogs, growth hormone, gonadotropins (follicle-stimulating hormone, luteinizing hormone, and human chorionic gonadotropin), thyroid-stimulating hormone, and parathyroid hormone. In contrast, some blood products are generated by cost-effective methodologies from the natural source. Nonetheless, herein, only recombinant blood products, and particularly blood factors, anticoagulants, and thrombolytic agents, will be addressed. Similarly, some therapeutic enzymes are natural and others are from recombinant origin and have several clinical applications. Each section of this chapter briefly addresses current therapeutic uses of the respective recombinant protein, and further discusses future applications.

2 Hormones

2.1 Insulin

Insulin is composed of 51-amino acids and produced by the β-cells of the islets of Langerhans [7]. The major function of this hormone is regulating the blood glucose levels, but it participates in other metabolic processes, promoting glycogen synthesis in the liver and muscles, fatty acid synthesis in the adipocytes and inhibiting the glycogenolysis and the gluconeogenesis [8–10]. Therapeutic use of insulin is classically for the management of type 1 diabetes, which is an autoimmune metabolic disorder characterized by a deficient or absent production of insulin. Nonetheless, in some cases, insulin can be used for type 2 diabetes, when is observed the production of ineffective insulin [8, 11–13].

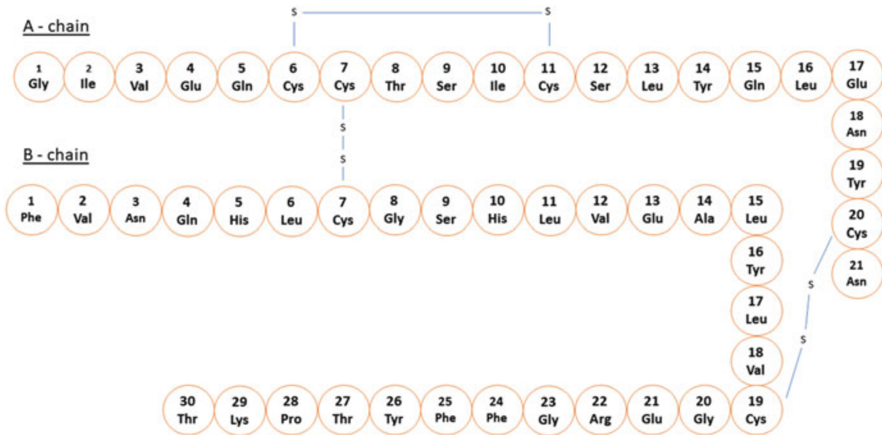


Fig. 1 Schematic representation of the human insulin. *Ala* alanine, *Arg* arginine, *Asn* asparagine, *Cys* cysteine, *Glu* glutamic acid, *Gln* glutamine, *Gly* glycine, *His* histidine, *Ile* isoleucine, *Leu* leucine, *Lys* lysine, *Phe* phenylalanine, *Pro* proline, *Ser* serine, *Thr* threonine, *Tyr* tyrosine, *Val* valine (adapted from [17])

Insulin manufacture started with the advent of recombinant-DNA technology. Before that therapeutic insulin was purified from bovine and porcine pancreas. Nonetheless, the use of animal-derived products presents disadvantages, including immunogenicity, difficulties to obtain enough supplies, and risk of contamination. Furthermore, the insulin amino acids sequence is different among the various species [8, 9, 14–16].

Figure 1 shows a schematic representation of human insulin, which has a dimeric structure formed by two polypeptide chains, A- and B-, linked by disulfide bonds between A^7–B^7, A^{20}–B^{19}, and A^6–A^{11}. A-chain consists of 21 amino acids, whereas B-chain contains 30 amino acids [9, 13, 16, 18, 19].

First human recombinant insulin was approved in 1982 (Humulin®), being the first drug produced by genetic engineering techniques in *Escherichia coli*. Since then, several recombinant insulins have been marketed, mostly produced in *Escherichia coli*, despite some are produced in *Saccharomyces cerevisiae* and *Pichia pastoris* [8, 15, 19–21]. Moreover, the use of genetic engineering techniques allowed the manufacture of different types of recombinant insulin (insulin analogs), changing the amino acid sequences of the native human insulin. These products have different pharmacokinetic and pharmacodynamic profiles and are called fast-acting or short-acting, intermediate-acting, and slow-acting or long-acting insulins. Fast-acting increases the blood insulin concentration more quickly, while slow-acting enters the bloodstream more slowly, but has a longer duration, helping to maintain basal levels of insulin in the blood. Intermediate acting insulin with specific activity was also produced [8, 13, 14, 19, 22].

Table 1 shows examples of marketed biological and biosimilar medicines containing recombinant human insulin and insulin analogs approved by the European Medicines Agency (EMA) and/or the Food and Drug Administration (FDA).

Apart from the medicines mentioned in Table 1, new insulins have been introduced in some countries. For example, Afrezza® (pulmonary insulin) was recently authorized in Brazil [72], and Glaritus® (insulin glargine) is only approved in some Asian and African countries [73].

Concerning insulin biosimilar medicines, the firsts were approved by EMA in 2014 (Abasaglar®) [54] and by FDA in 2015 (Basaglar®) [74, 75]. Later, more biosimilar insulins reached the market and some have been withdrawn (e.g., Solumarv® and Lusduna®) [33, 56].

In addition to regulating blood glucose levels, insulin also increases intestinal cells growth. In this sense, the orphan designation was granted by EMA to recombinant insulin for the treatment of short bowel syndrome, a condition where the body cannot absorb nutrients and fluids due to a missing of part of the small bowel. Thus, insulin can regenerate the intestine of patients with short bowel syndrome, improving the disease symptoms [76].

2.1.1 New Insulin Formulations

Excluding Afrezza®, current approved insulin medicines are for subcutaneous administration, but researchers have been looking for alternative non-invasive routes, improving patient's compliance [9, 77, 78]. The results seem promising and there are several products under clinical studies. For example, MidaForm® PharmFilm, a buccal film containing insulin, is under phase II clinical trials [78, 79]; and IN-105 or tregopil, which is an oral insulin analog (differs from insulin at position B^{29}) with improved stability against enzymatic degradation, is under phase II/III clinical trials [80].

Regarding the subcutaneous administration of insulin, advances in formulations have been made to improve the management of diabetes. In this sense, insulin pumps for subcutaneous implantation have been used. These devices contain a glucose sensor connected to an insulin delivery system that promotes an optimal glycemic control. However, limitations to control the postprandial hyperglycemia were observed. To circumvent this, human insulin rapid-acting analogs have been included in implantable pumps. Currently, the only pump available is the MIP 2007D that is marketed by Medtronic® in Europe. This titanium pump is surgically implanted into the abdominal wall [81–83]. Artificial pancreas or closed-loop system is the most promising insulin device, which consists of a pump and an infusion system with an integrated glucose sensor and a computer that analyzes the blood glucose level and adjusts the insulin flow rate [8, 78, 81, 84]. Recently, more accurate closed-loop devices, which allow the real-time monitoring of interstitial glucose levels, by means of transcutaneous glucose sensors (glucose-oxidase-based electrochemical sensors) and external insulin pumps, have been successfully tested for the management of type 1 diabetes in children. These systems contain an electronic device that measures the blood glucose level and adjusts the insulin infusion rate, maintaining glucose within the normal range [85].

Table 1 Examples of approved biological and biosimilar medicines containing recombinant human insulin and insulin analogs, and respective duration of action and structure

Duration of action	Recombinant protein	Structure	Marketed medicines	References
Short-acting, fast-acting, or rapid-acting	Human insulin	Identical to native human insulin	Humulin®; Humulin®R; Novolin®R; Insuman®; Actrapid®; Velosulin® BR; Exubera®; Insulin human Winthrop®; Afrezza®; Solumarv®[a, b]	[23–33]
	Insulin lispro	Engineered insulin: inversion of B^{28}–B^{29} proline–lysine sequence	Humalog®; Liprolog®; Insulin Lispro Sanofi®[a]; Admelog®[a]	[34–38]
	Insulin aspart	Engineered insulin: B^{28}proline is replaced by aspartic acid	NovoLog®/ NovoRapid®; Fiasp®	[39–41]
	Insulin aspart and insulin aspart protamine		NovoLog Mix®	[42]
	Insulin glulisine	Engineered insulin: B^{29} lysine is replaced by glutamic acid and B^{3} asparagine is replaced by lysine	Apidra®	[43, 44]
Long-acting or slow-acting	Human insulin	Identical to native human insulin	Protaphane®; Monotard®; Ultratard®	[45–47]
	Insulin glargine	Engineered insulin: A^{21} asparagine is replaced by glycine and B-chain elongated by two arginines	Lantus®; Toujeo®; Suliqua®/Soliqua®; Abasaglar®[a]; Lusduna®[a, b]; Semglee®[a]	[21, 48–56]
	Insulin detemir	Engineered insulin: 14 fatty acids are covalently attached to B^{29} lysine and B^{30} threonine is deleted	Levemir®	[57, 58]
	Insulin degludec	Engineered insulin: B^{30} threonine is deleted and B^{29} is coupled to hexadecanedionyl-γ-L-glutamate	Tresiba®; Xultophy®	[59–62]

(continued)

Table 1 (continued)

Duration of action	Recombinant protein	Structure	Marketed medicines	References
Intermediate acting, dual-acting, or long-acting combined with fast-acting	Human insulin	Identical to native human insulin	Actraphane®; Mixtard®; Insulatard®	[63–66]
	Insulin lispro and insulin lispro protamine	Engineered insulin: inversion of B^{28}–B^{29} proline–lysine sequence	Humalog® Mix; Liprolog Mix®	[67, 68]
	Insulin aspart and insulin aspart protamine	Engineered insulin: B^{28} proline is replaced by aspartic acid	NovoMix®	[69]
	Insulin degludec and insulin aspart	Engineered insulin: B^{30} threonine is deleted and B^{29} is coupled to hexadecanedionyl-γ-L-glutamate; B^{28} proline is replaced by aspartic acid	Ryzodeg® 70/30	[70, 71]

[a]Biosimilar medicine
[b]Medicine withdrawn

2.1.2 Insulin Analogs Under Development

Several ultra-fast-acting insulin analogs, showing higher physiological rate than native insulin and fast-acting insulin analogs, have been developed and some are under clinical evaluation [78, 81]. For example, VIAject®/Linjeta® acts faster than insulin lispro, due to the addition of ethylenediaminetetraacetic acid (EDTA) that chelates zinc and solubilizes insulin hexamers [81, 86–88]. Biodel insulin (BIOD-531) is a concentrated formulation of recombinant human insulin with improved post-meal glucose control, compared to Humalog®, due to the presence of EDTA, citrate, and magnesium that promote tissue dispersion of insulin [16, 89]. BioChaperone® Lispro contains insulin lispro and oligosaccharides that promote insulin absorption. Clinical studies with this type of insulin showed an increase of early insulin exposure in 168%, compared to Humalog®, and improved metabolic profile, compared to NovoLog® and Fiasp® [90, 91]. LY900014 which is an ultra-rapid insulin lispro that recently finished phase II clinical trials is waiting for regulatory review [92]. Ultra-fast-acting insulin aspart, containing nicotinamide to promote absorption and arginine as a stabilizer, showed earlier onset of action and exposure, compared to insulin aspart [89, 93, 94].

Another approach used to improve the onset of human insulin analogs involves combining the molecule with recombinant human hyaluronidase [78]. For example, a study in 14 healthy volunteers showed ultra-rapid profiles with a faster onset associated with reducing postprandial hyperglycemia for insulin analogs linked to hyaluronidase [81, 95]. Similar results were observed in another study, where was administered human insulin combined with hyaluronidase to 40 patients with type 1 diabetes [96].

Currently, some companies are conducting clinical trials with new insulins for subcutaneous administration in type 2 diabetes patients. For example, Eli Lilly is evaluating LY3209590, which is an engineered insulin fused to an antibody Fc domain that provides a long-acting basal profile, in comparison to glargine and degludec insulins [97, 98].

2.2 Glucagon

Glucagon is a 29 amino acids polypeptide synthetized by the α-cells of the islets of Langerhans and other related intestinal cells. Its major function is to prevent hypoglycemia, promoting liver glycogenolysis and gluconeogenesis, increasing the blood glucose level. Thereby, glucagon is frequently used to prevent hypoglycemia caused by insulin administration in patients with type 1 diabetes [99, 100]. In addition, glucagon precursors or glucagon analogs produced by intestinal endocrine L-cells have been identified, including glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2). The first has an opposite activity to glucagon, stimulating the β-cells proliferation with consequent insulin secretion and reduction of the blood glucose. In contrast, GLP-2 stimulates intestinal growth while slowing proximal bowel motility and secretion [101–103]. Similar to insulin, initial therapeutic glucagon was obtained directly from porcine and bovine pancreatic tissues, being GlucaGen® the first recombinant glucagon produced in *Saccharomyces cerevisiae* approved by FDA in 1998 [8, 104]. Table 2 shows examples of recombinant glucagon and glucagon analogs approved by EMA and/or FDA.

The pharmacokinetic and pharmacodynamic characteristics of dasiglucagon, a glucagon analog, were evaluated in comparison to GlucaGen®, in children with type 1 diabetes. The results showed increased blood glucose levels for the patients treated with dasiglucagon. From these findings, the authors suggested the use of dasiglucagon for hypoglycemia rescue therapy [122]. However, to the best of our knowledge, there are no marketed medicines available, although the EMA approved this indication in 2018 [123].

2.2.1 New Glucagon Formulations

A glucagon nasal powder formulation was successfully evaluated in phase III clinical trials for use in severe hypoglycemic episodes and is currently waiting for regulatory review. This new medicine is indicated for emergency situations, being the first needle-free treatment for hypoglycemia [97, 124]. A liquid glucagon rescue pen showed positive results in phase III clinical trials, where all the patients achieved optimal blood glucose levels up to 30 min after the administration. Thereby, this new device was suggested as an effective alternative for ready-to-use in severe hypoglycemia in type 1 diabetes patients, as compared to the currently marketed injectable glucagon [125].

Table 2 Examples of recombinant glucagon and glucagon analogs, therapeutic indications, and respective approved biological medicines

Recombinant glucagon	Marketed medicines	Therapeutic indications	References
Glucagon	GlucaGen®; Glucagon for injection™	Severe hypoglycemia in adult patients with diabetes type 1 treated with insulin Inhibition of the gastrointestinal tract motility for radiologic examinations	[104, 105]
	Orphan medicine	Noninsulinoma pancreatogenous hypoglycemia syndrome (excessive growth of pancreas cells with insulin overproduction and hypoglycemia episodes)	[106]
		Congenital hyperinsulinism (inherited disorder where occurs a not needed increase of insulin)	[107, 108]
Glucagon linked to human immunoglobulin Fc fragment			[109]
Liraglutide (GLP-1 receptor agonist)	Saxenda®	Adjunct to chronic weight management for obese and overweight patients undergoing diet and physical exercise	[110, 111]
	Victoza®	Adjunct to diet and exercise to improve glycemic control (stimulate insulin production) in patients with type 2 diabetes	[112, 113]
Semaglutide (GLP-1 receptor agonist)	Ozempic®		[114, 115]
Dulaglutide (GLP-1 receptor agonist)	Trulicity®		[116, 117]
Teduglutide (GLP-2 analogue)	Revestive®(orphan medicine)/Gattex®	Short bowel syndrome	[118, 119]
Apraglutide (GLP-2 analogue)	Orphan medicine		[120]
GLP-2 linked to human immunoglobulin Fc fragment			[121]

GLP-1 glucagon-like peptide 1, *GLP-2* glucagon-like peptide-2

2.3 Growth Hormone

Somatotropin, growth hormone (GH) or human growth hormone plays a major role in the regulation of body growth, cell metabolism, and circadian rhythm. This peptide hormone has 109 amino acids and is secreted by the hypothalamus anterior pituitary gland, in a process regulated by the GH-releasing factor or somatorelin (stimulatory peptide) and the GH-release inhibiting hormone or somatostatin (inhibitory peptide) [8, 126, 127].

GH biological effects include increase of bones, cartilage, and muscles growth, stimulation of protein synthesis, anti-insulin and lipolytic effects, and improved renal function. GH can act directly, binding to specific cell receptors, or indirectly,

binding to liver receptors that promote different growth effects in the body. The latter is mediated by the insulin growth factor-1 (IGF-1), which controls the secretion of GH [8, 126, 127].

Therapeutic use of GH started in the 1950s, being this hormone obtained directly from cadaveric human pituitaries, which presented limitations of safety and available amounts. Only in the 1980s, by means of recombinant DNA technology, was produced the first recombinant GH in *Escherichia coli* [8, 127, 128].

Clinically approved GH is usually indicated for the treatment of children with short stature caused by different conditions, including GH deficiency, Prader–Willi syndrome, Turner syndrome, homeobox-containing gene deficiency, or idiopathic. In addition, GH is used for the management of growth failure in children with chronic renal insufficiency, in children born small for gestational age, metabolism regulation in short bowel syndrome, acquired immunodeficiency syndrome (AIDS)-related cachexia, and for replacement therapy in adults with GH deficiency. There is also a widespread illicit use for athlete body building [8, 127, 129]. Table 3 shows examples of EMA and/or FDA approved medicines containing GH or somatotropin, where it can be seen that, despite all contain recombinant GH, the therapeutic indications of each medicine are not the same.

2.3.1 New Growth Hormone Formulations

New approaches in the development of medicines containing recombinant GH have focused on increasing the drug circulating half-life, reducing the required number of administrations from daily up to weekly or monthly [147]. Examples of formulations under clinical studies include: TransCon (prodrug containing GH bounded to a linker carrier) for weekly administration, which is in phase II (adults) and III (children) [148]; MOD-4023 that is a carboxy-terminal peptide-modified GH for weekly administration, which is in phase III (adults) [149]; GX-H9 (hybrid Fc-fused to GH) for twice-monthly administration that is in phase II (adults and children) [150]; Somapacitan (albumin-binding GH) for weekly administration that is in phase III (adults) [151].

2.4 Gonadotropins

Gonadotropins comprise a family of hormones secreted by gonadotrope cells of the anterior pituitary, including the follicle-stimulating hormone (FSH), luteinizing hormone (LH), and human chorionic gonadotropin (hCG). FSH and LH play a major role in the reproductive function regulation and sexual characteristics, while hCG has a central role during pregnancy. These dimeric hormones are formed by one α-polypeptide subunit of 92 amino acids and one β-polypeptide subunit with 111 FSH, 121 LH, and 145 hCG amino acids [8, 152, 153].

Table 3 Examples of approved biological and biosimilar medicines containing recombinant growth hormone (GH) or somatotropin and respective therapeutic indications

Recombinant GH	Marketed medicines	Therapeutic indications	References
Growth hormone or somatotropin	Humatrope®	Children with short stature associated with GH deficiency, Turner syndrome, homeobox-containing gene deficiency, or idiopathic and born small for gestational age	[130]
	Norditropin®; Tev-Tropin®; Somatropin Biopartners®[a]; Valtropin®[a, b]; Saizen® (orphan medicine)	Long-term treatment of GH deficiency	[131–137]
	Genotropin®; Zomacton®; Omnitrope®[b]	Children with short stature associated with GH deficiency, Turner syndrome, Prader–Willi syndrome, homeobox-containing gene deficiency, or idiopathic and born small for gestational age	[138–141]
	Nutropin AQ®	Children with short stature associated with GH deficiency, idiopathic short stature, Turner syndrome, and chronic kidney disease	[142, 143]
	Zorbtive® (orphan medicine)	Short bowel syndrome in patients receiving nutritional support	[144]
	Serostim®	HIV patients with wasting or cachexia	[145, 146]

[a]Medicine withdrawn
[b]Biosimilar medicine

In men, FSH is responsible for sperm production, targeting the testis Sertoli cells and regulating the early stages of spermatogenesis. In women, FSH stimulates the ovarian follicle maturation, participates in the synthesis of estrogen and glycosaminoglycans, and regulates the reproductive function. LH stimulates the women ovulation of mature follicles, and (together with FSH) participates in the conversion of androgens to estrogens. In men, LH is involved in the testosterone production and sperm maturation. In contrast, the hCG is produced by pregnant women, having an important function during the early phase of pregnancy [8, 154, 155].

Gonadotropin hormones have been used in assisted reproductive therapies and in the treatment of several women infertility disorders. For example, to induce or stimulate ovulation for support of natural conception or intrauterine insemination, and to induce multifollicular growth required for in vitro fertilization procedures. In men, FSH and hCG stimulate sperm synthesis and are used for the management of hypogonadotropic hypogonadism. There is also an illicit use of hCG by athletes to

stimulate testosterone production. First therapeutic FSH and LH were extracted from women menopausal urine, while hCG was obtained from the urine of pregnant women [8, 152, 155, 156]. Owing to the increase on the number of fertility treatments, the quantities of urinary gonadotropins available have been scarce. For this reason, recombinant gonadotropins (or gonadotropins analogs) have been produced in mammalian cell lines, such as Chinese hamster ovary (CHO) cells, among others. Nonetheless, urinary gonadotropins remain in clinical use [155, 156]. Table 4 shows examples of recombinant gonadotropins approved by EMA and/or FDA, respective therapeutic indications, and biological and biosimilar medicines.

From Table 4, it can be seen that only recombinant FSH has approved biosimilar medicines, although a recombinant hCG biosimilar was recently evaluated to induce ovulation in patients undergoing intrauterine dissemination. The results demonstrated clinical equivalence to Ovitrelle®, suggesting the potential of using this biosimilar as an alternative to the reference medicine [171].

Concerning the clinical applications of gonadotropins, there is an open field for therapeutic uses, including the treatment of polycystic ovary syndrome, management

Table 4 Examples of approved biological and biosimilar medicines containing recombinant gonadotropins (*FSH* follicle-stimulating hormone, *LH* luteinizing hormone, *hCG* human chorionic gonadotropin) and respective therapeutic indications

Recombinant gonadotropins	Marketed medicines	Therapeutic indications	References
Follitropin alfa (FSH)	Gonal-f®; Ovaleap®[a]; Bemfola®[a]	Women: induction of ovulation and pregnancy in anovulatory infertile patients; development of multiple ovulatory follicles in patients undergoing fertility treatments; patients with severe deficiencies of LH and FSH Men: stimulation of spermatogenesis in congenital or acquired hypogonadotropic hypogonadism	[157–160]
Follitropin beta (FSH)	Puregon®/ Follistim®; Fertavid®		[161–163]
Follitropin delta (FSH)	Rekovelle®	Development of multiple ovulatory follicles in women undergoing fertility treatments: in vitro fertilization or intracytoplasmic sperm injection	[164]
Corifollitropin alfa (long-acting FSH)	Elonva®		[165]
Lutropin alfa (LH)	Luveris®	Induction of ovulation in women with severe LH and FSH deficiencies	[166, 167]
Follitropin alfa/ lutropin alfa (FSH/LH)	Pergoveris®	Women with FSH and LH deficiencies: induction of ovulation (FSH) and eggs release (LH)	[168]
Choriogonadotropin alfa (hCG)	Ovitrelle®/ Ovidrel®	Women treated with LH and FSH to stimulate ovaries: trigger ovulation and development of the corpus luteum to support pregnancy Women undergoing fertility treatments (in vitro fertilization) Anovulatory or oligo-ovulatory women	[169, 170]

[a]Biosimilar medicine

of ovary and prostate cancers, prevention of postmenopausal symptoms (avoidance of bone loss and weight gain), and as contraceptives [156].

2.4.1 New Gonadotropins Formulations

Recombinant gonadotropins have been used clinically in fertility treatments, by means of injectable formulations. Nonetheless, some limitations have been pointed to these medicines, regarding the necessity of performing multiple administrations, lack of stability, and adverse effects, such as ovarian hyperstimulation syndrome. Researches have been focused in the development of long-acting recombinant gonadotropins with improved stability, achieving appropriate pharmacokinetic profiles and minimizing the number of administrations [156]. Several studies showed that corifollitropin alfa, a long-acting FSH analog, reduces the drawbacks of the in vitro fertilization treatments (Table 4) [172, 173]. In this sense, other strategies have been studied to develop more long-acting recombinant FSH molecules. Examples include the addition of glycosylated peptides or polyethylene glycol – PEG (PEGylation) to the gonadotropin molecule, and fusion of the gonadotropin with an immunoglobulin Fc domain (fusion protein) [153, 156].

2.5 Other Recombinant Hormones

In addition to insulin, glucagon, growth hormone, and gonadotropins there are other recombinant hormones under clinical use, which are the thyroid-stimulating hormone (TSH) and the parathyroid hormone (PTH). TSH or thyrotropin has a molecular structure that resembles gonadotropins, containing one β-subunit and one α-subunit. TSH is produced by the anterior pituitary and targets the thyroid gland, stimulating its functions, including iodine uptake, production of the iodine containing thyroid hormones (iodothyronines) triiodothyronine (T3) and thyroxine (T4), and promotion of thyroid growth. Furthermore, TSH participates in the prevention of thyroid cells apoptosis and in thyroid ontogenesis. Recombinant TSH produced in CHO cells has been used for the diagnostic of thyroid cancer and for the detection of thyroid remnants in post-thyroidectomy patients [8, 174]. PTH or human PTH is an 84 amino acids polypeptide that regulates extracellular phosphate and calcium metabolism. In bone, PTH has a catabolic effect, stimulating osteoblasts for bone formation. In kidneys, PTH promotes the synthesis of vitamin D that increases the intestinal calcium absorption and inhibits the renal phosphate reabsorption. Thereby, recombinant hPTH produced in *Escherichia coli* was approved for the management of osteoporosis in women post-menopausal and in men and for chronic hypoparathyroidism [8, 175]. Table 5 shows examples of

recombinant TSH and PTH approved by EMA and/or FDA, respective therapeutic indications, and biological and biosimilar medicines.

In addition to Table 5, other formulations containing recombinant PTH have been investigated. For example, TransCon PTH has successfully completed phase I clinical trials to treat hypoparathyroidism [184].

Current approved medicines containing PTH are for subcutaneous administration, which reduces patient compliance. Nasal and transdermal routes have been suggested as alternative routes for the administration of PTH in osteoporosis treatments. The results of in vitro studies are promising, although in vivo studies are required to confirm this application [185, 186].

3 Blood Products

Blood products of therapeutic interest are proteins that can be extracted directly from the natural source, i.e., from the blood red and white cells, platelets, and plasma. However, due to the large amount required for clinical use and some safety concerns, several therapeutic blood proteins have been produced by genetic engineering techniques, including recombinant blood factors, anticoagulants, and thrombolytic agents [187, 188]. Concerning the scope of this chapter, we focus only in recombinant blood products.

3.1 Blood Clotting Factors or Coagulation Factors

Blood clotting factors (or blood factors) have been used for the treatment of hemophilia, which is a rare bleeding inherited disorder that reduces the normal

Table 5 Examples of approved biological and biosimilar medicines containing recombinant thyroid-stimulating hormone (TSH) and parathyroid hormone (PTH) and respective therapeutic indications

Recombinant TSH and PTH	Marketed medicine	Therapeutic indications	References
Thyrotropin alfa (TSH)	Thyrogen®	Thyroid cancer: detection of thyroid tissue left after surgery; elimination of remaining thyroid tissue (in combination with radioactive iodine) in patients who removed the thyroid gland	[176, 177]
Teriparatide (PTH)	Forsteo®/Forteo®; Movymia®[a]/ Terrosa®[a]	Osteoporosis in postmenopausal women and in men with increased risk of fracture	[178–181]
PTH	Natpara®/ Natpar®(orphan medicine)	Hypocalcemia control in patients with chronic hypoparathyroidism	[182, 183]

[a]Biosimilar medicine

blood clotting process and causes severe consequences. People with this disease bruise very easily and show difficulty in blood coagulation after trauma, increasing the bleeding time. There are 12 different blood clotting factors and several cofactors that play a key role in the blood coagulation process, particularly, in the blood coagulation cascade. A genetic defect in the expression of blood factors (or anti-hemophilic factors) originates hemophilia, which is divided into subtypes A, B, and C. In hemophilia A (or classical hemophilia that occurs in about 90% of the cases) the blood clotting factor VIII is deficient or abnormal, whereas in hemophilia B the blood clotting factor IX is deficient or abnormal [188–191]. Both blood factors VIII and IX play vital roles in the coagulation cascade, being essential for the conversion of prothrombin into thrombin. Afterwards, thrombin converts fibrinogen to fibrin, a primordial fibrous protein of the blood coagulation process. Hemophilia C is a rare type that is characterized by a deficient or abnormal blood clotting factor XI [191–193].

Hemophilia treatment is performed by supplying the impaired blood clotting factor, which was firstly obtained directly from the blood donors. However, this process showed disadvantages related to the risk of virus infection that originates severe diseases, such as AIDS, hepatitis C and B, and others. Therefore, recombinant blood clotting factors, produced by DNA recombinant techniques in CHO cells, are used for the management of hemophilia [188, 189, 191]. In addition, some companies are conducting clinical trials with gene therapy products for the treatment of hemophilia A and B, which may be better than the chronic treatments with weekly injections of recombinant blood clotting factors. Examples of such companies are: BioMarin® [194], Sangamo Therapeutics [195], and uniQure [196].

Table 6 shows examples of recombinant blood products (blood factors, anticoagulants, and thrombolytic agents), therapeutic indications, and names of the respective approved biological and biosimilar medicines, by EMA and/or FDA.

Table 6 Examples of recombinant blood products (blood clotting factors, anticoagulants, and thrombolytic agents), therapeutic indications, and respective approved biological and biosimilar medicines

Recombinant blood products	Marketed medicines	Therapeutic indications	References
Blood clotting factors			
Eptacog alfa (factor VIIa)	Novoseven®; Novoseven RT®; Niastase®; Niastase RT®	Hemophilia A and B; factor VII deficiency; Glanzmann's thrombasthenia	[197, 198]
Octocog alfa (factor VIII)	Advate®; Bioclate®; Helixate FS®; Kogenate FS®; Kovaltry®; Recombinate®; Iblias®	Hemophilia A	[199–204]
Turoctocog alfa (factor VIII)	NovoEight®; Zonovate®	Hemophilia A	[199, 205]
Lonoctocog alfa (factor VIII)	Afstyla®	Hemophilia A	[199, 206]

(continued)

Table 6 (continued)

Recombinant blood products	Marketed medicines	Therapeutic indications	References
Susoctocog alfa (factor VIII)	Obizur®	Hemophilia A	[207]
Rurioctocog alfa pegol (factor VIII)	Adynovi®	Hemophilia A	[208]
Damoctocog alfa pegol (factor VIII)	Jivi®	Hemophilia A	[209]
Efmoroctocog alfa (factor VIII linked to Fc fusion protein)	Eloctate®/Elocta®	Hemophilia A	[199, 210]
Moroctocog alfa (factor VIII)	Refacto AF®; Xyntha®	Hemophilia A	[199, 211]
Simoctocog alfa (factor VIII)	Nuwiq®; Vihuma®	Hemophilia A	[199, 212, 213]
Pegylated factor VIII (factor VIII linked to PEG)	Adynovate®	Hemophilia A	[199]
Vonicog alfa (von Willebrand factor)	Veyvondi®	von Willebrand disease	[214]
Eftrenonacog alfa (factor IX linked to Fc fusion protein)	Alprolix® (Orphan medicine)	Hemophilia B	[210, 215]
Nonacog alfa (factor IX)	BeneFix®	Hemophilia B	[216]
Nonacog gamma (factor IX)	Rixubis®	Hemophilia B	[217]
Nonacog beta pegol (factor IX linked to PEG)	Refixia®	Hemophilia B	[218]
Albutrepenonacog alfa (factor IX linked to albumin)	Idelvion® (Orphan medicine)	Hemophilia B	[219, 220]
Factor IX	IXINITY®, RIXUBIS®	Hemophilia B	[197]
Andexanet alfa (factor Xa, inactivated-zhzo)	ANDEXXA®	Reversal of anticoagulation for patients treated with rivaroxaban and apixaban	[221, 222]
Catridecacog (factor XIIIa)	TRETTEN®; NovoThirteen®	Congenital factor XIIIa subunit deficiency	[223, 224]
Anticoagulants			
Lepirudin	Refludan®[a]	Heparin-induced thrombocytopenia type II and thromboembolic disease	[225, 226]
Desirudin	Revasc®/Iprivask®[a]	Prevention of thrombosis in patients undergoing hip or knee replacement	[2, 227, 228]

(continued)

Table 6 (continued)

Recombinant blood products	Marketed medicines	Therapeutic indications	References
		surgery, and for pulmonary embolus	
Antithrombin alfa	ATryn®	Prevention of thromboembolic events in congenital antithrombin deficiency	[229, 230]
Drotrecogin alfa	Xigris®[a]	Reduce risk of blood clots during sepsis by inhibition of clotting factors Va and VIIIa	[2, 231]
Thrombolytic agents			
Alteplase (tissue plasminogen activator)	Actilyse®; Cathflo® Activase®	Management of acute myocardial infarction	[232, 233]
Reteplase	Ecokinase®[a]; Retavase®; Rapilysin®		[234–236]
Tenecteplase	Metalyse®; Tenecteplase Boehringer Ingelheim Pharma GmbH Co. KG®[a]; TNKase®		[237–239]

[a]Medicine withdrawn; *PEG* polyethylene glycol

Recombinant human coagulation factor VIIa or eptacog alfa is a vitamin K-dependent glycoprotein that activates factor IX and factor X, which are essential for hemostasis. Thus, eptacog alfa is involved in the clotting process initiation and controls bleeding disorder, being used for the treatment of patients with hemophilia A and B, congenital factor VII deficiency, and Glanzmann's thrombasthenia (a rare bleeding disorder) [198, 240, 241]. Recently, Biron-Andreani and Schved revised the pharmacodynamics and pharmacokinetics data of eptacog beta, a new type of recombinant factor VIIa produced in the milk of transgenic rabbits. From their search the authors concluded that, when compared to eptacog alfa, eptacog beta requires a lower dose to obtain the same in vivo effect, being a less expensive alternative for the management of hemophilia [242].

Recombinant human coagulation factor VIII is used for the treatment of patients with congenital factor VIII deficiency (hemophilia A). Biological and biosimilar medicines available are shown in Table 6 and include octocog alfa, turoctocog alfa, lonoctocog alfa, susoctocog alfa, simoctocog alfa, rurioctocog alfa pegol, and damoctocog alfa pegol [200–209, 212, 213, 241, 243–248]. Efmoroctocog alfa is a recombinant factor VIII-Fc fusion protein used as an antihemorrhagic agent for the treatment and prophylaxis of acute bleeding episodes in patients with hemophilia A. The addition of the Fc protein (B-domain) to the recombinant factor VIII extended the drug half-life, compared to the recombinant factor VIII alone [191, 192, 210, 241, 247]. Pegylated factor VIII contains the human recombinant

factor VIII covalently linked to a PEG molecule, which increases the circulation half time, improving therapeutic efficacy [249, 250].

Vonicog alfa is a recombinant von Willebrand factor used to control bleeding in patients with von Willebrand disease (an inherited bleeding disorder), when desmopressin is not effective [214, 251]. The hemostatic efficacy of using vonicog alfa alone or in combination with recombinant factor VIII, in patients with severe von Willebrand disease that are undergoing surgery, was evaluated in a phase III clinical study. Researchers observed that the use of vonicog alfa alone originated hemostasis 6 h after the administration and the effect lasted from 72 up to 96 h. From these findings the authors concluded that, according to each patient risk factors, the treatment of von Willebrand disease should be performed with vonicog alfa alone or in combination with recombinant factor VIII [252].

Eftrenonacog alfa is a recombinant fusion protein containing the human factor IX covalently linked to the constant region (Fc) domain of the human immunoglobulin (IgG1), which is used for the treatment and prophylaxis of bleeding episodes in patients with hemophilia B. This molecule restores the levels of factor IX, promoting normal blood coagulation. Adding the Fc domain to factor IX increases its half-life, improving the bioavailability and, consequently, the therapeutic efficacy [193, 215, 241]. Albutrepenonacog alfa is a recombinant fusion protein that comprises human factor IX linked to albumin, which is indicated for patients with hemophilia B for the control and prevention of bleeding episodes [219, 220]. Nonacog alfa, nonacog gamma, and nonacog beta pegol are also recombinant human factor IX used for the treatment of hemophilia B [188, 197, 216–218].

FDA approved the recombinant coagulation factor Xa, inactivated-zhzo, or andexanet alfa to reverse the effects of factor Xa inhibitors, apixaban and rivaroxaban, when an anticoagulation effect is required [222]. Some studies suggested the use of andexanet alfa for reversing the effect of edoxaban, which is another factor Xa inhibitor, but this indication has not been approved yet [221, 253].

Patients with deficient or abnormal coagulation factor XIII can be treated with recombinant human factor XIIIa, also known as catridecacog [223, 224, 241, 254]. Recently, Sottilotta et al. performed a clinical study where was observed that the use of catridecacog is effective for continued prophylaxis and for conducting major surgical procedures in patients with congenital factor XIII deficiency [255].

3.2 Anticoagulants

Thrombus formation occurs by changes in the blood coagulation process within the vessels and causes serious health problems or even death. Anticoagulants are able to extend the coagulation cascade length and have been used to treat and prevent embolic events or thrombotic disorders, avoiding the changes in the blood clotting process [188, 256].

Heparin, dicoumarol, warfarin, and hirudin are the most studied anticoagulants. Heparin was first extracted from the liver, but current marketed medicines contain

enoxaparin sodium (low molecular weight heparin) obtained by alkaline depolymerization of the heparin benzyl ester that is extracted from porcine gastric mucosa. This heparin derivative activates the antithrombin III and, thus, inhibits the clotting factors Xa and IIa, being used to prevent and treat vein thrombosis, acute coronary syndromes, pulmonary embolism and for the prophylaxis of ischemic complications (angina and myocardial infarction). EMA and FDA approved biological (Lovenox®, Clexane®, Clexane T®, Clexane Forte®, Klexane®, Qualiop®, Enoxaparin Sanofi®, and Enoxaparine Sanofi®) and biosimilar (Inhixa® and Thorinane®) medicines containing enoxaparin sodium to prevent and treat conditions associated with blood clots. These include deep vein thrombosis (usually in the legs), unstable angina (related to impaired blood flow to the heart), and certain types of myocardial infarction or heart attack [241, 257–261]. When compared to recombinant biological medicines (Table 6), heparin and derivatives are less expensive. Nonetheless, disadvantages have been pointed, related to the risk of severe side effects, including bleeding and thrombocytopenia [262].

Dicoumarol and warfarin are obtained by chemical synthesis and will not be referred, because they are not biological medicines. Hirudin is a natural small polypeptide that exists in the saliva of bloodsucking parasites (*Hirudo medicinalis*) and has anticoagulants properties, binding and inhibiting thrombin [188, 256, 263, 264]. Regarding the small amount of hirudin available for removal from the natural source, lepirudin (Table 6), a recombinant hirudin variant-1, has been produced in *Saccharomyces cerevisiae*, and is indicated for preventing thrombus or clot formation. Nonetheless, this medicine is not currently available [188, 225, 226, 265, 266]. El-Mowafi et al. conducted a clinical trial where was observed that topically administered recombinant hirudin gel is effective and safe for the management of symptomatic hematomas [267]. Similar products have been produced by recombinant techniques. For example, desirudin produced in *Saccharomyces cerevisiae* was approved by EMA and FDA (Table 6) as a selective thrombin inhibitor that prevents deep venous thrombosis (in patients undergoing elective hip or knee replacement surgery) and decreases the risk of pulmonary embolus, but is not currently available [188, 227, 228].

Antithrombin is a plasma native inhibitor of the coagulation process that binds to thrombin (factor IIA), factor IXa and Xa, and interrupts the coagulation cascade. Antithrombin alfa (Table 6) was the first biological medicine produced in transgenic animals, being obtained from the milk of genetically modified goats [188, 268, 269]. Antithrombin alfa is used for the prevention of thromboembolic events in patients with congenital low levels of antithrombin [230]. Drotrecogin alfa (Table 6) is similar to the activated human protein C that inhibits the clotting factors Va and VIIIa and reduces the blood coagulation process, and was approved to avoid excessive blood clotting during severe sepsis, but is not currently available [188, 231].

3.3 Thrombolytic Agents

Thrombolytic agents or fibrinolytics convert zymogen plasminogen (glycoprotein synthesized by the kidneys) into plasmin, which is an active enzyme that promotes the proteolytic degradation of fibrin, dissolving blood clots. There are several recombinant thrombolytic agents used for the treatment of thromboembolic disease, including alteplase, reteplase, tenecteplase, and urokinase (Tables 6 and 7) [188, 191, 269, 300, 301].

Alteplase (Tables 6 and 7) is a human recombinant tissue plasminogen activator used for the management of acute myocardial infarction or acute stroke [232, 302]. Tenecteplase (Tables 6 and 7) and reteplase (Tables 6 and 7) are also recombinant forms of the human tissue plasminogen activator that have been indicated for the treatment of patients suspected or after having a heart attack. This enzyme acts by dissolving the clots formed inside the vessels, improving the blood flow into the heart. Compared to the other thrombolytic agents, reteplase has been showing better clinical results, due to its longer half-life [234–239, 303].

4 Therapeutic Enzymes

Concerning some of their properties, enzymes play an important role in the pharmaceutical field. For example, in diagnostic assays, due to the high affinity and specificity to bind targets, and for the management of several diseases and disorders. Some therapeutic enzymes are extracted from the natural source, while others have been produced through recombinant-DNA techniques. The latter have been originating higher yields, regarding the possibility of producing larger quantities of pure enzymes [188, 273, 304]. Nonetheless, when therapeutic enzymes can be easily withdrawn from the natural source, the production of similar recombinant enzymes is not required, which reduces costs of the final product. Examples of therapeutic enzymes obtained from natural sources include: asparaginase derived from *Escherichia coli* (Elspar®), which is used for the management of acute lymphoblastic leukemia [305]; collagenase *Clostridium histolyticum* (Xiapex®) extracted from the bacterium *Clostridium histolyticum*, which is used to break up collagen in patients suffering from Dupuytren's contracture and Peyronie's disease [306]. Digestive enzymes including lipases, proteases, and amylases (pancrelipase) that are used to treat pancreatic insufficiency, caused by cystic fibrosis or chronic pancreatitis (Creon®, Pancreaze®, Zenpep®, Pertzye®, Viokace®), are extracted from pig pancreas [307–311]. Nonetheless, concerning the aim of this chapter, only recombinant therapeutic enzymes are described.

Therapeutic enzymes obtained through biotechnological processes are widely used for several clinical applications (Fig. 2), often in enzyme replacement therapy, when the native enzyme is lacking, and as adjuvants to the management of severe diseases and disorders. Table 7 shows examples of EMA and/or FDA approved medicines containing recombinant enzymes, respective therapeutic indications, and mechanism of action.

Table 7 Examples of recombinant enzymes and respective approved biological medicines, therapeutic indications, and mechanism of action

Recombinant enzymes	Marketed medicines	Therapeutic indications	Mechanism of action	References
Alfagalsidase (alpha-galactosidase A)	Replagal®; Fabrazyme®	Fabry's disease: absent/insufficient alpha-galactosidase A that breaks down the globotriaosylceramide, which is a fatty compound that accumulates in the body and affects the nervous system, vascular endothelial cells, and major organs	Catalyzes the hydrolysis of globotriaosylceramide, reducing the body accumulation	[270–272]
Alteplase (tissue plasminogen activator)	Actilyse®; Cathflo® Activase®	Acute ischemic stroke or acute myocardial infarction	Converts plasminogen into plasmin, dissolving the blood clots responsible for the blockage of the coronary arteries	[232, 235, 273, 274]
Asparaginase (L-asparaginase)	Kidrolase®; Oncaspar®; Spectrila®	Acute lymphoblastic leukemia	Catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia Asparagine is fundamental for cancer cells proliferation and a reduction on its blood levels originates death of cancer cells. Normal cells are able to produce asparagine and, thus, are not affected by the administration of asparaginase	[273, 275–277]
Pegylated Erwinia chrysanthemi L-asparaginase	Erwinaze® (orphan medicine)	Acute lymphoblastic leukemia in patients who have developed hypersensitivity to native *E. coli* derived asparaginase	Catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia Asparagine is fundamental for cancer cells proliferation and a reduction on its blood levels originates death of cancer cells. Normal cells are able to produce asparagine and, thus, are not affected by the administration of asparaginase PEGylation reduces the clearance of asparaginase from the body	[273, 278, 279]

(continued)

Table 7 (continued)

Recombinant enzymes	Marketed medicines	Therapeutic indications	Mechanism of action	References
Asparaginase (L-asparaginase)	Graspa®[a]	Acute lymphoblastic leukemia	Catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia Asparagine is fundamental for cancer cells proliferation and a reduction on its blood levels originates death of cancer cells. Normal cells are able to produce asparagine and, thus, are not affected by the administration of asparaginase Contains asparaginase encapsulated in erythrocytes for increasing its circulation time and protection against blood degradation and immunological reactions	[280]
	Orphan medicine	Pancreatic cancer and acute myeloid leukemia		[281, 282]
Dornase alfa (deoxyribonuclease – DNase)	Pulmozyme®	Cystic fibrosis: retention of purulent and viscous secretions that affect lung function and cause infections	Catalyzes the cleavage of the phosphodiester linkages of the mucus DNA. This process reduces mucus viscosity and promotes secretions clearance	[2, 273, 283]
Elosulfase alfa (N-acetylgalactosamine-6-sulfatase)	Vimizim® (orphan medicine)	Mucopolysaccharidosis type IVA: lack of N-acetylgalactosamine-6-sulfatase, which breaks down glycosaminoglycans. Accumulation of glycosaminoglycans causes short bones, difficulty moving and difficulty breathing, clouding vision and hearing loss	Enzyme replacement therapy, breaking down glycosaminoglycans that stop building up in cells	[284, 285]
Galsulfase (N-acetylgalactosamine 4-sulfatase)	Aryplase®/ Naglazyme® (orphan medicine)	Mucopolysaccharidosis type VI: lack of N-acetylgalactosamine 4-sulfatase that breaks down glycosaminoglycans. Body accumulation of glycosaminoglycans originates macrocephaly, short body, difficult moving, impaired vision and hearing loss, reduced pulmonary function, cardiac abnormalities, etc.	Enzyme replacement therapy. Galsulfase is taken up by cells into lysosomes and catalyzes the cleavage of sulfate ester from terminal N-acetylgalactosamine 4-sulfate residues of glycosaminoglycan chondroitin 4-sulfate and dermatan sulfate, which increases the catabolism of glycosaminoglycans	[273, 286, 287]

Idursulfase (iduronate-2-sulfatase)	Elaprase® (orphan medicine)	Hunter syndrome or mucopolysaccharidosis type II: disease caused by insufficient levels of the enzyme iduronate-2-sulfatase. Patients are not able to degrade glycosaminoglycans, which accumulate in cells, originating difficulty breathing and difficulty walking	Enzyme replacement therapy. Cleaves the terminal 2-O-sulfate moieties from glycosaminoglycans dermatan sulfate and heparan sulfate, increasing the catabolism of glycosaminoglycans	[288, 289]
Laronidase (α-L-iduronidase)	Aldurazyme®	Non-neurological symptoms of mucopolysaccharidosis type I: α-L-iduronidase enzyme deficiency that promotes the body accumulation of glycosaminoglycans, causing several dysfunctions, including enlarged liver, difficult moving, reduced lung, and heart and eye diseases	Enzyme replacement therapy. Catalyzes the hydrolysis of the terminal α-L-iduronic acid residues from the glycosaminoglycans dermatan sulfate and heparin sulfate, which increases the catabolism of glycosaminoglycans	[290, 291]
Imiglucerase (glucocerebrosidase)	Cerezyme®	Type 1 and 3 Gaucher disease: lack of glucocerebrosidase or acid beta-glucosidase, an enzyme responsible for the degradation of the fatty waste product glucosylceramide, which builds up in the liver, spleen, and bone marrow. Typical disease symptoms include enlarged spleen and liver, anemia, thrombocytopenia, and bone disease	Enzyme replacement therapy. Catalyzes the hydrolysis of glucosylceramide to glucose and ceramide, stopping it building up in the body	[292, 293]
Velaglucerase alfa (glucocerebrosidase)	VPRIV® (orphan medicine)	Type 1 Gaucher disease: glucocerebrosidase deficiency that affects the liver, spleen, and bones. Originates liver malfunction, skeletal disorders, bone lesions, neurological complications, anemia, and low blood platelet count		[294, 295]
Rasburicase (urate oxidase)	Elitek®; Fasturtec®	Acute hyperuricemia: high levels of uric acid in the blood. Avoid kidney failure in patients undergoing chemotherapy	Catalyzes enzymatic oxidation of uric acid to allantoin, which is easily eliminated by the kidneys	[296, 297]

(continued)

Table 7 (continued)

Recombinant enzymes	Marketed medicines	Therapeutic indications	Mechanism of action	References
Velmanase alfa	Lamzede® (orphan medicine)	Mild to moderate alpha-mannosidosis: congenital absence of the enzyme alpha-mannosidase that breaks down glycosides. Body accumulation of oligosaccharides originates failure of body functions, such as breathing and movement	Enzyme replacement therapy. Degradation of glycoproteins, avoiding tissue accumulation of oligosaccharides	[298, 299]

[a]Medicine withdrawn

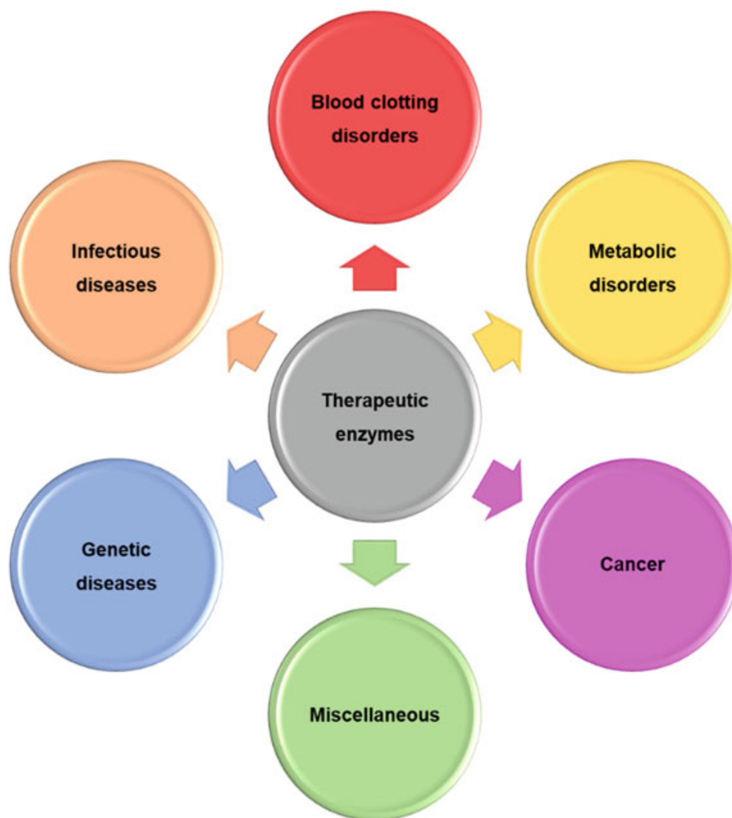


Fig. 2 Main clinical uses of therapeutic enzymes [273, 304]

5 Conclusion

From this review, we can conclude that biological medicines are currently a well-established therapeutic area, enabling the treatment of various incurable diseases.

Concerning the three different therapeutic groups addressed in this chapter, the hormones are the ones with highest number of marketed products (78 approved medicines), where 38 are insulin and analogs, 9 are glucagon and analogs, 12 contain GH, 12 contain different gonadotropins, 1 contains TSH, and 6 incorporate PTH. Furthermore, insulin has 4 biosimilars, 2 of which have been withdrawn and 1 has orphan designation. In contrast, no biosimilar glucagon was approved, which is surprising, as the first recombinant glucagon was approved in 1998. The same is observed for GH, which has only the first biosimilar medicine approved. Two orphan designations and 2 withdrawn medicines were noticed for GH, while gonadotropins have 2 approved biosimilars.

There are 49 approved biological medicines incorporating recombinant blood products, where 35 are blood factors (2 orphan designations), 5 are anticoagulants (3 withdrawn), and 9 are thrombolytic agents (2 withdrawn). None biosimilar has been approved for recombinant blood products, which is unexpected since they reached the market in the 1990s. Similarly, from the 22 approved therapeutic enzymes, none is a biosimilar, 3 are orphan medicines, and 1 was withdrawn from the market.

Current investigations on recombinant hormones, recombinant blood products, and therapeutic enzymes seem to follow the same directions, searching for alternative non-injectable administration routes, development of new recombinant molecules with improved pharmacokinetic properties and discovering new clinical applications for the approved medicines. These approaches are showing positive results and new medicines are expected to reach clinical approval in the coming years. Future prospects also include the approval of more biosimilar medicines.

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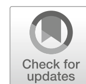
Advances in Vaccines



Helen H. Mao and Shoubai Chao

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Abstract Vaccines represent one of the most important advances in science and medicine, helping people around the world in preventing the spread of infectious diseases. However, there are still gaps in vaccination programs in many countries. Out of 11.2 million children born in EU region, more than 500,000 infants did not receive the complete three-dose series of diphtheria, pertussis, and tetanus vaccine before the first birthday. Data shows that there were more than 30,000 measles cases in the European region in recent years, and measles cases are rising in the USA. There are about 20 million children in the world still not getting adequate coverage of basic vaccines. Emerging infectious diseases such as malaria, Ebola virus disease, and Zika virus disease also threaten public health around the world. This chapter provides an overview of recent advances in vaccine development and technologies, manufacturing, characterization of various vaccines, challenges, and strategies in vaccine clinical development. It also provides an overview of recently approved major vaccines for human use.

Graphical Abstract

Keywords Characterization, Dengue vaccine, Ebola vaccine, Recombinant technology, Shingles vaccine, Vaccine clinical trials, Vaccine development, Vaccine manufacturing, Viral vaccine

1 Introduction

Vaccines represent one of the best advances in science and medicine, helping people around the world in eliminating and preventing the spread of infectious diseases. Although many human vaccines have been developed and are in use, infectious diseases are still threats to people's health, especially during epidemic outbreaks. During the 2003 SARS outbreaks in Asia, there were more than 8,000 cases, and more than 800 deaths occurred, and the economic impact of SARS exceeded US$50 billion, according to the World Health Organization (WHO) report [1]. During the largest Ebola outbreak in West Africa in 2014–2015, more than 28,000 cases were reported, and there occurred more than 11,000 fatalities. It is estimated that Guinea, Liberia, and Sierra Leone have endured more than US$2 billion loss in economic growth as a result of Ebola virus disease outbreaks [2]. Based on the experiences from West Africa Ebola epidemic outbreaks, the WHO published a prioritized list of 11 pathogens that likely to cause outbreak situations, including Ebola virus, Lassa virus, Marburg virus, MRSA, Zika virus, etc. [3]. Some of these epidemic infectious disease vaccines are currently under development. As pointed out by the WHO, it will be a common goal for all countries to provide equitable access to high-quality, safe, affordable vaccines and immunization services throughout the life course.

1.1 Most Effective Tools in Controlling Infectious Diseases

Vaccines have a long list of achievements in the past. Vaccines have been tremendously beneficial in protecting individuals and communities from serious infectious diseases and reducing healthcare costs around the world. In developed countries, vaccines are more readily available to infants, children, and adults. In the European region, more than 90% of children receive at least basic vaccination during infancy [4]. In China, overall vaccination rates for basic vaccines that are recommended to infants and children exceeded 90%, according to data published by the Chinese Center for Diseases Control and Prevention (CCDC) [5].

In the USA, the Advisory Committee on Immunization Practices (ACIP) recommends routine vaccination programs, and each vaccine is approved by the regulatory agency US FDA. The immunization schedules are published by US CDC for different age groups including infants and children, adolescents, and adults. For example, the immunization schedule for infants from birth to 24 months includes vaccines against 14 potentially serious illnesses. US CDC data from the 2017 National Immunization Survey-Child (NIS-Child) database show that the vaccination rates are higher than 80% in the USA nationally [6].

Huge progress in vaccination has been made in the last 30 years. Now about 85% of children worldwide (about 116 million) receive essential, lifesaving vaccines, protecting them from infectious diseases including measles, diphtheria, tetanus, pertussis, hepatitis B, and polio. As vaccination rates increase from about 20% in

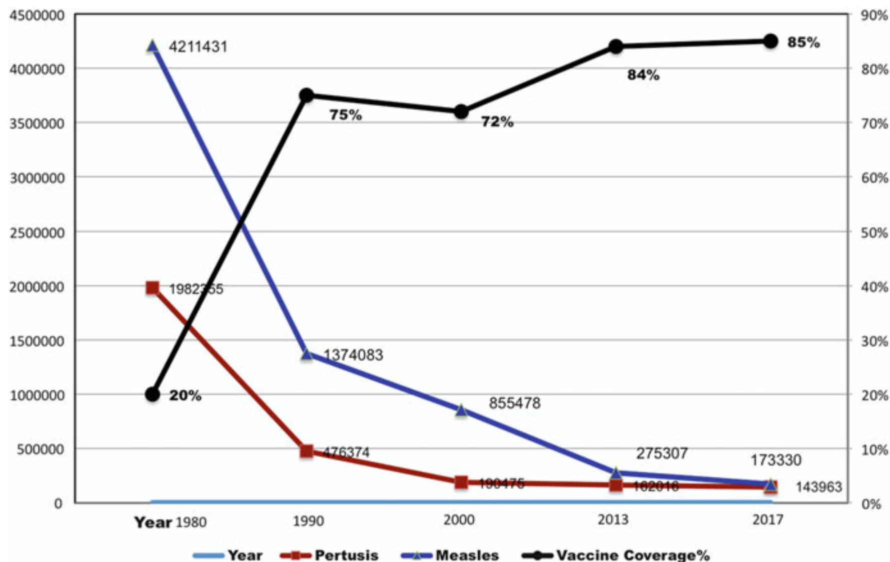

Fig. 1 Number of pertussis and measles cases and vaccine coverage % of measles, polio, and DTP3

1980 to about 85% in 2017 (as shown in Fig. 1), the numbers of cases for measles and pertussis have decreased significantly, according to the WHO report [7]. This represents dedication and hard work by all, including public health workers, researchers, pharmaceutical industries, policy makers, parents, and communities.

However, there are still gaps in vaccination programs in many countries. Out of 11.2 million children born in the EU region in 2012, more than 500,000 infants did not receive the complete three-dose series of diphtheria, pertussis, and tetanus vaccine before the first birthday. Data shows that there were more than 30,000 measles cases in the European region in recent years [4]. According to preliminary WHO data, measles increased by around 300% globally in the first 3 months of 2019, compared with the same time last year, with sizable increases in all regions of the world [7]. The reasons for children not getting their vaccines are diverse for different regions in the world; the major reasons are lack of access to vaccination services, and with Sub-Saharan Africa region that has the lowest coverage and the greatest burden of cases. There are about 20 million children in the world that are still not getting adequate coverage of basic vaccines.

As indicated in European Vaccine Action Plan 2015–2020 (EVAP) [4], EVAP's goal is to guide countries in the European region toward their joint vision of a region free of vaccine-preventable diseases. It establishes six goals which include sustaining polio-free status, eliminating measles and rubella, controlling hepatitis B infection, meeting regional vaccination coverage targets at all administrative levels throughout the region, making evidence-based decisions on introduction of new vaccines, and achieving financial sustainability of national immunization programs.

1.2 *Vaccine Development Life Cycle*

Vaccine development involves many stakeholders and multiple disciplines including sciences, medicine, public health, regulatory agencies, manufacturers, healthcare professionals, vaccine safety professionals, and consumers [8]. In many countries, the government agencies play important roles in vaccine innovation, development, and commercialization. For example, in the USA, the National Institutes of Health (NIH) conducts and supports basic research, translational research, and clinical evaluation to identify new vaccine targets and to advance new vaccine candidates through product development pipelines. The regulatory agency US FDA is involved in vaccine review and licensing, regulatory sciences, manufacturing inspection, and post-licensure safety monitoring. The US Centers for Disease Control and Prevention (CDC) identifies, controls, and prevents infectious diseases through surveillance, detection and response, vaccine use recommendations, vaccine purchasing and service delivery, health communications, and post-marketing vaccine safety and effectiveness monitoring. Vaccines are highly regulated products and require extensive safety monitoring.

In the USA, vaccines are regulated by the FDA Center for Biologics Evaluation and Research (CBER) and Office of Vaccines Research and Review (OVRR), where the authority resides in Section 351 of the US Public Health Service Act and the Federal Food, Drug, and Cosmetic Act. CBER conducts thorough review of laboratory, manufacturing, and clinical data to ensure the safety, efficacy, purity, and potency of the vaccine products. Figure 2 illustrates the typical process of vaccine development and licensure.

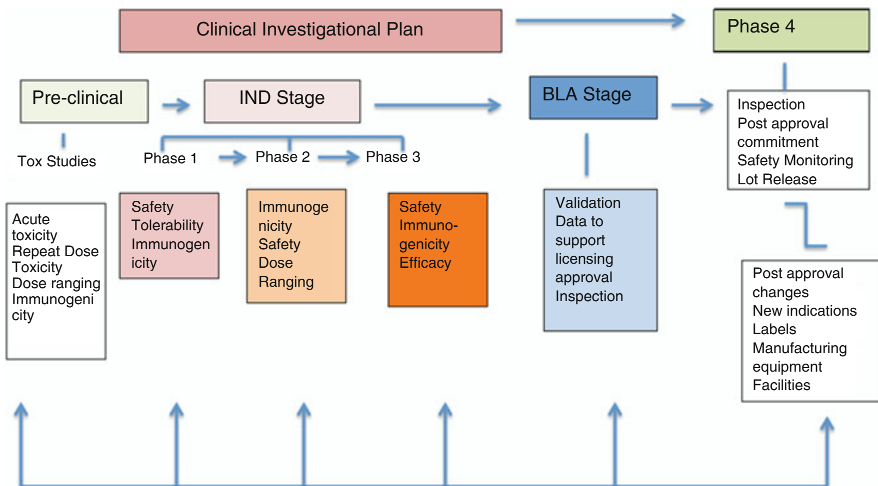

Fig. 2 Stages of vaccine review and regulation

1.3 Economics of Vaccine Development

Vaccine developments are long, expensive processes with high financial risks. It may take more than 10 years and more than US$1 billion to develop a new innovative vaccine. In fact, cost data of developing new vaccines is usually scarce. According to Dimitrios Gouglas [9], the average cost of successfully developing an epidemic infectious disease vaccine from preclinical to Phase 2a is estimated to be US$84–112 million (excluding the cost of facilities). Substantial investments are needed to develop the vaccines for these new targets. Innovation is the key to the future development of new vaccines to combat infectious diseases [10].

Although the cost of vaccine development is high, the benefits of vaccines are also significant. The development and licensure of pneumococcal conjugate vaccine (PCV) is a good example. The bacteria *S. pneumoniae* is the leading cause of pneumonia mortality globally and accounted for more deaths than all other causes (etiologies) combined in 2016. Most of these deaths occur in countries in Africa and Asia. Each year *Streptococcus pneumoniae* causes approximately 3,300 cases of meningitis, 100,000–135,000 cases of pneumonia requiring hospitalization, and six million cases of otitis media annually in the USA [11]. Pneumococcal conjugate vaccine 7-valent (PCV7) was approved in year 2000 for its use in the USA. It was designed to cover the seven serotypes that account for about 80% of invasive infections in children younger than 6 years of age. In 2007, the WHO published a position paper recommending all countries to include PCV as part of the routine infant immunization schedule. PCV7 and later PCV13 (13-valent pneumococcal conjugate vaccine) have been widely used in the world. There are 142 countries having put PCV13 into national immunization programs.

Following the introduction of the pneumococcal conjugate vaccines in the USA (PCV7 in 2000 and PCV13 in 2010), there are about 90% reduction of pneumococcal diseases. Invasive pneumococcal disease decreased from 100 cases per 100,000 people in 1998 to 9 cases per 100,000 in 2015, according to the data published by US CDC [6]. Currently there are two approved PCV vaccines in the USA, including PCV10 developed and marketed by GSK and PCV13 (Prevnar 13) developed and marketed by Wyeth Pharmaceuticals (Pfizer).

1.4 Promoting Innovation

Vaccine industries have achieved tremendous successes in the development of human vaccines that are currently in use. There are more than 200 vaccine clinical trials ongoing with more than 120 vaccine candidates for more than 40 infectious disease targets [12]. However, for the remaining targets, there are many significant challenges in developing new vaccines for these targets. The remaining infectious disease (ID) vaccine targets include those diseases affecting large populations such as respiratory syndrome virus (RSV) disease, human immunodeficiency virus (HIV),

malaria, etc. and emerging infectious diseases such as Lassa, Marburg, Ebola, MERS, and Zika listed in the WHO vaccine pipeline tracking sheet. As the development of new vaccines against these remaining targets faces significant challenges, the vaccine policy makers and the industries need to work together and encourage innovations through collaboration and support [13, 14].

To continue promote innovations in vaccine research and development, policy makers and regulatory agencies are recognizing the evolving development in sciences and medicine and using new approaches in vaccine review and approval (fast-track approach), as well as in the clinical trial design (demonstrated in the Ebola clinical trials in Africa during the 2018–2019 outbreaks) [15].

2 Manufacturing of Vaccines

2.1 General Considerations

Vaccine manufacturing is a complex process. Vaccines take a long time to manufacture, ranging typically from 6 months to 36 months. During the manufacturing process, more than 50% of production time is usually dedicated to the quality control tests. During a vaccine manufacturing process, several hundred quality control tests may be required before releasing a batch of vaccine products. The ability to manufacture at the commercial scale is also very important.

New vaccine commercialization is a complex and costly process [16]. Licensing of a new vaccine is based upon the demonstration of *safety and effectiveness* (through clinical evaluation) and the ability to be *manufactured in a consistent manner* (through process development and validation). The critical path toward developing safe and effective vaccines includes the following:

- Speedy development of new technologies
- Well-designed and well-operated facilities
- Improved manufacturing methods and scale-up
- Improved analytical evaluation tools and reference standards
- Streamlined preclinical and clinical evaluations
- Improved international cooperation
- Effective vaccine review and release process
- Improved product safety monitoring programs

In the early phases of vaccine development and manufacturing, the following information are critical for addressing product safety aspects:

- Source (biological seed or cell bank) characterization
- Raw materials including biologically derived materials (such as cell culture, media, etc.)
- Initial scalable process development
- Initial product characterization

- Testing/qualification/clearance of impurities, contaminants
- Process control especially for safety of the products (e.g., sterilization, virus clearance where applicable)

During the clinical development stages, the following chemical and manufacturing control (CMC) activities are important, and gradually phased-in approaches are usually taken:

- Process and product characterization
- Formulation development
- Raw material qualifications and supply management
- Component characterization
- Process and analytical method qualification
- Specification development
- Stability studies
- Manufacturing process scale-up and development
- Process control and validation
- Packaging development and label design
- Cold-chain establishment and vendor qualification

During the vaccine development and manufacturing process, applying current good manufacturing practice (cGMP) is very important to ensure product safety, efficacy, and consistency. It is highly recommended that cGMP be in effect for manufacture of products used in clinical studies – starting from Phase 1. One should follow the general approaches and principles that are broadly applicable, tailoring cGMP applications to specific product, process, and facilities by assessing potential risks and taking appropriate actions (risk-based approach).

2.2 Vaccine Technologies Development

A wide range of technologies have been used in developing successful vaccines, including:

- Live attenuated bacteria and viruses (e.g., BCG, MMR, etc.)
- Inactivated bacteria and viruses (e.g., whole-cell pertussis, IPV, etc.)
- Proteins (e.g., diphtheria and tetanus toxoids)
- Polysaccharides (PneumoVax)
- Conjugated polysaccharides (e.g., meningococcal conjugate vaccine, PCV13)
- Viruslike particles (VLPs)
- Recombinant proteins
- Application of mRNA
- Adjuvant development and applications

Many vaccines involve pathogens; thus there are special requirements for facility and equipment design depending on the biosafety levels. The manufacturing

methods include traditional egg-based influenza manufacturing or newly developed cell culture-based bioreactor manufacturing. Traditional egg-based processes use millions of eggs each year and a large number of manual handlings during manufacturing and testing. Advances in automation of these steps have greatly enhanced the process consistency and reduced the potential contamination from human intervention. Fermentation processes often use stainless steel fermenters for the manufacturing of many current bacterial vaccines on the markets. Cell culture-based production processes are used in many viral vaccine products. The investigational new vaccine candidates often use single-use bioreactors. The advantages of single-use bioreactors include faster and lower cost of initial installation, faster development cycle, and quicker delivery of clinical trial materials. The disadvantages include higher cost of operations (disposable bioreactors) and potential issues with mechanical strengths in the joints and ports of the bioreactors.

A typical vaccine manufacturing and product release process include the following steps:

1. Culture process: Selected bacterial strains or virus seeds or cells grow in appropriate culture media through fermentation or cell culture to generate the desired antigens.
2. Inactivation: Pathogens from the above culture are usually inactivated using chemical agents (e.g., formalin) or heat (e.g., 65°C).
3. Harvesting: Remove cells and cell debris from the product stream. Antigens are separated from the cells through filtration and/or centrifugation.
4. Purification: Impurities are removed from the harvest through purification methods such as ultrafiltration, chromatography, etc. The antigens are also concentrated during purification processes.
5. Detoxification of toxins: The pathogenicity is suppressed, and the immunogenicity is maintained in the product.
6. Bulk drug substance: The desired antigen is collected and stored under controlled temperatures for future use.
7. Formulation: Target antigens are assembled together with excipients to make final formulation.
8. Filling process: The final formulation is filled with automated filling machine under aseptic conditions into glass vials or prefilled syringes or other packaging containers.
9. Outer packaging: The filled vials or syringes are packaged into the secondary containers for future storage and shipping.
10. QC testing: Quality control laboratories conduct the quality control tests of the intermediates and the final product.
11. QA release: Quality assurance confirms that the product has been manufactured and tested according to approved specifications and procedures and can be released.
12. Final release for clinical trials: For clinical trials conducted in EU region, a qualified person (QP) releases the final product batch into the clinical trial usage.

13. Final release for distribution: For commercial vaccine products, many countries require that the National Regulatory Agency (NRA) release the final product for distribution into the market, such as in the USA, European Union, and China.
14. Product shipping: Most vaccine products currently are stored and shipped under cold-chain management, typically under 2–8°C, with very few products shipped under −60°C.
15. Product monitoring: After the product is released into the markets, product safety monitoring for serious adverse events (SAEs) is tracked and reported back to the manufacturers and to the regulatory agencies accordingly.

2.3 New Trends in Manufacturing of Vaccines

In recent years, there are new trends in the manufacturing of vaccine products, and these include:

1. Use of recombinant technology

It is more often that vaccine constructions are derived from recombinant technology, such as Shingrix vaccine, adenovirus-based Ebola vaccine (Ad5-EBOV), and rVSV-ZEBOV Ebola vaccine [17, 18]. Recombinant technology is used in a new recombinant pertussis vaccine development which provides higher product yield and less impurities.

2. Single-use technology

In recent years, single-use technology has been used more and more often in many new vaccine development and manufacturing processes. The advantages of using single-use technologies in vaccine development and manufacturing include:

- Minimizing potential contamination
- No need for equipment cleaning between batches
- No need or less requirement for cleaning validation
- Less initial capital costs
- Fast and easy installation

The disadvantages of single-use technologies include the following:

- Need mechanical strength to avoid component breakup.
- Installation of testing probes.
- Mixing may not be as good as in stainless steel tanks.
- Potential leachable and extractable materials from the bags.
- Scalability depending on the bioreactor design.
- More suitable for viral products than bacterial products.

3. Continuous manufacturing

Recent advances in disposable manufacturing technologies and process analytical technologies (PAT) have made the continuous manufacturing possible. The application of continuous process in antibody manufacturing has been reported in a number of conferences by companies such as WuXi Biologics and Amgen. The development of purification technologies has enabled integration of continuous processes from upstream through to downstream to final bulk drug substances manufacturing. Application of continuous manufacturing in vaccines has gained attention from nonprofit organizations such as Bill and Melinda Gates Foundation. It is reported (private conversation) that application of continuous manufacturing could result in a much lower cost of goods. The goal is to make vaccines affordable to everyone in the world. This will greatly improve the accessibility and affordability of vaccines in less developed nations, especially for GAVI (Global Alliance for Vaccines and Immunisation) countries.

4. Use of animal-free components

The raw materials and media used for vaccine fermentation and purification processes are mostly animal component-free to avoid the potential BSE/TSE risks, especially for final products.

2.4 Vaccine Manufacturing Challenges

One of the big challenges in the manufacturing of vaccine products is that materials used in Phase 3 clinical trials are typically manufactured in a commercial-scale facility. Many different technology platforms are used for various vaccines. It is difficult to standardize facilities and equipment. Unique facility and equipment are usually necessary for each vaccine or for each family of vaccines. There is a long lead time for building up a new commercial-scale manufacturing facility (typically 3–5 years), and large capital investment is often required.

The successful scale-up requires deep understanding of the processes and conducting well-designed experiments to complete process scale-up. As demonstrated in the assessment of safety and immunogenicity of two different lots of diphtheria, tetanus, pertussis, hepatitis B, and haemophilus influenzae type b vaccine manufactured using small- and large-scale manufacturing process, successful scale-up was completed and proved through clinical trial results [19].

During the manufacturing of biological products including vaccines, it is often considered that *process is product*, and the process/product is tightly linked with clinical experiences and outcomes. Major changes in manufacturing facilities or manufacturing processes may require regulatory approval or even conducting additional clinical trials. Thus for vaccine manufacturing, once a process is confirmed, it is usually not changed unless there is a strong reason to make a major change. This situation makes process improvements for existing vaccines challenging.

Another manufacturing challenge lies in the large-scale process validation. It is an expensive exercise to produce full-scale process validation lots to meet regulatory filing (e.g., line-specific real-time stability requirements for fill and finish in the USA). In addition, vaccines are biological products that are typically with low fill volume but very high throughput (with million to tens of million doses annually); thus it is challenging to run product filling lines under aseptic conditions for a long period of time. Employee training and/or utilizing isolation technology is important. Moreover, live viral vaccine (such as FluMist, measles vaccine) or live bacterial vaccine (such as BCG vaccine) requires dedicated fill-and-finish facility to avoid potential contaminations.

In addition, as current vaccines are typically temperature sensitive, thus they need cold-chain storage under 2–8°C or even lower temperatures (−20°C or −60°C). This generates significant challenges for handling in-process holding and final product storage as well as supply chain management. Large cold rooms are required in the manufacturing areas to meet the cold-chain storage requirements. Consistent electrical power supplies to these cold storage areas are essential to maintain the cold temperature control all the time. Most vaccine producers maintain emergency backup power supply in the event of power outages; the power supply can be switched to the backup power supply quickly.

Another significant challenge is the unpredictability of demand for seasonal vaccines (e.g., flu vaccines) and vaccines for emergency use (such as Ebola vaccine). The vaccine manufacturers will need advanced procurement and significant lead time to prepare raw materials, facilities and equipment, quality control, and human resources for the production of these vaccines.

3 Characterization of Vaccines

Vaccines, unlike other pharmaceutical products, are often perceived as being not well characterized due to their complex structures and properties. The implications are that Phase 3 clinical trials need to be conducted using clinical trial materials made in large-scale manufacturing facilities to ensure the consistency of Phase 3 clinical materials with the commercial products. The other approach is to conduct clinical bridging studies to demonstrate equivalence of Phase 3 clinical trial materials with commercial materials. The potential impact of this requirement is twofold: (1) delay in final facility readiness and (2) requirement of large capital investment in facilities much ahead of time. Thus it is very important to perform characterization of vaccine products as early as possible, to avoid or minimize costly changes later in the Phase 3 clinical trial stages.

In general, vaccines are very heterogeneous in structure. As greater characterization of vaccines becomes more prevalent, it may be possible to connect structural changes in the vaccine components with changes in potency and toxicity. This, in turn, may provide a better understanding of how certain vaccines function and

interact with the immune system. Information gained in this area will undoubtedly improve the effectiveness and safety of future vaccines.

Vaccines can be divided into three major categories: live vaccines, killed or attenuated vaccines, and component (subunit) vaccines. The component vaccines are generally the more easily characterized. They usually consist of a relatively small number of immunogenic components. The live or killed/attenuated vaccines include complex biological components such as attenuated or killed viruses and intact bacteria or multiple bacterial components. Advances in proteomics make the characterization of even these difficult vaccines more manageable.

3.1 Characterization of Bacterial Seeds

Characterization of vaccine generally involves analysis of bacterial strains, virus seeds, cell banks, intermediates, bulk drug substance, formulated final bulk, and finished product. Various physical, chemical, and biological tests are used for the analyses of these different biological materials. The purpose of these characterization tests is to ensure product quality and consistency.

For genetically modified bacterial strains used for vaccine manufacturing, the following tests need to be performed in addition to generally accepted characterization tests of bacterial strains:

- Genotype verification by PCR
- Verification of linear plasmid DNA
- Gene sequence verification
- Verification of flanking sequence

Table 1 is an example of quality tests on a pre-master seed for a genetically modified bacterial seed (recombinant pertussis vaccine under development) for GMP material manufacturing.

3.2 Characterization of Viral Seeds, Cell Banks, and Other Biological Materials Used in Viral Vaccine Manufacturing

Cell-based processes are used in viral vaccine development and manufacturing. The characterization of both viral seeds and cell lines is required. In addition, biological raw materials used in manufacturing processes should also be characterized with clear source of origin with potential risks of introduction of adventitious agents or viruses into the process and product stream. For viral product, US FDA guidance “Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease

Table 1 Quality control tests of a genetically modified seed lot

Items	Methods	Results
Culture characteristics	LB medium culture	No growth
	Bordet-Gengou medium culture	Small, round, smooth, convex, silver-gray, opaque colonies and without abnormal colonies
Biochemical tests	Glucose biochemical medium	No utilization of glucose
	Nitrate peptone water medium	No reduction of nitrate
	Urea medium	No urease reaction
	Citrate medium	No use of citrate as a carbon source and nitrogen source
	Semisolid nutrient agar medium	No flagellar power
Morphology	Gram stain	Gram negative
Serological tests	Serum agglutination test	Serum agglutination: positive
Phenotype tests	ELISA	Positive for target antigen
Genotype tests	PCR and restriction digestion	(1) The results of the recovered PCR products showed that the restriction enzyme map of the original strain is consistent with the size of the theoretical sequences. (2) The sequence is identical to the sequence of target strain
OD_{600}	Culture in flask	3.1
Target protein expression (μg/mL)	ELISA	>5
Skin necrosis test	Intradermal injection of bacterial suspension (rabbit model)	Positive

Indications" has clearly outlined the requirements for characterization of cell substrates, cell banking, viral seeds, vaccine intermediates, and biological raw materials at different stages of vaccine development [20].

For viral subunit vaccine product manufacturing, the most important factor is to prevent contaminations from adventitious materials and other contaminants in all stages of processes. It is important to demonstrate viral clearance through process validation.

However, live attenuated viruses, whole inactivated virus, or viruslike particles often cannot be purified as rigorously as viral subunit vaccines. Unlike the production of protein products, it is not possible to introduce validated viral inactivation or removal steps to mitigate these risks. Addressing these issues requires application of GMP principles and approaches in development programs at a very early stage, focusing on the history, purity, and stability of the viral seeds, cell banks, and biological raw materials.

For viral vector-based vaccine development, it is now common for a viral vector to be rescued from synthesized plasmids, allowing for traceability of the viral vector and full sequencing to be performed of the plasmids and resulting viral vector. Studies can also be performed to demonstrate genetic stability of vectors at an early stage of development. For manufacturing cell lines, it is essential that the origins are known with clear history and that they are free from adventitious agents and that generation of cell banks for both process development and production meet GMP requirements. In addition, it is very important to maintain segregation in the processes throughout development programs to prevent potential contamination of viral stocks and cell banks.

Although the production of viral vectors poses a number of technical challenges in cell culture, recovery, characterization, and analytical perspective, but with recombinant viral vector systems, where the virus is essentially used as a delivery vehicle and the manufacturing approach is independent of the genes it carries, a platform process can be developed. Therefore, the application of a platform can be developed for the production of those vaccines based on viral vectors, such as adenovirus, adeno-associated virus (AAV), and lentivirus.

For cell substrates (cell lines) intended for vaccine manufacturing, the following characterization tests or documentation needs to be provided:

- Cell substrate properties such as plasmid sequence, phenotype, and expression of antigens
- Source of the cell line including species of origin and the tissue type
- Donor’s medical history and the results of tests performed on the donor for the detection of adventitious agents
- Culture history of the cell line, including methods used for the isolation of the tissues from which the line was derived
- Passage history, medium used, and history of passage in animals
- Documentation of the history of human-derived and animal-derived materials used during passage of the cells
- Documentation of any genetic material introduced into the cell substrate
- Identity test, cytogenetic characteristics
- Results of all available adventitious agent testing
- Growth characteristics
- Expression characteristics
- Susceptibility to adventitious agents
- Generation of cell substrate
- Long-term storage conditions
- Stability of cell lines

Based on the above thorough understanding of the cell substrates, the cell banking systems with primary cell bank (PCB), master cell bank (MCB), and working cell bank (WCB) can be established.

The passage history and derivation history of viral seeds intended for vaccine manufacturing should also be well documented, including:

- Sourcing of each biological starting material (e.g., plasmids, parental viruses)
- Donor screening, testing, and donor medical history
- Any manipulation of the viral phenotype, such as cold adaptation and development of temperature sensitivity
- Any attenuation of virulence and genetic manipulations such as reassortment or recombination
- Nucleic acid sequences
- Growth characteristics on production cell substrate
- Genetic markers
- Long-term storage conditions
- Viability during storage
- Genetic stability through production
- Absence of adventitious agents

Other biological raw materials such as serum and other biologically sourced raw materials should also be controlled to prevent adventitious material contaminations.

3.3 Advances in Vaccine Characterization

The continuous development of safe, effective, and innovative vaccines around the world calls for new technologies, not only in the vaccine discovery areas but also the new technologies for characterization of vaccines.

The traditional methods of vaccine characterization rely on the study of physical-chemical properties using methods such as differential scanning colorimetry (DSC) and thermogravimetric analysis (TGA), pH, various stress conditions (agitation, freeze-thaw, etc.) based on particulate formation, and methods of quantitating protein content as well as elemental composition. While these methods are capable of determining whether or not the end product is consistent with previous batches, they are unable to detect small changes that can result in a vaccine with reduced or even lost immunogenicity. Not all changes to the structure of the vaccine components have physical consequences, but many of them result in reduced vaccine effectiveness. Most of these techniques lack sensitivity when it comes to detecting small changes in the structure of the vaccine components that can cause them to fail during use. Some changes can cause severe side reactions even in small quantities.

TGA and DSC are used to analyze the denaturing point of the vaccine protein or nucleotide. These tests generally give indirect indications of changes in the vaccine with time and stress. Changes in protein sequence or modifications can substantially affect denaturing kinetics, but these techniques provide no way to correlate these changes with actual changes in the structure of the molecule.

Appearance and pH are used to monitor major changes in the composition of the vaccines and are relatively insensitive to these changes. Other physical characteristics that affect vaccine function include particle size and particle size distribution. Clumping of the vaccine antigen can degrade the function of the vaccine and can

cause unwanted side effects. Specific tests for quantitating proteins or oligonucleotides, such as elemental analysis and total protein content (bicinchoninic acid or BCA) tests, can provide vital quality control data for troubleshooting manufacturing problems, but they are of limited value in analyzing degradation of the vaccines since elemental composition changes from degrades represent only a small percentage of the overall elemental composition. In addition, most protein degrades will still be identified as proteins in a total protein analysis. The conditions used for these assays also break up clumped proteins or oligonucleotides and are insensitive to most changes caused by minor structural modifications of these molecules.

Besides the traditional physical and chemical tests for vaccines, advanced technologies such as high-resolution mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, chromatography, and polymerase chain reaction (PCR) are used in the new vaccine development. ICP-MS, GC-MS, HPLC-MS, etc. are the examples of MS used for vaccine characterization. ICP-MS can be used for quantitatively measurement of metals in the vaccine intermediates and finished product. GC-MS and HPLC-MS can be used to determine the molecular weight of vaccines and detect if there are changes in the molecular structure. The mass spectrometry method can detect the structure change of functional proteins and lipids in vaccine with a high degree of accuracy, which is often linked with vaccine immunogenicity. Other biophysical tools such as florescence spectroscopy, light-scattering spectroscopy, etc. are also used for macromolecule characterization.

4 Clinical Aspects of Vaccine Development

Like other pharmaceutical products, the development of new vaccines will include clinical testing phases. Through well-controlled clinical trials, data will be collected to demonstrate that the vaccine product has an effect on clinical endpoint or a surrogate endpoint that is reasonably likely, based on clinical, serologic, epidemiologic, therapeutic, pathophysiologic, or other evidence, to provide clinical benefits.

4.1 Design of Clinical Trials for Vaccines

Generally, clinical trials for vaccines include clinical trials from Phase 1 to Phase 3 and Phase 4 post-approval. Phase 1 clinical trial is to test the initial safety and tolerability of the candidate vaccine; Phase 2 clinical trials test the safety, immunogenicity, and dose ranges; and Phase 3 clinical trials are for additional safety data, immunogenicity, and efficacy.

Prior to initiating Phase 3 clinical trials, it is recommended that the vaccine developer has continuous dialogue with regulatory agencies to discuss study details such as disease prevention or treatment; study sites; subject selection; choice of control group; trial design including endpoints, case definitions, diagnostic tests,

dose selection, dosing schedule, study duration, concomitant vaccinations, and medications; and safety assessments to ensure that they meet the intended goals for clinical trial and product licensure. Vaccines are approved for licensure from regulatory agencies if clinical trial data show that the product is safe, pure, and potent and that the manufacturing facility meets the standards designed to assure that the vaccine product continues to be safe, pure, effective, and non-inferior when administered with concomitant vaccines.

From regulatory point of view, the proof of effectiveness of a vaccine would consist of controlled clinical investigations that are adequate and well-controlled studies, unless waived as not applicable to the vaccine product or when an alternative method is adequate to substantiate effectiveness, such as using serological response data where a previously accepted correlation with clinical effectiveness exists [21].

The effectiveness may be proven by a well-designed, single clinical study. In the case of preventive vaccines, one adequate and well-controlled clinical trial may be supported by compelling animal challenge/protection models, human serological data, passive antibody data, or pathogenesis information.

4.2 Consistency Lots

Phase 3 vaccine clinical trials usually use the same scale of manufacturing and make clinical trial materials in the intended commercial manufacturing facilities. This requirement has made it challenging for the vaccine developers, as the facilities need to be ready long before the vaccine can be approved for commercial launch.

Unlike other therapeutic biological products, the efficacy of Phase 3 clinical trials for vaccines usually needs to demonstrate the protection of healthy people from a particular disease in the target population. Thus depending on the disease burden of a particular candidate vaccine, the Phase 3 efficacy clinical trial can be very large, enrolling several thousand or more healthy volunteers. For example, the recently approved Shingrix vaccine had Phase 3 clinical trials with more than 17,000 people enrolled. Thus Phase 3 clinical trials for preventive vaccine are usually very lengthy and costly. It is estimated that the cost of Phase 3 clinical trials of preventive vaccines may exceed US$100 million [9].

4.3 Vaccine Immunogenicity

Vaccine immunogenicity is often tested in the clinical trials. For example, during the clinical trials conducted for Ad5-EBOV Ebola virus disease vaccine, the Ebola-specific antibody responses against the vaccine-matched 2014 Zaire-Makona glycoprotein (GP) were assessed with enzyme-linked immunosorbent assay (ELISA) method, and anti-adenovirus type-5 neutralizing antibody titers were detected with a serum neutralization assay before and after vaccination. GP-specific IgG titers are

the important data for the immune response of the vaccine. Anti-Ad5 neutralizing antibody detection was used to analyze the vector immunity and the preexisting immunity influence on the vaccine's immunogenicity. Specific T-cell response was quantified by enzyme-linked immunospot (ELISpot) assay (IL-2, IFN-γ, and TNF-α), and IFN-γ, tumor necrosis factor-α (TNF-α), and interleukin-2 (IL-2) in peripheral blood mononuclear cells when phase I and phase Ib clinial trials were conducted in China. Another phase 2 clinical trial was conducted in Sierra Leone in 2015 [22–24]. The study results demonstrated that one shot of intramuscular injection with Ad5-EBOV vaccine could elicit strong humoral and cellular immune response in three clinical trials. Ad5-EBOV vaccine produced a considerable GP antibody response and GP-specific T-cell response in healthy African participants in China after 14 days [22]. GP antibody response peaked at day 28 and lasted more than 6 months. With a booster at the sixth month, the GP antibody response can last more than 12 months. The pre-exsiting immunity to Ad5 can be overcome with selected vaccine dosage.

Another example is Shingrix vaccine. Shingrix is a recombinant, subunit vaccine designed to restore VZV immunity in individuals who are at increased risk of developing Shingles due to age or immunodeficiency [25]. VZV gE is the most abundant envelope glycoprotein, predominantly expressed on the surface of virus-infected cells. The VZV gE protein plays a critical role in virus infectivity since it is involved in virus entry and cell-to-cell spread, harboring sites for N- and O-linked glycosylation. The subunit vaccine elicits stronger virus-specific CD4+ T-cell response as well as antibody B-cell response to gE, compared to the currently used live attenuated vaccine (Zostavax®). Evidence indicates that the gE protein induces both neutralizing antibodies and T-cell responses. The gE antigen component of Shingrix is derived from a VZV strain that was isolated from a patient with severe varicella disease. This antigen is a recombinant truncated form of VZV gE. The recombinant protein is then produced in Chinese hamster ovary (CHO) cells that were genetically modified to express the VZV gE gene [26].

There are many vaccine clinical trials ongoing for various vaccine candidates, and these are published in the website either at clinicaltrial.gov or at clinicaltrialsregister.eu.

4.4 Combination Vaccines

A combination vaccine consists of two or more live organisms, inactivated organisms, or purified antigens either combined by the manufacturer as a single product or mixed immediately before administration. It is intended to prevent multiple diseases or to prevent one disease caused by different strains or serotypes of the same organism. Meningococcal conjugated vaccine MCV (A, C, Y, W135), DTcP, and Prevnar 13 are good examples of combination vaccines.

The clinical studies are designed to demonstrate the safety, immunogenicity, and efficacy of combination vaccines through randomized, controlled studies. The

efficacy of each component should be demonstrated in clinical studies. Ideally, clinical trials will be prospective, randomized, and controlled. Endpoints used to evaluate efficacy in these trials can range from disease incidence to a well-established correlation of protection [27].

4.5 Special Populations

Vaccine development and clinical trials generally take stepwise approaches starting from adults to children. For routine vaccines aiming for pediatric applications, clinical trials in children's population groups are normally required, such as meningococcal vaccine, PCV vaccine, DTcP vaccine, etc. However, for some global infectious diseases such as malaria or Ebola virus disease, children suffer the same or even greater risks as their immune systems are not as strong as those of adults. It was reported that during the Ebola outbreaks in the Democratic Republic of the Congo, about one third of the reported cases were from children or adolescents under 18 years of age [28]. Thus it is important to include children and adolescents in the vaccine development programs for global infectious diseases unless a waiver is granted by the regulatory agencies. In some cases, if the course of the disease and the effects of the drug are sufficiently similar in adults and pediatric patients, the regulators may conclude that pediatric effectiveness can be extrapolated from adequate and well-controlled studies in adults, supplemented with other information obtained in pediatric subjects, such as immune response studies, as pointed out in the US FDA guidance [29].

For vaccine clinical trials including pregnant women in the population groups, reproductive toxicity studies need to be completed prior to including them.

4.6 Accelerated Approval

For certain global infectious diseases such as tuberculosis, malaria, and human immunodeficiency virus/acquired immunodeficiency syndrome (AIDS) that are serious and/or life-threatening, accelerated approval may be granted using a surrogate endpoint or a clinical endpoint other than survival or irreversible morbidity for a vaccine that provides meaningful clinical benefits for preventing the spread of infectious diseases or therapeutic benefits to patients over existing treatments [29].

For some special vaccine products, approval may be granted based on evidence of effectiveness from studies in animals when human efficacy studies are not ethical or feasible [30]. In such cases, after approval, a sponsor must conduct post-marketing studies, such as field studies, to verify and describe the biological product's clinical benefit and to assess its safety when used as indicated in circumstances where such studies are feasible and ethical. One example of applying "animal rule" is the approval of anthrax vaccine by the US FDA [31].

5 Overview of Recent Approved Vaccines

In recent years, several new vaccines are approved in different countries around the world. Brief descriptions are given in the following sections.

5.1 *Shingrix Vaccine*

Shingles causes painful rash of fluid-filled blisters and sometimes causes chronic pain. Shingles is a virus that results from reactivation of the varicella zoster virus (type 3 herpes zoster), the virus that causes chicken pox. Chicken pox is the initial infection, and shingles is the reactivation of the virus years later. Shingles may be developed at any age but most common for people aged over 50 years. Shingles causes substantial pain that can interfere with activities of daily living and reduce the quality of life. The estimated average overall incidence of shingles is about 3.4–4.8 per 1,000 person years which increases to more than 11 per 1,000 person years in those aged over 80 years in Europe, according to Robert W. Johnson's study [32]. Shingles is the third most common cause of chronic neuropathic pain in the USA, with an estimate of approximately 500,000 cases yearly.

Vaccination is an effective way to reduce the incidence of shingles. Two vaccines against shingles have been approved by the US FDA. Merck developed zoster vaccine live (ZVL, Zostavax), and it has been in use since 2006 for people 60 years and older.

The second vaccine for shingles has been developed by GSK Company. Shingrix is a recombinant zoster vaccine with adjuvant and is indicated for prevention of herpes zoster (shingles) in adults aged 50 years and older. It has two doses; the second dose is administered 2–6 months after the first dose [33].

Since its approval in 2017, Shingrix has obtained tremendous success in marketplace in the USA and internationally, as the sales of Shingrix exceeded US$1 billion in 2018. It is recommended by ACIP as the preferred shingles vaccine for people aged 50 years and older.

Shingrix is a suspension for injection supplied as a single-dose vial of lyophilized varicella zoster virus glycoprotein E (gE) antigen component reconstituted with the accompanying vial of AS01B adjuvant suspension component. After reconstitution, a single dose of Shingrix is 0.5 mL. The gE antigen is obtained by culturing genetically engineered Chinese hamster ovary (CHO) cells, which carry a truncated gE gene, in media containing amino acids, with no albumin, antibiotics, or animal-derived proteins. The gE protein is purified by several chromatographic steps, formulated with excipients, filled into vials, and lyophilized. The adjuvant suspension component is AS01B, which is composed of 3-O-desacyl-4′-monophosphoryl lipid A (MPL) from *Salmonella minnesota* and QS-21, a saponin purified from plant extract *Quillaja saponaria* Molina, combined in a liposomal formulation. The liposomes are composed of dioleoyl phosphatidylcholine (DOPC) and cholesterol

in phosphate-buffered saline solution containing disodium phosphate anhydrous, potassium dihydrogen phosphate, sodium chloride, and water for injection. After reconstitution, each 0.5 mL dose is formulated to contain 50 µg of the recombinant gE antigen, 50 µg of MPL, and 50 µg of QS-21. Each dose also contains 20 mg of sucrose (as stabilizer), 4.385 mg of sodium chloride, 1 mg of DOPC, 0.54 mg of potassium dihydrogen phosphate, 0.25 mg of cholesterol, 0.160 mg of sodium dihydrogen phosphate dihydrate, 0.15 mg of disodium phosphate anhydrous, 0.116 mg of dipotassium phosphate, and 0.08 mg of polysorbate 80. After reconstitution, Shingrix is a sterile, opalescent, and colorless to pale brownish liquid. Shingrix does not contain preservatives. Each dose may also contain residual amounts of host cell proteins ($\leq$3.0%) and DNA ($\leq$2.1 picogram) from the manufacturing process [33].

Shingrix has gone through multiple clinical trials. Overall, 17,041 adults aged 50 years and older received at least 1 dose of Shingrix in 17 clinical studies. The safety of Shingrix was evaluated by pooling data from 2 placebo-controlled clinical studies (Studies 1 and 2) involving 29,305 subjects aged 50 years and older who received at least 1 dose of Shingrix (n = 14,645) or saline placebo (n = 14,660) administered according to a 0- and 2-month schedule. Both studies were conducted in North America, Latin America, Europe, Asia, and Australia. In the overall 4 population, the majority of subjects were white (74.3%), followed by Asian (18.3%), black (1.4%), and other racial/ethnic groups (6.0%), and 58% were female. The safety profile was acceptable. Phase 3 clinical trials demonstrated that Shingrix has been shown to be highly efficacious with VE against shingles of 97.2% in subjects over 50 years of age and 91.3% in subjects over 70 years of age [34].

Shingrix is currently under significant shortage in the USA and around the world. This supply issue is a common problem with many successful vaccines. When the products do well because of their effectiveness, vaccine developers are struggling to supply. It is good to address these supply issues and how to mitigate it ahead of time. GSK announced in April 2019 that it plans to expand its manufacturing facility in Montana with a capital investment of US$100 million.

5.2 *Ebola Vaccine: Ad5-EBOV*

Ebola viruses (EBOVs) are enveloped, non-segmented, negative-stranded RNA viruses belonging to the family Filoviridae. They are known to cause lethal hemorrhagic fever in humans and nonhuman primates with a mortality rate of 40–90% [35, 36]. Ebola virus disease (EVD), formerly known as Ebola hemorrhagic fever, is a severe, often fatal illness in humans. EBOVs are transmitted among humans through close contact with infected blood, bodily fluids, or tissues; moreover, the intentional release of EBOVs would probably result in mucosal infection by small-particle aerosol dispersion.

From 1976 when the Ebola virus was first discovered to June 2019, there have been more than 30 epidemic outbreaks in Africa causing tens of thousands of deaths.

The species of Ebola viruses were mainly Zaire and Sultan types, and the outbreak sites were concentrated in Central Africa, in countries such as Congo, Sultan, Uganda, and South Africa. The 2014 outbreak in West Africa has been one of the worst Ebola epidemics. On August 8, 2014, the WHO director general declared this outbreak a Public Health Emergency of International Concern [37].

Many methods have been used to develop EBOV vaccines. Some of the vaccine candidates have been tested in nonhuman primates (NHP) and showed good protection. These vaccine candidates include inactivated vaccine, subunit vaccine, non-replicated virus vector vaccine, and replicated virus vector vaccine. Most of them were developed to protect against Zaire ebolavirus. In the past a few years, the protective mechanism of vaccine has also been studied, and the results showed that the antibody protection is essential.

A recombinant Ebola virus disease vaccine (Ad5-EBOV) has been successfully developed jointly by Beijing Institute of Biotechnology and CanSino Biologics Inc. (CanSinoBIO). The preclinical research of the recombinant Ebola virus disease vaccine (adenovirus type-5 vector) (Ad5-EBOV) was initiated in 2006. The key technology in vaccine preparation and evaluation was based on the Ebola GP antigen. A significant gene sequence variation of the GP antigen in the Zaire Ebola virus strain has been identified in the 2014 Ebola epidemic outbreaks in West Africa. To ensure the effectiveness of the vaccine, Ad5-EBOV vaccine was designed according to the 2014 Ebola virus genotype. The vaccine candidate was tested in animal models to confirm the immunogenicity, safety, and efficacy. Challenge studies in guinea pigs and NHPs (cynomolgus monkeys) conducted in biosafety level 4 (BL-4) lab in Public Health Agency of Canada also confirmed that the vaccine candidate is 100% effective in protecting guinea pigs and NHPs from Ebola virus infection.

CanSinoBIO started the development of recombinant Ebola virus disease vaccine at the end of 2014 as the Ebola virus disease outbreaks occurred in West Africa. The HEK293 cell line used for Ad5-EBOV production was licensed from National Research Council (NRC), Canada. The production process has been scaled up successfully. All key materials, intermediates, and final product (including virus seeds, cell banks, purified bulk, and final product) are QC tested and released internally. The entire production process for EBOV vaccine is animal component-free. The process of large-scale production of adenoviruses has been well characterized and optimized. Several vaccines and biological products have been developed using the Ad5 vector-based technology [38]. The platform technology applied to Ad5 vector-based production has been demonstrated by Ad5-EBOV to be robust and scalable and with high productivity.

In 2015, CanSino obtained a clinical trial permit from the Chinese Food Drug Administration (NMPA, formerly CFDA) and from the Government of Sierra Leone. The Phase 1 and Phase 2 clinical trials conducted in China and Sierra Leone involved a total of 681 subjects, and the clinical trial results indicated that the Ad5-EBOV vaccine is safe and immunogenic. The immune response level in human is comparable with that of rVZV-ZEBOV vaccine developed by Merck. Clinical trial results demonstrated that Ad5-EBOV is well tolerated with good safety

profile in tested subjects between ages 18 and 60. The GMTs of anti-GP antibody peaked around 28 days after vaccination regardless of the dose levels. Satisfactory immune response can be reached at dosage of 8×10^{10} VP per dose. Pre-existing immunity to Ad5 vector can be overcome by proper dose selection (8×10^{10} VP/dose), and the conversion rate is 100%. Ad5-EBOV response is fast and long-lasting, and these features could offer help in Ebola virus disease outbreak situation.

In October 2017, the new drug registration application (NDA) for recombinant Ebola virus vaccine (adenovirus type-5 vector) was approved by the Chinese Food and Drug Administration (CFDA). The manufacturing facilities, including QC testing center, are fully validated and in operation at CanSinoBIO.

5.3 Meningococcal Subgroup B Vaccine (MenB)

Currently approved meningococcal ACWY conjugate vaccine (MenACYW135) is given to preteens and teens at age 11 or 12 years with a booster dose at 16 years to protect against serotypes A, C, Y, and W135. In 2014–2015, another meningococcal subgroup B vaccine (Bexsero and Trumenba) [39, 40] were approved for adolescent and adult population aged 10–25 years who are at increased risk of meningococcal B diseases. Bexsero is an FDA-approved vaccine to prevent invasive disease caused by *Neisseria meningitidis* serogroup. It is not recommended for routine vaccination at this point.

In fact, meningitis subgroup B infection is very rare in the USA. Among all 11–23-year-old adolescents and young adults in the USA, between 50 and 60 cases are reported annually, with 5–10 deaths. According to the CDC, there were 7 outbreaks on college campuses from 2009 to 2013, with 41 cases and 3 deaths [41].

Bexsero was developed by Novartis and is now marketed by the GSK Company. It is a sterile suspension of three recombinant proteins and meningococcal outer membrane vesicles (OMV). The recombinant proteins neisserial adhesin A (NadA), neisserial heparin binding antigen (NHBA), and factor H binding protein (fHbp) are individually produced in *Escherichia coli* and purified. The OMV component is produced from *N. meningitidis* strain NZ98/254 (expressing outer membrane protein P or A serosubtype P1.4). The antigens are adsorbed onto aluminum hydroxide. Each 0.5 mL dose of Bexsero is formulated to contain 50 μg each of recombinant proteins NadA, NHBA, and fHbp, 25 μg of OMV, 1.5 mg aluminum hydroxide, 3.125 mg sodium chloride, 0.776 mg histidine, and 10 mg sucrose at pH 6.4–6.7 according to the product insert [39]. Bexsero is a suspension for intramuscular injection in 0.5 mL single-dose prefilled syringes. Two doses are given at least 1 month apart from the first dose.

Another approved meningococcal group B vaccine is Trumenba® developed by Pfizer, initially approved in 2014. Trumenba is a bivalent meningococcal group B vaccine that contains two factor H binding proteins (fHBP) from *Neisseria meningitidis* (*N. meningitidis*) serogroup B. fHBP is a conserved, outer membrane

lipoprotein and a virulence factor that contributes to the ability of the bacteria to avoid host defenses.

Trumenba is a sterile suspension of two recombinant lipidated factor H binding protein (fHBP) variants, one from each of the two antigenically distinct fHBP subfamilies subfamily A and subfamily B (A05 and B01, respectively). These proteins are also known as rLP2086 proteins. The proteins are individually produced in *Escherichia coli* and subsequently purified. Each 0.5 mL dose of Trumenba is formulated to contain 60 μg of each fHBP variant subtype (120 μg total protein), 0.018 mg of polysorbate 80, and 0.25 mg of Al3+ as AlPO4 in 10 mM histidine-buffered saline at pH 6.0, according to the product insert [40]. Trumenba is approved for use in individuals from 10 through 25 years of age.

Later in 2017, Trumenba vaccine was approved for three doses schedule (a dose administered at 0, 1–2, and 6 months) by US FDA. Additional clinical trial results also demonstrated that it is safe to co-administer Trumenba vaccine with meningococcal (groups A, C, Y, and W135) polysaccharide diphtheria toxoid conjugate vaccine and tetanus toxoid, reduced diphtheria toxoid, and acellular pertussis vaccine, adsorbed in persons 10 years to less than 13 years of age.

5.4 HPV Vaccine

Human papillomavirus (HPV) is a sexually transmitted virus. It is passed through genital contact or by skin-to-skin contact. HPV infection causes benign and malignant dysplastic anogenital disease in men and women. Nearly 100% of cervical cancers and 90% of anal cancers are caused by oncogenic HPV types.

GARDASIL 9, human papillomavirus 9-valent recombinant vaccine, was developed by Merck and initially approved by US FDA in 2014. Prior to the licensure of GARDASIL 9, Merck's 4-valent HPV vaccine, GARDASIL, was licensed in 2006. GARDASIL protects against disease caused by HPV types 6, 11, 16, and 18. GARDASIL 9 includes the original four HPV types in GARDASIL, plus an additional five types, HPV 31, 33, 45, 52, and 58. GARDASIL 9 is indicated in girls and women aged 9–26 years for the prevention of cervical, vulvar, vaginal, and anal cancer caused by human papillomavirus (HPV) types 16, 18, 31, 33, 45, 52, and 58 and genital warts (condyloma acuminata) caused by HPV types 6 and 11. GARDASIL 9 is also indicated in boys and men aged 9–26 years for the prevention of anal cancer caused by HPV types 16, 18, 31, 33, 45, 52, and 58 and genital warts (condyloma acuminata) caused by HPV types 6 and 11.

A 0.5 mL dose of GARDASIL 9 contains recombinant viruslike particles (VLPs) of the major capsid (L1) protein of HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58 adsorbed on preformed aluminum-containing adjuvant (amorphous aluminum hydroxyphosphate sulfate or AAHS). The amounts of HPV type L1 protein in each dose are as follows: 30 μg/40 μg/60 μg/40 μg/20 μg/20 μg/20 μg/20 μg/20 μg, respectively. It is available as a suspension in 0.5 mL single-dose vials or prefilled syringes, for intramuscular administration in two doses at months 0 and 2–6 month or three doses at months 0, 2, and 6 according to product insert [42].

5.5 Dengue Vaccine

Dengue infection is caused by dengue virus which includes four known serotypes (dengue virus 1, 2, 3, and 4), all transmitted primarily by *Aedes aegypti* mosquitos, as well as other members of the *Aedes* mosquito family. Annually, an estimated 390 million dengue infections occur worldwide, of which approximately 100 million are associated with clinical manifestations, 500,000 with hospitalization, and 20,000 with death [43]. Dengue disease is a major public health concern in more than 128 countries. It is endemic in Asia, the Pacific area, Africa, and Latin America with the four dengue virus serotypes found in tropical and subtropical regions, including some European territories. Dengue is endemic in the US territories of American Samoa, Guam, Puerto Rico, and the US Virgin Islands [44–46].

A dengue tetravalent vaccine is developed by Sanofi Pasteur Inc. and is approved by US FDA in May 2019. DENGVAXIA is a live, attenuated, tetravalent, chimeric virus vaccine, containing the replication genes and the capsid gene from the attenuated yellow fever [17D] virus and the Pre-M and ENV genes from each of the four serotypes (CYD). Each CYD virus is purified from Vero cells.

The indication of DENGVAXIA vaccine is for the prevention of dengue disease caused by dengue virus serotypes 1, 2, 3, and 4 in individuals 9 through 16 years of age with laboratory-confirmed previous dengue infection and living in endemic areas. Previous dengue infection can be assessed through a medical record of a previous laboratory-confirmed dengue infection or through current serotesting. The safety and effectiveness of the vaccine were determined in three randomized, placebo-controlled studies involving approximately 35,000 individuals in dengue-endemic areas, including Puerto Rico, Latin America, and the Asia-Pacific region. The vaccine was approximately 76% effective in preventing symptomatic, laboratory-confirmed dengue disease in population 9 through 16 years of age who previously had laboratory-confirmed dengue disease. DENGVAXIA has already been approved in 19 countries and has been approved by the European Union.

DENGVAXIA is supplied as a vial of lyophilized powder containing each of the four virus components that is reconstituted at the time of use with the supplied sodium chloride diluent (0.4% NaCl). After reconstitution, each 0.5 mL dose of DENGVAXIA is formulated to contain 4.5–6.0 log10 CCID50 of each of the CYD virus components. The reconstituted vaccine is administered subcutaneously in three doses at 6-month intervals (at day 0, month 6, and month 12) according to product insert [47].

5.6 EV71 Vaccine

Enterovirus 71 (EV71) is one of the major causative agents of outbreaks of hand, foot, and mouth disease or herpangina in Asia. An EV71 (Enterovirus 71) vaccine has been developed by Sinovac Biotech (China) using Vero cell and EV71 C4

subgenotype. The Phase 2 study of inactivated vaccine (Vero cell) against EV71 virus has been completed in December 2011 in China. The purpose of the Phase 2 clinical study was to demonstrate the safety and immunogenicity of EV71 vaccine in preventing hand, foot, and mouth disease caused by EV71 in a total of 10,000 healthy infant volunteers aged from 6 to 35 months old. The data from Phase 1 and 2 clinical studies suggested that the inactivated EV71 vaccine had clinically acceptable safety and good immunogenicity for healthy Chinese infants. A Phase 3 clinical trial was conducted in China in 2014 with 10,007 healthy infants and young children (6–35 months of age). The vaccine efficacy against EV71-associated hand, foot, and mouth disease or herpangina was 94.8%. Vaccine efficacies against EV71-associated hospitalization and hand, foot, and mouth disease with neurologic complications were both 100%. In the immunogenicity subgroup (1,291 children), an anti-EV71 immune response was elicited by the two-dose vaccine series in 98.8% of participants at day 56. An anti-EV71 neutralizing antibody titer of 1:16 was associated with protection against EV71-associated hand, foot, and mouth disease or herpangina [48–55]. EV71 vaccine was approved by the Chinese Drug Administration (formerly CFDA) in 2017.

5.7 HBV Recombinant Vaccine

Hepatitis B virus infection is a serious public health issue. More than 250 million persons are infected with hepatitis B virus (HBV) worldwide. Approximately 887,000 deaths worldwide were reported in 2015, mostly due to chronic hepatitis B and resultant end-stage liver disease and/or hepatocellular carcinoma [56, 57].

A new HBV vaccine, recombinant, with adjuvant (Heplisav-B) has been developed by Dynavax Technologies Corporation for preventing hepatitis B virus infections. The product contains 20 μg recombinant hepatitis B surface antigen with 3,000 μg adjuvant 1018, which is a novel cytosine phosphoguanine (CpG)-enriched oligodeoxynucleotide (ODN) phosphorothioate adjuvant. The indication and usage are for immunization against infection caused by all known subtypes of hepatitis B virus in adults 18 years of age and older. The product Heplisav-B (rHBsAg-1018 ISS), recombinant hepatitis B surface antigen (rHBsAg), subtype adw, is produced in yeast cells. The dosage contains two 0.5 mL doses administered 4 weeks apart.

Heplisav-B is supplied as single-use vials of 0.5 mL volume. Each 0.5 mL dose contains 20 μg HBsAg, 3,000 μg CpG 1018 adjuvant, 8 mM sodium phosphate, 154 mM sodium chloride, 0.01% w/w polysorbate 80, and pH 7.0 buffer. The vaccine does not contain preservatives. The shelf life of the final container product is 36 months at 5 ± 3°C from the date of manufacture according to the product insert [58–60].

The Heplisav-B vaccine was approved by the US FDA in 2017. This was the first hepatitis B vaccine approved in the USA for the last 25 years.

6 Vaccines Under Development

Many vaccines are under development around the world; below are a few examples of those vaccines under development.

6.1 rVSV-ZEBOV Ebola Vaccine

Although rVSV-EBOV has not been officially approved for licensing yet, it is in the process of applying licensure both with US FDA and with EMA in Europe. It has obtained fast-track review and breakthrough status. It has completed Phase 3 clinical trials and demonstrated its safety and efficacy [61].

In July 2015, clinical results of the Ebola ça Suffit ring vaccination Phase 3 cluster-randomized trial of the rVSV-ZEBOV vaccine in Guinea were obtained and published in Lancet. On the basis of interim analysis, the trial showed 100% vaccine efficacy, with 75.1% vaccine effectiveness at the cluster level, including herd immunity of unvaccinated members of clusters.

In March 2016, an rVSV-ZEBOV expanded access and compassionate use trial was conducted, named as “Ring vaccination with rVSV-ZEBOV under expanded access in response to an outbreak of Ebola virus disease in Guinea,” and the interim results were published in Lancet. The ring vaccination strategy was used between Ebola and the contacts. A total of 1,510 individuals were vaccinated in four rings in Guinea, including 303 individuals aged between 6 years and 17 years and 307 front-line workers. The results show that a ring vaccination strategy can be rapidly and safely implemented at scale in response to Ebola virus disease outbreaks in rural settings.

Although there are a lot of progress made in recent combat to Ebola outbreaks in Congo, and the data published by Gsell and Camacho [62] regarding the ring vaccination results demonstrated 100% efficacy of rVSV vaccine, it is worrisome that a survivor of Ebola virus disease previously in Guinea in the 2014 outbreak had Ebola virus after 531 days and caused another round of Ebola infections among the people he was in contact with. We need much better tools to monitor the situation [63].

In addition, despite significant progress in the characterization of the response to vaccination, correlation of protection (CoP) against Ebola virus infection has not been established in humans [64]. This holds true even for rVSV-ZEBOV, the most advanced Ebola vaccine candidate and the only one with demonstrated efficacy in humans so far when this chapter is published. This is especially challenging in emergency outbreaks settings, and major efforts would be required to ensure that samples and data are collected from the clinical trial participants. During emergency outbreaks, it is very difficult to track those people and to collect samples for further testing. Integration of data from preclinical and clinical vaccine studies together with data from disease survivors will thus be essential to identify Ebola vaccine correlates

of protection. The information generated for rVSV-ZEBOV may help identify the CoP of the other Ebola vaccine candidates, although these may also be different.

6.2 RSV Vaccine

Respiratory syncytial virus (RSV) is an important cause of viral lower respiratory tract illness in infants and children globally, but no vaccine is currently approved to protect these vulnerable populations. RSV is transmitted by direct and indirect contact with nasal or oral secretions and causes repeat infections throughout life and significant disease in pediatric and elderly populations [65–70]. The pathogen is an enveloped, non-segmented, single-stranded, negative-sense RNA pneumovirus belonging to the family *Paramyxoviridae*.

Many development programs for RSV vaccine are ongoing, some of them are in the preclinical stage, and 16 RSV candidate vaccines are in clinical development [71, 72]. RSV candidate vaccine developed by Novavax is currently in Phase 3 clinical trial stage. RSV vaccine development had a setback recently as Novavax Phase 3 efficacy trial for RSV F vaccine in maternal immunization did not reach its Phase 3 primary efficacy endpoint (www.novavax.com. Accessed 12 Apr 2019). 4,636 third-trimester pregnant women were enrolled in this large clinical trial. The clinical trial results demonstrated effectiveness in severe RSV cases. So far there is no approved RSV vaccine on the market yet. The development work is continuing, and more work remains to be done.

6.3 Malaria Vaccine

In 2013, there were an estimated 584,000 deaths and 198 million clinical illnesses due to malaria, the majority of which is in sub-Saharan Africa. Development of malaria vaccine has been ongoing for more than 40 years. The fact that malaria is caused by parasites that makes the development of effective vaccine against malaria more difficult than the development of vaccines against bacteria and viruses [73–78].

There is no vaccine approved on the market for prevention of malaria at present time. Clinical trials for malaria vaccines are ongoing.

6.4 Other Vaccines Under Development

Other vaccines currently under development include 15-valent and 20-valent pneumococcal conjugated vaccines, HIV vaccine, TB booster vaccine, Zika vaccine, Lassa vaccine, Middle East respiratory syndrome coronavirus (MERS-CoV)

vaccine, chikungunya vaccine, Nipah virus vaccine, and so on [78]. Development of mRNA vaccines has achieved significant progresses in recent years [79].

7 Challenges We Face

There are many challenges in providing adequate vaccination around the world. Firstly, vaccine hesitancy is still a problem in many countries including the USA, the UK, and also China [80]. The vaccine hesitancy does not include situations where vaccine uptake is low because of poor availability, e.g., lack of vaccine, lack of offer or access to vaccines, unacceptable travel/distances to reach immunization clinics, poor vaccine program communication, etc. Vaccine hesitancy reflects concerns about the decision to vaccinate oneself or one's children. Vaccine hesitancy and refusal, a growing trend in recent years, hinders the elimination and eradication of diseases. As of March 2019, there are multiple measles outbreaks in the USA, and parts of New York City declared emergency due to measles outbreak.

Although concerns about vaccine safety can be linked to vaccine hesitancy, it is only one factor that may be related to hesitancy; many other factors also contribute to the issue. Vaccine hesitancy and refusal factors may include:

- Compulsory nature of vaccines
- Coincidental temporal relationships to adverse health outcomes
- Unfamiliarity with vaccine-preventable diseases
- Lack of trust in corporations and public health agencies

In some areas, people are allowed to not participate in vaccination programs for religious reasons. In some other areas, people have limited access to education, and they do not know much about the benefits of vaccines. As a result, they refuse vaccination.

Overall, vaccine hesitancy is a complex and rapidly changing global problem that requires ongoing monitoring. Each country and each region need to come up with specific strategies to address the issue.

Another challenge is the resurgence of pertussis diseases. Despite the vaccination of DTP to infants and children over three decades, the occurrence of pertussis in adolescent and adult population is under recognized. Although the adolescents and adults do not have significant clinical symptoms when pertussis bacteria infect them, they can transmit the disease to infants and young children around them. Studies in European region (2019 ECDC Report on Pertussis) found that young adolescents older than 15 years old had the highest infection rate and young infants less than 6 months old had the most severe infections. The study results demonstrated that the majority of cases above the age of 30 years were unvaccinated. These data stress the need to refine vaccination strategies with the final aim of protecting infants. In the USA and many European countries, immunization of Tdap for adolescents and adults is recommended in the national immunization programs. Thus it is recommended that Tdap immunization of adolescents and adults should be

implemented in China and other countries that do not have Tdap immunization for adults and adolescents.

In summary, vaccines have made tremendous contributions to the society so far. However, we have more work to do to continue developing safe and effective vaccines to control the infectious diseases and help people around the world to live a healthy and long life [81–83].

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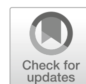
Human Pluripotent Stem Cells: Applications and Challenges for Regenerative Medicine and Disease Modeling



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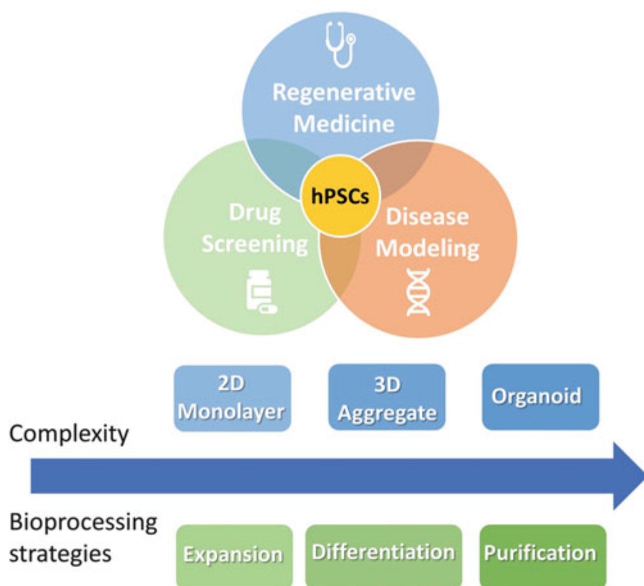
Abstract In recent years, human pluripotent stem (hPS) cells have started to emerge as a potential tool with application in fields such as regenerative medicine, disease modeling, and drug screening. In particular, the ability to differentiate human-induced pluripotent stem (hiPS) cells into different cell types and to mimic structures and functions of a specific target organ, resourcing to organoid technology, has introduced novel model systems for disease recapitulation while offering a powerful tool to provide a faster and reproducible approach in the process of drug discovery. All these technologies are expected to improve the overall quality of life of the humankind. Here, we highlight the main applications of hiPS cells and the main challenges associated with the translation of hPS cell derivatives into clinical settings and other biomedical applications, such as the costs of the process and the ability to mimic the complexity of the in vivo systems. Moreover, we focus on the bioprocessing approaches that can be applied towards the production of high numbers of cells as well as their efficient differentiation into the final product and further purification.

Graphical Abstract



Keywords Bioprocessing, Disease modeling, Human pluripotent stem cells, Organoids, Regenerative medicine

1 Introduction

The possibility of producing virtually any type of cell of the human body from human pluripotent stem (hPS) cells has introduced a myriad of possibilities regarding their applications in innovative fields such as regenerative medicine, disease modeling, and drug discovery.

Besides the self-renewal capacity and differentiation ability associated with stem cells in general, hPS cells provide the opportunity of generating every cell type derived from the three embryonic germ layers – endoderm, ectoderm, and mesoderm [1]. Importantly, the breakthrough discovery that somatic cells can be reprogrammed back to a pluripotent stem cell state, awarded with the Nobel Prize of Physiology or Medicine in 2012, has introduced the opportunity of using these cells in personalized medicine approaches, both including regenerative medicine and drug discovery [2].

The opportunity of generating organoids from hPS cells amplifies the relevance of this stem cell source within the fields of disease modeling and drug discovery [3]. Besides allowing the recapitulation of the inherent differentiation of embryo-like cells into complex and organized structures that are found within a normal embryonic development process, disease-specific hPS cell-derived organoids also provide a unique opportunity of understanding the underlying mechanisms of a particular disease and how they will affect the final phenotypic characteristics [3]. On the other hand, organoid technology opens the door for new methods of drug screening that may reduce the need to resort to animal models that do not fully represent a human response and that are associated with ethical issues [4].

Stem cell bioprocessing includes the main strategies for scaling up or scaling out of stem cell expansion, differentiation, and purification for the upcoming use of stem cells or their derivatives in the fields of regenerative medicine, disease modeling, and drug screening. To fulfill the previously described applications, scalable processes that involve the use of laboratory-scale (e.g., spinner flasks) and fully controlled bioreactors for the production of clinically relevant numbers of cells are currently under development and optimization. So far, several robust and efficient bioprocesses have been developed including the xeno-free scalable expansion of hPS cells while retaining their pluripotent potential [5] as well as the directed differentiation of human induced pluripotent stem (hiPS) cells into cardiomyocytes [6]. Moreover, scale-out approaches that envisage novel methodologies for drug screening and testing processes have been described and hopefully will be integrated in the drug discovery pipeline in a near future [7].

In this chapter, we address the main challenges and opportunities regarding the application of hPS cell derivatives towards regenerative medicine, disease modeling, and drug screening, and we revise the main bioprocessing tools that are necessary to take full advantage of the potential of hPS cells.

2 Human Pluripotent Stem Cells: The Tool

2.1 Methods for Isolation and Derivation of hES and hiPS Cells

The properties of human pluripotent stem cells confer them a remarkable potential towards biomedical and pharmaceutical applications. Embryonic stem (ES) cells were firstly isolated from mouse embryos, and their pluripotent potential was demonstrated by showing that these cells gave rise to teratocarcinomas when grafted into adult mice [8]. Later, in 1998, it was also possible to isolate ES cells from human embryos [1]. Soon – 4 to 5 days – after the fertilization of an egg in human embryos, there is the formation of the blastocyst, a hollow sphere composed of an outer layer of cells, the trophectoderm, and an inner cell mass, from where embryonic stem cells are isolated [9]. These cells are known for their pluripotency, as in vitro they can give rise to any type of cell originated from the three embryonic germ layers (ectoderm, mesoderm, and endoderm). Nevertheless, they are unable to form a whole new individual, since the placenta is derived from the trophectoderm and therefore cannot be formed [1]. The pluripotency of human ES (hES) cells can be assessed by embryoid body formation, directed differentiation into cells of the three embryonic germ layers, and teratoma formation assays [1].

Despite their enormous potential in cell therapy, drug discovery, and disease modeling, the requirement of destroying a human embryo for obtaining hES cells raises ethical concerns, namely, the ending of a potential human life. The ethical implications and reduced availability of hES cells led to search for alternative paths to obtain human pluripotent stem cells. Several methods were explored, the first ones being nuclear transfer [10] and cell fusion [11] which in spite of creating pluripotent stem cell lines retained the ethical concerns and the impossibility of clinical use, respectively. Soon after that, it was discovered another technology for pluripotency induction, capable of bypassing these two problems, along with the possibility of reducing the risk of immune rejection, hiPS cells, obtained by direct reprogramming of somatic cells by defined transcription factors [12]. Furthermore, hiPS cell technology offers the possibility of developing personalized medicine approaches, since hiPS cells can be induced from somatic cells of a specific patient.

The technique of nuclear transfer requires a somatic cell and an unfertilized oocyte from which the nucleus was removed. It consists in the transfer of the DNA content of the somatic cell into the oocyte, where the union will occur, leading to the creation of an "embryonic-like" cell [13]. However, this process still maintains the ethical concerns associated with hES cells, due to the origin of a cell that could theoretically form a human being.

Cell fusion, as the name indicates, involves the fusion between two cells, generally an embryonic stem cell and a somatic cell. The fusion comprises that the reprogramming factors that exist in the cytoplasm of embryonic stem cells will induce the somatic nucleus towards a pluripotent stem cell state, expressing Oct4, Sox2, and Nanog. However, their tetraploid nature prevents their use in clinical applications [13–15].

In the past few years, scientific research was directed towards bypassing the ethical concerns associated to human embryonic stem cells. Takahashi and Yamanaka [12] discovered in 2006 that terminally differentiated cells could be reprogrammed back to a state of pluripotency, therefore achieving an embryonic-like state [16]. This process was firstly performed using mouse embryonic fibroblasts, but 1 year later the process of cell reprogramming was also validated for human cells, by using adult human fibroblasts to generate human iPS cells for the first time [2]. In this study, four transcription factors were shown to be essential for the generation of iPS cells, those being Oct3/4, Sox2, *c*-Myc, and Klf4. These four factors were introduced into the somatic cells by viral vectors. One of the most surprising results of this work was that Nanog, a transcription factor present in ES cells, was not found to be essential in the reprogramming process. It is important to notice that the optimal duration of transgene expression will depend on the type of somatic cell that will be reprogrammed, but still, once the cell achieves the pluripotency hallmark, the endogenous expression of the factors is sufficient to maintain the pluripotent state [17].

Human iPS cells are identical to hES cells in terms of colony and cell morphology, gene expression, and in vitro differentiation ability. At the same time, these cell types share the expression of surface pluripotency markers SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81, as well as the transcription factors Oct3/4, Sox2, and Nanog [2, 18]. Despite the great similarity observed between hES and hiPS cells regarding morphologic characteristics and signaling pathways, there are some differences that distinguish these two types of cells. Firstly, there are some indications that differential promoter binding of the reprogramming factors leaves a signature in the gene expression of hiPS cells [19]. Also, hiPS cells exhibit a different methylation pattern when compared to hES cells. Moreover, it has also been argued that hiPS cells may retain somatic mutations acquired before reprogramming, which would make the reprogrammed cells genetically different from its embryonic equivalents. In addition, it was previously shown that hiPS cells have the ability of forming teratomas with an efficiency of 100% when transplanted subcutaneously or intratesticularly into immunocompromised mice, whereas this percentage decreases for 81 and 91% with hES cells, with subcutaneous and intratesticular implantation, respectively [20].

2.2 Safety and Efficiency of hiPS Cell Reprogramming

The production of hiPS cells without genomic integration of reprogramming factors is one key feature that facilitates their approval mainly for regenerative medicine applications. In fact, methods based on genomic integration can cause insertional mutagenesis and potentially transform the cells towards a malignant pathway [21].

Although the first report of derivation of hiPS cells was based on retroviral vectors that encoded key reprogramming factors with reprogramming efficiencies averaging 0.02% [2], several different approaches have also been reported that avoid

integration of the genetic material in the genome. Amid integration-free methods, episomal transfection has demonstrated the ability to generate iPS cells in a technically simple and reproducible manner [22, 23]. Despite that the reprogramming efficiencies of non-integrating methods tend to be lower when compared to viral vectors, averaging 0.01%, a recent study demonstrated that there are no significant differences in terms of DNA methylation pattern, marker expression levels, or developmental potential in hiPS cells derived using different non-integrating techniques [24]. Another way to evade introducing genetic material into the genome consists in the direct delivery of synthetic RNAs, modified to prevent antiviral response mechanisms from the cells, to reprogram somatic cells back to a pluripotent state with an efficiency of more than 2%, which translates to a reprogramming efficiency of two orders of magnitude higher comparing to viral methods [25].

Although hiPS cells represent an alternative for overcoming the ethical issues often associated with hES cells, they still retain the risk of forming teratomas if not efficiently differentiated. A safer approach may reside in using direct reprogramming to derive a somatic cell from another somatic cell type or from an induced tissue-specific stem (iTS) cell. These strategies allow bypassing of the pluripotent state, while at the same time reduce the duration of the periods of hiPS cell derivation and differentiation from several months to a few weeks [26]. As a good example of direct reprogramming from a somatic cell type to another, the reprogramming of mouse fibroblasts into cardiomyocytes was performed by overexpression of cardiac-specific factors Gata4, Mef2c, and Tbx5 [27]. The derivation of functional neurons from fibroblasts using lentiviral vectors to induce the expression of Ascl1, Brn2, and Myt1l was also reported [28]. Still, a major drawback of direct reprogramming into somatic cells relies on the reduced proliferative capacity of the derived cells, as well as a lack of population diversity, which in the end may compromise the regenerative medicine potential of the cells [29]. Another alternative relies on the use of iTS cells, which are derived by transfection of somatic cells with a plasmid that harbors the genes for the four pluripotency induction factors in combination with tissue-specific selection genes [30]. Using this process, mouse pancreatic and liver iTS cells were derived by using transient overexpression of reprogramming factors and combination with Pdx1$^+$ or HNF4α$^+$ cell selection, respectively. These cells expressed genetic markers for endoderm and pancreatic or hepatic progenitors and were able to further differentiate into insulin-producing cells and hepatocytes.

2.3 *Epigenetic Memory in hiPS Cells*

Induction of pluripotency of somatic cells also involves epigenetic changes in the DNA. Therefore, there is the need for specific DNA methylation and methylation/acetylation of histones that will allow the required genes for pluripotency to be expressed [17]. Although DNA methylation patterns are dynamic throughout development and cell differentiation, some of these patterns can be retained as a form of epigenetic memory.

The DNA methylation of Oct3/4 and the modification of histones observed by Takahashi and Yamanaka [12] were the first suggestion that iPS cells were caught in an intermediate epigenetic state between the initial somatic cell and an ES cell state. Later, Bar-Nur et al. observed that hiPS cells derived from pancreatic islet β cells had increased potential to differentiate into insulin-producing cells [31]. After analyzing the open chromatin structure, it was possible to determine that this hiPS cell line retained a methylation pattern and an open chromatin structure at key β-cell genes, introducing the hypothesis that methylation patterns in hiPS cell lines are influenced by their tissues of origin. Therefore, the epigenetic memory associated with the reprogramming process was also shown to improve the efficiency of differentiation into mature cells of the same lineage of the donor cell.

Nevertheless, the method of iPS cell generation can also influence the epigenetic memory of the cells. Kim et al. compared iPS cells derived by transcription factor-based treatment and by nuclear transfer [32]. While transcription factor-based reprogramming demonstrated some residual DNA methylation patterns characteristic of the somatic donor cells, reprogramming by nuclear transfer yielded DNA methylation signatures that closely resemble the ones of ES cells [32]. Still, in transcription factor-based approaches, the predisposal of iPS cells to favor differentiation towards the same lineage of the donor cell could be bypassed by sequential differentiation and reprogramming of iPS cells, as well as with treatment with chromatin-modifying drugs [19].

3 Human Pluripotent Stem Cells and Regenerative Medicine

3.1 *Clinical Challenges and Legislation*

Given that regenerative medicine products provide a high-risk-high-reward approach, some countries, including the United States and Japan, have begun to review the laws of delivery of new regenerative medicine-based products or even create new laws to fulfill the gaps in legislation.

Since late 2014, there are two laws in Japan that provide a fast track for product approval for the commercialization of cell therapy products within the country. Therefore, companies are allowed to receive a conditional marketing approval and commercialize cell-based products while clinical trials proceed to later stages. The first law, Act on the Safety of Regenerative Medicine (Law No. 85/2013), states the guidelines that allow the acceleration of clinical application and commercialization of innovative regenerative medicine products, covering clinical research and medical practice. The second law, Pharmaceuticals and Medical Device (PMD) Act (Law No. 84/2013), introduces a specific regulatory framework for regenerative medicine products, providing a provisory marketing approval of 7 years after exploratory clinical trials have demonstrated safety and basic efficiency data. During this period,

companies are required to continue to submit new clinical trials data to Japan's Pharmaceuticals and Medical Devices Agency and, in the end, apply for final marketing approval or withdrawal of the product. In this Act, regenerative medicine products include cell and gene therapy products, as well as tissue-engineered products.

In the United States, the Food and Drug Administration (FDA) approved a directive – the 21st Century Cures Acts – in order to accelerate the process of giving access to innovative products to patients in need and to reduce the regulatory hurdles for FDA approval. In this Act, there is also a pathway to accelerate FDA approval and market entry of regenerative medicine therapies, including cell therapies and other human tissue products, known as the regenerative medicine advanced therapies (RMAT).

Among the main challenges of hPS cell-based product applications in cell therapies, the high cost of the cell manufacturing process remains as one of the main obstacles to tackle [33]. For example, the costs of the preclinical studies of the first clinical trial using hES cell-derived oligodendrocyte progenitors to treat spinal cord injury were estimated to be 200 million dollars [34].

3.2 Cell Sources

Although hiPS cells offer the possibility of patient-specific cell therapies, the reprogramming, differentiation, and purification steps may require months of processing before reaching clinically relevant cell numbers with high quality and functionality. Thus, allogeneic therapy using universal hiPS cells from healthy donors can be used instead of customized hiPS cells. In fact, hiPS cells from donors with homozygous human leukocyte antigen (HLA) who match ~20% of the Japanese population at major HLA loci have been generated and banked and potentially provide an excellent alternative to autologous stem cell-based therapy [23]. It was already demonstrated that upon hiPS cell-derived dopamine neurons engraftment in nonhuman primates, major histocompatibility matching reduces the immunological response, through suppression of immune cells in the site of the graft and increases the survival of the grafted neurons [35].

3.3 Current Clinical and Preclinical Trials Involving hPS Cell-Derived Products

Most of the clinical trials that have employed hPS cell-based products are directed towards treatment of ophthalmologic, neurological, and neurodegenerative diseases. Furthermore, there is a clear observation that so far there has been more resourcing to hES cells than to hiPS cells for differentiation into the final cell product (Tables 1 and 2). One of the major debates concerning the clinical applications of hPS

Table 1 hES cell-based products in clinical trials

Disease	Company/ sponsor	ID	Status	Country
AMD	Southwest Hospital, China	NCT02749734	Phase I	China
AMD	Chinese Academy of Sciences	NCT02755428	Phase I	China
AMD	Chinese Academy of Sciences	NCT03046407	Early phase I	China
Dry-form AMD	Astellas Institute for Regenerative Medicine	NCT02463344	Follow-up of NCT01344993	USA
Dry-form AMD	Cell Cure Neurosciences	NCT02286089	Phase I/II	Israel/USA
Dry-form AMD	CHA Biotech	NCT01674829	Phase I/II	Korea
Dry-form AMD	Astellas Institute for Regenerative Medicine	NCT01344993	Phase I/II	USA
Dry-form AMD	Regenerative Patch Technologies	NCT02590692	Phase I/II	USA
Wet-form AMD	Pfizer	NCT01691261	Phase I	UK
Macular degenerative disease	Astellas Institute for Regenerative Medicine	NCT03167203	Phase I/II	USA
Outer retinal degenerations	Federal University of São Paulo	NCT02903576	Phase I/II	Brazil
Parkinson's disease	Chinese Academy of Sciences	NCT03119636	Phase I/II	China
Severe heart failure	Assistance Publique – Hôpitaux de Paris	NCT02057900	Phase I	France
Spinal cord injury	Asterias Biotherapeutics	NCT02302157	Phase I/II	USA
SMD	CHA Biotech	NCT01625559	Phase I	Korea
SMD	Astellas Institute for Regenerative Medicine	NCT02941991	Follow-up of phase I/II	UK
SMD	Astellas Institute for Regenerative Medicine	NCT01469832	Phase I/II	USA/UK
SMD	Astellas Institute for Regenerative Medicine	NCT01345006	Phase I/II	USA
SMD	Astellas Institute for Regenerative Medicine	NCT02445612	Long-term follow-up of NCT01345006	USA

(continued)

Table 1 (continued)

Disease	Company/ sponsor	ID	Status	Country
Type 1 diabetes mellitus	ViaCyte	NCT02239354	Phase I/II	USA/Canada
Type 1 diabetes mellitus	ViaCyte	NCT02939118	Observational, follow-up of NCT02239354	USA
Type 1 diabetes mellitus	ViaCyte	NCT03162926	Phase I	USA/Canada
Type 1 diabetes mellitus and hypoglycemia unawareness	ViaCyte	NCT03163511	Phase I/II	USA

AMD age-related macular degeneration, *SMD* Stargardt's macular dystrophy

Table 2 hiPS cell-based products in clinical trials

Disease	Company/ sponsor	ID	Status	Country	Type of therapy
AMD	Moorfields Eye Hospital NHS Foundation Trust	NCT02464956	Observational	UK	Autologous
Wet-AMD	RIKEN	UMIN000011929	Interventional	Japan	Autologous
Graft-vs-host disease	Cynata Therapeutics	NCT02923375	Phase I	Australia/ UK	Allogenic
Neovascular AMD	Kobe City Medical Center General Hospital	UMIN000026003	Interventional	Japan	Allogenic
Thalassemia	The Third Affiliated Hospital of Guangzhou Medical University	NCT03222453	Not applicable	China	Autologous

AMD age-related macular degeneration, *SMD* Stargardt's macular dystrophy, *Not applicable* trials without FDA-defined phases, including trials of devices or behavioral interventions

cell-based products resides in whether to transplant mature cells with full functionality or progenitor cells that still have some plasticity and may integrate better within the host tissues. Several examples of both scenarios have been reported, including the transplantation of fully differentiated cells, such as dopaminergic neurons [35] or cardiomyocytes [36], or the transplantation of dopaminergic [37], oligodendrocyte [38] or pancreatic [39] precursor cells.

The first clinical trial involving a hPS cell-based product dates back to 2014 and was started by Geron Corporation, in the United States. This study was directed towards spinal cord injury and relied on a preclinical trial that used hES cell-derived oligodendrocyte precursor (OP) cells to remyelinate rat spinal cords [40]. In fact, it

was demonstrated that OP cells were able to not only survive but also migrate and further differentiate into oligodendrocytes, restoring motor function. Currently, this trial is in phase I/II by Asterias Biotherapeutics, with the main objective of optimizing the injected cell dose.

Neurodegenerative diseases are potentially one of the main targets for iPS cell therapies. Parkinson's disease (PD), which is characterized by a neurological dysfunction based on the loss of dopaminergic neurons, was the first condition to be tackled using iPS cell-based therapy. In 2011, the first evidence that dopaminergic progenitor cells derived from hES cells were able to engraft and mature upon injection was attained using a rat model [41]. In addition, the mature dopaminergic neurons survived for long periods, retaining the ability to form synaptic connections and restoring motor functions that were lost due to PD [42].

A preclinical trial in monkeys was initially run to confirm the safety and efficacy of a treatment for PD resorting to hiPS cells [37]. In this study, dopaminergic precursor cells were derived from hiPS cells and injected into the putamen of macaque monkeys. For that, hiPS cells were firstly directed towards midbrain dopaminergic progenitor cells, and then cells expressing CORIN – a floor plate marker – were sorted in order to obtain a more purified population of cells expressing FOXA2 and Tuj1, markers for floor plate and immature neurons, respectively. In the end, this approach yielded more than 85% of $FOXA2^{+}Tuj1^{+}$ cells, while more than 15% of the cells expressed a marker of midbrain dopaminergic neurons (NURR1). Furthermore, no pluripotent Oct4+ cells were detected, as well as neural rosette-forming cells ($Sox1^{+}Pax6^{+}$), that might also contribute to tumor formation. Upon transplantation, implanted cells were able to survive for at least 2 years without harmful effects in the body and were able to even integrate with monkey's brain cells. In October 2018, the first clinical trial comprising the implantation of hiPS cell-derived dopaminergic precursor cells has been initiated in Japan. In the first approach of the procedure performed in a single individual, 2.4 million cells were implanted into 12 different sites known to contain dopamine activity, and, if no complications arise in the first 6 months, the procedure will be repeated. Until the end of 2020, there is the hope to confirm the safety and efficacy of the technique by treating six more patients with PD, with the final goal to make the therapy available by 2023 [43].

A rat model was used for validation of a preclinical trial to address radiation injury to the brain, a clinically important need for cancer survivors. The goal was to use hES cell-derived oligodendrocyte precursor (OP) cells that upon engraftment into the forebrain had the ability to remyelinate the brain and salvage cognitive deficits [38]. Also, upon injection of 250,000 O4+ cells in the cerebellum, there was an improvement in motor tasks of the rats, while there was no indication of teratoma formation. Further efficacy and safety data in preclinical animal studies has already demonstrated that OP cell distribution is limited to the spinal cord and that there were no adverse clinical observations [44], supporting the initiation of a Phase I/IIa clinical trial in the United States.

It was recently announced that in 2019 the first clinical trial to tackle spinal cord injury using hiPS cell-derived neural precursors will be held in Japan [45]. The

preclinical trial has taken place in 2012, during which it was demonstrated that the procedure, involving the injection of neural precursors 2–4 weeks after the injury, promoted regeneration of the spinal cords and increased the mobility in monkeys [46]. In the clinical trial that will take place, two million neural precursors will be injected within the same timeframe of injury, starting with a group of four patients.

Tissue engineering approaches combining the use of cells with scaffolds, biomolecules, or devices promise an evolution in the regenerative medicine field. Generation of pancreatic progenitor (PP) cells from hPS cells has promised to establish an almost unlimited source of islet cells for transplantation in diabetic patients [39]. Furthermore, these cells have demonstrated the ability of treating diabetes upon transplantation into mice [47]. ViaCyte, a biotechnology company, has been conducting a series of clinical trials in the past years that are currently on Phase I/II (Table 1). Their products comprise an islet-like cell population differentiated from hES cell-derived PPs, which is microencapsulated within a permeable device [48]. Upon transplantation into the patient, the islet-like cells can further mature in vivo and generate glucose responsive β-like cells.

Although heart disease represents the main cause of death worldwide, it was not until 2018 that the first clinical trial results started to emerge. The main aim of these therapies involves the replacement of cells lost due to acute heart failure and employs strategies such as cell sheets containing hPS cell-derived cardiomyocytes [49] or fibrin patches comprising hPS cell-derived endothelial and smooth muscle cells [50]. Recently, a clinical trial for the treatment of severe ischemic left ventricular dysfunction has demonstrated the safety of hES cell-derived cardiovascular progenitor (CVP) cells. In this safety study, a combination of hES cell-derived CVP cells embedded in a fibrin patch was delivered to patients during a coronary artery bypass, and it was demonstrated that this product improved the patient's symptoms while avoiding the formation of teratomas or the presence of arrhythmias, a common complication of cardiac cell therapy [51]. Importantly, a preclinical trial that tested the efficiency and safety in primates was already performed [36]. Here, it was observed that hiPS cell-derived cardiomyocytes were able to survive up to 12 weeks post-implantation and, importantly, were able to connect electrically with host cardiomyocytes and thus improve cardiac contractile function. This study will be one of the bases for the initiation of a small clinical trial led by the cardiac surgeon Yoshiki Sawa, which is supposed to start in early 2019. In this trial, allogeneic hiPS cell-derived cardiomyocyte sheets comprising 100 million cells each will be implanted onto the hearts of 3 patients suffering from advanced heart failure.

4 Bioprocesses for hPS Cell Expansion, Differentiation, and Purification

A bioprocess for production of hiPS cell derivatives, starting with the collection of donor somatic cells and ending with the delivery of a purified hiPS cell-derived product, is a long pathway that can take up to a few months. Most of these

bioprocesses still rely on culturing cells under 2D conditions, in the absence of control over culture parameters, such as pH and oxygen, and without automation, which can lead to operator variability and, consequently, low reproducibility and higher risks of contamination [52]. Therefore, to fulfill the potential of hPS cells in regenerative medicine, there is the need to develop robust and scalable platforms that can produce large numbers of high-quality hPS cells and/or hPS cell-derived products.

Most of the cells within the human body, upon in vitro culture, depend on the adhesion to a substrate to survive and proliferate. Under static conditions, this dependence can be addressed by coating tissue culture plates with the appropriate ECM molecules. However, in scaling up to dynamic culture systems, there is the need to increase the surface-to-volume ratio of the adhesion substrate and, at the same time, provide a system that can be used under dynamic conditions to afford a homogeneous environment to the cells. This can be attained using microcarriers. Although the use of solid microcarriers has been extensively studied for hPS cell expansion and differentiation either in spinner flasks [5, 53–55] or vertical wheel bioreactors [56], their use towards regenerative medicine applications requires an additional separation step. This limitation can be potentially overcome by the use of dissolvable microcarriers, which allow the integrated cell expansion and harvesting inside the bioreactor while avoiding the use of filtration procedures [54]. Nevertheless, although 2D culture can sustain the growth of almost all cell types, it fails to recapitulate in vivo conditions, as cells in vivo are usually surrounded either by other cells or ECM. Thus, 3D aggregate-based culture provides a better recapitulation of the in vivo microenvironment while avoiding the need for matrices that are typically required for cell adhesion, either in adherent or microcarrier cultures, hence reducing the number of components within the culture. Thus, in this chapter we will focus on this culture format for scalable expansion and differentiation of hiPS cells.

The first described method for aggregate generation was the hanging drop, which relies on the spontaneous aggregation of cells contained in droplets hanging from a surface [57]. By controlling the cell number and volume in each droplet, the size of the outcoming aggregate can be adjusted. Another technique relies on the seeding of V-shaped well plates, allowing generation of one aggregate per well [58]. This method can be adapted, in order to be reduced to a microscale level, where the droplets are formed in microwells, allowing the formation of smaller aggregates [59]. Moreover, the simplest technique involves the plating of a single-cell suspension into static or dynamic non-adherent surfaces, leading to spontaneous aggregation of the cells [60]. Cell density is an important factor, since the more cells are in the suspension, higher the probability of starting to form aggregates [61]. In addition, manually dissociated clumps can be passed through a mesh filter and fragmented into smaller aggregates [62]. Microfabrication methods can be used to produce microwell arrays made from a PDMS stamp in agarose that comprises an inverted pyramid shape where a cell suspension is seeded and, by centrifuging the plate containing the cells, will be entrapped in the microwell and generate size-controlled aggregates [63].

Scaling up of hPS cell culture exposes the cells to fluctuations in physicochemical parameters and to the hydrodynamic shear stress inside the bioreactors [64]. Importantly, homogeneity of size and morphology of hPS cell aggregates can be improved using forced aggregation in microwells [63], and the negative effect of shear stress under dynamic conditions can potentially be controlled by cell encapsulation [65]. Nevertheless, the use of dynamic suspension cultures also leads to increased spheroid homogeneity, as well as increased cell yields [66, 67]. However, it has to be considered that increased agitation speeds leads to an augmentation of shear forces, thus compromising the viability of the cells [61]. Polysulfated compounds have confirmed their capacity of reducing cell aggregation and promoting cell survival by reducing apoptosis [68]. Dextran sulfate has recently been demonstrated to improve aggregate size homogeneity on hPS cells without compromising their pluripotency, which will improve their efficiency in terms of expansion [69].

An important highlight on the scale-up of cell culture resides in the reduction of the overall cost of the process. For example, it has been demonstrated that scaling up of the production of β cells from hPS cells to provide insulin for type 1 diabetes would be able to reduce the cost per patient from $430,000 to $160,000 [70].

4.1 hPS Cell Expansion as 3D Aggregates

An initial approach towards expansion of hPS cells as suspension aggregates included the use of mouse embryonic fibroblast (MEF)-conditioned medium (MEF-CM), which was supplemented with bFGF and ROCKi [71], but this strategy carried the risk of contamination from xenogeneic products. A number of systems using animal-free conditions have emerged since, including human foreskin fibroblast-CM, which was used to scale up hPS cell expansion with a yield of eightfold in 7–10 days [72]. Amit et al. developed one of the first protocols for hPS cell expansion as suspension aggregates using serum-free media supplemented with interleukins, bFGF, and ROCKi [73]. This system was able to attain a 25-fold increase in 10 days, while maintaining the pluripotency and differentiation potential of the cells. At the same time, Steiner et al. developed a system that employed ECM-rich culture medium in combination with bFGF, activin A, and a serum replacement [74]. Since then, several defined culture systems have emerged and established major improvements in terms of robustness and reproducibility (Table 3), as well as compliance with good manufacturing practices (GMP) for eligibility for future clinical trials.

Culture of hPS cells as suspension aggregates in chemically defined conditions using commercially available culture media, such as mTeSR [61, 76, 78–80, 82, 85–87], StemPro [77, 88], and E8 [75, 80], has been extensively reported, leading to a maximum 25-fold increase in cell number after 6 days [78]. As another improvement to this culture system, single-cell inoculation and consequent aggregation of hPS cells have been used instead of passaging cells as aggregates, resulting in a higher level of expression of pluripotency markers [73, 76, 87]. 3D aggregate culture

Table 3 Scalable approaches for hPS cell expansion as suspension aggregates

Vessel				Expansion		
Type	Volume (mL)	Feeding strategy	Seeding density (cells/mL)	Media	Yield	Ref
Spinner flask	45	Repeated batch	4–5 × 10^5	E8	4.5-fold in 4 days	[75]
	50	Repeated batch	3 × 10^4 to 1 × 10^6	DMEM/ F12 + KOSR+ bFGF+IL6RIL6	25-fold in 10 days	[60]
	50	Repeated batch	0.33–1 × 10^6	mTeSR1	Sixfold in 4–7 days	[76]
	50	Repeated batch	0.3 × 10^5	DMEM/ F12 + Glutamax + bFGF	Eightfold in 7–10 days	[72]
	50	Repeated batch	1 × 10^6	mTeSR1	Twofold in 7 days	[61]
	60	Repeated batch	2.5 × 10^5	StemPro SFM	Fourfold in 4 days	[77]
	100	Repeated batch	1.8 × 10^4	mTeSR1	25-fold in 6 days	[78]
	100	Batch	2 × 10^4	mTeSR1	11–12-fold in 6 days	[79]
BioLevitator	30	Fed batch	0.3 × 10^5	mTeSR1	20-fold in 4 days	[80]
	30	Fed batch	0.3 × 10^5	E8	14-fold in 4 days	
3D hollow fiber bioreactors	17	Perfusion	2.9 × 10^6	mTeSR1	100-fold in 15 days	[81]
Controlled bioreactor	100	Repeated batch	4–5 × 10^5	mTeSR1	Fourfold in 7 days	[82]
	125	Perfusion	5 × 10^5	mTeSR1	Sixfold in 7 days	[83]
	150	Perfusion	5 × 10^5	E8	6.7-fold in 7 days	[84]

systems have been performed using several scalable culture platforms, including shaking flasks [60, 86], spinner flasks with a wide range of working volumes [61, 72, 73, 75–79, 82], and bioreactors with no impellers, using a gentle tube rotation [80], or using 3D hollow fibers [81]. In this type of systems, factorial design was used to maximize cell productivity, by allowing simultaneous optimization of the different culture parameters, including the agitation speed, and inoculation density. This mathematical tool allowed an increase in cell number of 12-fold after only 6 days of culture [79]. The feeding regimen of the reactor also plays an important role, with the use of perfusion yielding a final cell density of 2.85 × 10^6 cells/mL, which

represents an increase of 47% in cell yield when compared with batch cultures [83]. Furthermore, a recent approach by Manstein et al. describes the use of four parallel bioreactors that allows the generation of two million hPS cells using chemically defined medium under a perfusion feeding regimen during 7 days [84].

Importantly, the quality of hPS cells cultured in bioreactors and other scalable systems is closely monitored in terms of maintenance of the expression of pluripotency markers, assessment of pluripotent potential by multilineage differentiation or ability to generate teratomas in immunocompromised mice, and karyotypic analysis [75, 79].

4.2 *hPS Cell Differentiation as 3D Aggregates*

Although hPS cell-derived products represent an amazing opportunity for regenerative medicine, there is still the need to produce clinically relevant numbers of cells, which can attain up to 10^9 cells per patient [89]. The development of strategies for production of lineage-differentiated derivatives from hPS cells on a large scale has been one of the foci in the field of stem cell bioprocessing in the last years (Table 4). The use of small molecules and chemically defined and xeno-free culture media has increased the efficiency and robustness of differentiation protocols.

In the case of neural induction, it was determined that an average aggregate diameter of approximately 140 μm was optimal to efficiently generate neural progenitor (NP) cells from hiPS cells [90]. Furthermore, after only 6 days of induction using a dual SMAD inhibition protocol using small molecules – 10 μM SB431542 and 100 nM LDN193189 – nearly 14 million $Pax6^+$ NP cells were successfully derived from hiPS cells, in spinner flasks, under chemically defined conditions with efficiencies averaging 62% [91].

Table 4 Scalable approaches for hPS cell directed differentiation as suspension aggregates

Culture type	Differentiation		
Type	Directed lineage	Yield (%)	Ref
Spinner flask	Neural progenitors	~80	[90]
	Neural progenitors	~62	[91]
	Cardiomyocytes	27	[77]
	Hematopoietic progenitors	25–80	[75]
	Hematopoietic progenitors	5–6	[67]
	Cardiomyocytes	90	[6]
	Cardiomyocytes	80–99	[88]
	Islet cells	~90	[95]
Rotary orbital shaker	Cardiomyocytes	>60	[86]
	Pancreatic progenitors	7–32	[94]
Controlled bioreactor	Cardiomyocytes	85	[86]
	Cardiomyocytes	~80	[119]

As previously mentioned, the use of 3D conditions has numerous advantages comparing to adherent monolayer conditions. In the case of cardiomyocyte derivation from hPS cells, 3D conditions have demonstrated to promote an earlier mesendoderm lineage differentiation, as well as a faster maturation of the cardiomyocytes, either structurally or functionally [92].

Several groups have reported different methodologies that were able to generate large numbers of cardiomyocytes [6, 77, 85, 88] by culturing hiPS cells under 3D conditions in stirred bioreactors. The majority of these protocols are based in a Wnt signaling pathway modulation, as established by Lian et al. [93], which consists in an early Wnt signaling activation at early stages of differentiation by using a GSK3 inhibitor such as CHIR99021, followed by an inhibition of this signaling pathway by using IWP to specify cardiac differentiation of the mesendoderm-directed cells. Using this protocol for Wnt signaling modulation, hPS cell cardiac differentiation using a fully controlled stirred bioreactor has led to the production of up to 40 million cardiomyocytes with up to 95% of efficiency [85, 86].

Chen et al. highlighted the importance of size control of hPS cell aggregates before cardiac induction [88]. Here, an average hPS cell aggregate diameter of 200 ± 20 μm was shown to be less susceptible to changes in CHIR99021 concentrations, which could affect the efficiency of the differentiation protocol. Therefore, the optimized protocol was translated to a 1 L scale and was able to produce up to 96% of cardiac troponin T (cTnT) positive cells at a density of $1.4 \pm 0.4 \times 10^6$ cells per mL.

Furthermore, Fonoudi et al. developed a modified protocol that also used the initial inhibition of the Wnt signaling pathway but that, in the inhibition phase, also inhibited the TGF-β pathway and activated the sonic hedgehog (SHH) pathway [6]. Here, an optimal hPS cell initial aggregate diameter of 175 ± 25 μm promoted spheroid beating efficiency averaging almost 100% and was able to derive up to 90% of cardiomyocytes in only 10 days at a 100 mL bioreactor scale.

A four-stage protocol to differentiate pancreatic progenitors (PPs) from hPS cells was implemented under suspension conditions using ultra-low attachment plates and rotary agitation [94]. The first step involved the specification into definitive endoderm, by using a medium supplemented with activin A and Wnt3A for 1 day, following another day without Wnt3A. Then, KGF and TGF-β RI kinase inhibitor IV were used to promote primitive gut tube, and TTNPB, KAAD-cyclopamine, and Noggin supplementation favored posterior foregut formation. Finally, pancreatic and endocrine progenitors were generated by further supplementation with Noggin, KGF, and EGF. In the end, it was possible to obtain functional glucose-responsive, insulin-secreting cells. Nevertheless, this protocol still employs the use of fetal bovine serum in the first stages of differentiation, which will need to be replaced before considering this approach towards clinical applications. A similar protocol was translated to a 30 mL spinner flask, promoting the generation of islet-like cells with an efficiency of more than 90% of $PDX1^+$ cells that, upon engraftment in mice, were able to revert hyperglycemia [95].

4.3 Purification of hPS Cell-Derived Products

The major risk of the application of hPS cell-derived products resides in the possibility of teratoma formation due to an uncontrolled proliferation of a small number of pluripotent cells that remain in the final cell therapy product. In order to prevent this, clinical-scale purification of hPS cell derivatives for cell-based therapies needs to be addressed [96, 97].

Fluorescent-activated cell sorting (FACS) is probably the most widely used and more accurate method for purification of cells. This technique involves labeling the target cells with an antibody linked to a fluorescent molecule and has demonstrated efficiencies of separation above 95% [98]. For example, Kikuchi et al. included a sorting step at day 12 of neural induction of hPS cells that allowed the selection of more than 90% CORIN-positive cells, which would further differentiate into midbrain dopaminergic neurons [37]. In this study, on day 26 of differentiation, there were less than 1% of cells expressing pluripotency markers. FACS sorting of O4-positive oligodendrocyte progenitors led to an enriched cell population containing more than 93% of these cells that upon animal engraftment did not originate teratomas [38].

A widely used example of a target antigen for cell purification is SSEA-1, because this surface marker is absent from hPS cells [99]. Since the efficiency of the differentiation protocol of hES cells into CVPs only led to approximately 64% of efficiency, Menasche et al. integrated within the production pipeline a purification step taking advantage of this marker [51, 100]. The purification strategy consists in the depletion of hPS cells in culture by magnetically labeling cells with SSEA-1 and then using magnetic activated cell sorting (MACS) to select SSEA-1-positive cells, which had lost their pluripotency. Furthermore, the final product, which was required to contain less than 0.1% of undifferentiated cells, was also tested for the expression of Nanog.

For the treatment of Stargardt's macular dystrophy (SMD) and dry-age-related macular degeneration (AMD), hES cell-derived retinal pigment epithelium (RPE) cells were purified by exposure to type IV collagenase followed by manual isolation of RPE using a glass pipette [101]. Still, long-term safety studies in preclinical models have not detected any uncontrolled cell proliferation, indicating that no teratomas were generated upon implantation of hES cell-derived RPE [102]. An approach to address spinal cord injury by using hES cell-derived OP cells has demonstrated that a final product containing up to 5% of undifferentiated hES cells did not lead to teratoma formation [103]. However, the OP cell differentiation protocol has an efficiency that leads to less than 1% of hES cells in the final product.

Metabolic purification of hPS cell-derived products has also been focused in the last few years, mainly due to its reduced cost and high technical feasibility, which relies on the supplementation of the culture medium with components that are already present in most culture media formulation. Furthermore, these methods can be easily integrated within bioreactor culture systems without the need for cell singularization, contrary to methods like FACS or MACS. One good example of the

application of these methods is for hPSC-derived cardiomyocyte purification. In fact, the two main substrates for energy production by cardiomyocytes are glucose and fatty acids, but cardiomyocytes can also use lactate as an energy source in anaerobic metabolism [104]. In the first report of hPS cell-derived cardiomyocyte purification using a metabolic approach, a glucose-depleted and lactate-supplemented culture medium was capable of purifying the cardiomyocyte population up to 99%, both in mouse and human cells [105]. Still, glucose remains the main substrate for primary ATP production in immature cardiomyocytes, whereas fatty acids play an important role on the energy demand of mature cardiomyocytes. It has been demonstrated that glucose depletion associated with fatty acid supplementation is capable of reducing the number of hPS cells in the final product while increasing the maturation of hPS cell-derived cardiomyocytes [106].

Contrary to hPS cells, hPS-differentiated cells have mechanisms that prevent excessive uptake of L-alanine – an amino acid present in the human body – which would lead to cell swelling and, consequently, death. A novel approach towards selective elimination of hPS cells relies on the use of high concentrations of L-alanine to deplete the culture from pluripotent stem cells, and that can be a cost-effective alternative for purification of hPS cell-derived products to use in cell therapies [107].

4.4 Bioengineering Strategies for the Improvement of hPS Cell-Derived Organoids

Organoids are complex structures that can be generated from hPS cells or from organ-specific progenitor cells and that are capable of differentiating into multiple cell types while self-organizing themselves in a structure similar to the represented organ and, at the same time, recapitulating some of the organ functions, such as neural activity or contraction [108].

The application of bioprocessing strategies is crucial towards overcoming the limitations often associated with organoids. One of the main limitations resides in the heterogeneity among different organoids often associated with poor efficiency of differentiation of hPS cells into a specific lineage. It was already demonstrated that the initial hPS cell aggregate diameter is critical for the optimal differentiation into a specific cell lineage [90, 109]. For that, strategies that employ the use of microwells to control hPS cell aggregate size have proven effective to direct hPS cells into cardiomyocytes [63]. Arora et al. demonstrated that the passage from a spheroid containing hindgut progenitor cells into a pre-organoid is favored by the size and morphology of the spheroid [110]. Here, the sorting of aggregates with diameters greater than 75 μm favored the further development into intestinal organoids in 3.8-fold when compared with non-sorted populations.

As the size of the organoids increases, mass transport to the inner areas of the organoid becomes critical, since diffusional limitations often arise in spheroids with

diameters greater than 300 μm [111]. The use of agitated systems, such as spinner flasks, that enhance the mixing of nutrients and promote gas transfer has led to a rapid development of brain organoids [3, 112]. Nevertheless, in the absence of medium agitation, a vascularized liver organoid has already been developed, by combining hPS cell-derived hepatic endoderm with mesenchymal stem cells and human umbilical vein endothelial cells [113].

Another limitation of organoid technology relies on organoid encapsulation, since many of the processes developed so far depend on the embedding of organoids in a supporting extracellular matrix that helps maintaining the organoid in a favorable microenvironment for growth and maintenance [3]. Still, mechanical properties of the substrate in which the organoids are embed also play an important role. Although intestinal organoids present self-organization, embedding the organoids within a collagen gel allowed the inner cystic structures to align, generating macroscopic hollow tubes composed of multiple cells, a process that was not observed without this matrix [114]. A bioprocessing approach that employs the use of a two-fluidic electrostatic co-spraying technique has tackled the issue of large-scale production of encapsulated organoids [115]. Here, the organoids are encapsulated in a Matrigel core-shell that promotes cell growth and structural support and an outer alginate shell that protects the capsule from shear stress in suspension culture inside bioreactors.

Lastly, the main challenge associated with organoids and other tissues derived from hPS cells remains in the display of fetal-like characteristics [116], which may not adequately model adult diseases. Human intestinal organoids derived from hPS cells have demonstrated to resemble human fetal intestine instead of adult tissue [117], but upon in vivo transplantation are able to further mature and become more adult-like intestinal tissue. For cardiac derivatives generated from hPS cells, physical conditioning using electromechanical stimuli over a period of 4 weeks in culture was sufficient to accelerate cardiomyocyte maturation into adult-like human cardiac tissue [118].

5 Human Pluripotent Stem Cells and Disease Modeling

The ability to generate disease-specific hiPS cells has brought the opportunity of unveiling disease mechanisms that until recently were unknown. In the past few years, and mainly by using a combination between hiPS cells and organoid technologies, a myriad of disease models have been developed, from neurological [3, 120] to cardiac [121] or gut [122] diseases.

5.1 Modeled Diseases

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder where loss of motor neurons in the spinal cord and motor cortex induces progressive paralysis and, ultimately, death [123]. One of the first reports using hiPS cell technology consisted in the generation of ALS-specific hiPS cell lines that efficiently differentiated into motor neurons [124]. Still, only recently a model that incorporates 3D skeletal muscle bundles with light-sensitive channelrhodopsin-2-induced motor neuron spheroids derived from ALS patients was developed [125]. Using this platform, it is possible to analyze muscular contraction through light activation of the motor neurons. When ALS-specific motor neurons were used instead of healthy cells, there was less muscular contraction, together with more motor neuron degradation and decrease in their viability.

Second to Alzheimer's disease, Parkinson's disease (PD) is the most common chronic progressive neurodegenerative disorder, characterized by loss of dopaminergic neurons. Although patient-specific PD hiPS cells were already derived [126], since the onset of PD occurs on average at 50 years of a human life, additional cues – such as exposure to oxidative stress or neurotoxins – are needed to replicate the pathological mechanisms of PD in vitro.

Spinal muscular atrophy (SMA) is a genetic disorder based on the mutation of the *SMN1* gene and is characterized by selective degeneration of lower α-motor neurons [127]. Derivation of hiPS cells from a child with SMA followed by neural differentiation into motor neurons demonstrated deficits in either cell number or size when compared with a control from a parent [128].

Rett syndrome, a neurological disease commonly caused by mutations in the *MECP2* gene of the X chromosome, is related with impairment of motor function and autistic-like behavior [120]. Using patient cells and hiPS cell technology, it was possible to recapitulate an altered process of neurogenesis in cells of these patients, as well as the presence of an inferior number of neurons and less complex neurites [129].

Familial dysautonomia, or Riley-Day syndrome, is a fatal autosomal recessive disease caused by a mutation in the *IKBKAP* gene and characterized by the degeneration of sensory and autonomic neurons [130]. Neural induction of patient-specific hiPS cells has demonstrated that in vitro these cultures present neural crest precursors with lower Tuj1^{+} neuron differentiation potential, in addition to defects in migration behavior [131].

Schizophrenia affects around 1% of the worldwide population and possesses a hereditary genetic component in almost 85% of the cases [132]. It was shown that neural differentiation of hiPS cells from schizophrenic patients led to decreased neural connectivity and decreased number of neurites and synaptic protein levels while presenting a normal electrophysiological behavior and spontaneous calcium transient activity [133].

Heart diseases have also been addressed as a target for disease modeling using hiPS cells. Recapitulation of familial long QT syndrome was achieved by generating

hiPS cell-derived cardiomyocytes from patients, recreating electrophysiological features of the disorder, such as prolonged action potentials [121].

Dilated cardiomyopathy occurs in 1 of 250 adults and is characterized by progressive left ventricular dilation and, eventually, heart failure [134]. This disease may arise from mutations that truncate the sarcomere protein titin, which was verified by derivation of disease-specific hiPS cells that, upon cardiac differentiation, resulted in the generation of cardiomyocytes with sarcomere deficiency, combined with decreased responses to stress, as well as to activation from growth factors [135].

Another example of a heart disease modeled using hiPS cell technology consists in Duchenne muscular dystrophy (DMD), which is characterized by the progressive weakness of the skeletal and cardiac muscle [136]. Cardiomyocytes derived from patients with DMD displayed dystrophin deficiency when compared with controls from healthy donors. Furthermore, disease-specific cardiomyocytes demonstrated higher levels of apoptotic markers, including increased levels of cytosolic Ca^{2+} or caspase-3, since these cells are more sensitive to stress-induced damages [137].

5.2 Organoids and Disease Modeling

Neural differentiation of hPS cells has been thoroughly investigated, since neuroectoderm represents the default differentiation pathway upon withdrawal of pluripotency maintenance culture conditions [138]. Brain organoids derived from hPS cells were the first complex 3D models that were able to recapitulate certain aspects of in vivo brain development such as progenitor zone organization and the organization of outer radial glial stem cells [3]. Using Matrigel as an extracellular matrix to support the process of self-organization, it was possible to obtain brain organoids that exhibited cortical self-organization, as well as specification of hind-brain and forebrain regions. Since then, other brain-specific organoids have been recreated using specific morphogen gradients, including the cerebellum [139] and the optic cup [140], as well as organoids from other tissues, such as the lung [116] or even liver buds [113]. Furthermore, it was recently demonstrated that brain organoids from different regions could be fused to mimic interactions between brain regions. The coculture of brain organoids with dorsal and ventral forebrain identities led to the generation of a model of a dorsal-ventral axis that demonstrated the migration of CXCR4-dependent GABAergic interneurons from ventral to dorsal forebrain [141].

The combination between hiPS cell differentiation and organoid technology has opened the possibility of using patient-specific cells to recreate disease mechanisms in vitro using more relevant models of in vivo tissues, as certain structural characteristics of a disease can also be replicated in a 3D structure. Several disease models have been developed using organoids, ranging from neurological diseases [3, 142–144], using brain organoids, to heart conditions, using cardiac organoids [121, 135], or cystic fibrosis [145], using lung organoids, as listed in Table 5.

Table 5 Disease modeling using hiPS cells and respective observed in vitro characteristics

Modeled disease	Model characteristics	Ref
Alagille syndrome	• Impaired chloride transport	[144]
Autism	• Normal early neuronal differentiation • Imbalance in inhibitory GABAergic neurons over glutamatergic	[143]
Cystic fibrosis	• Impaired forskolin-induced swelling	[145]
Dilated cardiomyopathy	• Sarcomere insufficiency • Impaired responses to mechanical and β-adrenergic stress	[135]
Familial adenomatous polyposis	• Increased cell proliferation • Increased nuclear localization of β-catenin	[122]
Familial dysautonomia	• Defects in neural differentiation	[131]
Familial long QT syndrome	• Prolonged action potential in cardiomyocytes	[121]
Hirschsprung's disease	• Impaired organization of enteric nervous system	[146]
Microcephaly	• Premature neuroepithelial differentiation • Aberrant glial orientation • Smaller areas of differentiated tissue	[3]
Miller-Dieker syndrome	• Poor neurite growth • Apoptosis in the ventricular zone • Impaired neuronal migration	[142]
Rett syndrome	• Impaired neurogenesis • Reduced neuronal migration	[120]
Schizophrenia	• Decreased neural connectivity, neurite formation, and synaptic protein expression	[133]
Spinal muscular atrophy	• Motor neuron differentiation	[128]

In the field of neurological diseases, the study of microcephaly was the first that was addressed using hiPS cell-derived brain organoids [3]. Using fibroblasts from a patient with microcephaly, caused by truncating mutations in *CDK5RAP2*, it was possible to generate a patient-specific hiPS cell line that, under pluripotency maintenance conditions, behaved similar to a hiPS cell control line. Still, upon induction to neuroectoderm as a 3D structure, a smaller neuroepithelial tissue with fewer progenitor zones and an increased premature neuronal outgrowth was observed.

Autism spectrum disorders affect 1 in every 60–70 children and have as main symptoms behavioral deficits in social interaction, communication, and interests, along with repetitive behaviors [147]. Using patient-specific hiPS cell-derived neural organoids, normal neuronal differentiation was observed, while there was an imbalance in the number of inhibitory GABAergic neurons over glutamatergic neurons, which suggests an underlying mechanism for this neurological disease [143].

Miller-Dieker syndrome is a genetic neurological disorder caused by large heterozygous deletions of human band 17p13.3, and it is characterized by a major absence of cortical folding of the brain, leading to microcephaly, mental retardation, and intractable epilepsy [148]. Using brain organoids derived from patient-specific hiPS cells, Bershteyn et al. demonstrated that there were apoptotic areas in the subventricular zone, poor neurite growth, and impaired neural migration [142].

The enteric nervous system (ENS) of the gastrointestinal tract is crucial for the functions of this organ including motility, secretion, and blood flow [149]. The development of a normal intestinal enteric nervous system has been recapitulated by merging hiPS cell-derived neural crest cells and human intestinal organoids, which were able to mediate contractile waves [146]. Using this model, it was possible to recapitulate Hirschsprung's disease, characterized by congenital lack of enteric ganglia [150], and to verify that in vitro there was an impaired neural crest and ENS development [146].

A model of vascular disease based on blood vessel damage induced by diabetes was recently developed [151]. Here, endothelial cells were derived from hiPS cells, and, using a basement membrane, blood vessel organoids were created. Furthermore, upon transplantation to mouse models and exposure to a diabetic environment, there was thickening of vascular basement membrane, characteristic of diabetic vasculopathy.

Cystic fibrosis (CF) is a genetic disease that affects several organs, such as the liver and intestine, but mostly the lungs. Contrary to other lineage specifications from hPS cells, differentiation protocols towards cells of the respiratory track tend to be complex. Still, directed differentiation of hPS cells into NKX2.1+ airway progenitor cells followed by low Wnt signaling activation led to the generation of lung organoids that contained cells from the goblet, basal, and secretory lineages [145]. Furthermore, using hiPS cells derived from CF patients, it was possible to recapitulate key features of the disease, including impaired forskolin-induced swelling.

5.3 Disease Modeling Using CRISPR/Cas9

Despite the several examples of genetic diseases modeled using hiPS cell technology (Table 5), a new challenge that emerges is how to clearly discriminate if the differences observed in the phenotype are due to the mutation or to the individual's genetic background. A state-of-the-art approach relies on the use of genetic manipulation tools that correct the mutation of a given clone, creating an isogenic hiPS cell line. The most used tool is the clustered regularly interspaced short palindromic repeats (CRISPR) modified with two components – Cas9, which is the enzyme responsible for DNA cleavage, and guide RNA, which binds to Cas9 and pairs with the desired DNA site [152]. The CRISPR/Cas9 technology allows a footprint-free gene modification and at the same time has the advantage of being a simpler manipulation procedure comparing to other gene editing techniques such as zinc finger nucleases [153]. In addition to this strategy, CRISPR/Cas9 can also be used for the insertion of a mutation to recreate a mutated phenotype.

Huntington disease (HD) is caused by a CAG repeat in *HTT* and results in impaired neural rosette formation and deficits in mitochondrial respiration. Using a HD-specific hiPS cell line and a corrected isogenic hiPS cell line, it was possible not only to differentiate the cells into forebrain neurons but also to rescue phenotypic

abnormalities in the isogenic hiPS cell line [154]. Several other genetic diseases, like amyotrophic lateral sclerosis [155], Alzheimer's disease [156], and β-thalassemia [157], among others, have been studied using this approach. On the other hand, CRISPR/Cas9 has also been used to introduce the CCR5del32 mutation in hiPS cells, which originated monocytes resistant to HIV infection [158].

Despite all the apparent advantages, CRISP/Cas9 technology has been thoroughly debated, since recent reports have demonstrated that this methodology may not be as specific as initially presented, since significant mutations near the target site have been detected [159].

5.4 Drug Discovery

Both organoids and hiPS cell-derived cells are currently part of a paradigm change in the industry of drug screening and development. Apart from the use of such technologies for studying the response of in vitro human tissues to specific drugs, the possibility of attaining personalized treatments is also possible due to hiPS cell technology.

We have previously reported the use of hiPS cell-derived NPs to predict the effect of commonly used compounds, such as valproic acid (VPA), during the early stages of the process of neurodevelopment [7]. Exposure of cells to VPA during the initial stage of brain tissue formation, replicated in vitro using hiPS cell spheroids, has led to disorganized neural rosette structures, decreased cell viability, and delayed neuronal differentiation.

As previously mentioned in this chapter, using a combination of 3D skeletal muscle bundles and light sensitive spheroids of motor neurons derived from hiPS cells of an ALS patient, it was possible to analyze muscular contraction based on light activation of the motor neurons [125]. This platform was used to test the efficacy of drug candidates to treat ALS, such as rapamycin and bosutinib. In the presence of these drugs, the levels of caspase-3/7 decreased, indicating fewer cell death, which may suggest a neuroprotection mechanism of such drugs.

Another application of hiPS cell-based products relies on the validation of drugs to inhibit viral infection of neural tissues. Derivation of NP cells from hiPS cells has also been addressed to screen for compounds that block Zika virus infection and, at the same time, clear the virus from already infected NP cells [160].

Acute myeloid leukemia (AML) is an oncologic disease that is caused by a translocation in the *MLL* gene and that compromises normal hematopoiesis, by uncontrolled proliferation of myeloid progenitor cells. iPS cells derived from AML patients were able to differentiate into hematopoietic progenitors, while still retaining their malignant characteristics [161]. Importantly, this study has demonstrated that clonal differences in different hiPS cell lines derived from patients with AML can represent a powerful tool to understand drug sensibility of individual hiPS cell-derived subclones.

Although the efficiency of a drug is important, other aspects comprised within the drug metabolism process cannot be neglected. Intestinal drug absorption is important for the oral drug delivery system, and, for that, using hiPS cell-derived epithelial cells, a model of prediction of drug absorption was developed [162]. The derived epithelial cells displayed similar characteristics to their in vivo counterparts, such as the presence of thigh junctions, metabolic enzymes, and drug transporters, that closely mimic the mechanisms of oral drug absorption, which makes this platform a powerful tool towards the prediction of drug absorption in vivo.

Organ-on-a chip platforms provide invaluable tools for drug screening, since they combine the opportunity of recapitulating the multicellular architectures of an organoid with the possibility of testing different drugs on a high-throughput scale. The derivation of multi-organ platforms from hPS cells and their application for drug screening have already been reviewed in Miranda et al. [4]. Still, we highlight the development of a multi-organ platform that incorporates three different modules containing organoids from the liver, heart, and lung [163]. This device has the great advantage of allowing to analyze an individual response to a drug for each organoid or to combine the response between the organoids from different tissues, which gives a more complete preview of the in vivo behavior. Here, the effect of bleomycin – a chemotherapeutic drug used to treat lung cancer – was studied in separated organ modules and in an interconnected manner. Using an isolated module setting, as predictable, there was an increase in inflammatory markers in the lung module and no cardiotoxic effect in the separate heart module. Still, in a three-organ setting, it was possible to observe a decrease in heart rate due to inflammatory factor-driven cardiotoxicity, which highlights the importance of a combined response between different organs in order to efficiently predict cell behavior upon drug exposure.

Overall, although organ-on-a-chip platforms provide an opportunity towards drug discovery applications, most of them still uses primary cell sources and immortalized cell lines. Despite the fact that primary cells retain similar characteristics, such as metabolic profiles and functional properties to the original tissues, they tend to lose these properties when cultured in vitro for prolonged periods of time [164]. On the other hand, immortalized cell lines frequently fail to recapitulate the original tissue and are known for the accumulation of karyotypic abnormalities that may compromise their response to stimuli [165]. Therefore, hPS cell derivatives can address some of these issues, adding the possibility of generating disease- and patient-specific cells that promote a more realistic approach to drug screening applications.

6 Conclusions and Future Perspectives

The advancement in hiPS cell technology has been impressive since the first derivation of these cells, providing great opportunities in the fields of regenerative medicine, disease modeling, and drug screening. Here, we summarized recent progresses in the biomedical applications of hiPS cells as well as the bioprocessing challenges that have to be surpassed to fulfill these applications, namely, to allow

their translation either into clinical settings or for study of disease mechanisms or towards drug discovery.

Some limitations still need to be addressed prior to a complete shift towards these systems. First, there is the need to keep developing robust protocols for hiPS cell lineage specification, since even protocols that employ the use of defined culture medium and small molecules are not always reproducible. Moreover, there is the need to develop robust, safe, and scalable protocols for the large-scale production of these derivatives with high quality and functionality. Second, even though patient-specific hiPS cell lines can be derived, the individual hiPS cell clones may display some variability. Furthermore, although isogenic hiPS cell lines are considered state of the art, it must be considered that the single-cell cloning procedures may result in clones that possess different properties from the original cells.

Currently, most of the hPS cell-derived organoids represent only some aspects of the target organ, displaying a limited number of cell types [166], which can hinder a correct structural organization and thus function of the tissue. In the future, it will be necessary to introduce protocols that will differentiate hPS cells into a representative number of cell types, in order to accurately represent the physical interactions and organization between cells. Future approaches also include further maturation of organoids, since current organoids obtained from hPS cells represent an early developmental stage, often compared with fetal tissues [167]. It is expected that hiPS cell-based disease models using organoids will improve preclinical drug screening and accelerate candidate therapies into clinical trials. Incorporation of organoid technology combined with organ-on-a-chip technology will accelerate the readouts of each experiment, while maintaining a humanized response in comparison with the currently available animal models.

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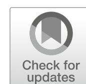
Addressing the Manufacturing Challenges of Cell-Based Therapies



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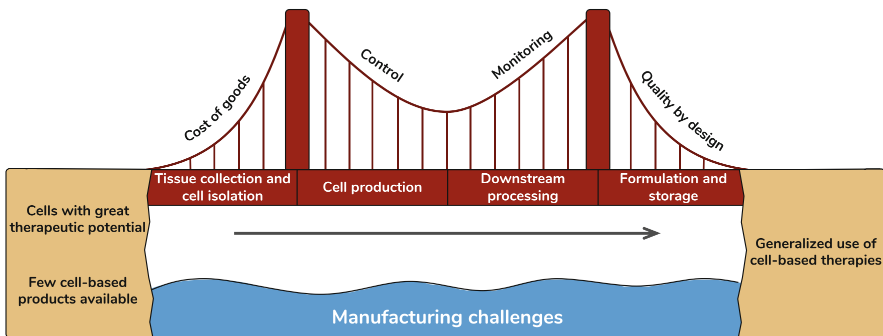
Abstract Exciting developments in the cell therapy field over the last decades have led to an increasing number of clinical trials and the first cell products receiving marketing authorization. In spite of substantial progress in the field, manufacturing of cell-based therapies presents multiple challenges that need to be addressed in order to assure the development of safe, efficacious, and cost-effective cell therapies.

The manufacturing process of cell-based therapies generally requires tissue collection, cell isolation, culture and expansion (upstream processing), cell harvest, separation and purification (downstream processing), and, finally, product formulation and storage. Each one of these stages presents significant challenges that have been the focus of study over the years, leading to innovative and groundbreaking technological advances, as discussed throughout this chapter.

Delivery of cell-based therapies relies on defining product targets while controlling process variable impact on cellular features. Moreover, commercial viability is a critical issue that has had damaging consequences for some therapies. Implementation of cost-effectiveness measures facilitates healthy process development, potentially being able to influence end product pricing.

Although cell-based therapies represent a new level in bioprocessing complexity in every manufacturing stage, they also show unprecedented levels of therapeutic potential, already radically changing the landscape of medical care.

Graphical Abstract



Keywords Bioreactor, Cell therapy, Hematopoietic stem/progenitor cells (HSPC), Manufacturing, Mesenchymal stem/stromal cells (MSC), Process engineering

1 The Landscape of Cell-Based Therapies

1.1 Hematopoietic Cell Transplantation: A Landmark Cell-Based Therapy

The development of cellular therapies began with the establishment of hematopoietic cell transplantation (HCT). Early work in murine models led to the observation that

supralethal radiation could be survived if affected mice were infused with a bone marrow (BM) graft [1]. BM aspirates containing hematopoietic stem/progenitor cells (HSPC) were able to migrate to the affected BM after radiation-derived myeloablative treatment and reconstitute the entire hematopoietic system.

After proving to treat radiation injury, BM transplantation was considered as a possible treatment for leukemia. In 1956, Barnes and colleagues were able to infuse normal BM grafts on leukemic mice as a proof of concept [2]. Knocking out a murine hematopoietic system also meant eliminating its blood-related malignancies. Transplants of healthy grafts would then repopulate the BM and form a new hematopoietic system. By transposing this knowledge to humans, Thomas and colleagues were the first to successfully perform HCT in human acute leukemia patients, paving the way toward showing the feasibility of cell therapies [3].

HCT development demonstrated that a main and contemporary threat in cell therapies, known as rejection, could be overcome. Indeed, early on, Medawar and colleagues were crucial in identifying rejection as an immunologic response by observing the progressive degradation of skin grafts [4]. In 1953, Billingham and colleagues described a breakthrough that brought down the inevitability of rejection. The concept of acquired tolerance was described when lack of rejection was seen in mice that were previously infused with donor cells during fetal development, originating chimeras [5]. HCT was able to surpass rejection through acquired tolerance with elimination of the existing immunological response by radiation pre-conditioning.

Rapidly after the discovery of acquired tolerance, another major concern related with cell therapies arose. Billingham and Brent observed that even with spleen or BM cells infused to induce tolerance, skin grafts continued to fail as the age of the recipients increased [6]. The reason behind this adverse reaction was another immunological response, brought upon by the actual graft. Active immune cells from the donor that accompanied the grafts were not tolerant toward the recipient. Consequently, these would launch a hostile immune response mirroring rejection mechanisms. However, this reaction is limited to HCT, not occurring in solid organ transplantation, where the only mechanism of rejection is the attack triggered by the recipient's immune system against the transplanted organ. Initially termed runt disease, since affected mice would not develop into their adult life, this immune-based effect is presently known as graft versus host disease (GVHD) [7].

Rejection and GVHD possess similar modes of action, being based on an immunologic response. Circumventing these complications in a transplant scenario meant finding the basis of immunological identity, namely, genes encoding for the human leukocyte antigens (HLA) [8]. With the development of HLA typing, transplants could be performed between HLA-matched patients, with the hope of avoiding rejection and GVHD. This led to the first successful HLA-matched HCT between two siblings performed by Gatti and colleagues in 1968 to treat severe combined immunodeficiency (SCID) [9], thus demonstrating the potential of HCT to treat illnesses other than cancer.

HCT was the first cell therapy to be adopted in a clinical setting, ultimately gaining widespread acceptance for treatment of genetic or oncological disease with

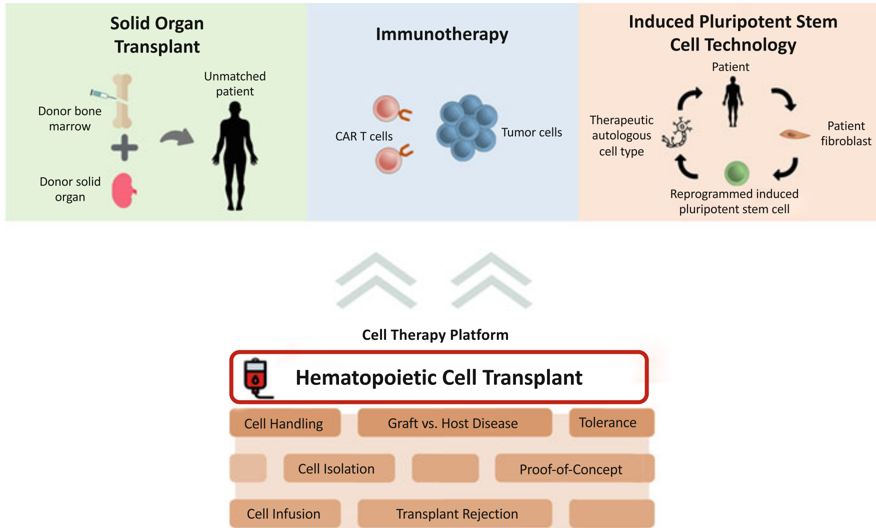


Fig. 1 HCT as a platform for the development of cell-based therapies. Pioneering experience in using cells as therapeutic agents laid the foundation for the diversification of cell therapies. HCT has been challenged with most of the crucial obstacles of cell-based therapies, from technical hurdles due to handling, isolation, or infusion of cells to unprecedented biological concepts (e.g., rejection) transversal to any therapy involving cells as therapeutic agents. Success in solid organ transplants was reliant on understanding compatibility between patients. Identifying the main agents of the immunological system was imperative to the development of immunotherapies. Also, induced pluripotent stem cell technology gained substantial impact since it showed that lack of compatibility could be overcome by creating autologous sources for any type of cell. *HCT* hematopoietic cell transplantation

therapeutic success. The implementation of most current cell therapies can be traced back to knowledge gained by the establishment of HCT (Fig. 1). For instances, the field of solid organ transplantation, which is very limited by tissue tolerance, has also benefited from HCT experience [10]. Joint transplantation of organs with their respective BM graft was able to cause acquired tolerance toward the solid organ and overcome HLA mismatch [11]. Gained knowledge of the potential and limits of allogenic and autologous approaches has facilitated therapeutic objective delineation in skin grafts. Using an allogenic source for the production of dermal and composite substitutes (e.g., Dermagraft® and Apligraf®) has limited grafts to serve only as a temporary barrier that promotes wound healing, without any permanent engraftment [12, 13]. On the other hand, autologous skin grafts (e.g., EpiCel® and PermaDerm®) integrate the skin of the patient while continuously promoting wound closure, reducing scar tissue formation, and mitigating an inflammatory microenvironment [14, 15].

Besides GVHD, HCT also demonstrates a graft vs. leukemia (GVL) effect against residual malignant cells that defied conditioning treatments. This behavior has been associated with lymphoid constituents of the transplant, which has heightened

interest in adoptive T-cell therapy. Donor lymphocyte infusions (DLI) of specific T-cell subsets or, more recently, enhancement of their antitumoral capabilities through chimeric antigen receptor T (CAR-T) cell technology are approaches that have shown great success in both treating cancer and mitigating GVHD in patients [16, 17].

Instead of tackling donor compatibility, rejection and GVHD could be avoided altogether. Induced pluripotent stem cell (iPSC) technology has opened up the possibility of using patients as cell sources for their own therapies. Although very promising and prompting considerable investment, this technology is yet at its infancy, with very few clinical trials to date and still requires significant maturation before moving to clinical practice [18]. Nonetheless, its potential for cell therapies and disruptive nature are founded on compatibility issues learned from HCT.

HSPC are the most studied cells in clinical trials for multiple innovative applications [19], with over 3,500 clinical trials taking place worldwide, the vast majority in the USA followed by Europe (Fig. 2a) (clinicaltrials.gov, accessed on 29 May 2019, using the search term "hematopoietic stem cell OR hematopoietic progenitor cell"). Neoplasms by histologic type, immune system diseases, leukemia, lymphoproliferative disorders, and immunoproliferative disorders are the top five conditions with the highest numbers of undergoing clinical trials worldwide.

1.2 Mesenchymal Stem/Stromal Cells as a New Paradigm for Paracrine Cell Therapy

Following initial developments in HCT, the BM was once more the source for the discovery of yet another promising stem cell population, named mesenchymal stem cells (MSC) by Caplan in 1991 [20]. The foundations for the discovery of these stem cells can be traced back to the nineteenth century, when studies on the BM transplantation to heterotopic anatomical sites resulted in de novo generation of ectopic bone and marrow [21, 22]. However, it was only later that the work of Tavassoli and Crosby clearly provided evidence of an inherent osteogenic potential associated with the BM [23]. In the 1960s–1970s, Friedenstein and colleagues isolated and characterized a subpopulation of adherent spindle-shaped cells from mouse BM that were responsible for the previously described osteogenic potential [24, 25]. Moreover, they also demonstrated that BM cell suspensions could generate colony-forming unit-fibroblasts (CFU-F). These cells were then later designated as "mesenchymal stem cells" and shown to have multilineage differentiation potential, including the osteogenic, adipogenic, and chondrogenic lineages [20, 26].

Over the following decades, questions were raised over the usage of the term "mesenchymal stem cells," and alternative nomenclatures have been proposed by different authors. This is due to unfractionated plastic-adherent marrow cells being quite heterogeneous and current data being insufficient to characterize them as stem cells. In order to address the inconsistency in the nomenclature and account for the

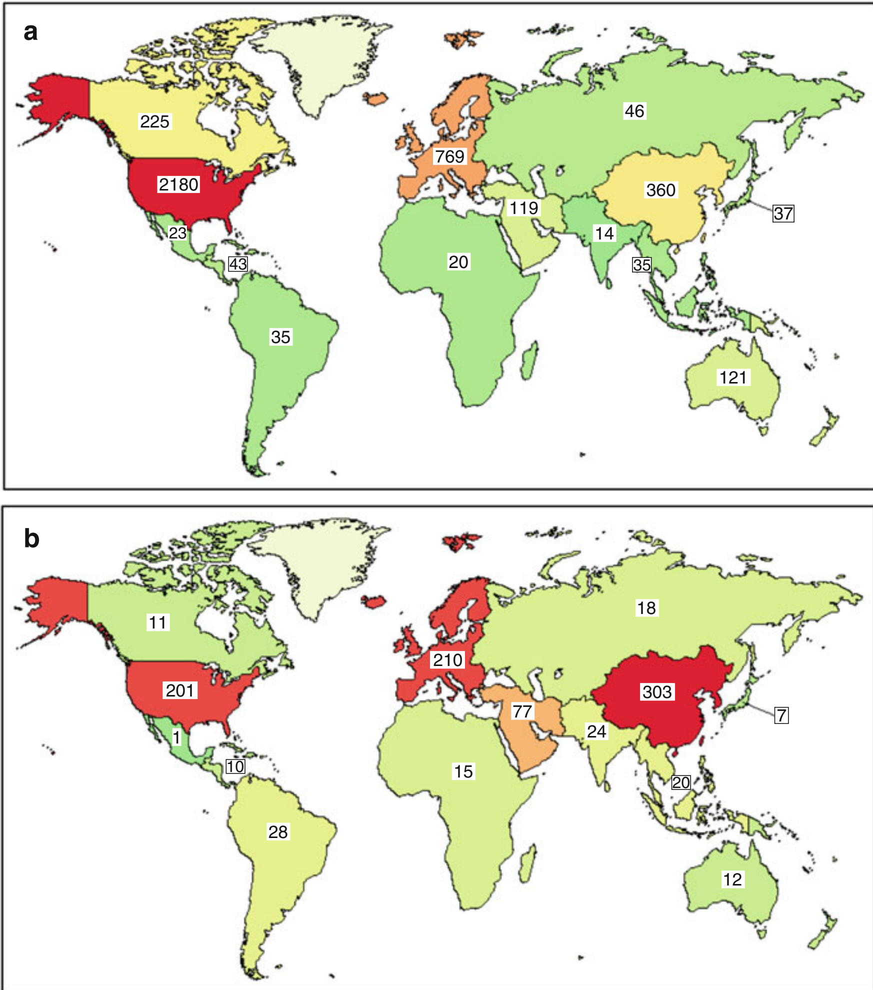


Fig. 2 Worldwide distribution of clinical trials obtained from "clinicaltrials.gov" on 29 May 2019, using the terms "hematopoietic stem cell OR hematopoietic progenitor cell" (**a**) and "mesenchymal stem cell OR mesenchymal stromal cell" (**b**)

biological properties of these cells, the International Society for Cellular Therapy (ISCT) proposed that plastic-adherent cells described as mesenchymal stem cells should be termed multipotent mesenchymal stromal cells, maintaining the acronym MSC [27].

The controversy in the appropriate nomenclature is accompanied by the inconsistency between investigators on the set of characteristics that define MSC. Laboratories have developed different methods of isolation and expansion, as well as different approaches to characterize these cells. Thus, an appropriate comparison between studies may be difficult to achieve. In order to address this issue, the ISCT

has proposed minimal criteria to define human MSC: (1) adherence to plastic; (2) expression of CD73, CD90, and CD105 and lack of expression of CD14 or CD11b, CD79α or CD19, CD34, CD45, and HLA-DR; and (3) osteogenic, adipogenic, and chondrogenic differentiation potential under standard culture conditions [28]. Similarly, minimal criteria for the definition of adipose tissue (AT)-derived stromal/stem cells have also been recently established by a combined panel from ISCT and the International Federation for Adipose Therapeutics and Science (IFATS) [29].

MSC present additional characteristics that make them attractive for therapeutic purposes, other than their ability to give rise to different mesenchymal phenotypes. The secretion of a broad range of bioactive molecules, such as growth factors, cytokines, and chemokines, renders them with immunomodulatory and trophic activities, acting both in a paracrine and autocrine manner [30, 31]. MSC trophic activity relies on bioactive factors that assist in repair and regeneration processes. MSC are able to inhibit scarring (fibrosis) and apoptosis, promote angiogenesis, and support growth and differentiation of progenitor cells into functional regenerative units [30, 31].

The panoply of beneficial effects ascribed to MSC has made them the second most studied cells in clinical trials, immediately after HSPC [19], with over 900 clinical trials taking place worldwide, receiving a special focus in China, Europe, and the USA (Fig. 2b) (clinicaltrials.gov, accessed on 29 May 2019, using the search term “mesenchymal stem cell OR mesenchymal stromal cell”). MSC are promising candidates for the treatment of a wide range of diseases, which is clearly observed from the great diversity of conditions targeted in clinical trials. Musculoskeletal diseases, immune system diseases, wounds and injuries, central nervous system diseases, and vascular diseases are the top five conditions with the highest numbers of undergoing clinical trials worldwide.

Many other cell types are being studied in clinical trials including lymphocytes, dendritic cells, hepatocytes, and endothelial cells [19]. Nonetheless, a special focus will be given to HSPC and MSC throughout this chapter.

1.3 Clinical Application and Challenges of Cell-Based Therapies

Since 2009, 12 cell-based therapies have been approved and received marketing authorization in the European Union (EU) and the USA combined (Table 1) [32–34]. The first successfully approved product was ChondroCelect, from TiGenix, despite being withdrawn in 2016 due to commercial reasons. This product consisted in autologous cartilage cells expanded ex vivo to treat knee cartilage defects. Holoclar (Chiesi Farmaceutici) was the first approved stem cell product (2015), consisting in ex vivo expanded autologous human corneal epithelial cells containing stem cells to treat severe limbal stem cell deficiency. Other approved products

Table 1 Cell-based therapies that received MA in the USA and EU by September 2018 [32–34]

Product (MA holder)	Product description	Therapeutic indication	Date approved
Alofisel (Takeda Pharma A/S)	Expanded allogeneic mesenchymal adult stem cells extracted from adipose tissue	Perianal fistulas in patients with Crohn's disease	2018 (EU)
Yescarta (Kite Pharma)	Autologous T cells genetically modified by retroviral transduction to encode an anti-CD19 chimeric antigen receptor (CAR)	Large B-cell lymphoma	2018 (EU) 2017 (USA)
Kymriah (Novartis)	Autologous T cells genetically modified using a lentiviral vector to encode an anti-CD19 CAR	Acute lymphoblastic leukemia; large B-cell lymphoma	2018 (EU) 2017 (USA)
Spherox (co.don AG)	Spheroids of human autologous matrix-associated chondrocytes	Knee cartilage defects	2017 (EU)
Strimvelis (Orchard Therapeutics)	Autologous $CD34^{+}$ cells transduced with an engineered retroviral vector encoding the human adenosine deaminase sequence	Severe combined immunodeficiency	2016 (EU)
Zalmoxis (MolMed)	Allogeneic T cells genetically modified to express a truncated form of the human low affinity nerve growth factor receptor and the herpes simplex I virus thymidine kinase	Control mechanism for graft-versus-host disease after hematopoietic cell transplantation	2016 (EU)
Holoclar (Chiesi Farmaceutici)	Ex vivo expanded autologous human corneal epithelial cells containing stem cells	Severe limbal stem cell deficiency	2015 (EU)
Provenge (Dendreon)	Autologous peripheral-blood mononuclear cells activated with prostatic acid phosphatase granulocyte-macrophage colony-stimulating factor	Metastatic prostate cancer	2013 (EU) (withdrawn from EU in 2015) 2010 (USA)
Maci (Vericel)	Autologous cultured chondrocytes	Knee cartilage defects	2016 (USA) 2013 (EU) (suspended in EU in 2014)
GINTUIT (Organogenesis)	Allogeneic cultured keratinocytes and fibroblasts in bovine collagen	Mucogingival conditions	2012 (USA)
Laviv (Fibrocell Technologies)	Autologous fibroblasts	Severe nasolabial fold wrinkles	2011 (USA)
ChondroCelect (TiGenix)	Autologous cartilage cells expanded ex vivo expressing specific marker proteins	Knee cartilage defects	2009 (EU) (withdrawn in 2016)

MA marketing authorization, *USA* United States of America, *EU* European Union

include the first CAR-T cell therapies for liquid cancers, Kymriah (Novartis) and Yescarta (Kite Pharma), approved in 2017 in the USA and in 2018 in the EU, and more recently, Alofisel (Takeda Pharma) that consists in expanded allogeneic AT-derived MSC to treat perianal fistulas in patients with Crohn's disease.

Due to their uniqueness, cell therapies have earned their own category in regulatory agencies with special directives concerning approval candidature. Cell-based therapies are considered advanced therapy medicinal products (ATMP), defined by the European Medicines Agency (EMA) as medicines for human use that are based on genes, tissues, or cells, offering groundbreaking new opportunities for the treatment of disease and injury [33].

In spite of the establishment of guidelines and regulations applying to cell therapies, a number of unresolved issues remain, making the regulatory path toward clinical approval a challenge [35]. Certain important requirements often lack in clarity, and regulation is not specific enough. This results in products where the appropriate classification is not entirely certain [36, 37]. Furthermore, discrepancies between regulatory agencies from different countries hinder companies trying to reach the market at an international level [37]. The challenging regulatory environment contributes to the need to endure over long time periods before reaching the market. Often, cell products only gain market access 15–20 years after the company was founded [37]. Nevertheless, the regulatory environment is gradually improving. In order to continue this path and make wise development choices, it will be crucial to promote a cross talk between scientists, companies developing cell therapies, and regulators [37].

Although this millennium has been marked with considerate advances, with regulatory victories for several ATMP, cell therapy development has a long and considerable track record. Recent success is due to much effort in the past uncovering and understanding all the obstacles that stood between the establishments of therapeutic options based on cells. HCT was decisive as a vehicle of problem-solving and thus has deserved its recognition as a foundation for cell therapy development [10].

With multiple cell-based therapies already reaching the market, one of the most pressing issues will be addressing the challenges in manufacturing these products. Most cell-based therapies are costly and target widespread medical conditions. The robust and scalable cell manufacturing for the cost-effective delivery of safe and potent cell-derived ATMP (either with autologous origin (i.e., cells from the patient) or allogeneic) relies on process engineering tools to understand the impact of cellular features (biological, biochemical, etc.) on cell product function and performance and how process variables influence the critical quality attributes of the cell product. In general, the manufacturing process of cell-based therapies consists of several stages: tissue collection, cell isolation, culture and expansion (upstream processing), cell harvest, separation and purification (downstream processing), and finally product formulation and storage (Fig. 3). The main advances made in the field and future challenges will be addressed in this text, for each of the manufacturing stages.

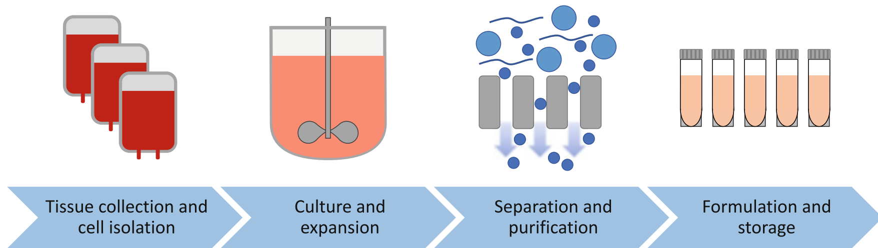


Fig. 3 Manufacturing process for cell-based therapeutic products

2 Source and Isolation of Cells for Therapeutic Use

From a manufacturing perspective, cell-based therapies have transformed cells and tissues themselves into a bioprocess raw material. Consequently, securing their supply is an unprecedented initial challenge in a production pipeline, differing from previously established engineered cell factories. For instance, cell retrieval from human tissues can be problematic. Although the appropriation of biological waste can minimize this issue, some cell sources may be very difficult to reach, while potentially posing health risks for a donor or patient. Thus, management of this supply chain has a level of complexity that is very case specific, depending on the cellular component of the therapy [38].

2.1 Sources and Tissue Collection

Cell-based therapies depend primarily on obtaining the appropriate material from which cells with possible therapeutic application can be isolated. So far, multiple human tissues have been used as sources to obtain cells with therapeutic potential [39].

Home to the hematopoiesis process, the BM harbors multiple cell types that closely interact together, forming the so-called BM hematopoietic niche, encompassing bone, osteoblasts, osteoclasts, HSPC, MSC, macrophages, blood vessels, and extracellular matrix (ECM) [40]. However, the harvesting of BM requires an invasive procedure, allowing a relatively small cell yield, which declines with donor age [41, 42]. For example, HSPC ($CD34^+$) frequency from BM aspirates after leukapheresis is only 1.5% [43], and MSC frequency in a BM aspirate is only 0.001–0.01% [26].

Mobilized peripheral blood (PB) is an alternative source of HSPC. Donors are treated with granulocyte-colony stimulating factor (G-CSF) that temporarily shifts HSPC from extravascular BM sites into the circulating blood. This allows a painless and less invasive harvesting procedure compared to BM aspiration. Hematopoietic recovery from mobilized PB transplantation is similar to BM [44, 45]. However, the

risk of GVHD is lower with BM transplantation compared with PB [44, 45], and the frequency of $CD34^+$ cells in the PB is only 0.5%, which is lower than in BM [43].

AT obtained from subcutaneous tissue represents an abundant source for isolating MSC reliably using simple techniques. Liposuction, the technique generally used for harvesting AT, has the advantage of being less invasive than BM aspiration and is associated with high MSC isolation yields [46]. Specifically, liposuction allowed a yield of stromal vascular cells of 0.5×10^6 to 0.7×10^6 cell/g AT, and between 0.4 and 1.9% of the cells were able to adhere and proliferate in culture [46]. Moreover, liposuction material is considered medical waste, thus being an attractive alternative source. The expansion potential, differentiation capacity, and immunophenotype of MSC derived from AT are nearly identical to those isolated from BM [42].

Neonatal tissues, such as the umbilical cord and placenta, are promising alternative sources to adult ones. The umbilical cord is a rich source of HSPC [47, 48] and has been shown to be a rich source of MSC [49]. For example, about 0.6 million MSC were obtained per gram of umbilical cord [50]. Harvesting the umbilical cord requires a painless and noninvasive procedure. The umbilical cord is considered medical waste and is usually discarded after birth, thus being an attractive alternative source. Within the umbilical cord, HSPC are collected from the umbilical cord blood (UCB). A large-scale study of 126,341 red blood cell-depleted UCB units in the USA inventory revealed that the median frequency of $CD34^+$ cells was 0.34% [51]. MSC on the other hand can be isolated from the UCB as well as from the Wharton's jelly, the connective tissue surrounding umbilical vessels [52]. Most studies are performed with MSC derived from Wharton's jelly, which is commonly referred as the umbilical cord matrix (UCM). Umbilical cord-derived MSC expand at a higher rate when compared to BM- and AT-derived MSC [42, 53]. Furthermore, UCB enables a better repopulation efficiency upon HCT, when compared to BM and mobilized PB, as assessed through quantitative in vivo severe combined immunodeficiency (SCID)-repopulating cell assay [54], despite the limited cell number per unit, which has set UCB particularly suited for pediatric patients.

Possibly due to their broad definition, MSC have been successfully isolated from a number of tissues other than the previously mentioned, including synovial membrane [55], placenta [56], dental pulp [57], brain, liver, kidney, lung, muscle, thymus, and pancreas [58].

Notably, cells show different therapeutic capacity depending on the source they were isolated from. For example, MSC isolated from BM, AT, and UCM revealed different ability to suppress PB natural killer and B and T cells, when co-cultured with phytohemagglutinin-stimulated PB mononuclear cells [59].

2.2 *Isolation of Target Cell Populations*

Depending on the nature of a specific cell therapy, assuring source availability and succeeding in tissue collection may be enough to proceed to the following bioprocessing stage. For minimally manipulated cell products, such as HCT,

heterogeneous populations are isolated and directly infused into the patient. However, newer and more advanced cell therapies are becoming ever more population specific. Thus, bulk populations that normally result from harvesting procedures need funneling techniques that isolate a desired cell type [60].

Still, the most commonly used method to isolate MSC is very simplistic, relying solely on the ability that MSC have to adhere to plastic surfaces [28]. After tissue collection, cells are plated on polystyrene-based tissue culture flasks. MSC will adhere to the plastic surface, while contaminating cells, such as the ones from hematopoietic lineages, are washed away after medium change and passaging [61, 62]. Typically, when MSC are obtained from tissues such as UCM, AT, or synovial membrane, these can be either enzymatically digested using collagenase solutions [46, 62, 63] or simply plated directly onto plastic surfaces as explants [64–66].

More sophisticated techniques can be used to isolate specific cell populations following tissue collection, typically relying on affinity-based and centrifugation-based separations. Although affinity-based separation has gained significant momentum in cell therapy manufacturing, classical centrifugation techniques are still part of typical bioproduction processes. When blood or marrow samples are used (e.g., BM, PB, and UCB), a first isolation step with density gradient centrifugation, using a polymeric solution (e.g., ficoll or percoll), separates the mononuclear cell (MNC) fraction from other constituents such as plasma and erythrocytes [61]. A considerable part of cell therapies are centered on hematopoietic subpopulations (e.g., CAR-T cells, regulatory T cells (T_{reg}), monocytes, and HSPC), whose mentioned isolation consists of centrifugation approaches for separation and extraction of MNC.

Several Sepax (originally developed by BIOSAFE, now GE Healthcare) cell processing systems have brought a fully closed and automated centrifugation unit to cell therapy production pipelines [67]. More advanced centrifugation platforms combine different physical forces to achieve higher isolation recovery and purity. Terumo BCT has established a continuous centrifugation system (Elutra®) that joins centrifugal forces with counterflow [68]. Using blood, initial centrifugation separates the MNC layer from plasma and erythrocytes. Fluid moving in counterflow separates the buffy coat into different fractions. By achieving cell population separation based on size and density, these platforms are able to reach much higher resolution in separation [69]. Monocyte isolation from peripheral blood mononuclear cells using this technology managed to concentrate monocytes in a single elutriation fraction with a recovery of 78% and a purity of 62%. Nevertheless, in combination with a post-elutriation density gradient, monocyte purity rose to 91% without affecting the initial recovery [69].

Cell isolation through affinity is an ever-growing alternative due to its separation criteria being based on biological instead of physical characteristics. Cell population immunophenotype is commonly used to isolate specific cells from their original sources, such as HSPC ($CD34^+$ selection) [70, 71] and MSC ($Stro\text{-}1^+$ selection) [72, 73]. Typically mediated by antibody-antigen interactions, fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) occupy

leading roles in affinity-based separation. Through fluorescent-labeled antibodies, FACS is able to separate cell populations based on their surface marker expression. This technology allows for multiple marker selection with high selectivity due to single-cell analysis [74]. In combination with cell separation, multiparametric studies can be performed simultaneously, allowing for identity and quality control.

MACS shares the same separation criteria as FACS (i.e., immunophenotype) but achieves cell sorting with antibodies coupled to magnetic particles. While these techniques are not novel, direct and thorough comparison has only recently been established [75]. Whereas FACS dominates selectivity and subpopulation purity, the respective cell sorter is not inherently prepared for a clinical setting. An expensive hardware system combined with lack of parallelization, sterility issues, and time-consuming protocols are some constraints that contribute against its translation. Due to its column-based system, MACS is able to separate cells at a much faster rate with possibility for parallel operation and is compatible with current good manufacturing practice (cGMP) guidelines. Closed versions of MACS (e.g., CliniMACS Plus® by Miltenyi Biotec and CTS™ DynaMag™ by Thermo Fisher) have been developed for clinical-scale cell isolation [76, 77]. The trade-off between purity and recovery has burdened many isolation techniques and protocols. Natural killer cells purified by MACS were able to achieve a 95% purity while only reaching a median recovery of 37% [76]. Nevertheless, lack of bead detachment from cells after isolation is a significant drawback for MACS as a cell therapy bioprocessing unit. Besides particle contamination during the production process, epitope restrain by antibody-magnetic particle complexes can affect cell performance and undermine therapeutic value.

Interestingly, both techniques are complementary and therefore selection of cell sorting technique is application dependent. Still, for cell therapy process development, there is a clear tendency toward MACS due to previously mentioned arguments. Recently, efforts have been made by both sides to overcome their limitations.

New platforms for FACS that include disposable microfluidic cartridges provide optimism for its adaptation into production pipelines. WOLF (NanoCellect Biomedical) and On-chip Sort (On-chip Biotechnologies) are two cell sorters that use this concept to overcome typical FACS limitations. The microfluidic cartridges allow for a closed circuit that includes the optical path and sterile sorting containers, minimizing contamination risks. Still, these systems have intrinsically low sorting rates that combined with demanding clinical cell dose targets make their use unrealistic [74].

Development of the MACSQuant Tyto (Miltenyi Biotec) system has been the latest contribution toward FACS adoption into the cell therapy bioproduction process. With sorting speeds reaching 30,000 cells/s, MACSQuant Tyto outmaneuvers its microchip competitors by a factor of 100 [74].

Although not possessing an established clinical platform, vortex actuated cell sorting (VACS) has come forward as a potential challenger for MACSQuant Tyto. This technology uses the same principles for cell sorting as FACS but possesses a different sorting mechanism. The valves or deflection plates used to separate distinctive cell populations are substituted by a microfluidic thermal vapor bubble

actuator that deflects cells due to the formation of an inertial vortex in the flow. Designed by Cellular Highways, the prototype sorter named Highway 1 is currently being developed, with sorting rates reaching 43,000 cells/s. It combines full automation with closed circuits, possibility for multiplexing and a respectable sorting speed [78].

On the other hand, strategies for MACS improvement regarding disruption of cell-magnetic particle complexes have also been explored, focusing on the interaction between antibody molecules and magnetic particles. Thermo Fisher's Dynabeads® FlowComp™ combine streptavidin-coated beads with biotin-conjugated antibodies, enabling a post-isolation bead removal mechanism [79]. STEMCELL Technologies established their own product, termed Releasable RapidSpheres™, that possess a tetrameric antibody complex which assures cell and particle interaction [80]. Following cell isolation, a mild dissociation agent cleaves the tetrameric complex, releasing the magnetic particles. Successful particle removal will also help alleviate regulatory issues over end product safety. Consequences of nano- and microparticle contaminations are still debatable, with magnetic bead internalization being a validated concern.

Instead of improving existing techniques, novel approaches for affinity-based cell isolation have also been investigated. Since cell therapies possess more stringent safety criteria, delivering cells without any by-products due to bioprocessing is crucial. Therefore, antibody removal after affinity separation is of considerable interest.

Traceless affinity cell selection (Fab-TACS®) is an innovative technology that explores a reversible antibody-antigen interaction. Antigen-binding fragments (Fab) are combined with a short peptide tag (Strep-tag®II) with significant affinity toward a derivative of streptavidin (Strep-Tactin®). By having a Strep-Tactin®-coated agarose matrix, desired cells are retained in a Fab-TACS® column [81]. Using biotin analogs, cells are eluted from the column due to interaction competition. Since the antibody fragments have low affinity toward the selected antigen, Fab release occurs, leaving the isolated cells without any separation by-product or trace. This technology has been translated into an automated commercial device (FABian® by IBA Lifesciences) [81].

A combinatorial approach for cell isolation has been explored by Akadeum Life Sciences. Instead of improving or developing new techniques, an innovative method has been devised that brings centrifugation and affinity-based separation together. Buoyancy-activated cell sorting (BACS™) brings several above-mentioned concepts together in a novel manner [82]. Biotinylated antibodies are introduced in a cell suspension to target undesired cells (negative selection). Glass-shelled microbubbles coated with streptavidin are mixed to capture antibody-tagged cells. These microbubbles are separated from the remaining cell populations through centrifugation by flotation [82]. Purity and recovery values higher than 80% have been reported for this novel technique [83].

Isolation of a target cell population can have different impact depending on a specific cell therapy, with products ranging from bulk and heterogeneous populations to very selective subpopulations with a defined phenotype. Adequate

selection of a separation method is also dependent on the prioritization of opposing purification concepts, such as purity and recovery [38].

3 Cell Production

The relatively low frequency of cells with therapeutic potential within the native tissues, followed by harvesting procedures and eventually successive isolation steps, yield a substantially low number of cells in the end. Therefore, in order to use these cells in a clinical context, it is usually required additional steps of manipulation and propagation ex vivo, which depend on choosing the appropriate culture medium conditions, physicochemical parameters, and culture platforms [84].

3.1 Culture Medium Formulation

The maintenance and propagation of animal cells in vitro require a cell culture medium, supplying nutrients and inorganic salts, as well as providing the appropriate physicochemical conditions. Generally, medium components include glucose (carbon source), amino acids (nitrogen source), vitamins (cofactors), inorganic salts (maintains electrolyte balance), sodium bicarbonate (buffer, to maintain pH at 7.4), sodium chloride (adjusts osmotic pressure), antibiotics (prevent microorganism contamination), phenol red (visual pH indicator), and growth factors and hormones (growth stimulation) [85, 86]. These components are provided in commercially available basal medium formulations, such as Eagle's medium and derivatives (e.g., Dulbecco's Modified Eagle's medium (DMEM), Minimum Essential Medium Eagle alpha (αMEM)), medium from Roswell Park Memorial Institute (RPMI), and several other well-established media, which have been subject of improvement over the years [86].

Generally, the addition of certain molecules to basal medium formulations is required. For cell therapies based on hematopoietic lineages, cytokines play an important part as a culture medium component. These soluble factors have a great role in influencing cellular signaling pathways. In the context of HSPC expansion, factors present in the medium prevent cell differentiation while promoting the self-renewal capability of cells. Stem cell factor, thrombopoietin, granulocyte-colony stimulating factor, fms-like tyrosine kinase 3 ligand, and interleukin-6 are some cytokines currently used in expansion protocols for the manufacturing of UCB expanded grafts under study in several clinical trials [87–89].

Culture medium formulations usually require the addition of a protein-rich supplement, containing growth and adhesion factors. The most commonly used culture supplement is animal serum, especially fetal bovine serum (FBS). Serum is a source of amino acids, proteins, vitamins, carbohydrates, lipids, hormones, growth factors, and inorganic salts [86]. Moreover, it enhances cell adhesion, improves

the pH-buffering capacity of the medium, and helps reduce shear stress during cell manipulation. However, it presents significant disadvantages such as being ill-defined, wide batch-to-batch variability, risk of contamination with virus and prions, and ability to transmit xenogeneic antigens, leading to increased immunogenicity of cultured cells, thus limiting FBS application in the clinical setting [90, 91]. Besides the cell biological perspective, ethical concerns and animal welfare issues arise from the use of animal serum, as serum collection causes animal suffering [90]. Furthermore, the global supply of FBS is declining over the years, and this tendency is expected to continue [90]. This will eventually result in a FBS supply that will not be able to meet the increasing demand. Therefore, given its disadvantages, using FBS for cell culture of clinically applied cell products is discouraged and should be avoided. By complying with current international guidelines and regulatory frameworks [92–94], there is need for developing alternative culture supplements.

In the last decade, the development of serum-/xeno(geneic)-free (S/XF) culture formulations (i.e., without serum or animal origin components) has been a priority for the field of cell therapies. Although these media represent a valuable alternative to FBS, as they are more consistent and standardized, they still contain a cocktail of growth factors, proteins, and hormones derived from human serum or even plant hydrolysates, classified as chemically undefined [95]. Chemically defined, animal component-free media, on the other hand, consist exclusively of well-defined and characterized components and entirely free of animal (including human) derived products. These include purified recombinant proteins and synthetic bioactive molecules [95].

One of the well-established supplements used in S/XF media, proposed as an alternative to FBS, is human platelet lysate (hPL). As early as the 1980s, hPL-supplemented medium was found to support proliferation of established cell lines and primary fibroblasts [96, 97]. hPL is usually prepared from fresh blood or platelet concentrates, containing bioactive molecules such as growth factors, adhesion molecules, and chemokines, which originate primarily in the α-granules of platelets [98]. Preparation of hPL from platelet concentrates can be achieved either by repeated freeze/thaw cycles, sonication, induced platelet activation by addition of thrombin or $CaCl_2$, or solvent/detergent treatment [98].

Blood banks routinely prepare pooled allogeneic platelets from human blood donations. When these are not used for transfusion, they are used for further manufacturing into hPL, thus allowing a steady supply for manufacturing an allogeneic "off-the-shelf" hPL product for cell culture [98, 99].

Multiple studies have demonstrated hPL-supplemented media to be efficient for the isolation and expansion of MSC from various origins [100–102], cultured both in static and dynamic systems [103, 104], already with several ongoing and completed clinical trials (clinicaltrials.gov). Moreover, it has been shown that both allogeneic and autologous hPL-supplemented media allow improved cell proliferation when compared to FBS-containing media [100, 102, 105, 106]. The main differences in hPL protein content compared to FBS are the higher content of immunoglobulins and the possible presence of fibrinogen and other coagulation factors, when hPL is produced without thrombin activation [98].

In addition to its application for MSC expansion, hPL has been evidenced as an efficient growth medium supplement for ex vivo expansion of other cell types such as human gingival fibroblasts [107], chondrocytes [108], osteocytes, myocytes and tenocytes [109], as well as endothelial cells [110], indicating its potential applicability in multiple areas of cell therapies and regenerative medicine. Although hPL is considered safer than FBS by the scientific community and regulatory agencies, it still poses some constraints, such as the risk of transmission of human diseases by known or unknown viruses, ill-definition, and the possibility of triggering immune responses [111]. Nonetheless, hPL products derived from pooled units and produced in large scale are already commercially available [99] and seem to be the most promising alternative to FBS supplementation in cell culture medium in the near future. Moreover, novel gamma-irradiated hPL products have been developed toward pathogen reduction. Results showed that gamma radiation allowed 4 $\log_{10}$ reduction of viral titer with low impacts on the potency for cell expansion [112].

There are other commercially available S/XF media that have been successfully applied for cell culture. StemPro® MSC SFM (Life Technologies) and MesenCult™-XF (STEMCELL™ Technologies) are two chemically undefined S/XF media that have been used to successfully expand MSC from different sources [113–116].

Considering the expansion of human HSPC, most current protocols are targeting the use of serum-free medium formulations supplemented with cytokines. StemSpan™ H3000 (STEMCELL™ Technologies) is a S/XF medium with human-derived components and has been used for expansion of HSPC [117]. Although being chemically undefined, containing human-derived components, these media formulations represent an improvement for cell culture, due to better definition and lower batch-to-batch variability when compared to FBS- and hPL-supplemented media.

The ideal candidate for production of clinical-grade cell-based therapies would be a chemically defined, animal component-free media (including human), composed exclusively of well-defined factors that could replace serum and serum-derived products. These include synthetic bioactive molecules and purified recombinant proteins [95]. There are multiple factors that can be combined in order to replace serum, such as growth factors (e.g., EGF, FGF, TGF), hormones (e.g., growth hormone, insulin), carrier proteins (e.g., albumin, transferrin), lipids (e.g., cholesterol, fatty acids), transition metals (e.g., Se, Fe, Cu, Zn), vitamins, adhesion factors (e.g., fibronectin, laminin), polyamines, and reductants (e.g., 2-mercaptoethanol) [86]. The number of possibilities that result from the combination of these components is enormous, making the selection of the most appropriate ones and their respective concentration in a medium formulation an extremely difficult task. For that purpose, design of experiments is possibly the best strategy to find the optimal concentration of each component in a culture medium, especially considering likely interactions between the components [86]. Consequently, S/XF media, especially chemically defined culture media, are often cell type specific.

The chemically defined medium TheraPEAK™ MSCGM-CD™ (Lonza) has been used for the expansion of MSC [118]. Successful expansion of T cells was

also achieved using chemically defined S/XF media, relying, for instance, on the CTS™ Immune Cell Serum Replacement supplement [119, 120].

In order to disseminate the development and application of serum-free media for cell culture, "FCS-free Database," a freely accessible serum-free media database, is available online (https://fcs-free.org/), providing an overview of FBS-free media for cell culture.

3.2 Physicochemical Parameters

Besides biochemical factors such as nutrient/metabolite concentration and growth factors, physicochemical parameters such as pH, temperature, osmolality, and oxygen tension are equally important for the maintenance of animal cell cultures. The optimal values for each of these physicochemical parameters will differ depending on the cell product, which poses an additional challenge in cell manufacturing.

Most cell lines grow successfully at pH 7.2–7.4. However, the optimum culture pH depends on the intended application. For example, differentiation of human MSC into osteoblasts can be improved by changing the pH of culture medium from normal to alkaline medium [121]. The differentiation of erythroid progenitors can also progressively increase as pH is increased from 6.95 to 7.4 and 7.6 [122]. Usually the pH is controlled in cell culture by using the CO_2/HCO_3^- buffer system. Cells are typically cultured in humidified incubators with gas phase CO_2 at 5% and sodium bicarbonate as a medium additive. The CO_2 dissolved in the aqueous phase stays in equilibrium with HCO_3^-, adjusting the pH [86].

The optimal temperature to cultivate human and warm-blooded animal cells is 37°C. However, cell culture at different temperatures may be advantageous for certain purposes. For example, culturing MSC at 32°C decreased the accumulation of oxidative damage and improved their osteogenic differentiation ability, when compared to 37°C [123].

Although different cells have different optimal osmolality values, most cells grow well in the range between 290 and 310 mOsm [124, 125]. As previously mentioned (Sect. 3.1), the osmolality is mainly defined by the sodium chloride content in the medium.

Most animal cell cultures are performed at an atmospheric oxygen level (21% O_2). However, the oxygen concentration in most tissues is lower than the atmospheric one, due to gas transfer phenomena. Therefore, mimicking the in vivo oxygen concentration might have a positive impact in cell culture as well as on the therapeutic potential of the cultured cells. One canonical example would be the BM, which is characterized by a hypoxic environment, with oxygen concentration ranging in the interval between 1 and 6% [126, 127].

In light of this observation, several studies were performed by exposing MSC to hypoxic conditions. Hypoxic conditions were found to have an advantage for MSC expansion as well as in terms of differentiation [128–130]. A study performed by

Oliveira and colleagues revealed that both BM MSC and AT MSC cultured in hypoxic conditions experienced an immediate and concerted downregulation of genes involved in DNA repair and damage response pathways [131]. Moreover, it revealed that AT MSC reacted to hypoxic environment more slowly than BM MSC, as different characteristics of each cell niche (e.g., degree of vascularization, oxygen tension, cell-cell interactions) determine distinct sensitivities to hypoxia ex vivo [131]. Likewise, hypoxia (5% O_2) enhanced the proliferation of UCB-derived HSPC, when compared to normoxia (21% O_2), as well as allowing a better preservation of bone marrow repopulation in SCID mice [132]. In another study, the ex vivo expansion of UCB-derived HSPC in co-culture with BM MSC was maximized in a 10% O_2 atmosphere, when compared to other hypoxia conditions and normoxia levels [133].

Besides the need to establish the most appropriate physicochemical conditions for a certain cell-therapy manufacturing process, maintaining these parameters at the correct values throughout culture is equally important. In traditional culture systems, these parameters are often observed, but rarely controlled, thus decreasing the robustness of the manufacturing process. This issue will be addressed in more detail in Sect. 3.5.

3.3 Scalable Culture Vessels

Whether isolated cell populations need to undergo differentiation or expansion, appropriate cell culture vessels and systems are necessary.

In terms of complexity, at the rear end of cell culture technology are simple plasticware containers. Different geometries make up a broad collection of vessels in order to cover any cell type and their projected application. Petri dishes, T-flasks, roller bottles, and multiwell plates all incorporate cell culture plasticware and are typically made of polystyrene that is previously treated either chemically or physically in order to gain hydrophilic functional groups (e.g., ketones, aldehydes, hydroxyl, and carboxyl groups) [134]. Indeed, surface treatment has a dramatic impact on adherent cell culture, with proper cell adhesion being a main concern. Unfortunately, when using S/XF culture media, cell adhesion can be compromised due to deficiency in serum-derived adhesion factors [135]. Commercially enhanced plasma treatment plasticware (e.g., CellBIND® by Corning Life Sciences) and xeno-free surface coatings (e.g., CELLstart™ by Thermo Fisher Scientific and Synthemax® by Corning Life Sciences) have been developed to address this issue [136, 137].

Besides allowing gas exchange through the cap region and having excellent optical clarity, commonly used vessels are seriously limited regarding any type of monitoring and control. Conventional plasticware as culture flasks also lack an agitation mechanism, not being able to assure fully homogenized cell cultures. Since their design was directed mainly toward research purposes, manufacturers quickly identified scalability issues for large-scale production. Advanced and

scalable culture systems based on plasticware were created to avoid laborious and unsustainable scale-out.

Although very simplistic, plastic malleable bags have a consolidated place in cell culture. Being integrated in basic plasticware, they offer a simple closed system solution which is critical for manufacturing under cGMP. However, limited culture control and poor agitation severely limit their application in optimized processes. Nevertheless, therapies based on hematopoietic cells (e.g., tumor-infiltrating lymphocytes, CAR-T, and HSPC) have relied on these platforms for cell culture, reaching human use in clinical trials [87, 138, 139].

Multilayered flasks (e.g., Nunc™ Cell Factory™ System by Thermo Fisher Scientific) were designed to increase culture area while reducing volumetric footprint of using multiple individual flasks. Additionally, closed versions with perfusion mechanisms of these flasks were also developed to overcome the open nature of conventional flasks. Large-scale expansion of MSC in serum-free conditions was achieved using HYPERStack system (Corning Life Sciences), yielding an average cell density of 2×10^4 cell/cm^2, corresponding to a fourfold increase in total cell number after 4 days [140]. Proprietary gas permeable films improve gas diffusion, which do not compromise cell viability in high-density adherent cultures of tightly packed multilayered flasks. Flask potential has been pushed further with the commercialization of the CellCube® by Corning Life Sciences, a closed system comprising of densely packed thin individual surfaces with continuous medium supply in laminar flow, reaching 85,000 cm^2 (39 cm $\times$ 25 cm) for adherent cell culture [141]. The Xpansion® multiplate system designed by Pall Corporation takes advantage of the same concept, aside from assuming a cylindrical geometry with capacity for up to 122,400 cm^2 of culture surface. Xpansion®-50 was used for large-scale expansion of human periosteum-derived stem cells for the treatment of bone defects, achieving a final cell density of 1.75×10^4 cell/cm^2, corresponding to a 3.9-fold change in total cell number after 7 days, and presenting a final recovery efficiency of 45% [142].

Roller bottles have also been optimized for large-scale manufacturing of cells. Cord blood-derived MNC were isolated and expanded in multiple 500 mL roller bottles with rotation assured by a bottle roller [143]. Further improvement led to the design of RollerCell™ by Cellon, a system capable of simultaneously holding 40 roller bottles with automated robotic processors for cell handling. RollerCell™ comparison with CellCube® for cell line production yielded similar results [144].

Although planar systems have evolved to closed and scalable systems with possibility for dynamic regimen through continuous fluid flow (e.g., CellCube® and Xpansion®), bioreactors have been the ultimate objective for cell therapy manufacturing, seeing that they incorporate monitoring and control, reduce process footprint, and minimize cell handling.

Incorporating highlighted challenges of a cell-centered process requires platforms capable of dealing with parameter complexity to deliver a safe and reproducible cell-based product. Table 2 enumerates current cell-based therapies in clinical trials that involve bioreactors in their production process. Innovative bioreactor designs have come forward to challenge more classical versions.

Table 2 List of clinical trials using bioreactors for cell based-therapies

Study name	Type of bioreactor	Cells	Condition	Phase
Extracorporeal Immune Support System (EISS) for the Treatment of Septic Patients (EISS-1)[a]	EISS-immune-cell bioreactor device	Human donor granulocytes	Severe sepsis and septic shock	Phase 1 and phase 2
Safety of Intramuscular Injection of Allogeneic PLX-PAD Cells for the Treatment of Critical Limb Ischemia[a]	PluriX™ 3D bioreactor system	Placental adherent stromal cells	Critical limb ischemia	Phase 1
Expansion of Invariant NKT Cells for a Cell Immunotherapeutic Approach Allowing the Control of GvHD and Preserving the Graft Versus Leukemia Effect After Allogeneic HSC Transplantation[b]	Bioreactor	NKT cells	Allogeneic hematopoietic stem cell (HSC) transplantation	Not available
Laryngo-tracheal Tissue-Engineered Clinical Transplantation[c]	Stem-cell seeded bioartificial tracheal scaffold	Autologous stem cells	Tracheal diseases	Not applicable

Clinical trials were obtained from "clinicaltrials.gov," on 21 March 2019, using the term "bioreactor" and selected for cell therapy applications
Clinical trial status: [a]completed; [b]not yet recruiting; [c]unknown

Stirred tank bioreactors (Fig. 4a) maintain widespread use, with their simpler and more standardized geometry. With extensive experience in what concerns the production of traditional biopharmaceuticals, much knowledge regarding these bioreactors has been transposed to cell-based therapies. These systems have mechanical impellers that are responsible for appropriate mixing and assuring dynamic flow. High compatibility with monitoring probes and respective modules has made culture control an intrinsic part of this bioreactor. Internal sparging mechanisms allow for efficient gas transfer, although shear stress associated with bubbling can be an issue to sensitive cells [145]. Exhaustive knowledge on fluid profiles based on computational fluid dynamics (CFD) models have given significant predictive control on culture estimates.

While being naturally prone for suspension cultures [146], adherent cell culture has been adapted through microcarrier development. These spherical particles provide the surface area for cell adhesion to occur. A broad variety of materials, porosity levels, and surface coatings have been developed to fulfill specific cell needs. The high variety of microcarriers has been extensively reviewed [147, 148].

Of notice, we have pioneered in the development of clinical-grade expansion of MSC of different human sources (i.e., BM and AT) in scalable microcarrier-based bioreactors using S/XF culture components, achieving the production of 1.1×10^8 and 4.5×10^7 cells for BM MSC and AT MSC, respectively, after 7 days of culture

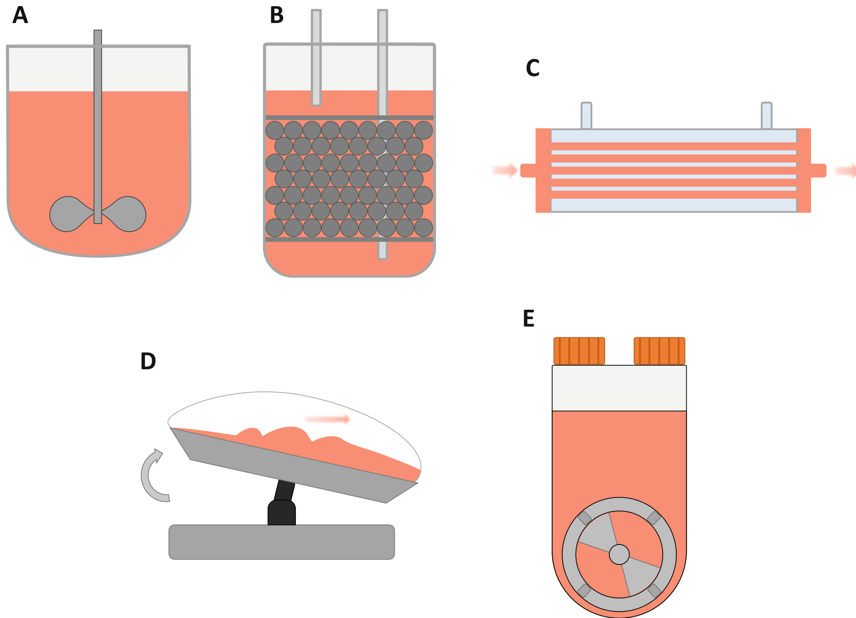


Fig. 4 Schematic representations of bioreactor configurations that can be potentially used in the manufacturing of cell-based therapies: (**a**) stirred tank bioreactor, (**b**) packed bed bioreactor, (**c**) hollow fiber bioreactor, (**d**) wave bioreactor, and (**e**) Vertical-Wheel™ bioreactor

[149]. Building on this platform, we have concentrated efforts in maximizing cell productivity by changing different culture parameters. We have previously optimized feeding and agitation regimes and performed microcarrier screening [114]. Furthermore, we have successfully incorporated an alternative MSC tissue source (i.e., UCM) [150] and have implemented a different bioreactor configuration with a vertical agitation design (Vertical-Wheel™) (Sect. 3.4) [104].

The scalability potential of stirred tank bioreactors for cell-based therapies has been embodied by development of MSC expansion processes. While initial studies restricted their culture scale to spinner flasks, Rafiq and colleagues managed to scale up MSC expansion to a 5 L stirred tank bioreactor, achieving a cell concentration of 1.7×10^5 cell/mL, corresponding to over a sixfold expansion in total number of cells [151]. Subsequently, Lawson and colleagues pushed the scalability of stirred tank MSC culture forward by successfully expanding human MSC in a 50 L bioreactor, being able to produce 177 clinical doses (70 million cells/patient assuming a 70 kg patient) in a single run [152]. In contrast to the above-mentioned scale-up, with contributions of multiple groups to ever-increasing culture dimensions, Schirmaier and colleagues were able to perform an entire stepwise scale-up of AT MSC expansion from spinner flasks to 35 L cultures, yielding 1×10^{10} cells at the end (35 L scale) [153].

Consequently, both adherent and suspension cultures are firmly established for cell culture in stirred tank bioreactors. Commercial versions of stirred tank bioreactors include the Celligene® series by Eppendorf and the Finesse series by Thermo Fisher Scientific.

Mammalian cells are known to be more shear sensitive which stimulated efforts to develop non-abrasive environments during cell culture. Packed bed bioreactors (Fig. 4b) provide a fixed chamber where microcarriers or scaffolds are located [154]. Adhered cells that populate the chamber have translational movements restricted, thus being able to better mimic solid tissue presence. Their constrained movement also promotes structured organization and cell-cell interaction, leading to high-density cultures. Low-velocity fluid flow guarantees dynamic culture without causing shear damage to cells. Culture medium has access to the chamber providing necessary nutrients and removing metabolites. Diffusion limitations or nutrient deficiency can occur due to 3D culture organization. Furthermore, significant cellular organization can result in beneficial biological outcomes but will normally complicate cell extraction and subsequent downstream processes. Expansion of MSC in a 2.5 L CelliGen® bioreactor (New Brunswick Scientific) with Fibra-Cel® (Eppendorf) disks demonstrated large-scale manufacturing potential for packed bed bioreactors, achieving 9.2×10^7 cells after 9 days of culture, corresponding to a 9.2-fold increase in total cell number [155].

Increasing available area for cell culture while protecting cells from harsh conditions has inspired innovative bioreactor designs. Hollow fiber bioreactors (Fig. 4c) fulfill those requirements by joining thousands of hollow fibers. These fibers are made of thin and porous material that provide a selective passage of nutrients. Culture medium recirculates through the fibers producing interesting tangential flow, mimicking vasculature to some extent [154]. However, significant quantity of fibers originates successive diffusion barriers that cause concentration gradients for nutrients, signaling factors, or gases. Similar to packed bed bioreactors, cell extraction processes are challenging to perform due to high cell interaction and difficulty in reaching cells uniformly inside the bioreactor.

With unprecedented tight regulatory measures, the field of bioreactors has moved toward disposable and single-use versions. In order to avoid clean-in-place (CIP) and steam-in-place (SIP) procedures and assure contamination-free product quality, conventional stainless steel or other reusable bioreactors are being substituted by plastic single-use bioreactors (SUB). They reduce cross-contamination and can be combined with limited monitoring probes. Disposable technology has been able to successfully adapt existing geometries, such as the Mobius series by EMD Millipore for stirred tank bioreactors and the Quantum® bioreactor by Terumo BCT for hollow fiber bioreactors. The latter bioreactor has been validated with adherent AT MSC, BM MSC, periosteum-derived MSC, and neural stem cells [156–160]. However, novel designs, such as the wave bioreactor (Fig. 4d) and the Vertical-Wheel™ bioreactor (Fig. 4e), have also shown that there is space for bioreactor innovation that integrate single-use technology. Recently, an overview of SUB and their applicability toward cell therapy have been investigated [161]. It was observed that SUB designs have evolved, currently integrating well-known principles of mass transfer and mixing. Their versatility and single-use nature align with cost

reduction and demanding regulatory guidelines associated with cell therapies. However, culture monitoring remains a challenge, and long-term bag stability must be assured.

Numerous bioreactor designs exist for performing cell culture; nevertheless selecting the correct culture vessel with an appropriate scalability strategy is the actual challenge for the manufacture of cell therapies. Achieving parallelization of individual units (scale-out) tends to be more associated with autologous therapies, while increasing bioreactor size and maintaining culture conditions (scale-up) is more adequate for an allogeneic production. A compromise between scalability and optimal culture conditions is deemed necessary.

3.4 Agitation

One of the crucial factors for successful cell expansion is culture medium homogenization. Bioreactors require sustained agitation of the culture system, in order to allow an appropriate mass transfer of nutrients and oxygen to the cells, as well as a removal of waste products derived from cell metabolism. For that purpose, cells must be maintained in suspension homogeneously, independently of whether the cells are cultured freely in suspension, as cellular aggregates or adherent to microcarriers/scaffolds.

However, agitation may have an impact on cellular physiology, due to increased shear stress. In this context, shear stress can be defined as the force component acting tangentially to a material, due to fluid motion [162]. Therefore, in bioreactor processing, cells are exposed to shear stress originating from fluid agitation. Shear stress has been described to have a significant impact on cell phenotype, which can be either negative or beneficial depending on the final application. In fact, it has been long established that animal cells in general are sensitive to shear stress, which compromises their viability above certain levels [163–165]. Additionally, agitation affects HSPC surface marker expression, including cytokine receptors [166] and CD34 [167], which impacts cell expansion by enriching specific HSPC populations in culture. On the other hand, shear stress has been demonstrated to induce osteogenic differentiation of BM MSC through increased expression of osteogenic factors such as bone morphogenetic protein-2 (BMP-2), bone sialoprotein (BSP), and osteopontin (OP) [168] and also resulted in increased intracellular Ca^{2+} levels [169]. Shear stress also improved the angiogenic potential of human AT MSC through stimulation of vascular endothelial growth factor (VEGF) secretion [170].

The importance of agitation and the impact it has on culture outcome have led to the development of new technologies and bioreactor configurations that specifically target this issue. Wave bioreactors (Fig. 4d) are suitable for the manufacturing of shear-sensitive cells. Their agitation through rocking motion prevents the use of an impeller exerting high shear forces directly in the cells. Very low level of shear stress was found in wave bioreactors compared to classical stirred tank reactors

[171]. Wave bioreactor implementation for culture of suspension cells, with emphasis to hematopoietic lineages, is well-known [172, 173].

In the same line, Vertical-Wheel™ bioreactors (Fig. 4e), developed by PBS Biotech, incorporate a vertically rotating wheel, allowing a more efficient mixing than the traditional horizontal stirring solutions. By allowing lower agitation rates, they are able to minimize shear stress effects. The vertical mixing allows a higher mass transfer rate and more homogenous and gentle particle suspension, favorable for anchorage-dependent cells on microcarriers [174]. Moreover, this technology is fully scalable, being available at working volumes that range from 60 mL up to 500 L. Vertical-Wheel™ bioreactors have been successfully applied in microcarrier-based cell culture systems for the expansion of MSC from multiple sources [104, 175], as well as for human iPSC [176].

In summary, agitation can modulate culture conditions and have a significant impact on the characteristics of expanded cells. Different agitation rates and configurations can be used to influence the cell culture outcome. An appropriate balance needs to be found at an agitation rate that allows adequate mass transfer for cell growth, without compromising cell integrity or stem cell fate due to excessive shear stress. Different bioreactor technologies and configurations are available to fine-tune cell culture agitation for each specific application.

3.5 Culture Monitoring

Monitoring of culture conditions is essential in any cell manufacturing process. An ideal continuous gathering of information from every bioprocess stage would allow for real-time informed decision-making, complete control and oversight, extensive knowledge of the whole manufacture process, and model estimation with response simulation. Naturally, any cell therapy manufacturing process would hugely benefit from such observational power, especially due to inherent complexity of being based on living organisms. The dynamic nature of cells is a significant source of instability for process control [177].

Monitoring has been exposed to ever-increasing difficulties associated with more advanced processes and products. Cell therapies have turned the spotlight toward cells, and monitoring has not been able to fully respond to newfound needs. However, regulatory agencies have implemented guidelines in order to stimulate the improvement of monitoring tools, establishing the process analytical technology (PAT) framework [178, 179]. It highly recommends design, incorporation, and control of innovative analytical tools for continuous improvement of cell therapy manufacturing. In order to ensure product quality and facilitate monitoring variable selection strategies and prioritization, critical process parameters (CPP) must be identified and closely followed.

As previously mentioned, cell therapies possess conventional physicochemical process parameters (e.g., pH, temperature, and agitation speed). Nevertheless, introduction of cells has brought a significant amount of unprecedented process

parameters, whose nature revolves around cellular-based concepts. For a cellular product, assurance of cell viability and cellular fitness involves controlling a micro-environment based on complex nutrient formulations and dissolved gas concentrations. Besides parameters related with cellular well-being, complexity in cell therapy production monitoring is associated with information on cell state, including phenotype and functionality. Identity of a cell population during production must meet desired standards and quality control, thus surface marker expression, transcriptomics, and metabolic profile are also important monitoring targets. Concisely, having cells as therapeutic products has considerably extended the list of CPP in both length and complexity, forcing PAT to advance and to try to develop novel tools at an unprecedented and unmanageable rate [177].

With the identification of process parameters present in cell therapy production that can be difficult to measure, versatility in monitoring might facilitate early development of new PAT tools. Measurement of process parameters should preferably be performed in situ, avoiding lag phases and delays in information gathering that can endanger the whole bioprocess [180]. By measuring directly inside the manufacturing unit, these sensors provide real-time data and do not compromise the sterility barrier. However, another possibility includes online measurement, which requires sample displacement and return to the unit through a bypass mechanism but also maintains vessel sterility [181].

For techniques that have not been developed for in situ integration, off-line and at-line monitoring are possible alternatives. These require destructive sampling accoupled with external sample preparation and analysis. The difference between both is related with the distance of the assay equipment, with at-line having the advantage of close proximity to the respective manufacturing unit. Consequently, any process control with these monitoring strategies will be performed in retrospective with delayed information [181].

Currently, cell therapy manufacturing possesses process parameters with different kinds of monitoring methods [181]. Nevertheless, advancement of PAT aspires for universal in situ monitoring (Fig. 5).

Conventional CPP have monitoring techniques that have existed for several decades. The lack of modernization associated with industry resistance in applying PAT advancements has crippled much needed innovation in cell therapy manufacturing. Instead of having monitoring development accompany efforts in making standard bioprocessing units, cell-centered manufacturers have paradoxically opposed it [182]. This is clearly evident for traditional univariable parameters, whose monitoring is based on outdated techniques.

Historically, pH and dissolved oxygen (DO) tracking is performed using electrochemical sensors [183, 184]. Due to the requirement of a reference electrode, pH glass electrodes tend to be bulky, and their glass envelope displays some fragility. Likewise, DO electrodes are also limited, since consumption of local oxygen requires constant flow around the sensor and low membrane stability restricts shelf-life [182]. Furthermore, mostly fixed geometry configurations have impaired adaptation of traditional electrochemical electrodes into novel bioreactor systems.

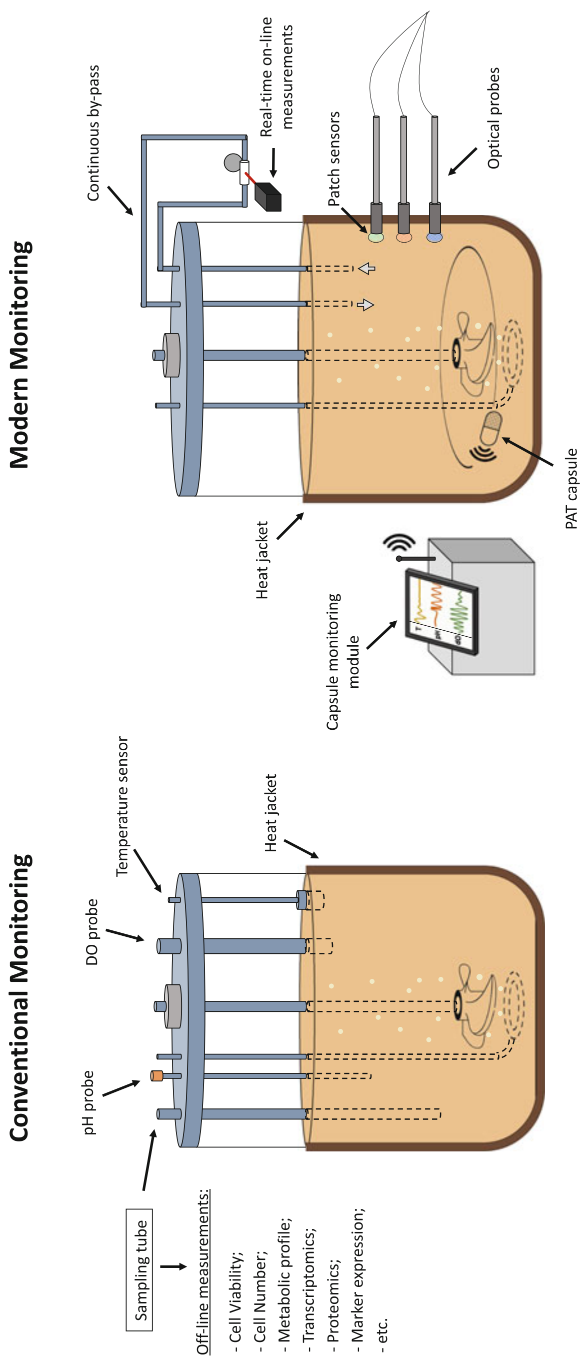


Fig. 5 Comparison of different monitoring paradigms. Conventional and established systems are outdated and based mostly on off-line methods. Innovative approaches have envisioned a new perspective that aspires to reach universal online monitoring toward a quality-assured cell-based product

Nevertheless, manufacturers have extensive knowledge regarding these sensors, and their considerable reliability has made their adoption widespread.

Fortunately, advancements in optical fibers have made it possible to follow pH and DO without the need of electrochemical probes. Optical sensors are able to quantify these parameters through the presence of indicator or fluorescent dyes [181]. Continuous optical monitoring of pH in a perfusion bioreactor for a baby hamster kidney (BHK-21) cell culture was achieved using phenol red as an indicator [185]. Additionally, instead of having bulk media constituents as indicators, dye adsorption on a solid matrix combined with patch technology has originated viable products and systems for cell culture during manufacture (e.g., Optical pH and DO sensors by PreSens Precision Sensing GmbH or Ocean Optics). Optical patch sensors were used to monitor pH and DO in a high-throughput system for simultaneous operation of 12 bioreactors [186]. Versatility, easy implementation, and miniaturization potential of patch sensors have also been exploited in disposable culture technology. Single-use bioreactors, such as BIOSTAT STR® produced by Sartorius, have integrated patch sensors for both DO and pH. This system has been used for scalability studies on AT MSC expansion using microcarriers in a stirred bioreactor [153]. Although optical sensors exhibit great adaptability and allow for continuous external monitoring, their dyes are vulnerable to photobleaching. Nevertheless, a comparison between electrochemical and optical pH and DO sensors highlighted considerable correlation between parameter measurements, easing concerns regarding lesser robustness of optical sensors [187].

Cell-related CPP demand pioneering approaches and instruments that are capable of following complex and multivariate data. For certain parameters it would be impossible to individually follow each specific component in a parallel manner. Culture medium formulation and cell transcriptomics are some variables that require a widespread perspective. Spectroscopy is an attractive technique to address multiparametric needs since a broad range of wavelengths can be covered [181]. Additionally, electromagnetic radiation interacts with any type of matter, which makes spectroscopy also applicable to biological parameters [180]. Partition of this wide-reaching field originates multiple techniques, with several being adapted as PAT tools for monitoring.

Ultraviolet (UV)/visible spectroscopy focuses on consequences of sample or analyte excitation through a UV or visible light source. Information for cell-based bioprocessing monitoring can be retrieved by observing two different radiation phenomena, namely, scattering and absorption. Measurement of light absorption through a specific path length is the basis for optical density (OD) and absorbance techniques [180]. Although restricted to suspension cultures, medium turbidity can be correlated with cell concentration. Furthermore, significant absorption targets for this range of the spectrum include aromatic molecules, such as fluorophores, chromophores, and aromatic amino acids. The latter can be extremely useful for protein quantification. Quality of cytokines used in medium supplementation can be certified using this technique.

Instead of absorption, light scattering is another event that can provide parameter information. Biomass from aggregate-based or suspension cell cultures can be

followed by the amount of scattering of an incoming light source. Growth of Chinese hamster ovary (CHO) cells was successfully followed with a backward light scattering platform [188]. While cell proliferation can be monitored, potential of UV/visible spectroscopy for cell therapy manufacturing is limited, since high aromatic compound selectivity cannot be fully exploited in a cell-centered bioprocess.

Moving to higher wavelengths in the spectral window leads to infrared (IR) spectroscopy. Lower-energy radiation associated with IR spectroscopy affects the vibrational states of molecules. Fortunately, each molecule after excitation emits its own unique radiation fingerprint [180]. Spectral monitoring of culture media allows for multiple quantification of culture medium components and metabolic by-products in a noninvasive manner. Unfortunately, water molecules present can also interfere with data acquisition. Deconstruction of these measured spectra is necessary to distinguish between individual analytes [189]. Thus, there is a significant dependence on chemometric algorithms to unravel incoming data. An in situ near-infrared spectroscopy probe was validated in determining analyte concentrations in a CHO-K1 cell culture [190]. Fourier transform infrared (FTIR) spectrometers are a third generation of instruments to reach the field of IR spectroscopy. With a higher signal-to-noise ratio, this technique has also been successfully used to follow MSC osteogenic differentiation and the metabolic profile of MSC expanded on microcarriers in a XF culture system [191, 192].

Molecular vibrational interactions are also responsible for originating Raman spectroscopy. This technique is based on sporadic inelastic scattering of incident light. In contrast to IR spectroscopy, it is less affected by water interference, and therefore substantial focus is being given to Raman spectroscopy [180]. This method combines possibility for versatile in situ probes, continuous and noninvasive real-time monitoring, sensitivity for most culture components, and a high signal-to-noise ratio. Cellular events aside from proliferation are also potential targets for Raman spectroscopy, with differentiation of adipose-derived MSC in multiple lineages being followed with an in situ probe [193].

Radiation-based monitoring strategies have an extensive application potential and have unquestionably expanded monitoring capabilities. Further spectroscopy techniques that have been explored as PAT tools include fluorescence spectroscopy and dielectric spectroscopy [194]. In situ probes based on the latter (e.g., iBiomass by Fogale Biotech and Incyte by Hamilton) have been developed for cell density measurements and are compatible with microcarrier-based cultures [195, 196].

With an ever-growing listing of CPP, the demand for techniques or instruments that incorporate multiple culture parameters in an efficient manner is growing. YSI 2950D by YSI is a metabolite analyzer that has been used to simultaneously measure up to six different culture parameters using proprietary immobilized enzyme electrodes [142, 197]. The leading instrument BioProfile Flex2 by Nova Biomedical has challenged the boundaries of parameter parallelization with monitoring capacity for 16 different parameters, ranging from glucose concentration to cell viability [198]. However, these devices do not fulfill in-line monitoring ambitions. In trying to solve this issue, a prototype capsule (PATsule) currently in development has heightened hopes for a real-time multiparametric in-line device [182].

The development of these techniques hopes to give PAT significant observational power, helping assure cell therapy manufacturing needs. The need for PAT advancement emphasized beforehand has culminated with a momentum in solving this issue. Recently, Raman spectroscopy was employed as a PAT tool in an autologous immunotherapy model for cell therapy bioprocessing [199].

4 Downstream Processing

For traditional biopharmaceuticals, cellular contribution ends after upstream processing, where cells are taken advantage of as miniature factories to produce a desired product. Downstream units from cell-centered bioprocesses have an unprecedented challenge in trying to design purification methods that give special care to cell sensitivity without changing cell identity and potency [84]. Fortunately, cell separation is a common practice in research, thus manufacturing units can try to scale existing technology or develop entirely novel techniques.

After upstream manipulation, cells must be harvested from their respective vessels to proceed to downstream processing. While cells grown in single-cell suspensions can be easily recovered, adherent cells require surface detaching techniques. Enzymatic methods disrupt cell-surface interactions by causing proteolytic cleavage of integrins, which are proteins responsible for cell adhesion and contribute to cell signaling by transducing ECM stimuli [200]. While damaging integrins can affect cell phenotype or function, enzymatic detachment of cells is common in cell-related processes [201–204], with possibilities varying between Trypsin-EDTA, TrypLE™, and Accutase™. In order to avoid disrupting cell phenotype, approaches focused on reversible adherence to microcarriers have been pursued.

Considering that many cell therapies will be administered intravenously, the presence of particulates or intact microcarriers in the final cell product represents a major safety risk [205]. In this context, different technologies have emerged that may address this concern. For instance, Advanced Corning® Dissolvable Microcarriers (Corning Life Sciences) have been developed to facilitate adherent cell harvesting. These carriers are comprised of polygalacturonic acid chains cross-linked by calcium ions, which can be dissolved with exposure to ethylenediaminetetraacetic acid (EDTA) and pectinase. Human iPSC expanded in a Vertical-Wheel™ bioreactor were successfully extracted from dissolvable microcarriers [206]. The use of dissolvable microcarriers for expansion of iPSC led to a detachment efficiency of $92 \pm 4\%$ compared with $45 \pm 3\%$ when using plastic microcarriers with common detachment techniques.

Microcarrier functionalization is an alternative strategy to achieve reversible microcarrier adherence. Poly(*N*-isopropylacrylamide) is a thermal-responsive polymer which dramatically alters its conformation based on temperature fluctuations [207]. Different microcarrier functionalization methods using this polymer with cell culture validation of various cell types have been reviewed [207]. Recently, the same concept was explored using carriers with larger pores (i.e., macrocarriers) for human

dermal fibroblast and MSC culture. Considering the difficulties in harvesting adherent cells from macroporous carriers, this technology may give these carriers new potential for implementation in cell therapy processes [208].

Considering their different size and density, cell-carrier separation has been explored by filtration or centrifugation techniques. These selected approaches must be compatible with manufacturing processes of considerate scale, while respecting cell sensitivity. Dead-end filtration represents the scalable version for small-scale mesh filters, with commercial versions (Opticap® series by Merck Millipore) being applied for efficient separation of MSC from respective microcarriers after cell expansion and detachment [209]. A screening of several filter mesh sizes between 30 and 100 μm was performed after detachment of cells from the microcarriers. Interestingly, 30 μm pore sizes originated a significantly reduced cell recovery (approximately 67%) when compared with the performance of meshes with 80 and 100 μm pore sizes (>90%) [209]. In order to avoid fouling and membrane clogging associated with normal flow filtration, tangential flow filtration (TFF) might be an improved alternative for such separation. Hollow fibers (developed by Repligen or GE Healthcare) allow for separation with less pressure due to feed flow occurring tangential to the membrane.

Being reliant on pressure for separation, filtration methods can cause cellular stress. Therefore, centrifugation techniques are a suitable alternative for such separations. Aforementioned counterflow centrifugation or fluidized bed centrifugation minimize shear stress during cell separation and can also be implemented for downstream purposes [210]. However, the more demanding volume scale might limit application of previously described instruments. kSep systems developed by Sartorius explore the same centrifugation principles, having been designed to be able to process larger volumes and also allow for continuous cell isolation [211].

With both cell types in suspension, cell purification stages may be required after cell manipulation. These techniques were extensively covered in Sect. 2.2, and their application is also ever-present during downstream phases of the manufacturing process. Depending on the production method, cell-based therapies may demand several cell purification units throughout the pipeline.

At this point, both adherent and suspension cells converge in their respective downstream pipeline, requiring concentration and washing units before entering the formulation and filling stage. Fortunately, reducing volume possesses coincident methods with cell-carrier separation. Previously mentioned TFF and counterflow centrifugation are both viable options for concentration. TFF implementation for MSC concentration after microcarrier detachment and clarification was studied with a systematic breakdown of hollow fiber characteristics [209] (i.e., material and pore size) and filtration operation modes [212]. TFF under continuous and discontinuous operation resulted in cell recovery rates higher than 80% with a cell viability of 95%.

Although downstream stages appear to be dominated by filtration and centrifugation processes, disruptive separation mechanisms are being explored for cell therapy manufacturing applications. FloDesign Sonics has harnessed acoustic waves to influence cell movement [213]. Using acoustophoresis, cells are restrained in produced acoustic waves, which allows for washing and volume reduction

without negatively affecting cells. Ekko represents a continuous, closed, and scalable platform that has been commercially developed by FloDesign Sonics for cell therapy manufacture.

Downstream flowcharts for cell therapy manufacturing vary between adherent and suspension cultures. However, established filtration and centrifugation techniques are mostly transversal throughout different stages. Still, the limited amount of approaches for downstream processing is a critical issue for cell therapy manufacturing.

5 Formulation and Storage

When purified cells are compliant with quality control objectives, their formulation and filling is required for commercialization. Transport of cell-based products is much more demanding than conventional biopharmaceuticals. For minimally manipulated therapies, such as transplants, cells are delivered fresh in cold storage. Autologous products can follow similar formulation and filling principles, since their shelf-life is typically low. However, allogeneic cell therapies that serve a one-fit-all business model depend heavily on cryopreservation methods. The storage of cells at extremely low temperatures (e.g., ranges between −135 and −190°C, using liquid/vapor phase nitrogen tanks) drastically minimizes metabolic activity, allowing the preservation of cell viability over prolonged storage. However, cryopreserved products are a logistic burden as specialized infrastructure and equipment are necessary for handling and transport [84].

Moreover, cryopreservation processes are prone to inflict cell damage through multiple mechanisms [214]. These include abrupt temperature changes and unwanted thermal fluctuations. Therefore, appropriate cooling rates and temperature maintenance are important parameters for a successful cryopreservation. Cell injury can be associated with extracellular and intracellular ice formation. In order to prevent ice formation, cryoprotectants are usually added to cryopreserved suspensions. However, cryoprotectants can also be toxic to cells. Finally, cells are also subject to thawing injury, as a result of intracellular recrystallization.

The median viability of PB-derived HSPC cells can decrease from 98–99% (at the time of harvest) to 71–76% after thawing these cells from a liquid/vapor phase nitrogen freezer [215, 216]. UCM MSC, isolated through enzymatic digestion, presented a mean viability after thawing ranging from 75 to 81%, depending on the cryopreservation period, while viability prior to freezing was 83% [217]. When isolated through explant culture, UCM MSC presented a mean viability after thawing ranging from 71 to 83%, while viability of fresh cells was 93%.

In order to minimize cryopreservation-induced cell damage and enhance product reproducibility, automated and quantitative tools can be used for cryopreservation and thawing [218]. Programmable controlled rate freezers (CRFs) employing liquid nitrogen as a refrigerant offer greater control and customizability of cooling rates. New freezing systems that do not require liquid nitrogen have recently emerged such

as the Asymptote VIA Quad, Duo, and Research freezers [218]. These are suitable for GMP cleanroom facilities where the use of liquid nitrogen tanks leads to risks in terms of contamination and air quality.

Automated dry-thawing devices can eliminate the risks associated with manual thawing in a water bath, such as water-borne contaminants and operator variability [218]. Examples of this technology include the Asymptote VIA Thaw SC2 and CB1000, Biocision ThawSTAR, Medcision ThawCB, and Sarstedt Sahara.

To some extent, it might be desirable to avoid cryopreservation at all, either to prevent cryo-induced cell damage or to circumvent laborious and expensive freeze and thawing procedures. Storage at 2–8°C can be sufficient, especially in situations that only require short-term storage. Specialized hypothermic storage media such as HypoThermosol (BioLife Solutions) allow an increased product stability at hypothermic temperatures (e.g., 2–8°C), avoiding the need for cryopreservation procedures [219]. In 2012, TiGenix completed the first phase I clinical trial with AT MSC (i.e., Alofisel) stored and administered in HypoThermosol (NCT01743222) [220].

An innovative technique has been developed that could have major implications on tissue and cell preservation. Osiris Therapeutics has designed a lyophilization technology (Prestige Lyotechnology) to preserve living cells and tissue. A proof-of-concept was performed with placental tissue, which can be used as biological dressings for treatment of burns and deep wounds [221]. Lyophilized tissue was compared directly with cryopreserved samples concerning cell viability, ECM components, and tissue organization, showing comparable results. Lyopreservation of cells has major consequences, as lyophilized samples can be stored at room temperature without the need for expensive cryopreservation equipment. For cell therapy manufacturing, this breakthrough might imply considerable cost reductions, with deep structural changes to formulation and filling.

6 Cost of Goods

Feasibility is a key concept concerning cell therapy development. Although possessing great therapeutic potential, cell therapies have inherently complex manufacturing processes that can impact their commercial viability. The introduction of cells as a therapeutic product has caused a paradigm shift in bioproduction. Every manufacture stage has had difficulties in translating typical manufacturing units to cell-based products. More sophisticated microenvironments during upstream production are necessary when producing cell-based therapies, which can include feeder layers and biomaterial-based scaffolds. During the downstream phase, sensitivity of living cells to typical separation and purification processes demands innovative approaches, which usually are costly. Also, end-stage product transportation cannot yet fully rely on more conventional lyophilization options, since cells still depend either on fresh or cryopreserved storage for transport [84].

In turn, production has a high risk of becoming exceptionally expensive, leading to an unsustainable commercial product. Even after achieving regulatory approval, recent products have suffered with suspicions against long-term commercial sustainability. CAR-T therapies, such as Yescarta and Kymriah, have battled with health insurers to achieve coverage deals in several countries [222]. In England, its health cost-effectiveness watchdog (NICE) had initially ruled against acceptance of Yescarta into the national healthcare system, claiming £300,000 per patient was an excessive strain on its healthcare budget [223]. However, progress was made when Yescarta-producing Gilead offered a confidential discount on the listed price, leading to the approval of Yescarta for treatment of adult patients with diffuse large B-cell lymphoma [224]. Being potential cures for blood malignancies, their pricing must recognize a less favorable commercial and manufacturing scenario associated with one-time treatments. Additionally, their therapeutic indication has been for cancer patients who have shown resistance to chemotherapy and have ruled out bone marrow transplants. Thus, expected demand for such cell therapies is relatively low. Every one of these constraints is a threat against reliable commercialization of these potentially life-saving cell therapies.

CAR-T cell therapies are not isolated cases, since approved Alofisel has also suffered from similar concerns. In early 2019, NICE released its final appraisal on this cell-based product, advising against its adoption for treatment of perianal fistulas in adults with non-active or mildly active luminal Crohn's disease [225]. Although possessing very promising clinical trial results with 50% of patients treated with Alofisel during its Phase 3 trial showing fistula remission [226], lack of long-term remission studies has raised suspicions on the durability of its treatment benefit. Consequently, the proposed therapeutic value of Alofisel reflected in its pricing (list price – £13,500/vial with one dose consisting in four vials) cannot be assured, leading to rejection of coverage by NICE.

In order to assure the sustainability of approved therapies, with regulatory consent and adoption by healthcare providers, detection of cost reductions at any stage of a cell therapy bioprocess is crucial [35].

Incorporation of cost of goods (COG) analysis allows for a systemic search for cost drivers and should be included during any decision related with the manufacture process [39]. Assessing COG at a preliminary level facilitates process modifications that would become laborious and critically expensive at a later stage. In the case of cell therapies, there are some inherent characteristics that differentiate their production backbone and are responsible for immediate distinctions in the COG analysis approach.

Interestingly, cell origin has a profound impact on manufacture, commercialization, and business model choice. Production of allogeneic therapies is an economy of scale, which becomes increasingly cost-effective as the demand increases. Aligned with common concerns regarding the need of high cell doses for most cell therapies, donor to patient treatments have a desired production profile. Scale-up allows for reductions of consumable costs and operating labor. However, allogeneic therapies have yet to fully harness the potential for COG reduction of an economy of scale [39]. The above-mentioned lack of automation and closed production pipelines for

“off-the-shelf” allogeneic products have been holding these therapies back. Nevertheless, these concerns have been identified, and efforts are being made to overcome them. Decisional tools for the manufacture process based on risk management analysis that incorporate COG breakdown have been developed for allogeneic cell-based therapies [227]. Recently, an open-source bioprocess economics tool revealed that the Vertical-Wheel™ bioreactor system would allow cost savings in the manufacturing of MSC for cell therapy purposes [104].

Autologous therapies have their own distinct scenario concerning COG. Being patient specific, each produced lot is restricted to only one patient. Instead of implementing a scale-up strategy, these therapies require parallelization as their manufacturing dogma. This has clear consequences in COG analysis, with single-use modular options becoming more attractive than traditional larger-scale production vessels [39]. Furthermore, autologous cell therapies do not follow conventional demand-supply relationships. In this case, demand is simultaneously supply, since the patient possesses the cellular component for its own cell therapy. This significantly reduces any implementation of cost-effectiveness measures due to demand predictability. Although demand can be tracked, increasing cell therapy stock is not possible for autologous therapies, as they are not an “off-the-shelf” product. In terms of manufacturing facility policy, autologous therapies require prioritized point-of-care. Since cells from the patient are extracted, altered/expanded, and reinfused, proximity to the patient is critical. Accordingly, the concept of decentralized facilities for COG reduction in autologous approaches has been explored [228].

Tailored COG analysis models are necessary to accurately reduce costs in autologous and allogeneic therapies. However, there have been attempts to increase cell therapy versatility by changing its cell source requirement. By removing the endogenous T-cell receptor (TCR) from allogeneic T-cells, advances were made toward the creation of a universal CAR-T product [229]. Consequently, an allogeneic COG model was able to be developed for such a cell therapy [230]. Altering the nature of tissue procurement may be a COG solution when trying to convert an unsustainable cell therapy into a commercially available product. Although cell source is able to deeply condition cost drivers due to different production models, other manufacturing parameters also have impact on commercial sustainability.

Having recognized the importance of COG analysis in cell therapy manufacturing, the ISCT conducted a survey for its members to quantify and discriminate costs in their production pipelines [39]. Acquisition of material and consumables had the highest mean response, being responsible for 31% of the total costs. Labor-related costs followed, occupying 20% of the cost burden. Processing and facility costs shared a similar slice, having been attributed 16% and 17%, respectively. Quality control and distribution were at the rear, representing 11% and 5% of total manufacturing costs. Although this breakdown cannot be applied as a universal template for cell therapies, it helps in comprehending their COG scenario.

Production of an MSC-based therapy was used as a case study in order to explore the full potential of cost optimization through COG analysis. As mentioned beforehand, allogeneic therapies benefit from an economy of scale and an “off-the-shelf”

business model. By predicting increases in annual demand and batch sizes, COG layout recommended adoption of specific expansion strategies [227]. While multiplate bioreactors and multilayered flasks competed at annual demands between 10^{10}–10^{11} cells and at a batch size of 10^{9} cells, single-use bioreactors in combination with microcarriers dominated higher targets in annual demand and batch size. Although planar technologies are normally chosen as the initial expansion platform, this simulation demonstrated that single-use bioreactors have comparable costs even at the lower values of demand and batch size. This observation was an improvement of a previous study that only considered adoption of bioreactors when confronted with infeasible scenarios of planar technology [231]. Additionally, considerable differences were pointed out concerning the labor associated with the explored platforms. For the first 50 days of an annual production of 100 batches consisting of 5×10^{10} cells each, multilayered flasks required 14 full-time equivalents with a labor load surpassing 80 h in a single day [227]. In the same manufacturing conditions, single-use bioreactors needed only two full-time equivalents with daily labor loads constantly under 10 h. Both selection of expansion platform and labor requirements were shown to be highly influenced by a COG breakdown as cost drivers.

This methodology enhances decision-making, by simulating possible scenarios and COG reaction to manufacture alterations. COG optimization should be pursued throughout the whole bioprocess, even as development advances.

7 Quality by Design

Having cells taking the central role in a therapy has broaden therapeutic angles to tackle innumerous diseases. However, ensuring high-quality products with consistency is more challenging for such cell-based therapies. The complexity of the cellular component associated with lack of complete comprehension of its machineries demands even more stringent quality measures during manufacture.

After initial proof-of-concept research, bioprocess development must include product quality guidelines to guarantee cellular therapeutic attributes, avoid manufacturing failure and alleviate regulatory concerns regarding safety. Quality assurance in the biopharmaceutical field was introduced by the US Food and Drug Administration (FDA) to tackle alarming waste rates, nonexistence of predictable models, and insufficient production control [232]. cGMP was delineated to renew the pharmaceutical sector to address this issue. Quality management of biopharmaceuticals changed with the creation of the Quality by Design (QbD) model [233]. Due to its increasing impact, the FDA and EMA launched a joint pilot program with parallel application assessments in order to harmonize and integrate QbD guidelines [234]. A holistic view of the bioprocess allied with extensive scientific knowledge of each production component and a systematic and iterative method of improvement serve as the basis of QbD.

Cell therapies have followed traditional biopharmaceutical development mistakes regarding manufacturing development. Inability to apply QbD and COG analysis

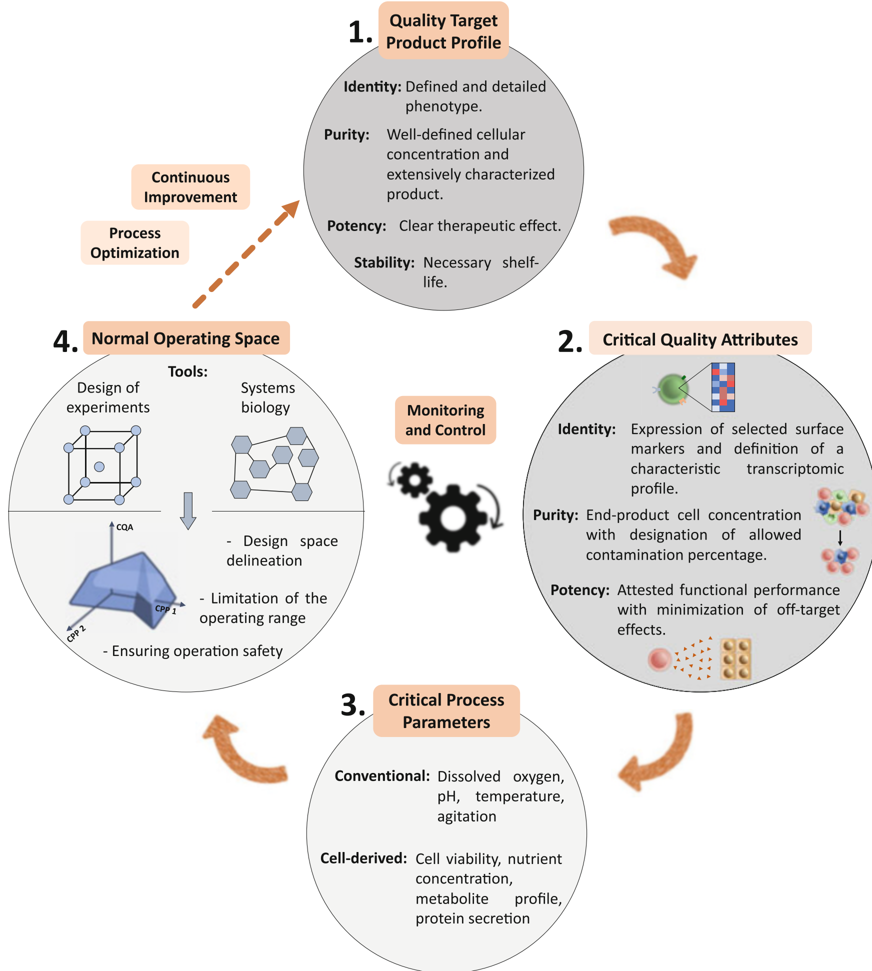


Fig. 6 Global description of the QbD process. An initial QTPP requires defining end product objectives that satisfy therapeutic needs. Translation of these objectives to their cellular features uncovers process CQA. In turn, process variables that are responsible for influencing CQA are identified as CPP. Controlled variation of these parameters originates a normal operating space, which limits process operability. Continuous process monitoring and control facilitates improvement implementation, creating a cycle of process optimization. *QbD* quality by design, *QTPP* quality target product profile, *CQA* critical quality attribute, *CPP* critical process parameters

has increased manufacturing failures and unsustainable bioprocesses. Translation of prominent clinical trial results to a scalable and cost-effective bioprocess that has led to several letdowns with development suspension and product withdrawal [235, 236].

A logical sequence of steps is delineated by QbD in order to advise manufacturers regarding intelligent production pipeline development for cell therapies (Fig. 6).

Initial guidelines concern the end product, with the identification of therapeutic objectives. These should describe with great detail cutoff goals for concepts such as identity, potency, and purity [237]. High degree of clarity and detail in defining end product properties will facilitate identification of critical production processes and problem localization and solving. By creating a quality target product profile (QTPP), the framework for QbD is established.

With the target profile set, the entire process needs to be broken down to identify critical variables that have a direct effect on the QTPP. Raw material attributes (RMA) need to be controlled for quality with selected checkpoints so that variability and lack of quality cannot compromise the bioprocess from the start [233]. After controlling process inputs, continuous monitoring with PAT throughout the entire process should exist to assure correct transformation or manipulation of raw materials into the final product.

Considering that following every possible variable is unrealistic, critical quality attributes (CQA) should be selected. For cell-based therapies, these attributes correspond to cellular features. Since living cells are multivariate systems, isolating inputs (e.g., signaling factors) and controlling outputs (e.g., cell expansion) are not trivial. Knowledge on cell networks is increasing, but a complete map of cellular machinery is still far from reach. Thus, identification of CQA, which are crucial for QbD, can be difficult.

Throughout the bioprocess, each unit has an impact on cells and is accountable for altering their characteristics to some extent. Controlling CQA will depend on identifying which CPP are responsible for changing those same features. Control strategies should build on CPP discovery, which serve as directives for selection of monitoring techniques [233]. The holistic nature of QbD demands whole process overview, with parallel interventions as the production pipeline moves forward.

After definition of individual CQA and respective CPP, studying their interactions should follow. By uncovering and exploring networks, process knowledge increases greatly. In turn, reaction to process variability can be achieved quickly and pragmatically by tweaking CPP. Rapid process correction is particularly relevant for cell therapy manufacturing. Even with RMA tightly controlled, cells have inherent complexity that leads to aberrant and unpredictable behavior. Therefore, discerning connections between CQA and CPP will increase process mapping and improve decision-making [238].

Interactive effects can be revealed by analyzing a defined design space. The range of variability of CQA caused by fine-tuning of CPP or RMA will define boundaries for a normal operating range in the design space. Working inside the normal operating range admits changes in CPP without compromising end product quality. CQA-CPP connection can be very laborious and difficult to obtain, with a substantial need for varied experimental data. In order to circumvent excess labor and costly optimizations, design of experiments and systems biology are effective tools that originate the same results using up only a fraction of expected time and resources [237, 239].

Design of experiments is dedicated to evidencing relationships between input and response variables in an optimized manner. Extensive multivariable exploration

inside a selected design window is performed using the least amount of experimental conditions. Factorial design is responsible for generating necessary experimental combinations. Obtained experimental data allows delineation of a response surface for every CPP. These surfaces are described by behavior functions, which model the above-mentioned CQA-CPP relationships [240]. Besides enhancing process knowledge, behavior functions enable CPP optimization resulting in CQA improvement. Implementation of design of experiments has caused process improvements from pluripotent stem cell cultivation to ex vivo HSPC expansion [241–243].

Systems biology tools can also contribute to discerning CQA-CPP interactions. Omics-based techniques, such as gene expression and protein production, can contribute to QTTP review, updating end product identity, and potency. Also, metabolic pathways can derive similar information without directly compromising cells. In this context, metabolic by-products were used to construct a fed-batch platform for HSPC expansion in order to avoid inhibitory feedback signaling [244]. Intrinsically, large amounts of generated data can also yield reduced dimension mechanistic models, similar to behavior functions. Overall, the impact of any CPP modifications on CQA can be more easily detected.

As QbD requires the combination of extensive fundamental knowledge with engineering principles, applying it to cell therapies is a daunting task. Biological variability is inherent, making standardization and quality control a struggle for any process that includes cells. However, QbD guidelines serve as a backbone for correct cell therapy manufacturing development. Its implementation is critical to any future bioprocess, assuring quality and robustness throughout each unit. Additionally, continuous improvement associated with QbD brings process evolution to the production pipeline, making it dynamically resistant to unsustainability [237].

8 The New Cell-Free Therapeutic Paradigm

Recent advances in cell biology have led to a better understanding of cell communication processes. Cells are able to interact with their surroundings, influencing other cells and tissues through paracrine and endocrine signaling. Multiple studies have been developed using cellular secretome for therapeutic purposes. For instance, MSC-conditioned medium improved hepatocellular regeneration in a rat model of acute liver injury [245]. On the other hand, conditioned medium from genetically modified MSC was able to limit infarct size and improve ventricular function in mice infarcted hearts [246]. In addition, the secretome obtained from bioreactor cultures of human BM MSC was able to induce neurogenesis and increase neuronal cell differentiation in vivo [247].

The secretome consists of every macromolecule a cell transports to the extracellular space at a given point in time. The secretome contains mainly proteins such as growth factors, hormones, cytokines, chemokines, and interferons, as well as extracellular vesicles [248, 249]. In particular, extracellular vesicles (EVs) have been given great attention recently by the scientific community, due to their potential both

as diagnostic and therapeutic tools. EVs are lipid membrane enclosed structures actively secreted by most cell types. These vesicles have emerged as relevant mediators of intercellular communication, through the transfer of a cargo of proteins and RNA (i.e., microRNA and mRNA), which prompt alterations on recipient cells [250–252].

Depending on their biogenesis, EVs are broadly categorized as exosomes, microvesicles, or apoptotic bodies. Of notice, exosomes (50–150 nm) are generated via the endosomal pathway [253, 254], microvesicles (50–1,000 nm) by outward budding of the plasma membrane [253, 254], and apoptotic bodies (up to 5 μm) released as blebs of cells undergoing apoptosis [255]. EVs are associated with multiple physiological processes, such as immune surveillance [250] and tissue repair [256, 257] but also in the pathology underlying several diseases, such as cancer [258–260] and neurodegenerative diseases [261].

Given the importance of EVs in cell communication, the scientific community soon realized their potential to target diseased cells. EV membranes resemble the cell membrane, allowing high biocompatibility to target cells for therapeutic purposes. Moreover, EVs show specific targeting activity [262] and small size, ideal to cross biological barriers, such as the blood-brain barrier [263].

The therapeutic use of EVs is twofold. On the one hand, EVs were found to mediate some of the therapeutic effects from their original cells [264, 265]. Therefore, these could be potentially used in substitution of their cell of origin, as a cell-free therapy triggering equivalent therapeutic effect. On the other hand, EVs can be used as drug delivery vehicles, through loading of EVs with therapeutic cargo [266].

Engineered MSC-derived EVs were able to successfully target and regenerate ischemic myocardium in mice [267]. Dendritic cell-derived EVs were able to deliver siRNA to the brain in mice, demonstrating their potential use as targeted therapy for neurodegenerative diseases [268]. Multiple studies have successfully developed EVs as drug delivery vehicles for cancer therapy [269–272]. As an example, intravenously injected exosomes from immature dendritic cells delivered doxorubicin specifically to tumor tissues in mice, leading to inhibition of tumor growth without overt toxicity [269].

Despite the promising potential of EVs for therapeutic applications, robust manufacturing processes that would increase the consistency and scalability of EV production are still lacking, both in EV production (upstream processing) and further isolation (downstream processing) [273, 274]. Furthermore, higher efficiency drug loading approaches and strategies to increase EV cell-specific targeting need to be developed [274].

In conclusion, cell-derived products such as EVs are emerging as novel therapeutic opportunities. These cell-free therapies present significant advantages, avoiding the complexity and safety issues in utilizing cells themselves as therapeutic systems in a clinical context [266, 275]. Nonetheless, this field is still in a very early development stage and requires substantial research before being successfully translated into clinics.

9 Concluding Remarks and Future Perspectives

Exciting developments in the cell therapy field over the last decades has led to an increasing number of clinical trials testing multiple cell products for therapeutic purposes. This has been followed by an increasing (although still small) number of products receiving marketing authorization by regulatory agencies. Even though there was a substantial progress in the field, manufacturing of cell-based therapies still presents multiple challenges that need to be addressed in order to assure the development of safe, efficacious, and cost-effective cell therapies.

Multiple human tissues have been used to derive cells for therapy, showing different therapeutic capacity depending on the source that cells were isolated from. Identifying the most appropriate cell source for each application would allow increased therapeutic efficacy. Moreover, most cells isolated from human sources are extremely heterogeneous. Identifying and expanding specific functional cell subpopulations could allow the development of more efficacious and reproducible products for a specific application.

FBS and other animal-derived products comprise the majority of cell culture media supplements used for cell manufacturing. These supplements are ill-defined, increase the risk of contamination with virus and prions, and lead to poor reproducibility, limiting their application in the clinical setting. The development and application of S/XF culture media formulations aiming at a chemically defined medium are essential for the clinical translation of cell-based products.

Bioreactor technology has allowed the establishment of scalable manufacturing processes for cell expansion. However, these dynamic systems also present challenges for cell culture, namely, shear stress, which may impact product features. New agitation designs such as the Vertical-Wheel™ and wave bioreactors have been developed to provide a more homogenous and gentler particle suspension at lower agitation rates. These bioreactors also present the opportunity to implement single-use technologies, more suitable for the establishment of GMP-compliant processes. Furthermore, bioreactors could be designed for a specific application and further optimized relying on simulation techniques.

Computer-monitored culture systems, capable of controlling feeding regimes and maintaining concentrations of physicochemical parameters (e.g., O_2 and pH) and certain molecules (e.g., growth factors and cytokines) within an optimal range, would offer a great improvement in the robustness of the manufacturing process.

Currently, isolation and expansion protocols are performed in open systems, involving a two-stage protocol, which lead to increased risk of contamination, thus compromising the safety and standardization of the final cell product. Ideally, cell isolation and expansion could be performed in a single-stage, closed system. This can be achieved using the Quantum® bioreactor and other strategies such as the one developed by Papadimitropoulos and colleagues to culture freshly isolated BM nucleated cells within 3D porous scaffolds in a perfusion-based bioreactor system [276].

The implementation of automated and closed systems would provide a major contribution to achieve a GMP-compliant, clinical-grade cell manufacturing.

CliniMACS Prodigy, developed by Miltenyi Biotec, has successfully shown how manufacturing automation and closed systems can be applied to cell therapies. This platform has integrated an entire manufacturing process in an automated manner. Upstream stages (cell fractionation, isolation, and cultivation) were combined with downstream units (cell purification, formulation, and filling) to enable a fully closed clinical scale cell therapy platform [277].

Advancements in cell therapy manufacturing and their individual process units are fruitless if the overall production pipeline is not cost-effective. Commercial sustainability will ultimately be responsible for dictating the future of a certain cell therapy. Continuous COG analysis represents the most pertinent tool for identifying critical cost drivers, which should be flagged and made priorities for improvement.

Early process development by design can avoid considerable and costly time constraints with manufacture innovation being focused for ensuring product quality. Continuous monitoring improves process knowledge, and respective control assures maximized pipeline stability, promoting reproducibility of cell therapy production. Furthermore, QbD cooperation with COG analysis can assist in parameter targeting for optimization, contributing toward cyclical process improvements.

Although cell-based therapies represent a new level in bioprocessing complexity in every manufacturing stage, these also show unprecedented levels of therapeutic potential, already radically changing the landscape of medical care.

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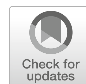
Bioprinting Technologies in Tissue Engineering



Bengi Yilmaz, Aydin Tahmasebifar, and Erkan Türker Baran

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Abstract Bioprinting technology is a strong tool in producing living functional tissues and organs from cells, biomaterial-based bioinks, and growth factors in computer-controlled platform. The aim of this chapter is to present recent progresses in bioprinting of nerve, skin, cardiac, bone, cartilage, skeletal muscle, and other soft tissues and highlight the challenges in these applications. Various composite bioinks

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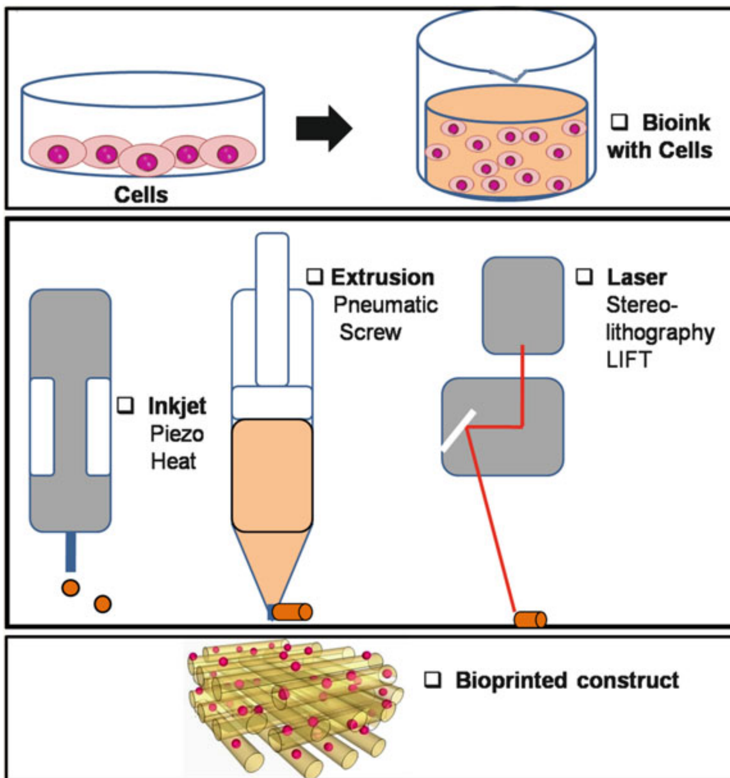
with bioactive ceramic-based scaffolds having patient-specific design and controlled micro-architectures were used at clinical and preclinical applications successfully for regeneration of bone. In nerve tissue engineering, bioprinting of alginate- and gelatin-based gel bioinks by extrusion presented a controllable 3D microstructures and showed satisfactory cytocompatibility and axonal regeneration. Bioprinting of cardiac progenitors in biopolymers resulted in limited success, while the use of bioinks from extracellular matrix induced satisfactory results in cardiac regeneration. Osteochondral scaffold bioprinting is challenging due to the complex hierarchical structure and limited chondral regeneration. Therefore, current approaches focused on osteochondral scaffold with vascular network and mimicking hierarchical structures. The applications of bioprinting in other types of tissues were also studied, and results showed significant potentials in regeneration of tissues such as cornea, liver, and urinary bladder.

Graphical Abstract



Keywords Bioprinting, Bone, Cardiac, Cartilage, Nerve, Skin, Tissue engineering

1 Introduction

Bioprinting is a variant of 3D printing and refers to the three-dimensional (3D) manufacturing of biological constructs through the layer-by-layer deposition of inks with living cells. It can also be defined as the use of material science and production techniques to build tissues and organs with viable cells and biomolecules in specific organization to perform a particular biological function [1]. The first international conference on bioprinting was held at the University of Manchester, UK, in September 2004, and the definition of bioprinting was first proposed in this conference as: "the use of material transfer processes for patterning and assembling biologically relevant materials – molecules, cells, tissues, and biodegradable biomaterials – with a prescribed organization to accomplish one or more biological functions" [2]. Since the ultimate goal of bioprinting is to produce living functional tissues and organs to be transplanted, Mironov et al. [3] proposed another term "organ printing" which is defined as: "computer aided 3D tissue engineering of living organs based on the simultaneous deposition of cells and hydrogels with the principles of self-assembly."

Bioprinting, or 3D bioprinting, was defined in "Workshop on Bioprinting, Biopatterning and Bioassembly" at the University of Manchester, UK, in 2004, as: "The use of material transfer processes for patterning and assembling biologically relevant materials—molecules, cells, tissues, and biodegradable biomaterials—with a prescribed organization to accomplish one or more biological functions" [4]. 3D bioprinting for the production of biological structures typically involves layer-by-layer deposition of bioink to form 3D tissue or organ structures with an input from a computer-aided design (CAD) program [5]. 3D bioprinting has been gathering enormous attention from the scientific community of regenerative medicine due to its ability to manufacture organs from native cells. Figure 1 demonstrates the increasing scientific interest to bioprinting and shows the related scientific categories according to Web of Science database. Although the bio-additive production of a transplantable entire organ has not been achieved yet, this technology is advancing, and it is thought that it will soon resolve the crisis of organ shortages [6].

According to the Organ Procurement and Transplantation Network of US Department of Health and Human Services, 114,417 people are actively waiting for a lifesaving organ transplant in the USA, and only 30,415 organ transplants were performed in 2018; see Fig. 2 [7]. Regenerative medicine gives hope to fill the gap between the number of organ transplants and organ need through the engineering of functional tissues or organs to improve or modify abnormal and necrotic tissues and organs [8]. Furthermore, 3D printed human tissues can be a better model for preclinical testing of potential drugs than in vivo testing on animal models in order to predict the clinical outcome in humans [9].

There are three basic variables that should be considered in order to be able to produce living and functional tissue structures successfully by using the bioprinting method. The first is the cellular component which is the living part of the structure and containing at least one or multiple cell types. The second one is the scaffolding

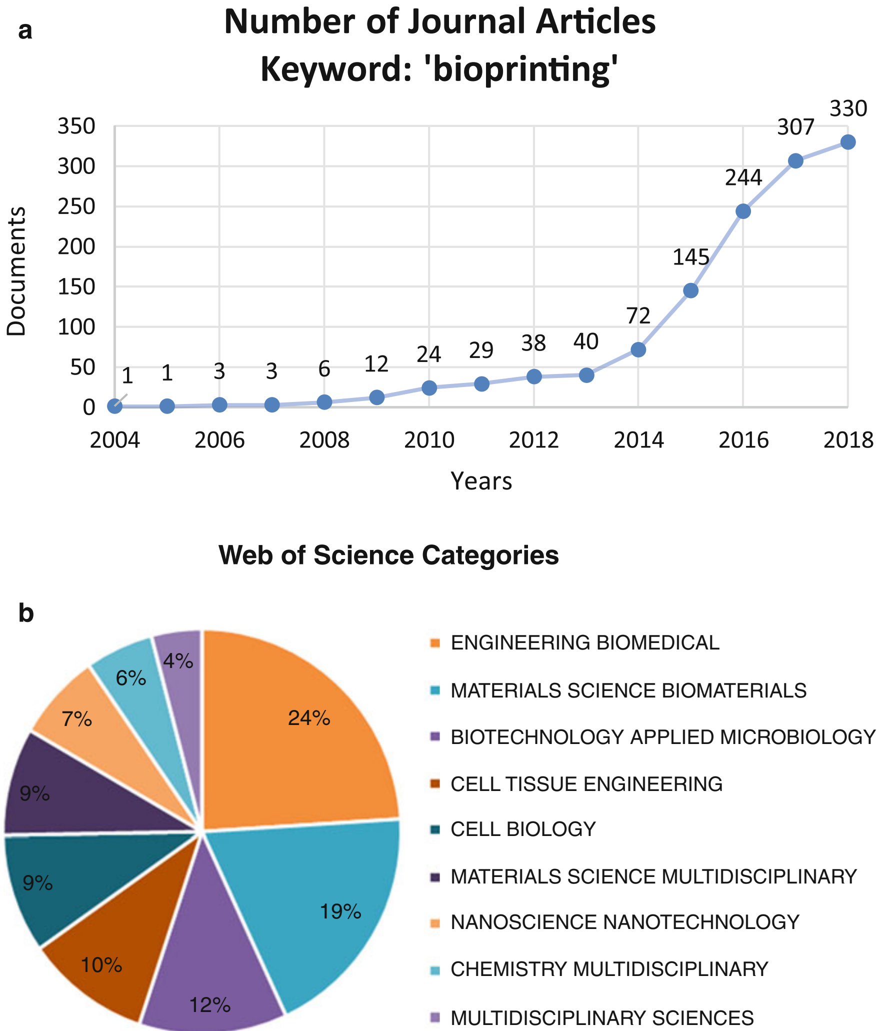


Fig. 1 (**a**) Number of records for "bioprinting" in academic publications by year; (**b**) scientific categories related to bioprinting. Source: Web of Science, December 2018

or supportive biomaterials comprising mostly proteins and polymers that can provide support and protection to cells during and after the manufacturing process. These biomaterials can mimic the physical environment and the biochemical signal cells as in the body and serve as a bioink bridge between cells and hardware. Third is the actual bioprinting device in which the manufacturing process is performed. Most importantly, the biomaterial used is a necessary bridge between the bioink, cells, and hardware [9].

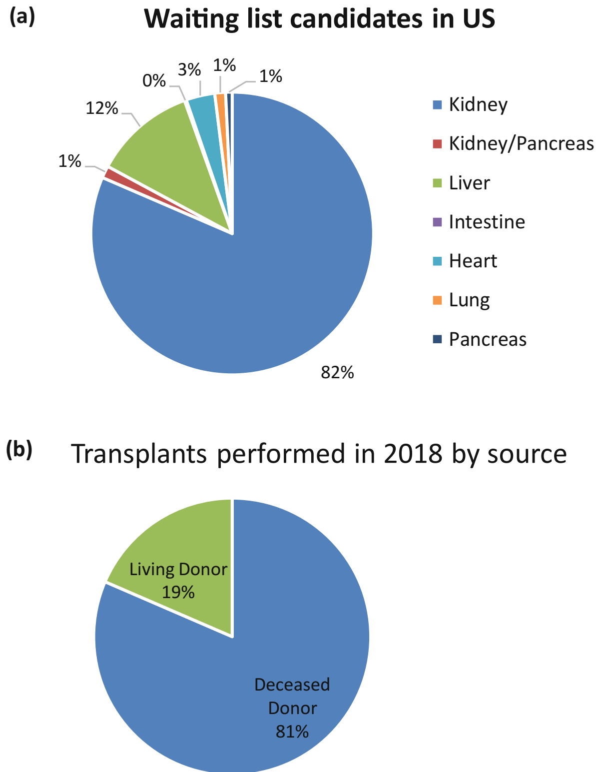


Fig. 2 According to Organ Procurement and Transplantation Network: (**a**) number of waiting list candidates by organ type as of December 2018 in the USA; (**b**) source of transplants performed in January to October 2018 in the USA [7]

In a historical point of view, Wyn Kelly Swainson owned a patent for a method and an apparatus for the first time in 1971 to produce 3D structures in a medium which is selectively sensitive to different parameters of electromagnetic radiation [10]. Later, in 1984, Charles W. Hull patented an apparatus for the production of three-dimensional objects by stereolithography [11], which was considered as the world's first 3D printer [12].

The use of 3D printing in healthcare applications was started with customized and patient-specific surgical guides and implants in orthopedics [13]. In 2002, Envisiontec GmbH began to sell a bioprinter named Bioplotter which was able to produce scaffold structures from various biomaterials for tissue engineering [14]. In 2010, a research center Chemical Institute of Sarria (IQS – Instituto Químico de Sarrià) announced two hydroxyapatite (HA) formulations for use in 3D printers [14]. In 2011, Objet introduced a biocompatible colorless photopolymer material, MED610, for medical and dental markets [15]. In 2012, Organovo Holdings and Autodesk announced a collaboration to create the first commercial 3D bioprinting software [14]. Organovo's NovoGen MMX Bioprinter was the first commercial bioprinter tested for 3D-printed tissues [16, 17].

2 3D Bioprinting

Custom-made and patient-specific scaffolds/tissues/organs can be printed according to the 3D models constructed from the data acquired by medical imaging techniques, such as 3D computed tomography (CT) and magnetic resonance imaging (MRI), or generated digitally by using many CAD software. Figure 3 shows the flow diagram for the manufacturing of bioprinted tissues that can be used either directly in animal models or patients or as in vitro constructs that can be used for drug testing and disease modeling.

3D bioprinting is a comprehensive process that requires adjustment of various design parameters, such as imaging, modeling, printer choice, selection of bioink, culture conditions, and development of 3D construct [8]. The manufacturing process can be categorized in three different steps: pre-bioprinting, bioprinting, and post-bioprinting [18]. The pre-bioprinting (modeling) step involves acquisition of 3D images by using techniques like 3D scanner, CT, and MRI, designing a digital 3D model suitable for printing from these imaging modalities or by using CAD-CAM and mathematical modeling techniques, and finally the selection of bioink, biomaterial, and cells. In the bioprinting step, 3D-rendered model is sliced into customizable horizontal portions that are imported into the bioprinter system. One or more bioinks, which are composed of cells, bioactive molecules, and biomaterials, are

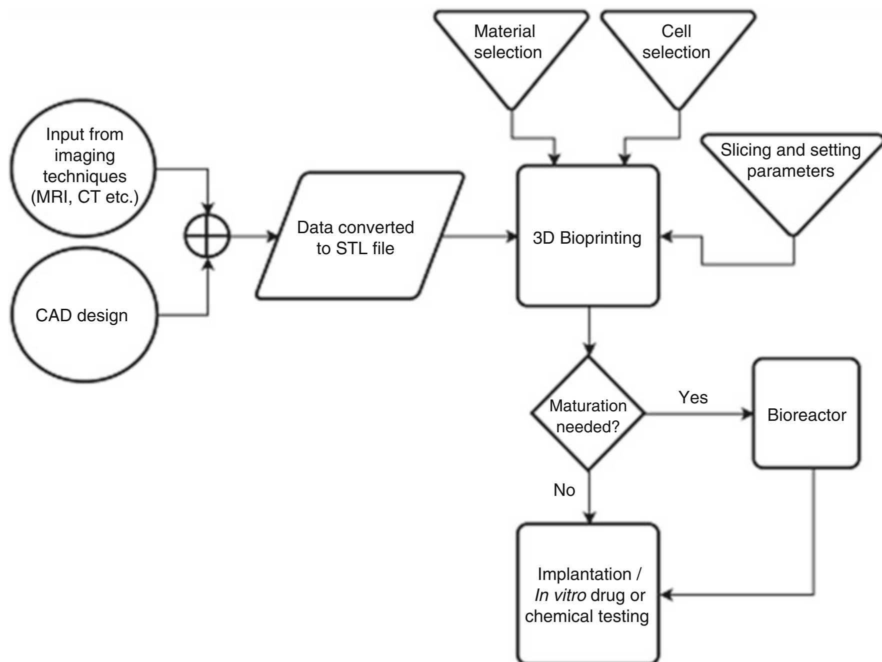


Fig. 3 A typical production flowchart for bioprinting

Table 1 Comparison of bioprinter types [19]

	Inkjet	Extrusion	Laser assisted
Material viscosities	3.5–12 mPa/s	30 mPa/s to >6 × 107 mPa/s	1–300 mPa/s
Gelation methods	Chemical, photo-cross-linking	Chemical, photo-cross-linking, sheer thinning, temperature	Chemical, photo-cross-linking
Preparation time	Low	Low to medium	Medium to high
Print speed	Fast (1–10,000 droplets per s)	Slow (10–50 μm/s)	Medium-fast (200–1,600 mm/s)
Resolution or droplet size	<1 pL to >300 pL droplets, 50 μm wide	5 μm to millimeters wide	Microscale resolution
Cell viability	>85%	40–80%	>95%
Cell densities	Low, <106 cells/mL	High, cell spheroids	Medium, 108 cells/mL
Printer cost	Low	Medium	High

placed in the printer cartridges and printed layer by layer in aseptic conditions. The post-printing, which is biomimetic remodeling of tissue with the help of mechanical and chemical signals, is also an important step for obtaining mechanical integrity and biological functionality.

The main technologies used for 3D bioprinting with biological materials are inkjet, micro-extrusion, and laser-assisted printing [19]. Table 1 summarizes the different features of these technologies.

2.1 *Inkjet Bioprinting*

2D inkjet printing is a noncontact technique that accepts a digital signal representing an image and deposits tiny ink drops to form the image onto a substrate, which is mostly paper. It is possible to fabricate 3D tissues by using a technology very similar to that of a common inkjet printer. In fact, the first attempts of bioprinting started with working on the hardware and software necessary to convert commercial tabletop inkjet printers into cell printers [20], and the first patent for printing viable cells with inkjet printer came in 2006 by Thomas Boland and coworkers at Clemson University [21]. One of the early researchers Anthony Atala (the director of the Wake Forest Institute for Regenerative Medicine in Winston-Salem, NC, USA) also began on a desktop inkjet printer that was modified to print 3D structures [22].

Droplet-based inkjet bioprinting works in a similar manner to the inkjet technology as described above. The most common types of inkjet printing devices that are adapted to bioprinting have thermal and piezoelectric mechanisms to generate the droplets in drop-on-demand principle (see Fig. 4). The devices with a thermal printhead have an electric heating unit that vaporizes the binder material to form a

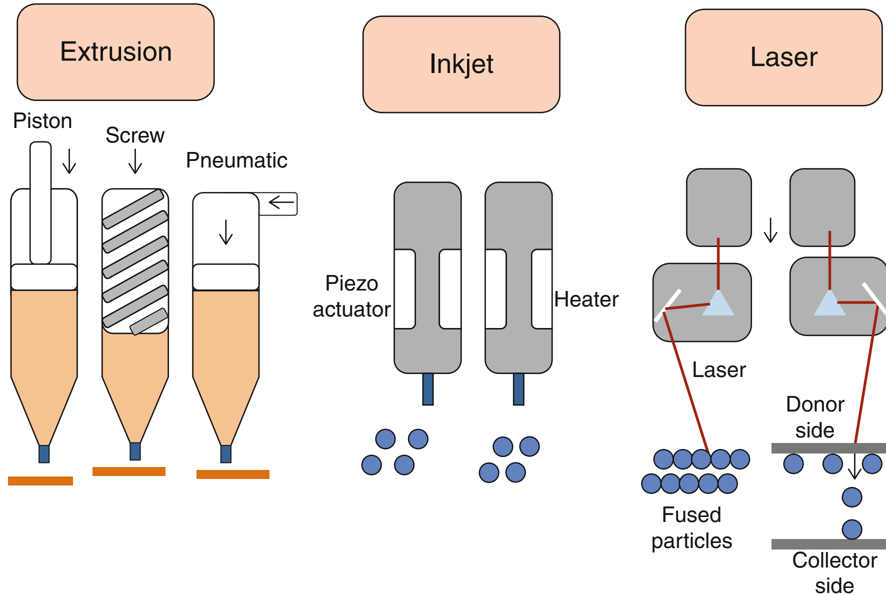


Fig. 4 Inkjet, extrusion, and laser-assisted bioprinters

vapor bubble. This vapor bubble expands due to pressure and exits as a droplet from the printhead. Although the temperature rises to 200–300°C while forming a bubble, the printhead temperature increases only about 10°C, because the process only takes a few microseconds (about 2 μs) [8]. In the devices using a printhead with a piezoelectric actuator, the voltage pulse in the printhead causes a rapid shape change in the piezo-material, and this generates a pressure pulse in the binding fluid, resulting in a droplet. These printers ensure fast and precise deposition of the binder liquid.

2.2 *Extrusion Bioprinting*

Fused deposition modeling (FDM) or fused filament fabrication (FFF) was developed in the early 1990s, and this extrusion-based 3D printing strategy is the most common and inexpensive type of additive manufacturing technology [8, 23]. FDM technique is based on extrusion of molten thermoplastic polymer filaments or small granules from a small nozzle, which then hardens to form a solid construct (see Fig. 5) [24]. FDM offers many advantages, such as easy material switching, low maintenance costs, compact size, and flexibility in working temperatures; however, the narrow range of printable materials limits its applications [25].

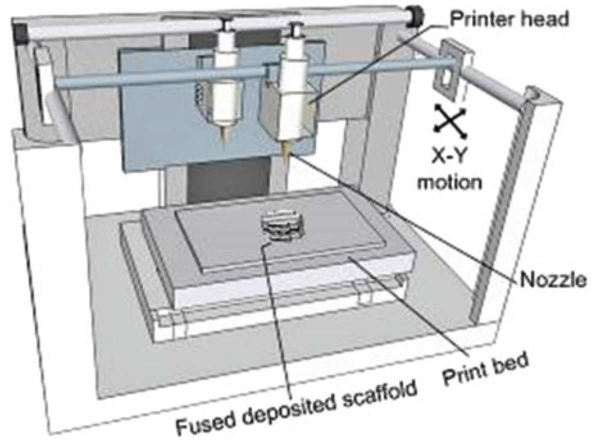


Fig. 5 A schematic diagram showing fused deposition modeling

Extrusion-based (dispensing or direct writing) bioprinting originates from FDM printing technology. These devices use pneumatic-, mechanical-, or electromagnetic-driven needle-syringe-type systems to deposit cells and biomaterials [8]. The cell-laden bioinks, which are mostly composed of hydrogels and some bioactive components, are dispensed by the printhead to form the desired 3D structures. In order to produce 3D structures with high accuracy, bioinks must be designed with shear-thinning or fast-solidifying properties. It is possible to obtain multiwalled and core-shell fibers from different bioinks by using multi-compartment type of nozzles and to adapt microfluidic strategy to extrusion bioprinting [26].

2.3 Laser-Assisted Bioprinting

In 1999, Odde and Renn [27] reported the possible tissue engineering applications of a laser-guided direct-writing system which has the ability to organize cells spatially into well-defined 3D arrays. Laser-assisted bioprinting is also a droplet-based system which is also known as laser-induced forward transfer (LIFT). This system consists of a pulsed laser source; a focusing system; a ribbon, which contains a layer of biological material to be printed and a glass covered with a laser-energy-absorbing layer (such as gold or titanium); and a substrate that collects the printed material [19, 28]; see Fig. 4. The metal film is vaporized by a laser pulse, and this produces a jet of liquid solution of organic materials (cells and molecules) which is then deposited onto the facing substrate [29]. Although laser-assisted bioprinting is less common than other bioprinting techniques, it has many advantages, such as having high spatial resolution and capability of printing a variety of biological materials and high cell viability.

2.4 Stereolithography (SLA)

Stereolithography was developed as a solid free-form manufacturing technique in the early 1980s [30]. Today, most 3D printers and recent bioprinters accept input files in the STL format. The acronym "STL" stands for STereoLithography, since it was the first 3D printing process, and the STL file format emerged as a native file format from a CAD software [31]. This STL file is sliced to generate a G-code, which provides instructions for the stereolithography apparatus (SLA).

The main components of the system are a reservoir filled with a photocurable polymer solution or resin, a *x*-*y* axis controlled laser, and a fabrication stage with *z*-axis control [9]. A SLA is depicted in Fig. 6. Stereolithography is a laser-assisted additive manufacturing technology that utilizes an ultraviolet (UV) laser to photopolymerize the surface of a bath of photosensitive polymer. The stage is gradually lowered, allowing layers to be polymerized on top of each other, thereby forming 3D structures in a bottom-up manner. The unscanned areas stay in the liquid phase.

The use of SLA in bioprinting was reported by the study of Dhariwala and coworkers in 2004 for the first time [32]. They filled a vat with cells and a photocurable hydrogel, which consists of poly(ethylene oxide) and poly(ethylene glycol dimethacrylate) in a ratio of 3:2 and a photoinitiator (Irgacure). The vat was placed on a table where the movement could be controlled in the vertical axis. UV light cured designated places in the vat according to the CAD file. For each layer, the process was repeated to produce 3D structures. High cell viability was achieved with a laser of UV light at 365 nm. Today, there are various applications of SLA from bioprinting to other rapid prototyping purposes.

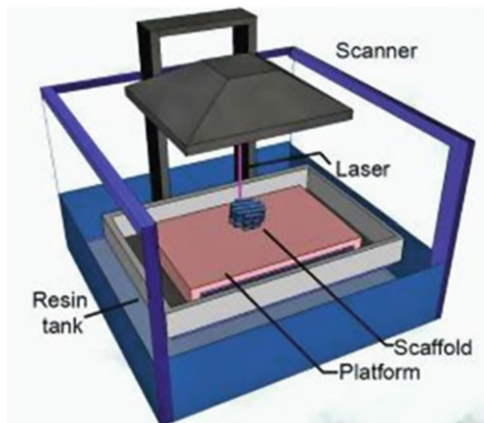


Fig. 6 A schematic diagram showing stereolithography apparatus

2.5 Microvalve-Based Bioprinting

Microvalve-based bioprinting is a kind of drop-on-demand systems which is mostly preferred for hydrogel dispensation [33]. This technology was developed by Demirci and Montesano in 2007. The main focus of their custom-made microvalve-based bioprinting is cell printing [34]. A conventional microvalve bioprinting system is composed of robotic platform and electromechanical printheads which are connected to a gas regulator [34, 35]. The gas regulator provides positive pressure on the system as it is in conventional systems. The solenoid coil and the plunger movement controls the microvalve opening time as low as 0.1 ms [34, 35]. The valve can be controlled by mechanical, electrical, and magnetic forces. Thus, the positive pressure and valve opening time controls allocating of bioink during printing [35].

The pressure, which is required for dispensing of material from the nozzle, leads to significant shear stress on cells. In addition, the parameters such as nozzle diameter and the viscosity of bioink have considerable effect on shear stress [36]. Although the moderate shear stress plays a crucial role in cell signaling and protein expression and improves stem cell differentiation, the disproportionate shear stress may rapture cell membrane [36]. The narrow range of printable viscosity is the main limitation of microvalve-based bioprinting systems (1–70 Mpa) [37].

3 4D Bioprinting

4D printing technology was invented by the Self-Assembly Laboratory at the Massachusetts Institute of Technology (MIT) in collaboration with Stratasys Ltd. [38]. The main difference from 3D printing is the production of multi-material prints with shape-changing ability over time or the use of customized materials which can be changed from one shape to another after being taken from the print bed. 3D bioprinting technology relies on the assumption that the printed cells can rapidly form tissues and start to synthesize the extracellular matrices, which can provide geometrical shape and mechanical support. 4D bioprinting, where time is the fourth dimension, the printed materials or living cellular constructs continue to evolve over time after being printed [39] (see Fig. 7). The stimuli for these constructs to evolve can be one or more of specific triggers, such as temperature, pH, humidity, electricity, magnetic field, light, and acoustics [40]. Integrating new smart materials, which can respond to these stimuli and transform with time, into 3D printing is the basis of 4D printing concept. One such smart feature is the shear-thinning property of bioinks that allows reversible changes in the shear viscosity of printable inks to assist 4D printing [41].

Since bioprinting requires the use of viable cells, 4D printable smart materials should be biocompatible, and rheological properties should be adequate in order to maintain high cell viability at optimum printability [42]. 4D printed cell-laden structures face many challenges as it is in 3D printing technique: (1) cell damage

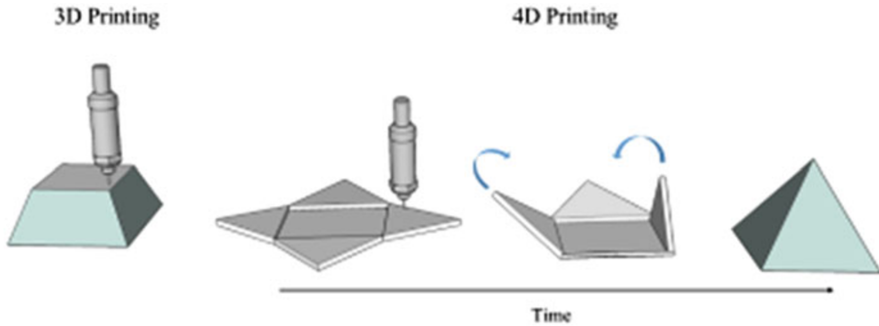


Fig. 7 Schematic depiction of printing of 3D and 4D objects

after printing, (2) deterioration of cell proliferation, and (3) deposition of low or excess cell population [43].

The number of sensitive materials, which can be printed by using the 4D printing technique, is very limited, and most are capable of responding to only one type of stimulus. Furthermore, the deformation ability of the 4D bioprinted structures formed with these materials is also limited to simple deformations such as folding/unfolding or self-organization. The precise control of the spatiotemporal evolution of the printed structure is restricted because these deformations can occur only in macroscale [39]. The 4D printing technique also needs to overcome the major difficulties in mimicking the complex dynamic deformation of natural tissues such as the blood pumping of the heart and the peristaltic movement of the esophagus or intestine [44].

4 Tissue Engineering Applications of Bioprinting

4.1 Bone Tissue

Bone tissue provides mechanical strength, creates a structural framework, and allows the body to move. It also serves as a mineral storage and plays a vital role in homeostasis and regulation of blood pH [45]. Bone is a connective tissue with a complex hierarchical structure. It is composed mainly of a mineral phase, which constitutes between 60 and 70 wt% of its total mass, and water between 5 and 10 wt%, while the remaining part is an organic matrix of collagen and other proteins [46]. Collagen and agglomerated HA mineral crystals intertwine to form several hundred nanometers of mineralized fibrils [47].

Bone is the second most widely transplanted tissue around the world, and more than four million operations are performed each year by using natural or synthetic bone grafts to treat bone defects [48]. The main solutions to facilitate bone repair are still the autograft and allograft techniques, even though autografting is expensive,

invasive, and subject to infections and hematoma, frequently affecting both donor sites and surgical sites, and allografts may cause an immune reaction followed by a rejection [49]. The role of biomaterials and tissue-engineered constructs has become more important in the last several decades. Among the most challenging and one of the most studied tissues to reconstruct is bone tissue. The design of bone tissue engineering scaffold should comprise a three-dimensional structure with interconnected pore network for cell growth and transport of nutrients/waste. The scaffold should certainly be biocompatible, and its surface chemistry should be suitable for cell attachment, proliferation, and differentiation. Finally, the absorbable scaffold should degrade at a rate that matches the tissue regeneration while keeping its mechanical properties very close to that of the host tissue [50].

Several techniques previously used in bone tissue engineering to obtain an ideal cell-laden scaffold did not yield significant successes due to many limiting factors, majorly vascularity [51]. Another substantial challenge is the critical size of the construct while meeting the clinical requirements of structural support, osteoinductive property, and controllable biodegradability [52].

The main focus of bioprinting for bone tissue engineering is the advancement in printing methods and the development of compatible ink materials. Various materials have been developed for use in 3D printing to create new alternatives to conventional bone grafts. The material groups, such as polymers, ceramics, and hydrogels, cannot fully mimic the properties of bone when used alone. For example, in order to increase the bioactivity, hydrogels are generally combined with osteoconductive elements such as calcium phosphates, tricalcium phosphate (TCP), biphasic calcium phosphate (BCP), hydroxyapatite (HAp), silicate, or silica and bioactive glass nanoparticles. Also soluble molecules such as bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF) are used to induce angiogenesis and osteogenesis [51]. The simultaneous integration of these temperature-sensitive biological agents, living cells, and the bioink materials to create a 3D artificial bone implant or a complex bone tissue construct with controlled cell distribution, geometry, and biodegradability, good mechanical properties, and high osteoinductivity and osteoconductivity is today’s most important challenge in 3D bioprinting. Table 2 reviews the studies investigating these possible bioink formulations for specific fabrication methods and their osteogenic, osteochondral, or vasculogenic/angiogenic potential.

Compared to 3D printing of constructs, which are added in the post-printing, cell-laden 3D bioprinting, as mentioned above, involves more complex factors, such as narrow set of printing materials, the strategy of gelling, cell viability, and some technical issues [62]. On the other hand, cell-free inks, or, in more accurate words, 3D printed biomaterials, offer a wide range of possible scaffolding materials and more unconstrained operating parameters, such as high temperature. However, controlling the cell distribution and seeding density on prefabricated 3D scaffolds is a very hard constraint, and the results are generally poor formations of extracellular matrix (ECM) microenvironment.

The advantage of using various 3D printable materials to construct cell-free scaffolds also makes this approach attractive to bone tissue engineering applications.

Table 2 Bioprinted cells and bioinks for bone tissue engineering

Bioinks[a, b]	Cells	Fabrication method	3D printer	Main outcomes about osteogenic potential[b]
Alginate hydrogel (3% w/v in PBS); alginate-HAp (20 mg/mL) + $CaCl_2$ (2% v/v as cross-linker); chitosan hydrogel (2% w/v in acetic acid); and chitosan-HAp (20 mg/mL) [53]	Mouse calvaria pre-osteoblast cell line (MC3T3-E1)	Syringe extrusion	Fab@Home Model 3, Seraph Robotics, USA	• Chitosan was superior to alginate • The deposition of 48 mg Ca ion per 1 g of chitosan – HAp hydrogel was detected on 21st day of culture
VEGF-conjugated (5% w/v) GelMA with low methacryloyl substitution (in core) and VEGF-conjugated (10% w/v) GelMA with high methacryloyl substitution and loaded with silicate nanoplatelets (around) [54]	Human umbilical vein endothelial cells (HUVECs), human mesenchymal stem cells (hMSCs)	Pneumatic-based syringe extrusion	NovoGen MMX Bioprinter, Organovo, USA	• The encapsulated hMSCs formed a mature bone niche after 21 days of culture under the medium perfused condition • Medium perfusion resulted in significant increases in gene expression levels of osteogenic differentiation markers with respect to nonperfused construct
Alginate (50 mg/mL)-gelatin (50 mg/mL) hydrogel + $CaCl_2$ (0.4% w/v as cross-linking solution) cell-laden bioink and agarose hydrogel (0.3% in growth medium) with Ca^{2+}-polyP complex (100 μM) as overlay layer [55]	Human primary osteogenic sarcoma (SaOS-2) cells	Pneumatic syringe extrusion	3D-Bioplotter, EnvisionTEC, Germany	• Ca^{2+}-polyP complex caused the cells to proliferate faster with an average generation time of approximately 47–55 h during the 6 days incubation period • After 7 days of culturing, the cells exposed to the osteogenic medium and Ca^{2+}-polyP complex exhibited significantly higher mineralization with respect to the cells that were not exposed to Ca^{2+}-polyP complex but treated with the osteogenic medium
GMs of two different methacrylation degree (7 wt% and 5 wt%)-HAM (1 wt%)-HAp (5 wt%) in PBS + LAP (0.135 wt% of polymer mass as photoinitiator) [56]	Primary human adipose-derived stem cells (hASCs)	Volumetric dispensing (extrusion)	TR300 tabletop robot, Unitechnologies SA, Switzerland	• The changes in rheological characteristics are attributed to extensive matrix production in the hydrogels by the encapsulated cells • The expression of bone matrix

				components, such as collagen I, fibronectin ALP, and osteopontin, was shown to increase with the presence of HAp but even more with the osteogenic media supplements after 28 days of culture
PEGDA (20% w/v)-Laponite XLG nanoclay (3, 5, 7, 10% w/v) + Irgacure 1173 (3 wt% relative to the mass of PEGDA cross-linker as photoinitiator); HA (20% w/v) in PBS [57]	Primary rat osteoblasts (ROBs)	Two-channel pneumatic extrusion	BioScaffolder 3.1, GeSiM, Germany	• 7% nanoclay was selected as the optimal concentration to add to the 20% PEGDA aqueous solutions • The viability of the printed cells was higher than 95% • The release of Mg^{2+} and Si^{4+} bioactive ions from the PEG-clay scaffolds stimulated the osteogenic differentiation of ROBs • ROB-laden PEG-clay constructs exhibited excellent osteogenic capability both in vitro and in vivo
Alginate (3 w/v % in PBS)-MC powder (9 w/v %) [58]	Immortalized human mesenchymal stem cell line expressing human telomerase reverse transcriptase (hTERT-MSC)	Pneumatic extrusion	BioScaffolder 3.1, GeSiM, Germany	• Calcium phosphate cement and bioink biphasic constructs were printed • Initial and local decrease in cell viability was detected at the interface of calcium phosphate cements, and cells started to migrate from the alginate-MC bioink to the calcium phosphate cement strands between day 7 and day 21
Type B gelatin (30%), alginate (5%), and Ca-free DMEM [59]	Wharton's jelly mesenchymal stem cells (WJMSCs), human umbilical vein endothelial cells (HUVECs)	Pneumatic syringe and melt extrusion	BioScaffolder 3.1, GeSiM, Germany	• The HUVEC-laden bioink and molten PDACS/PCL were dispensed alternately in each layer of structure, and WJMSCs were ejected by a piezoelectric nozzle on PDACS/PCL layers • Expressed ion levels of both angiogenic markers (vWF and Ang-1) of HUVECs increased at day 7

(continued)

Table 2 (continued)

Bioinks[a, b]	Cells	Fabrication method	3D printer	Main outcomes about osteogenic potential[b]
				• ALP, BSP, and OC protein expression levels of WJMSCs were highest on the PDASC/PCL with WJMSC and alginate-gelatin hydrogels with HUVEC group for 14 days
Matrigel (9 mg/mL in PBS) [60]	Human adipose stem cells (ASCs)	Pneumatic syringe extrusion	Custom-modified Prusa I3 A Pro with two additional syringes, Geeetech, China	• ASCs suspended in Matrigel bioink were printed with PCL/bioactive borate glass composite in chloroform • The live/dead assay showed 58 ± 11% viable ASCs on the scaffold after 1 week of incubation
Alginate-PVA-HAp (2.5% w/v)-collagen (5.0–6.5% w/v) + $CaCl_2$ (0.5 and 1.0% as cross-linker) [61]	Mouse calvaria pre-osteoblast cell line (MC3T3-E1)	Ball screw-driven extrusion	System 30 3D printer with a modified EMO-25 extruder, HyRel 3D, USA	• The constructs were unable to promote cell proliferation over 10 days • Further in vitro differentiation and in vivo studies are needed

[a]Different cell-laden bioink formulations are separated with semicolons (;)

[b]*PBS* phosphate-buffered saline, *HAp* hydroxyapatite, *VEGF* vascular endothelial growth factor, *GelMA/GM* gelatin methacryloyl/gelatin methacrylate, *polyP* poly(phosphate), *PVA* poly(vinyl alcohol), *HAM* methacrylated hyaluronic acid, *LAP* lithium phenyl-2,4,6-trimethylbenzoylphosphinate, *ALP* alkaline phosphatase, *PEGDA* poly(ethylene glycol diacrylate), *Laponite XLG* ($[Mg_{5.34}Li_{0.66}Si_8O_{20}(OH)_4]Na_{0.66}$, *HA* hyaluronic acid sodium salt, *MC* methylcellulose, *DMEM* Dulbecco's modified eagle medium, *PDACS* polydopamine-modified calcium silicate, *BSP* bone sialoprotein, *OC* osteocalcin, *β-TCP* β-tricalcium phosphate

There are various polymers that were 3D printed with CaPs or other biomaterials to form bone tissue engineering scaffolds. For example, poly(ε-caprolactone) (PCL) and (1) Ca^{2+}-poly(phosphate) microparticles (at a ratio of 2:1 w/w) [63] and (2) silanated silica particles (5, 10, 20 wt%) [64] were printed by pneumatic melt extrusion. Poly(lactic acid) (10 wt%) was previously printed with β-TCP (5 wt%) with four different hydrogels by syringe extrusion method [65]. Natural polymers, such as chitosan, are also of interest of bone tissue engineering area, for example, nano-sized HAp-chitosan-silica (15/50/35 molar ratio, respectively) and chitosan-silica (50/35 molar ratio, respectively) were previously printed by using pneumatic syringe extrusion [66]. Several forms of coating were also applied on 3D printed bone scaffolds. HAp/β-TCP (at a mass ratio of 60/40) (67% w/w) and 20 wt% Pluronic F-127 solution (33% w/w) was previously used as 3D printed biomaterial, and RGD (Arg-Gly-Asp)-phage nanofibers (10^{14} pfu/mL) and chitosan (1 mg/mL) were used as coating material [67].

4.2 Osteochondral and Cartilage

Osteochondral tissue is composed of hyaline cartilage and subchondral bone and is found at synovial fluid joints [68]. The main responsibility of hyaline cartilage is to decrease surface friction and act as a shock absorber on the joint during daily activity, while the subchondral bone tolerates load and supports hyaline cartilage [68]. Osteochondral tissue can be degenerated through tumor, aging, or disease that can result in poor life quality. The vascular structure of articular cartilage has limited self-reparability, so the presence of defect in articular cartilage can progress to the underlying subchondral bone creating osteochondral defects.

Osteochondral tissue has a hierarchical and organizational structure which is a big challenge in designing of scaffold for osteochondral defects [69]. Articular cartilage is composed of four different zones which, respectively, are the calcified, deep, middle, and superficial zones [70]. These zones have different extracellular matrix composition, collagen orientation, and chondrocyte phenotype [70]. The orientation of collagen fibers is changed in these zones as follows: (a) parallel alignment to the articular surface (superficial zone), (b) random alignment in the middle zone, and (c) perpendicular form to the articular surface (deep zone); the calcified zone has collagen X [70]. The orientation of collagen fibers has a key role in mechanical properties of articular cartilage.

The surgical treatments are usually required in osteochondral defects [68]. There are clinical treatments such as debridement, microfracture, autologous chondrocyte implantation, matrix-induced autologous chondrocyte implantation, and osteochondral autografting and allografting, but these are effective in early stages and only postpone tissue degeneration. All of these are complicated and costly treatments and did not result in proper biomechanical restoration [71].

Table 3 The list of commercial tissue-engineered cartilage scaffolds

Product name	Company	Biomaterial	Cell
BioCart™II	Histogenics, Waltham, MA	Fibrinogen/ hyaluronic acid	Chondrocytes
BioSeed®-C	BioTissue Technologies GmbH, Freiburg, Germany	Polyglactin 910/poly-p-dioxanone fleece	Fibrin and expanded autologous chondrocytes
Cartipatch®	Tissue Bank of France, Lyon, France	Agarose-alginate hydrogel	Chondrocytes
Chondrosphere®	Co.don AG, Teltow, Germany	Spheroids of neocartilage	Chondrocytes
Hyalograft® C	Anika therapeutics, Bedford, MA	Hyaluronic acid	Chondrocytes
Matrix-induced autologous chondrocyte implantation	MACI, Vericel, Cambridge, MA	Collagen II/III	Chondrocytes
NeoCart®	Histogenics, Waltham, MA	Bovine collagen I	Chondrocytes
NOVOCART® 3D	TETEC, Melsungen, Germany	Biphasic collagen I	Chondrocytes

There are different tissue-engineered cartilage products which are under clinical trials to overcome limitations of current treatments. Table 3 reviews the list of cartilage products [71].

Recently, there are significant research in tissue engineering approach for osteochondral defect treatment and cartilage scaffold design [68]. The architecture of scaffold plays an important role in vascularization, mechanical properties, cell proliferation, and infiltration. The 3D printing technology is widely used for printing of osteochondral and cartilage scaffolds due to its ability to create complex structures and control pore size (Table 4).

4.3 Nerve

In the treatment of peripheral nerve and spine injuries, neural conduits have been proposed as an effective neural regeneration matrix that support and guide neural cells using tissue engineering approaches. Neural tissue harbors neurons and glial cells (microglia, astrocytes, oligodendrocytes), while the vascular component consists of endothelial cells, pericytes, and vascular smooth muscle cells [83]. Bioprinting offers a relatively recent approach for engineering of controllable 3D scaffolds for neural tissues with diverse cell types and complex microscale features [28]. The major points for the design of nerve guide conduits are (1) mechanical support while aligning the proximal and distal nerve ends and prevent nerve compression, (2) permeability to nutrients and waste products through the conduit

Table 4 Bioprinted biomaterials and cells for osteochondral and cartilage

Biomaterial	Cells	Fabrication method	3D printer	Main outcome
15% methacrylated gelatin (GelMA) hydrogel for cartilage on top layer, a combination of 20% GelMA and 3% nanohydroxyapatite (nHA) (20/3% GelMA/nHA) hydrogel for interfacial layer, a 30/3% GelMA/nHA hydrogel for subchondral bone at bottom layer [68]	Bone marrow mesenchymal stem cells	Pneumatic dispensing	Customized, pneumatic dispensing system	The in vivo results showed that GelMa/nHA-based tri-layered scaffold is useful in repairing of hyaline cartilage and subchondral bone
$L_2C_4S_4$ apatite (1.8 g) + sodium alginate (0.1 g) + Pluronic F-127 (1.8 g) [72]	Rabbit chondrocytes, rabbit bone marrow stem cells	Three-axis positioning system	3D scaffold printer	The $L_2C_4S_4$ extracts promote osteogenic differentiation and bioactivity
HA + polycaprolactone (in different ratios) [73]	–	Extrusion printing	3D-Bioplotter; EnvisionTEC, Gladbeck, Germany	The porosity is more effective than addition of HA in terms of mechanical strain
PLGA + 1% ß-TCP 3D printed as a subchondral part and articular cartilage part was prepared from fresh bovine limb by freeze-drying [74]	Mesenchymal stem cell from the femoral marrow cavity	Pneumatic printing	3D biological printer	The advantages of this system include high cell utility efficiency, easy production, and precise tissue repair, which could provide a promising alternative to current approaches to repair joints defects
PLLA filament used for 3D printing + 6% gelatin membrane prepared by electrospinning + Osteogenon drug [75]	Murine fibroblasts L929 (ATCC, USA)	Fused deposition modeling	–	Gelatin concentration has significant effect on mechanical strength and also 1.5% of Osteogenon promote mechanical properties of scaffold
PCL + 20% GelMa + 1:1 GelMa/cell suspension [76]	Mesenchymal stem cell + chondrocyte	Microextrusion and inkjet printing	3D discovery, RegenHU, Switzerland	The reinforced polymeric framework by printing of spheroids and the collagen natural structure was mimicked

(continued)

Table 4 (continued)

Biomaterial	Cells	Fabrication method	3D printer	Main outcome
LCS ($Li_2Ca_2Si_2O_7$) powders, sodium alginate + LCS powder (5:100) added to 20% F-127 solution [77]	Rabbit chondrocytes	Pneumatic printing	–	The in vitro studies proved that release of Li and Si augmented osteogenic differentiation of rabbit chondrocytes
HAMA-GelMa + photoinitiator as a shell bioink HAMA-GelMa + cells as a core bioink [78]	Allogeneic adipose-derived mesenchymal stem cells	Core-shell extrusion printing	BioPen (custom-made)	The pilot study proved that real-time 3D printing promotes cartilage regeneration The shell thickness should be enough to protect cells from UV light
Nanocellulose + alginate [79]	IPSC	Microextrusion printing	3D discovery, RegenHU, Switzerland	The nanocellulose+ alginate bioink is suitable for cartilage tissue; also cell density in bioink plays important role in cartilage regeneration
Gelatin methacrylate (GelMa) [80]	–	Pneumatic printing	3D-Bioplotter; EnvisionTEC, Germany	The architecture of scaffold has an important role in mechanical properties
Nanocellulose/alginate bioink + cells (10:1) [81]	Human nasal chondrocytes	–	–	The new cell/bioink mixing system (passive mixing) was used and proved that this system increase cell viability in comparison with other conventional mixing
10% GelMA + different concentrations of polyethylene glycol diacrylate + photoinitiator + TGFβ1 [82]	Human bone marrow mesenchymal stem cell	Stereolithography-based 3D printing	Custom-made	The stereolithography-based 3D printing system promotes cell viability and protects growth factors after printing

wall, (3) low immunogenicity, and (4) biodegradability to eliminate the need for secondary surgery [84].

Among the printing techniques used in neural conduit construction, extrusion printing has been used frequently as gels can be deposited conveniently with high speed. However, laser-based bioprinting, such as stereolithography and inkjet printing, allows better print resolution, which is a significant advantage in mimicking anisotropic architecture of nerve tissue. The selection of bioink has a tremendous effect on neural regeneration as neural cells are relatively more sensitive to their extracellular milieu; hence neural tissue applications should mimic the natural ECM of the target tissue [85]. Table 5 summarizes recent studies that reported application of bioinks in neural conduit bioprinting.

Alginate, a seaweed polysaccharide, can form stable hydrogels upon ionic cross-link with bivalent ions like Ca^{+2} and Mg^{+2}. In a recent study, Naghieh et al. constructed alginate scaffolds at low concentration to enable effective neural network formation by using indirect bioprinting technique [86]. After a sacrificial gelatin framework was printed by extrusion-based device, a low-concentration alginate incorporating primary rat Schwann cells (PRSCs) was casted into the framework. Later, the gelatin framework was removed, and low-concentration alginate scaffold was exposed. However, although low alginate provided favorable conditions for cells, it was not mechanically stable, and cell viability decreased with time.

Recent studies showed that the cell compatibility of alginate bioink would be increased by using cell adhesion factors. In one of such studies, Sarker et al. tested the potential of RGD or YIGSR peptide in increasing cytocompatibility of alginate in bioprinting application [87]. An extrusion-based system was used to bioprint RGD, YIGSR, or both peptide-conjugated sodium alginate with Schwann cells into 3D scaffolds. After 9 days of culture, the printed hydrogel constructs contained more cells and supported long-term cell proliferation compared to 2D culture conditions (petri dish) with respect to control alginate construct. Furthermore, the conjugation of both RGD and YIGSR peptides in the same alginate molecules increased the proliferation of neural cells in bioprinted constructs significantly. To increase alginate biocompatibility, fibrin, hyaluronic acid, and RGD peptide were mixed [88]. A 3D plotter was used to construct a grid-type scaffold by extruding the blended biopolymer solution mixed with Schwann cells, which was later stabilized with a cross-linking solution containing $CaCl_2$ and thrombin that cross-link alginate and fibroin, respectively. As indicated by in vitro techniques, cells were able to sustain viability and proliferation in the scaffolds effectively. Similarly, the composite technique was used by Li et al. to increase the cell compatibility of alginate by using gelatin [89]. With the use of a bioplotter, Schwann cells were co-extruded in alginate-gelatin into a grid-type scaffold structure. In vitro culture of the printed hydrogel scaffolds showed that more cells were present in the scaffolds and the composite gel support long-term cell proliferation compared to 2D culture (petri dish) conditions.

Hyaluronate, a highly hydrophilic polysaccharide, is also known to not support cells like alginate. England et al. suggested the use of fibrin-factor XIII in

Table 5 The bioinks and bioprinting methods used in neural conduit construction

Bioink	Fabrication method	Neural regeneration outcome
Alginate [86]	An indirect bioprinting process of alginate with primary rat Schwann cells (PRSCs)	Decreased PRSCs viability with culture time. The scaffold with low alginate concentration showed mechanical unstability during culture
Alginate conjugated with RGD or YIGSR peptide [87]	3D gel extrusion of alginate peptides with Schwann cells	Cell viability was over 90% after 1 week for RGD and YIGSR-gel composites, while lower viability was observed without peptides
Composite hydrogels of alginate, fibrin, hyaluronic acid, and/or RGD peptide [88]	Extrusion of gel with Schwann cell using 3D-Bioplotter	Cells were able to sustain viability and proliferate in the scaffolds
Composite alginate-gelatin hydrogel [89]	Rat Schwann cell in composite gel was printed with an extrusion-based bioprinter	The printed hydrogel constructs contained more cells and support long-term cell proliferation (after day 9)
Fibrin-factor XIII-hyaluronate hydrogel [90]	3D gel extrusion with a bioplotter	Schwann cells encapsulated within these scaffolds during fabrication were viable and proliferated in culture. Extrusion induced longitudinal alignment of fine fibrin fibers which in turn enabled the alignment of encapsulated Schwann cells and dorsal root ganglion neurites along the 3D printed strands
(a) Gelatin methacrylate (GelMA) and gelatin mixed with fibrin (GEL/FIB) hydrogels (b) Commercial extracellular matrix (Matrigel) [91]	Extrusion-based multi-material 3D bioprinting with iPSC-derived neural progenitor cells for bioengineering of spinal cord	(a) The printed cells did not proliferate, and axon propagation was not observed in the matrix (b) When printed in 50% Matrigel concentration as the cell-laden bioink, the overall cell viability over 4 days in culture was >75% for both iPSC-derived sNPCs and oligodendrocyte progenitor cells (OPCs)
Water-based biodegradable polyurethane dispersions which may later form gel at mild conditions [92]	3D dispersion extrusion with fused deposition manufacturing equipment	Murine neural stem cells (NSC) which was extruded in hydrogels would proliferate and differentiate. NSC-laden hydrogels could reestablish the function of impaired nervous system in the zebrafish embryo

(continued)

Table 5 (continued)

Bioink	Fabrication method	Neural regeneration outcome
GelMA, PEGDA [93]	Engineered nerve guidance conduit using digital light processing (DLP)-based rapid continuous 3D-printing platform	Complete sciatic nerve transections of mouse models demonstrated directional guidance of regenerating sciatic nerves via branching into the microchannels and extended toward the distal end of the injury site

hyaluronate gel precursor for the extrusion of Schwann cells [90]. A bioplotter extruded hyaluronan-fibrin with Schwann cells into a 3D structure by stacking of longitudinal strands. Schwann cells encapsulated within these scaffolds during fabrication were seen viable and proliferated in culture. Interestingly, extrusion-induced longitudinal alignment of fine fibrin fibers in gel enabled the alignment of encapsulated Schwann cells and dorsal root ganglion neurites along the 3D printed strands.

GelMA gels, on the other hand, were proven to be not effective in keeping the viability and proliferation of induced pluripotent stem cell-derived neural progenitor cells, which are known to depend on extracellular matrix [91]. Either GelMA or gelatin blended with fibrin gels was bioprinted via extrusion-based plotter for construction of scaffold, intended for spinal cord regeneration. In vitro tests showed that the printed cells did not proliferate, and axon propagation was not observed in the matrix. Therefore, in the same study, Matrigel, which is composed of growth factors and proteins that mimic the basement membrane and extracellular matrix from mouse sarcoma cells, was used in bioprinting of neural progenitors [91]. The results showed that after extrusion plotting of cell-laden bioink, which was prepared at 50% Matrigel concentration, the overall cell viability over 4 days culture was >75% for both iPSC-derived sNPCs and oligodendrocyte progenitor cells (OPCs).

Water-based biodegradable polyurethane dispersions were used in bioprinting of murine neural stem cells (NSCs) which may later form gel at mild conditions [92]. NSCs were embedded into the polyurethane nanoparticle dispersions before gelation and extruded by using fused deposition manufacturing equipment. In vitro results reported that NSCs could proliferate and differentiate and hence showed the mild effect of bioprinting of cells with PU bioink. In addition, NSC-laden hydrogels that were injected into the zebrafish embryo neural injury model could reestablish the function of impaired nervous system.

Hydrogels based on collagen and gelatin have been proposed as an efficient bioink for neural regeneration. Due to insolubility of collagen at neutral pH, gelatin, which is a shorter-chain, denatured collagen product, is preferred candidate with high water solubility at physiologic pH. Gelatin itself has a random coil structure in solution and forms a helix below 35°C where helix-chain aggregation causes gelation [94]. Especially, the acrylated gelatin, gelatin methacryloyl (GelMA), has found particular interest as viable cells could be encapsulated via

photopolymerization during bioprinting process. Tao et al. attempted bioprinting of GelMA with poly(ethylene glycol)-poly(3-caprolactone) (MPEG-PCH) nanoparticles which encapsulated Hippo pathway inhibitor that can block MST1/2 kinase activity [95]. The bioprinted nerve conduit-drug construct induced higher rate of recovery of sciatic injuries with respect to morphology, histopathology, and functions in in vivo rat sciatic nerve model. In a similar approach, dopamine (DA) was conjugated to GelMA molecules and used in bioprinting of a scaffold for culturing of neural stem cells (NSCs) [96]. A custom-made stereolithography was used in bioprinting of GelMA-DA, and UV laser beam was effective in gelation at 25 μJ intensity. The observation of a significant neural network on the 3D printed GelMA-DA scaffolds after 12 days of culture indicated a stimulatory effect of conjugated dopamine on neural progenitor cell when compared to unconjugated GelMA scaffolds. In another study, GelMA was blended with poly(ethylene glycol diacrylate) (PEGDA) for the engineering of nerve conduit [93]. A digital light processing (DLP) apparatus, which could generate the desired patterns, was adapted to the laser beam processing in a rapid continuous 3D-printing platform for engineering of nerve guidance conduit. The implantation of the conduits with microchannels and sleeve design into mouse models of complete sciatic nerve transections demonstrated directional guidance of regenerating sciatic nerves via branching into the microchannels and extension toward the distal end of the injury site.

4.4 Skin

The skin being the outermost organ is susceptible to injuries, infections, and environmental effects. Skin tissue is composed of the upper epidermis and inner dermal layers with their respective cells of keratinocytes and fibroblasts. Tissue engineering of skin grafts is a lifesaving approach for patients having major injuries, burns, and skin diseases. The membrane preparation techniques, such as electrospinning, freeze-drying, and solvent casting, have long been used for skin graft constructions conveniently. Bioprinting of the skin attracted ever-growing interest due to its sophisticated and controlled production properties which would be rather difficult with conventional skin graft production methods. Vijayavenkataraman et al. pointed out commercial initiatives and collaborations among cosmetic companies (L'Oréal USA, NovoGen, Procter & Gamble) and bioprinter producers (Organovo, Rokit) mainly aimed at fulfilling the huge demand in production of skin products to test cosmetic products [97]. The potentials of 3D bioprinting technique for skin tissue engineering have been investigated recently by several researchers (see Table 6). Bioprinting facilitates the specific deposition of multiple types of skin cells simultaneously [105]. In addition, 3D bioprinting enables the precise localization of multiple cell types and appendages within a construct [106]. However, there are challenges that limit skin bioprinting, such as the technical difficulties associated with nozzle blockage and shearing stresses on the cells

Table 6 Bioprinted skin scaffolds with various bioinks and cell sources

Bioink	Fabrication method	Skin regeneration outcome
Sodium alginate [98]	Extrusion by 3D plotting	Bioprinted patches were produced according to patient's wound size and contour
Type I collagen [99]	Pneumatic extrusion by a 3D plotter	The bioprinted skin structure showed the dermal and epidermal layers as well as the terminal differentiation of the KC that formed the stratum corneum. MC-containing epidermal layer showed freckle-like pigmentations at the dermal-epidermal junction
Collagen [100]	The 3D bioprinted constructs were fabricated using a bioplotter with multiple microvalve-based printheads in two separate steps: 1. Bioprinting of collagen fibroblast matrices 2. Keratinocytes and melanocytes were directly printed onto the bioprinted collagen fibroblast matrices	Skin constructs had a higher degree of resemblance to native skin tissue in terms of the presence of well-developed stratified epidermal layers and the presence of a continuous layer of basement membrane proteins as compared to the manually cast samples
Bovine fibrinogen-thrombin [101]	3D cell spraying with a bioplotter	Bioprinted layered human dermal fibroblasts and epidermal keratinocytes in a hydrogel showed rapid wound closure, reduced contraction, and accelerated reepithelialization in nude mice model
Adipose-derived ECM mixed with bovine fibrinogen mixture for hypodermal compartment. Skin-derived ECM (dECM) and fibrinogen mixture for dermal compartment. Gelatin for vascular channels [102]	The simultaneous inkjet and extrusion 3D printing	Perfusable channels could supply nutrients throughout the dECM-based construct. Printed HUVEC could cover the surface of channel, forming endothelium
Cells embedded in collagen gel or a mixture of blood plasma and alginate [103]	Cell lines of fibroblasts and keratinocytes embedded in collagen were printed in 3D as a simple example for skin tissue. On the principle of laser-induced forward transfer	Printed fibroblasts and keratinocytes proliferated and were vital. Extensive formation of intercellular adherens junctions between keratinocytes and their minor formation between fibroblasts, which proves the tissue formation, were observed

(continued)

Table 6 (continued)

Bioink	Fabrication method	Skin regeneration outcome
Amniotic fluid-derived stem (AFS) cells and mesenchymal stem cells (MSCs) were separately suspended in the fibrinogen/collagen solution [104]	Pneumatic-driven and extrusion-based 3D hydrogel solutions, with or without cells	Bioprinted constructs of collagen/fibrin gel with AFS or MSC cells had faster wound closure rate than the gels without cells in vivo. High vessel formation per unit area was detected for both kinds of cells as compared to constructs without cells in vivo

[107]. Patient-specific construction of scaffolds is one of the most important advantages of 3D printing technology. There is one recent study that investigated segmenting chronic wounds and transmitting the coordinates to a bioprinter robot to facilitate the treatment of chronic wounds [98]. As a result, semiautomatic segmentation of wound images and image processing methods improved the control of bioprinting process through more accurate coordinates.

For increased vascularization and efficient epidermal growth, a perfusable, vascularized full-thickness skin equivalent composed of epidermis, dermis, and hypodermis was investigated by Kim et al. [102]. It was observed that the printed HUVECs were covered on the surface of vascular channel, forming endothelium resembling vascular structures. The results showed that keratin 10 and filaggrin were expressed at the early and late stages of differentiation of the epidermis. Within the dermal compartment, laminin and dermal ECMs were expressed at the boundary between the dermis and epidermis similar to natural process.

In a different approach, Koch et al. deposited 20 layers of fibroblasts (mouse NIH-3T3) and 20 layers of keratinocytes (human HaCaT) embedded in collagen gel onto a sheet of MatriDerm® (decellularized dermal matrix) by laser-assisted bioprinting for constructing dermis and epidermis layers, respectively [103]. The printed constructs could show the presence of intercellular junctions, such as cadherins and connexin 43 (Cx43), in the epidermis after 10 days of cultivation in culture.

In addition, bioprinting of pigmented skin was tested to obtain a better resemblance to native skin [100]. Primary human keratinocytes, melanocytes, and fibroblasts were used in bioprinting process, while a two-step drop-on-demand bioprinting strategy was used to deposit the cell droplets to form the melanin units of epidermal layer. As a result, 3D bioprinted skin constructs had a better resemblance to native skin as the printed tissue had well-developed stratified epidermal layers and a continuous layer of basement membrane proteins as compared to the manually cast samples.

Skin regeneration potential of amniotic fluid-derived stem (AFS) cells and bone marrow-derived mesenchymal stem cells (MSCs) was compared by suspending cells in fibrin-collagen gel and printing over the wound site [104]. A bioprinter with pneumatic-driven nozzles was used to deposit layers of fibrin-collagen and thrombin

gel solutions containing AFS cells or MSCs deposited over full-thickness wounds, which were created in nude mice models. In vivo tests showed that the constructs of collagen/fibrin gel with AFS cells accelerated closure of full-thickness wounds faster than the construct without cells, and they were as effective as the gels with MSC. In addition, the vessel formation per unit area, which is a significant indication of regeneration, was significantly higher with both kinds of cells than only-gel printed grafts as the histology staining revealed.

As a conclusion, the bioprinting technology has enabled controlled fabrication platform for construction of artificial skin, which potential has yet to be realized in skin tissue engineering [108]. However, the resolution, vascularity, optimal cell and scaffold combinations, and cost of bioprinted skin are some issues that should be overcome to harness the new technology [109]. Histological complexity of the skin, such as neural and immuno-components and hair follicles, should also be considered in bioprinting of the skin to obtain fully functional native skin [108, 110].

4.5 Cardiac and Skeletal Muscle

Irreversible loss of cardiomyocytes due to ischemic heart attack leads to lethal heart diseases and high mortality rates. Therefore, the regeneration of damaged cardiac muscle is the only way to restore heart functions. Cellular cardiomyoplasty is the implantation *of* in vitro cultured cardiomyocytes into the damaged myocardium, to facilitate regeneration [111]. However, when injected to the heart directly, low cell retention and viability of cardiomyocytes are encountered. Tissue engineering of heart muscle by using bioprinting is carrying significant potential as the scaffold matrix architecture would be customized while cardiac cells are placed in situ. In this part of the chapter, the skeletal tissue engineering by bioprinting approach is also discussed. Table 7 summarizes recent applications of bioprinting in cardiac and skeletal tissue engineering.

Adams et al. studied bioprinting of silicone rubber and poly(caprolactone) (PCL), which are known as printable and biocompatible materials, into scaffolds for cardiac patch construction [111]. A grid-type scaffold was 3D printed with pneumatic syringe extrusion from silicone rubber or PCL bioink. In vitro tests showed that PCL scaffolds had more efficacy as there was a higher percentage of adult primary human cardiomyocyte (pHCM) attachment and more cell migration, compared to the silicone rubber. In addition, an increase in cell density of cardiac cells was observed on the PCL scaffold after electrical stimulation.

Laser-induced forward transfer (LIFT) cell printing technique performs controlled transfer of inorganic and biological materials in conjunction with proteins, peptides, DNA, RNA, and cells in three-dimensional (3D) patterns with survival rates of printed cells at nearly 100% [112]. Poly(ester urethane urea) (PEUU) cardiac patch immersed in Matrigel was used to collect patterned cells from laser beam focused on the donor slide. Human mesenchymal stem cells (hMSCs) and endothelial cells (EC) were patterned in such a way that the rectangular islands of hMSC

Table 7 Bioprinted muscle scaffolds with various bioinks and cell sources

Bioink	Fabrication method	Muscle regeneration outcome
Silicone rubber and poly (caprolactone) (PCL) [111]	3D bioprinter with pneumatic syringe extrusion was used in the deposition of silicone rubber or PCL in a grid structure	PCL scaffolds showed more efficacy as there was a higher percentage of adult primary human cardiomyocyte (pHCM)) attachment and more cell migration, compared to the silicone rubber. Electrical stimulation increased cell density on the PCL scaffold
Poly(ester urethane urea) (PEUU) cardiac patch immersed in Matrigel was used to collect patterned cells [112]	Laser-induced forward transfer (LIFT) cell (human mesenchymal stem cells) printing technique	Increased vessel formation and found significant functional improvement of infarcted hearts following transplantation of a LIFT tissue-engineered cardiac patch in immune-deficient rat model
Carbon nanotubes (CNT)-incorporated alginate and methacrylated collagen (MeCol) [113]	A UV-integrated pneumatic 3D-Bioplotter system was employed to create hybrid cell-laden MeCol. Alginate was printed as the implant framework to contain cell-laden MeCol within its spaces in each printed layer	Human coronary artery endothelial cells in MeCol gel presented significant cellular proliferation, migration, and differentiation (lumen-like formation) over 10 days of incubation in vitro
Heart tissue-derived decellularized extracellular matrix (hdECM) bioink [114]	Human cardiac progenitor cells (hCPCs) or endothelial cells were extruded in a disk-shape structures (8 mm in diameter and 0.5 mm in thickness) by a bioplotter	MixC/M (with VEFGs and MSCs) and patternC/M (generated patches by patterning of CPCs and MSCs with VEGFs) greatly promoted vascularization compared with the CPC patch after 4 weeks implantation; the patterned patch exhibited enhanced cardiac functions, reduced cardiac hypertrophy and fibrosis, increased migration from patch to the infarct area, and neo-muscle and capillary formation in rat myocardial infarction model
Gelatin methacrylate (GelMA) [115]	Human embryonic stem cell-derived cardiomyocytes (hESC-CMs) were mixed with GelMA printed into a scaffold by using micro-continuous optical printing (μCOP)	A contractable cardiac tissue was obtained. hESC-CMs printed in isotropic slabs beat synchronously; however they contract without directional preference, whereas hESC-CMs encapsulated in the parallel line pattern contract in the direction of patterning

(continued)

Table 7 (continued)

Bioink	Fabrication method	Muscle regeneration outcome
No bioink was used [116]	Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), fibroblasts (FB), and endothelial cells (EC) were aggregated to create mixed cell spheroids. The 3D bioprinter picks up individual cardiospheres using vacuum suction and loads them onto a needle array	Patches exhibited ventricular-like action potential waveforms and uniform electrical conduction throughout the patch. In vivo implantations of assembled cardiac patch showed vascularization and engraftment of 3D bioprinted cardiac patches
Fibrin-based (containing gelatin and hyaluronic acid) bioink [117]	Primary cardiomyocytes suspended in a fibrin-based bioink and cell-laden hydrogel were sequentially printed with a sacrificial hydrogel and a supporting polymeric frame by using 3D pneumatic printing	Dense, uniformly aligned, and electromechanically coupled cardiac cells were observed after 3 weeks of culture
The decellularized skeletal muscle extracellular matrix (dECM) and vascular dECM (vdECM) bioinks [118]	Human skeletal muscle cells (hSKMs)-encapsulated bioink solution was 3D extruded into 3D scaffold by a plotter. Prevascularized muscle constructs were fabricated through coaxial nozzle printing with dECM and vdECM bioinks	Improved cell viability, myotube formation, and de novo myofiber regeneration in rat models of volumetric muscle loss (VML). Improved de novo muscle fiber formation and vascularization were observed in rats
PEG-fibrinogen and alginate [119]	Microfluidic printing head with coaxial needle was used to extrude muscle precursor cells (C2C12) into 3D constructs composed of aligned hydrogel fibers	An enhanced myogenic differentiation with the formation of parallel aligned, long-range, tightly packed, and completely striated myotubes

were surrounded by the EC lanes, mimicking vascular tissue model on a PEUU cardiac patch that was immersed in Matrigel. The therapeutic potential of the patch in cardiac regeneration was tested in immune-deficient Rowett nude rat left anterior descending ligation model with respect to cell survival, infarct wall thickness, angiogenesis, cardiac remodeling, and functional improvement. Increased vessel formation and significant functional improvement of infarcted hearts were observed in in vivo results.

Carbon nanotubes (CNT) were incorporated into alginate and methacrylated collagen (MeCol) bioink for building cardiac patch with electrical, mechanical attributes [113]. A UV-integrated pneumatic 3D-Bioplotter system was used to construct human coronary artery endothelial cells (HCAECs) encapsulated in MeCol. An indirect bioprinting technique was used for MeCol bioink. Alginate was printed as the implant framework in order to hold cell-laden MeCol within its spaces in each printed layer. As a result, HCAECs in MeCol gel presented significant

cellular proliferation, migration, and differentiation (lumen-like formation) over 10 days of incubation in in vitro cell culture.

Jang et al. tested heart tissue-derived decellularized extracellular matrix (hdECM) bioink to encapsulate and print human cardiac progenitor cells (hCPCs) [114]. Human cardiac progenitor cells (hCPCs) or endothelial cells were extruded in a disk-shape structures (8 mm in diameter and 0.5 mm in thickness) by using a bioplotter. Three patch types were constructed as: (1) only CPCs (CPC), (2) randomly mixed of both CPCs and human turbinate tissue-derived mesenchymal stem cells (MSCs) with vascular endothelial growth factors (VEGFs) (mixC/M), or (3) generated patches by patterning of CPCs and MSCs with VEGFs alternatively (patternC/M). Neovascularization and tissue formation potential of cell-laden constructs were studied in Balb/c nude mice model, while hdECM patch without cells was used at the epicardium of the rat myocardial infarct (MI) model to study the functional benefits of hdECM. The tests of vascularization and tissue formation of stem cell patch in vivo showed that mixC/M and patternC/M greatly promoted vascularization compared with the CPC patch after 4 weeks implantation. The mixC/M and patternC/M patches both seemed to induce a potent angiogenic response in the host tissues: blood vessels developed in the implanted patches and appeared to be functional vessels because red blood cells could be observed in the lumens. The cardiac functions in rat myocardial infarction model revealed that the patterned patch could exhibit enhanced cardiac functions, reduced cardiac hypertrophy and fibrosis, increased migration from patch to the infarct area, and neo-muscle and capillary formation.

In another study, gelatin methacrylate (GelMA) bioink was used in bioprinting of human embryonic stem cell-derived cardiomyocytes (hESC-CMs) [115]. With the aim of cardiac patch construction, hESC-CMs were mixed GelMA and bioprinted by using Micro-continuous optical printing (μCOP) platform. μCOP system consists of programmable digital masks to control millions of individual mirrors to pattern light onto a liquid volume. The digital patterns are casted on photopolymerizable polymer on a high-precision stage, which is computer controlled, to create 3D scaffolds. hESC-CMs were mixed with GelMA and polymerized into micropatterned (parallel lines) cardiac patch. After 3–7 days of cell culture, a contractable cardiac tissue was obtained. hESC-CMs printed in isotropic slabs beat synchronously; however they contract without directional preference, whereas hESC-CMs encapsulated in the parallel lines pattern contract in the direction of patterning.

In a different approach, a vacuum-assisted bioprinter device, which picks up individual cardiospheres using vacuum suction and loads them onto a needle array, could be used to construct a cardiac patch without using any bioink material [116]. Cardiospheres that were produced by cell aggregation of human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), fibroblast (FB), and endothelial cells (EC) were assembled into a cardiac patch by pickup function of dispenser needles of the bioprinter. Electrophysiological results indicated that patches exhibited ventricular-like action potential waveforms and uniform electrical conduction throughout the patch.

Wang et al. tested fibrin-based (containing gelatin and hyaluronic acid) bioink for primary cardiomyocytes (infant rat hearts) by co-printing with sacrificial gel and supportive frames [117]. Three dispensing modules for cell-laden hydrogel, sacrificial hydrogel, and PCL were operated separately. Primary cardiomyocytes suspended in a fibrin-based bioink and cell-laden hydrogel were bioprinted along with supporting frame (PCL) and a sacrificial hydrogel by using 3D pneumatic printing. Bioprinted cardiac tissue constructs had a spontaneous synchronous contraction in culture medium. Progressive cardiac tissue development was confirmed by immunostaining for actinin and a calcium influx spreading across the entire tissue after 1 week of culture. After 3 weeks, cardiac tissues were formed with uniformly aligned, dense, and electromechanically coupled cardiac cells.

In skeletal tissue engineering, Choi et al. used decellularized skeletal muscle extracellular matrix (dECM) and vascular dECM (vdECM) as bioinks for printing vascularized human skeletal muscle cells [118]. hSKMs were mixed in dECM while endothels (HUVECs) were mixed in vdECM bioinks for coaxial bioprinting of them into thick constructs by using extrusion-based 3D plotting system. The 3D muscle constructs showed enhanced cell viability, myotube formation, and de novo myofiber regeneration for rat models of volumetric muscle loss (VML). In vivo results showed that coaxial nozzle printing mimicked the hierarchical architecture of vascularized muscles, and allogenic human cells in the constructs improved de novo muscle fiber formation, vascularization, and innervation, as well as 85% of functional recovery could be observed in VML injury. In another muscle bioprinting trial, Constantini et al. used PEG-fibrinogen- and alginate-based bioinks by extrusion-based construction of aligned hydrogel fibers encapsulating muscle precursor cells (C2C12) [119]. With a unique approach, the printing head had a microfluidic device with a coaxial needle that can be used to extrude C2C12 cells into 3D constructs while producing aligned hydrogel fibers. By this method an enhanced myogenic differentiation with the formation of parallel aligned, long-range, tightly packed, and completely striated myotube structures could be achieved.

4.6 Other Soft Tissues

The 3D printing technique gives the chance to control the size and features at micro-/nanoscale for various types of soft tissues. The printing of complex tissues, such as liver and cornea, is possible, but many issues exist until today. In printing of multicellular tissues, cells and matrix materials should be printed together to mimic tissue micro-architecture [120, 121]. Therefore, many tissue scaffolds have been designed to mimic native tissue with complex 3D morphology. Table 8 shows some important researches that investigated those challenging soft tissues such as liver, cornea, and urinary bladder.

Table 8 Biomaterials and cells used for bioprinting of selected soft tissues

Tissue	Biomaterials	Cells	Fabrication method	3D printer	Main outcome
Liver [122]	GelMa + PEG-bis-chloroacetate + photoinitiator + cells	Human hepatic stellate cells, HepaRG cells	Cellbricks printing-stereolithography	–	It was proved liver organoid can be printed by using stereolithography technique and hydrogel as a bioink
Liver [123]	2% w/v alginate, 3% w/v gelatin, 0; 0.25; 0.5; 1, or 2 mg/mL w/v hECM, 0.03 M $CaSO_4$, and 7×10^6 mature HepaRG cells/mL	Human bipotent hepatic progenitor cells, human epithelial lung carcinoma cells	Microextrusion printer	Inkredible, Cellink, Gothenburg, Sweden	The desirable bioprinting was achieved in alginate-gelatin with 0.5 and 1 mg/mL hECM bioink
Urinary bladder [124]	Bone marrow-derived cell spheroids	Bone marrow-derived cells	–	Regenova, Cyfuse Biomedical K.K., Tokyo, Japan	The urinary bladder can be printed by using bone marrow stem cells, and vascularization is seen after transplantation to rabbit
Cornea [125]	Sodium alginate + methacrylated collagen	Human corneal stromal cells	Microextrusion printer	Inkredible, Cellink, Gothenburg, Sweden	The bioinks with low viscosity are suitable for 3D printing of cornea
Hepatic structures [126]	3% alginate + cells	Mouse embryonic fibroblasts (MEFs)	Pneumatic printing	3D bioprinting system, Korea Institute of Machinery and Materials, South Korea	The collagen is not printable alone, and alginate was added to modify its pH and printing temperature
Cornea [127]	Gelatin + lyophilized bovine AM	Human corneal endothelial cells (HCEC)	Extrusion printing	INVIVO, Rokit, Seoul, Korea	3D bioprinted HCEC-laden AM grafts being well engrafted and demonstrated significantly improved corneal thickness and edema compared to the control conditions

Human intestinal tissues [128]	Human intestinal myofibroblast (IMF) interstitium, human intestinal epithelial cells (HIEC)	Human primary intestinal epithelial cells, myofibroblast	Extrusion printing	Organovo, 3D NovoGen Bioprinter, San Diego, USA	Fully human 3D intestinal tissue model composed of primary cells with increased complexity and function compared with standard in vitro models can be designed
Photoreceptor-retinal tissue [129]	Human retinal pigmented epithelial cell line, human retinoblastoma cell line	Human retinal pigmented epithelial cell line, human retinoblastoma cell line	Microvalve-based bioprinting	RegenHU bioprinter, Switzerland	The microvalve-based bioprinting is efficient and accurate to build the in vitro tissue models with the potential to mimic natural tissue
Cornea [130]	Collagen I, ethylenediaminetetraacetic acid, human female AB blood plasma, human recombinant laminin-521, hyaluronic acid sodium salt	Limbal epithelial stem cells, human adipose-derived stem cells	Laser-assisted bioprinting	–	The laser-assisted bioprinting systems can be used in cell printing by optimizing laser source power

5 Conclusion

The significant factors which should be taken into account in designing of biological substitutes are pore size, mechanical integrity, architecture, and mass transportation. Thus, the success of biological substitute requires a deep knowledge about contribution of mechanical properties, biological factors, and material composition during regeneration period. The 3D print technology gives opportunity to accurately control the architecture of printing substitute which gives the ability of mimicking natural tissues. In addition, the bioink composition has a tremendous effect on success of bioprinting. The bioinks that mimic extracellular matrix of target tissue are a promising approach in bioprinting. Therefore, formulations of tissue-specific bioink for each type of tissue will become an effective strategy. 3D printing of complex and large-scale constructs with high spatial resolution is a major issue that needs to be investigated. Further inventions on these issues will unblock challenges in bioprinting of functional and complex tissues with high mechanical integrity.

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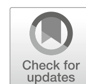
Gene Therapy



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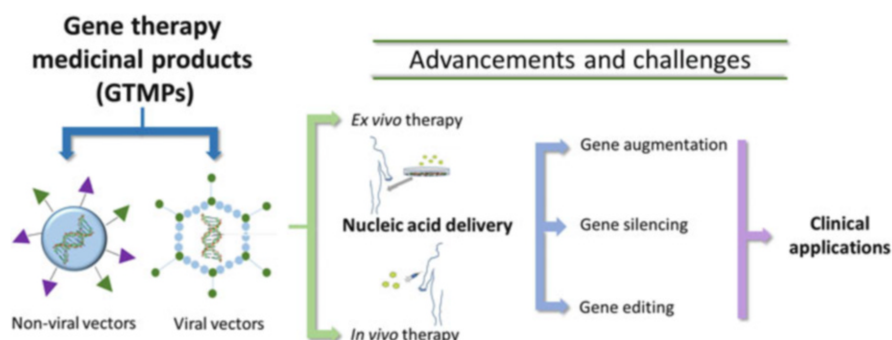
Abstract Gene therapy medicinal products (GTMPs) are one of the most promising biopharmaceuticals, which are beginning to show encouraging results. The broad clinical research activity has been addressed mainly to cancer, primarily to those cancers that do not respond well to conventional treatment. GTMPs to treat rare disorders caused by single-gene mutations have also made important advancements toward market availability, with eye and hematopoietic system diseases as the main applications.

Nucleic acid-marketed products are based on both in vivo and ex vivo strategies. Apart from DNA-based therapies, antisense oligonucleotides, small interfering RNA, and, recently, T-cell-based therapies have been also marketed. Moreover, the gene-editing tool CRISPR is boosting the development of new gene therapy-based medicines, and it is expected to have a substantial impact on the gene therapy biopharmaceutical market in the near future.

However, despite the important advancements of gene therapy, many challenges have still to be overcome, which are discussed in this book chapter. Issues such as efficacy and safety of the gene delivery systems and manufacturing capacity of biotechnological companies to produce viral vectors are usually considered, but problems related to cost and patient affordability must be also faced to ensure the success of this emerging therapy.

Graphical Abstract



Keywords Delivery vectors, Ex vivo, Gene therapy medicinal product, In vivo, Manufacturing, Quality control

1 Introduction

Biopharmaceuticals, pharmaceuticals produced in biotechnological processes by molecular biology methods, have become one of the most effective clinical treatments for a wide variety of diseases. The biopharmaceutical market includes

gene therapy products, based on the use of nucleic acids as active pharmaceutical ingredients for the modulation of the gene expression. Gene therapy based on the administration of DNA and messenger RNA (mRNA) acts by means of therapeutic protein expression, whereas the use of small interfering RNA (siRNA), microRNA, oligonucleotides, or aptamers provides posttranslational gene silencing. An emerging area in this field is genome editing, which corrects the disease by replacing a sequence of a defective gene by a healthy copy in order to restore the "wild-type" DNA. Most of those nucleic acids are produced as biopharmaceuticals, although some of them, such as antisense oligonucleotides, are made by chemistry means.

Despite that gene therapy entered clinical trials in the early 1990s, the first nucleic acid-based product registered in the European Union was Glybera in 2012, for lipoprotein lipase deficiency. Currently, only 15 gene therapy medicinal products have received approval worldwide; nevertheless, since 1989 almost 2,700 gene therapy-based clinical trials have been completed, are ongoing, or have been approved for a broad range of applications. Therefore, it is expected that nucleic acid-based products will have a substantial impact on the biopharmaceutical market in the near future.

2 Concept of Gene Therapy

Gene therapy is a novel approach to treat, cure, or ultimately prevent disease by changing the expression of a person's gene [1]. According to the European Medicines Agency (EMA), a gene therapy medicinal product generally consists of a vector or delivery formulation/system containing a genetic construct engineered to express a specific transgene ("therapeutic sequence") for the regulation, repair, replacement, addition, or deletion of a genetic sequence [2]. By far the most common vector systems used for gene therapy to date have been viral vectors and plasmid DNA vectors. Gene therapy works by repairing, deactivating, or replacing dysfunctional genes that cause disease with the aim of (re)establishing normal function.

Gene therapy has long been regarded a promising treatment for many diseases, including those inherited through a genetic disorder (such as hemophilia, human severe combined immunodeficiency, spinal muscular atrophy, or cystic fibrosis) or acquired (such as cancers of different kinds).

Depending on the cell type to be modified, gene therapy can be classified into two categories: somatic and germline gene therapy [3]. Somatic gene therapy targets to body somatic cells such as bone marrow or blood cells. This type of gene therapy cannot be passed on to descendants. In germline gene therapy, egg and sperm cells (germ cells) are the objective of therapy, and the inserted gene passes on to future generations. The idea of germline gene therapy is controversial due to ethical concerns, and it is not permitted in many countries. In spite of that, at the end of 2018, a research team in China announced the birth of two babies whose genomes had been edited, and there may be a third one [4]. These practices have been widely condemned as irresponsible and as failing to conform with international norms [5].

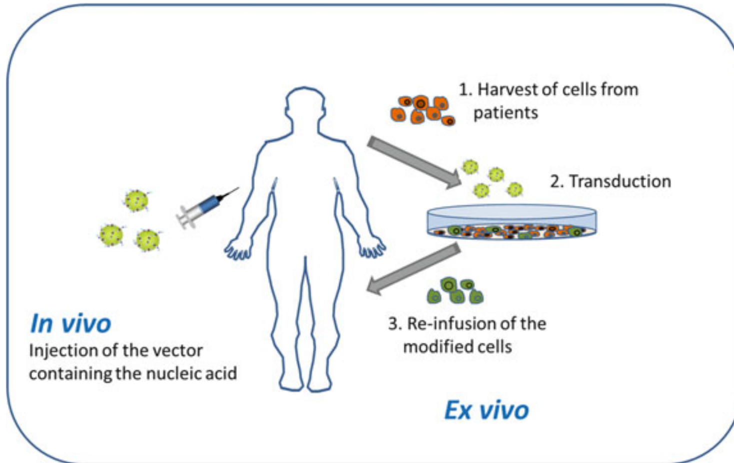


Fig. 1 Ex vivo and in vivo approaches to gene therapy

Two fundamental strategies have evolved to restore or modify target cell function: ex vivo or in vivo gene delivery [6]. In ex vivo therapy, cells from the patient or a donor are harvested, and the therapeutic gene is then transduced in a cell therapy manufacturing setting. The modified cells are later reinfused into the patient. In vivo gene therapy consists on functional modification of targets by direct transgene injection into the patient. Figure 1 features a scheme with the ex vivo and in vivo approaches to gene therapy.

Thanks to the advances of genetics and bioengineering, gene therapy has become possible, although at present, this is predominantly an experimental area. However, in the last 5 years, enormous advances have occurred, with the approval of a few drug products by the Food and Drug Administration (FDA) and the EMA and others that are expected to be in the near future.

3 Nucleic Acids for Gene Therapy

Historically many gene therapy approaches have been based on expression of a transgene encoding a functional protein (i.e., the transgene product). However, newer tools including directly acting nucleic acid sequences such as microRNA, interference RNA (RNAi) via short hairpin RNAs (shRNA) or short interference RNA (siRNA), molecular scissor and gene-editing approaches such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISP)-associated nuclease Cas9 (CRISPR/Cas9) are being extensively applied in research and for developing new medicinal products. The development of this emerging field is still maturing, although there are many nucleic acid-based drugs in clinical trials, and some of them

have been already approved. There are also other promising candidates to be used in a variety of genetic and nongenetic disorders such a monogenic, infectious, cardiovascular, inflammatory, neurodegenerative, and a wide variety of cancers.

Nucleic acids are negatively charged and high molecular weight molecules, with physical-chemical properties very different to that of conventional drugs. They present limited stability in the biological medium and must access to an intracellular compartment (the cytoplasm or the nucleus); these two characteristics contribute to the difficulties to develop a medicinal product.

Depending on the application, the objective of the therapy can be gene augmentation, gene silencing, or gene editing [7].

3.1 Gene Augmentation

The objective of gene augmentation is to restore normal cellular function by delivering a functional copy of a gene (DNA) or messenger RNA (mRNA).

3.1.1 DNA

Typically, the gene of interest is inserted into a plasmid or expression cassette, which is a high molecular weight, double-stranded DNA construct containing transgenes, which encode specific proteins [8]. Plasmids also contain a promoter and a terminator signal to drive and end gene transcription, respectively [9]. Transfection with DNA leads to much higher protein and persistent expression than those obtained with mRNA. However, with mRNA, once it reaches the cytoplasm, translation starts instantly, without the need to enter the nucleus to be functional [10]; on the contrary, DNA has to reach the nucleus of the target cell, being this process one of the most limiting steps for transfection.

Apart from plasmid DNA, minicircle DNA (mcDNA) are emerging due to their safety and persistent transgene expression in both quiescent and actively dividing cells [11]. mcDNA are episomal, covalently closed circular gene expression systems, generally biosynthesized in recombinant bacteria, that consist in minimalistic backbones with potential to meet the clinical requirements for safe and long-lasting expression [12]. An alternative approach to sustain prolonged gene expression is the inclusion of scaffold matrix attachment regions (S/MAR) moieties in mcDNA constructs. Among others, mcDNA has been proposed as a potential therapeutic strategy for cancer [13].

3.1.2 Messenger RNA

Messenger RNA (mRNA) is the template for the synthesis of proteins. The use of synthetic mRNA to produce a desired protein in cells is a very promising technology to apply in clinic. Contrary to plasmid DNA, mRNA-based therapeutics are still in their

infancy, in spite of important advantages. mRNA must only be delivered to the cytoplasm where cellular translation machinery is located. From a therapeutic perspective, protein expression arising from mRNA is more transient than that from DNA. From a safety point of view, mRNA does not integrate into the genome and so poses no risk of insertional mutagenesis [14]. Moreover, mRNA gene therapy circumvents the need for selecting a specific promoter, and thus the transfection process is relatively efficient and simple [15, 16]. Another advantage of mRNA refers to the production process, raw material synthesis, and the quality product, which are more easily standardized than that for DNA, that leads to higher reproducibility [17].

Foreign RNA possesses inherent immune-activating adjuvant properties, and this effect has been studied for the intracellular delivery of mRNAs coding for specific antigens, with potential application in cancer immunotherapy [18], prophylactic vaccines [19], and allergy tolerization [20].

Since the protein expression profile of mRNA and DNA is completely different, the codelivery of these two nucleic acids has been proposed to take advantage of both [6].

3.2 Gene Silencing

3.2.1 Antisense Oligonucleotides

Single-stranded antisense oligonucleotides (ASO) are short sequences of modified DNA or RNA that can be used as therapeutic tools through (1) activation of RNase H to achieve specific knockdown of the target transcript or (2) modulation of pre-mRNA splicing to enable the restoration of a (partially) functional protein or alternatively a protein with reduced toxicity [21]. The ASO's unprecedented specificity for transcripts makes them unique as a therapeutic entity as it allows very specific targeting and provides the opportunity to, for example, correct genetic defects for rare genetic diseases with a current unmet medical need, modulate splice defects in autoimmune or neurodegenerative diseases, or target transcripts expressed by tumors or viruses.

The utilization of synthetic ASOs is increasing in areas ranging from clinical diagnostics to novel pharmaceutical therapeutics, and the efficacy and safety of ASOs are being investigated for the treatment of various genetic disorders where no treatment is currently available. Recently, several first-in-class ASO drugs have been approved by the FDA or EMA, including mipomersen for the treatment of familial hypercholesterolemia, eteplirsen for the treatment of Duchenne muscular dystrophy, and nusinersen for the treatment of spinal muscular atrophy.

3.2.2 Aptamers

Aptamers are short single-stranded RNA or DNA oligonucleotides, normally 15–80 nucleotides, with the capacity to fold in stable three-dimensional structures. These molecules present very high affinity with nucleic acids through structural recognition

and bind to them through electrostatic interactions, hydrogen bonding, van der Waals forces, base stacking, or a combination of them [22].

Aptamers recognize and bind targets of interest just like antibodies and have important advantages over conventional antibodies: (1) easy to synthesize by automated methods; (2) easy to modify to improve the stability, binding strength, and specificity to the target nucleic acid; (3) structure very flexible; and (4) display low to no immunogenicity when administered in preclinical doses 1,000-fold greater than doses used in animal and human therapeutic applications [23].

Due to the molecular recognition of their targets, aptamers have a variety of diagnostic and therapeutic applications, such as biosensors and target inhibitors. Due to simple preparation, easy modification, and stability, aptamers have been used in diverse areas within molecular biology, biotechnology, and biomedicine [24]. However, up to now, the introduction of aptamers into the market has not been very successful, and only one aptamer-based product have been approved for clinical use, Macugen® (pegaptanib), for the treatment of age-related macular degeneration.

3.2.3 RNA Interference

RNA interference (RNAi) is a posttranscriptional mechanism of gene silencing through chromatin remodeling, inhibition of protein translation, or direct mRNA degradation. RNAi is a naturally occurring process of gene regulation present in plants and mammalian cells, and it can be used to downregulate disease-causing genes. Typically, there are three different types of commonly used RNAi molecules [25]:

– MicroRNA (miRNA)
– Short interfering RNA (siRNA)
– Short hairpin RNA (shRNA), also called expressed RNAi activators

miRNA

miRNA and their role in regulating normal physiological processes were discovered in the last decade, as well as their involvement in pathological disorders such as cancer [26]. They are noncoding RNA molecules of 18–25 nucleotide in length that regulate at posttranscriptional level the expression of genes by binding to the 3′-UTR of target genes [27]. A miRNA can regulate different mRNAs, because they are not specific to a single mRNA [28]. miRNA is transcribed from DNA as primary miRNA (pri-miRNA), which is later processed into a precursor miRNA (pre-miRNA) by two proteins: Pasha and Drosha. The pre-miRNA is transported to the cytoplasm, where it is processed by Dicer to obtain the miRNA, which is incorporated into the RNA-induced silencing complex (RISC), where a helicase unwinds the miRNA. The resulting antisense stand guides the RISC to its complementary mRNA, which is cleaved (Fig. 2).

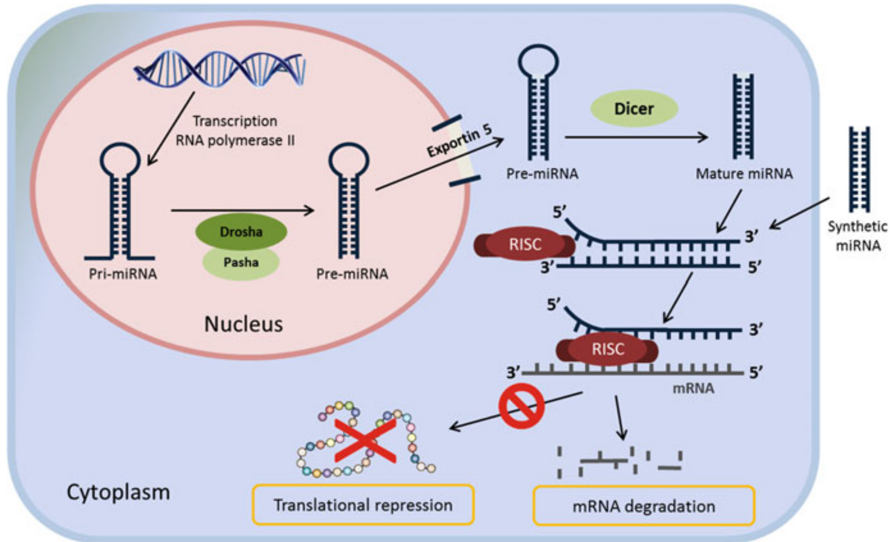


Fig. 2 Schematic representation of the mechanism of action of miRNA. Pri-miRNA is transcribed from DNA and is later processed into pre-miRNA. In the cytoplasm, the pre-miRNA is processed to give the miRNA, which incorporates into the RNA-induced silencing complex (RISC), where a helicase unwinds the miRNA. Finally, the resulting antisense strand binds to its complementary mRNA and cleaves it

miRNAs have emerged as key players in a wide array of biological processes, and changes in their expression and/or function have been associated with plethora of human diseases, such as myocardial infarction and stroke [29], Parkinson's disease [30], or cancer. The application of miRNA in cancer therapy is based on the finding that miRNA expression is deregulated in cancer tissues and also due to the ability of miRNA to target multiple genes and alter cancer phenotypes [31]. In fact, in neoplastic diseases, miRNA can be downregulated when they function as tumor suppressors or overexpressed when they function as oncogenes [32].

siRNA

siRNAs are short double-stranded RNA segments with 21–23 nucleotides and are complementary to the mRNA sequence of the protein whose transcription is to be blocked. siRNA molecules are incorporated into the RISC complex, which bind to the mRNA of interest and stimulate degradation of mRNA or the suppression of the translation process [22]. Figure 3 shows the mechanism of siRNA.

The main advantage of synthesized siRNA is that these molecules do not need to reach the nucleus to induce the therapeutic effect. As a drawback, stability must be improved in order to optimize the efficacy [22].

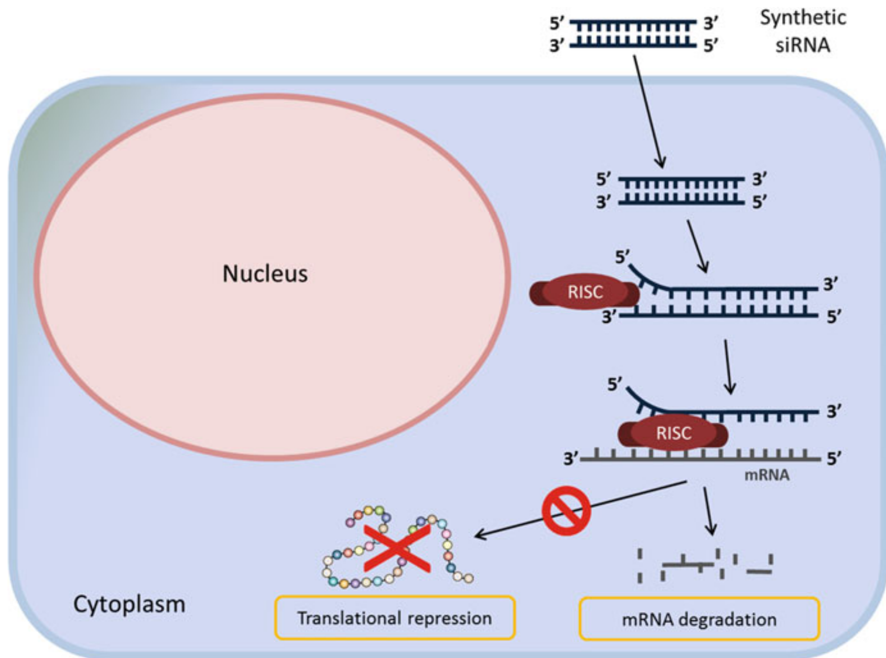


Fig. 3 Schematic representation of the mechanism of action of siRNA. The molecules of siRNA are incorporated into the RNA-induced silencing complex (RISC), where a helicase unwinds it. Finally, the resulting antisense strand binds to its complementary mRNA and cleaves it

Because of their small size and low potential to elicit adaptive immune responses, several antihuman immunodeficiency virus (HIV) RNAs have advanced to clinical trials. A potential advantage of anti-HIV-1 siRNAs over current therapies is that their sequences could be tailored to target a patient's particular viral strains and provide a personalized approach to therapy [33]. A major challenge for the development of anti-HIV-1 siRNAs is that lymphocytes, which represent the major cell type for HIV-1 replication, are widely distributed in the body and extremely difficult to penetrate with existing siRNA delivery technologies [34]. Other viral infections with limited treatment options and more easily accessible target that could be treated with siRNA include hepatitis B virus, Ebola, and respiratory syncytial virus [29]. Other indications of siRNA under clinical investigation are hepatocellular carcinoma, hepatic fibrosis, dry eye syndrome, melanoma, and pancreatic ductal adenocarcinoma.

At present there is a siRNA-based therapy (patisiran) recently approved by the FDA and the EMA for the treatment of hereditary transthyretin-mediated amyloidosis, a rapidly progressive, heterogeneous disease caused by the accumulation of misfolded transthyretin protein as amyloid fibrils at multiple sites and characterized by peripheral sensorimotor neuropathy, autonomic neuropathy, and/or cardiomyopathy [35]. Another product, inclisiran, is an experimental therapeutic agent for the treatment of hypercholesterolemia, which is being tested in late-stage clinical trials.

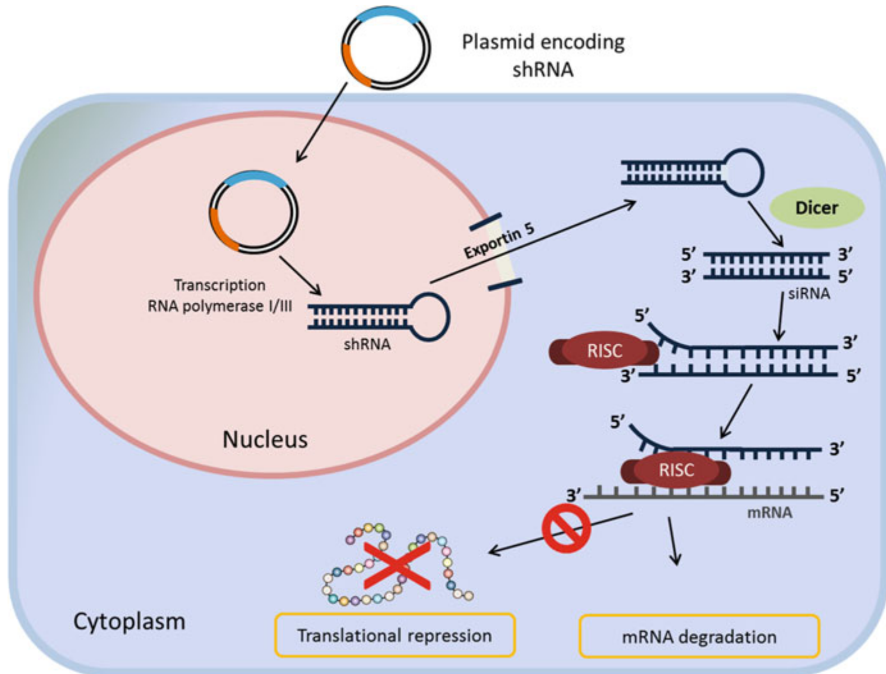


Fig. 4 Schematic representation of the mechanism of action of shRNA. The molecules of shRNA are transcribed in the nucleus and are exported to the cytoplasm, where the complex Dicer processes them to form a double-stranded siRNA. The siRNA is later incorporated into the RNA-induced silencing complex (RISC), where a helicase unwinds it. Finally, the resulting antisense strand binds to its complementary mRNA and cleaves it

Short Hairpin RNA

Short hairpin RNA (shRNA), also called expressed RNAi activator, is a plasmid-coded RNA that needs to be transcribed in the nucleus to downregulate the expression of a desired gene. It can be transcribed through either RNA polymerase II or III. The first transcript generates a hairpin-like stem-loop structure and is then processed in the nucleus by a complex containing the RNase II enzyme Drosha. The individual pre-shRNAs generated are finally transported to the cytoplasm by exportin 5. Once in the cytoplasm, the complex Dicer processes the loop of the hairpin to form a double-stranded siRNA [36]. Figure 4 features a scheme with the mechanism of action of shRNA. Since shRNA is constantly synthesized in the target cells, more durable gene silencing is achieved in comparison to other forms of RNAi [37]. shRNAs represent an important tool in the assessment of gene function in mammals and are largely used as a research tool. Although shRNA has been assayed to develop new therapies for retinal diseases [38], viral infections [22, 33], or cancer [39], no therapeutic product based on shRNA has been approved.

3.3 Gene Editing

Gene-editing technology has recently emerged as a new treatment modality for a variety of diseases, including hereditary, infectious, and neoplastic diseases. This technology is based on programmable nucleases, which consist of a nuclease that can be reprogrammed to cleave a precise target sequence [40, 41]. Consequently, they induce a double-strand break (DSB) at a specific and desired location.

There are four types of gene-editing nucleases: meganucleases, ZFN, TALEN, and CRISPR/Cas9 [36]. Meganucleases, also called homing endonucleases, stimulate the cellular recombination and repair of DNA to fix the break by simply copying the gene encoding them (the homing endonuclease gene) and flanking DNA into the broken chromosome [42]. ZFN contains zinc finger proteins, the most common class of DNA-binding proteins across all of biology, and *Fok*I nuclease as the DNA-binding and cleavage domains. ZFNs have now been widely used for genome editing in many species and cell types for basic science, biotechnology, and medical applications, such as targeted disruption of the CCR5 gene for HIV-1 therapy [43]. One important disadvantage of ZFN is that it is an expensive and time-consuming technology. TALEN use the same *Fok*I-derived nuclease domain as ZFN, but differ in that they employ distinctive DNA-binding arrays: TALE effector repeat arrays [44]. TALEN technology for the recognition of a wider range or target gene sequences requires complicated engineering that is a matter of concern. The CRISPR/Cas9 system is based on a prokaryotic antiviral mechanism in which the bacteria insert a partial gene sequence from an infection source, such as bacteriophage, into their own genomes to defend against repeat infection [45]. This system includes a RNA guide to bind to a complementary sequence in a target gene, which is recognized and cut by the Cas9 [46]. Once the target gene is cleaved by the programmed nuclease, the reparation of the DSB can be done by two different mechanisms: nonhomologous end joining (NHEJ) and homology-dependent repair (HDR) [43]. In the NHEJ, the target region is eliminated by joining the DSB, and it can be used to silence or correct a pathogenic gene. On the contrary, with the HDR modality, a homologous sequence can be introduced into the DSB, enabling donor DNA to be inserted to either correct an existing gene or add a new one. Figure 5 shows the different modalities of DSB reparation.

Contrary to gene augmentation and suppression, therapeutics based on gene editing can lead to a permanent effect at the genome, and therefore, this recent technology represents a key development of gene therapy [37].

In the last years, there has been tremendous progress in gene editing, mainly with CRISPR/Cas9, thanks to the simplicity of the manufacturing procedures in comparison to the earlier tools, meganucleases, ZFN, and TALEN. However, it is important to remark that in comparison to standard gene transfer approaches, genome editing, particularly that based on CRISPR/Cas9, is in its translational and clinical infancy. In spite of that, several clinical trials with gene-editing technologies have been completed or are undergoing [47]. For instance, engineering ZFN have been tested in clinical trials to disrupt CCR5 (C-C motif chemokine receptor type 5) expressed in human T cells and

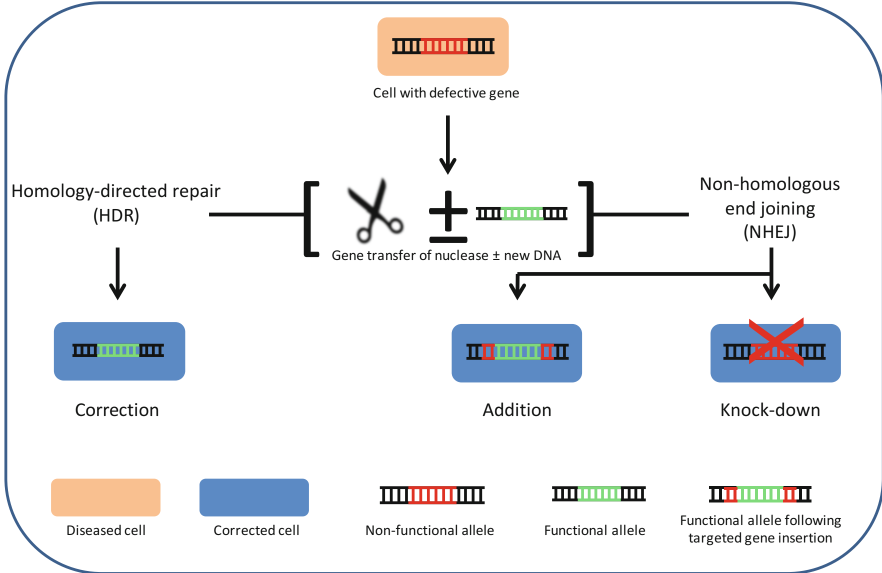


Fig. 5 Schematic representation of the different modalities of gene repair with nucleases

hematopoietic stem cells to provide to them resistance to human immunodeficiency virus (HIV) infection [48]. Other clinical trials with ZFN have been approved for delivering the factor IX gene for hemophilia, the α-iduronidase gene for mucopolysaccharidosis I, and the iuronidate-2-sulfatase gene for mucopolysaccharidosis II. The first-in-human use of TALEN gene-edited T cells in two infants with refractory relapsed B-cell acute lymphoblastic leukemia led to a successful induction of molecular remission ahead of allogeneic stem cell transplantation [49].

The rapid technological advances in genome editing have allowed manipulating germ cells, gametes, zygotes, or embryos. In a recent study [50], the CRISPR/Cas9 technology repaired in an embryo DSBs induced at the mutant paternal allele, by predominantly using the homologous wild-type maternal allele instead of a synthetic DNA template. The authors were able to avoid mosaicism in cleaving embryos and achieve a high yield of homozygous embryos carrying the wild-type *MYBPC*3 gene (whose mutation causes hypertrophic cardiomyopathy), without evidence of off-target mutation. In spite of the potential use for the correction of heritable mutations in human embryos, much remains to be considered before clinical applications, including the reproducibility of the technique with other heterozygous mutations.

Human application of these technologies still presents a substantial challenge. Collaboration between regulatory bodies and the scientists developing these life-changing treatments is very important for gene editing to progress to the clinic, with special emphasis to the critical assessment of risk vs benefit [41]. Among all the potential applications of gene editing, it is impossible to predict which approaches will ultimately be successful, both in the clinic and on the market, but it is expected that the application of gene-editing technologies is poised to change the practice of

medicine dramatically in the years to come [51]. The long-term follow-up of patients who will participate in genome-editing clinical trials will likely provide invaluable insight into the in vivo activity and specificity of programmable nucleases.

4 Delivery Systems Used in Gene Therapy

One of the main challenges of gene therapy is the development of safe and effective administration systems that are able to overcome the main limitations of nucleic acids when they are administered in the body [52]. Therefore, a fundamental aspect for the success of gene therapy is the availability of delivery systems capable of protecting the genetic material from degradation, facilitating its internalization in target cells, and releasing them intracellularly. The ideal delivery system depends on the target cells, the kind of nucleic acid to be delivered, and the duration of expression [53]. The delivery systems are classified into two large groups: viral and non-viral vectors.

Viral vectors are prepared from genetically modified viruses so that they are not able to replicate in the target cells, but they express the therapeutic gene they transport. Viral vectors allow high transfection efficiencies; however, they present important safety limitations due to the oncogenic and immunogenic potential (due to viral proteins). Another problem associated with viral vectors is the inability to transport large nucleic acids.

Non-viral vectors are safer, simpler, cheaper, and more reproducible systems. In addition, they do not present limitations regarding the size of the genetic material they can incorporate. Nevertheless, a disadvantage of non-viral systems is that their transfection efficiency is lower compared to viral vectors, although in recent years new non-viral systems have been developed with materials that exhibit higher transfection efficiencies. In fact, the number of clinical trials with products based on non-viral vectors (Fig. 6) has increased in the last decade, and those based on lipid nanocarriers (lipofection) are used in 4.1% of all trials [54].

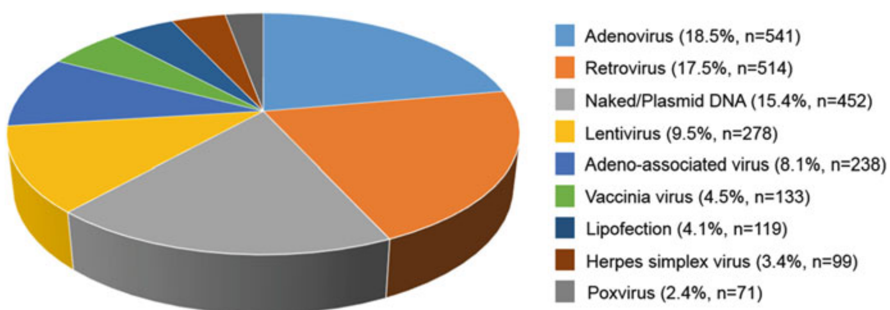


Fig. 6 Vectors used in gene therapy clinical trials. Data consulted in gene therapy clinical trials worldwide [54]

Table 1 Features of the most studied viral vectors in gene therapy

	Retroviruses	Lentiviruses	Adenoviruses	AAV
Viral genome	RNA	RNA	DNA	DNA
Target cells	Dividing cells	Dividing and nondividing cells	Dividing and nondividing cells	Dividing and nondividing cells
Integration in the host genome	Yes	Yes	No	Yes
Response	Long-term	Long-term	Short-term	Long-term
Size of the genetic material to be carried	8 kb	8 kb	7.5–30 kb	4.5 kb

AAV adeno-associated viruses

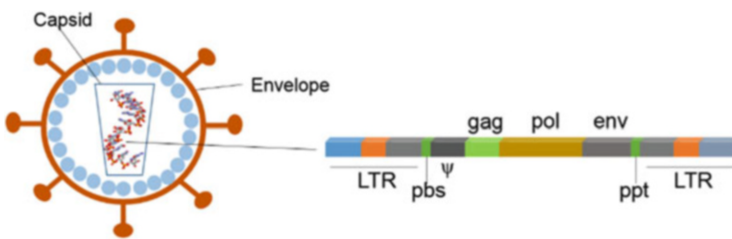


Fig. 7 General structure of retroviruses and retroviral genome. *LTR* long terminal repeats, *pbs* first binding site, *ppt* poly-purine sequence, *ψ* packaging signal

4.1 Viral Vectors

Viruses used as delivery systems of genetic material include adenoviruses, adeno-associated viruses (AAV), retroviruses, and lentiviruses among the most evaluated in clinical trials. Other viruses, such as those derived from herpesvirus or poxvirus, have also been studied as possible viral vectors.

The selection of the most suitable viral system in each case depends on different factors: the organ or the target cell, the ability to integrate the genetic material carried by the vector in the genome of the host cell, the duration of expression gene over time (short-term or long-term response), or the size of therapeutic nucleic acid. Table 1 shows the main characteristics of the most frequently studied viral vectors, to be taken into account for their application in gene therapy.

4.1.1 Retroviral Vectors

Retroviruses are RNA viruses that contain two strands of RNA enveloped by an icosahedral capsid of peptide nature (Fig. 7). The capsid is surrounded by a phospholipid envelope, in which different types of glycoproteins act as ligands for specific receptors on cell surfaces and therefore determine the tropism of the virus.

The genome of retroviruses (Fig. 7) contains three types of genes: *gag* genes that encode capsid proteins, *pol* genes that encode the enzymes necessary for the replicative cycle of the virus (protease, integrase, reverse transcriptase), and *env* genes that encode the envelope glycoproteins. This genome also contains a packaging signal, ψ, thanks to which the RNA molecules bind to the capsid proteins and are effectively packaged, and the long terminal repeats (LTR) at each end of the viral genome. The left LTR contains a region for the start of transcription (U3 promoter) and a first binding site (pbs) for the start of reverse transcription. The right LTR contains a poly-purine sequence (ppt) for replication of the second strand. For application in gene therapy, the retroviral vectors are generated by the substitution of the gag, pol, and env sequences of the viral genome by the therapeutic gene. As a consequence, the therapeutic sequence in this case cannot be greater than 7–8 kb.

The replicative cycle of a retrovirus begins with entry into the cell, which is mediated by receptors [55]. Once inside the cell, the viral reverse transcriptase enzyme produces a DNA molecule from the viral RNA. Subsequently, the DNA is integrated into the genome of the host cell, and the transcription yields different RNAs, which are exported to the cellular cytoplasm, where they are translated into structural proteins of the virus, and directs synthesis of new virion nucleocapsids. The nucleocapsids leave the cell and keep enveloped by a plasma membrane-derived outer coat.

It is important to point out that the DNA obtained by reverse transcription from the RNA is not able to cross the nuclear membrane of the cell to be treated. Therefore, retroviral vectors only transfect efficiently dividing cells, because the DNA takes advantage of the disruption of the nuclear envelope during the mitosis, to reach the interior of the nucleus [56].

On the other hand, the integrase enzyme allows the integration of the genetic material in the genome of the host cell. Thanks to this, it is possible to obtain a long-lasting expression of the therapeutic sequence; however, the insertion into the genome of the target cell is also one of the major problems of viral gene therapy, since if it takes place in an unwanted region of the genome of the transfected cells, there is a risk of mutagenesis and oncogenesis. In fact, in clinical trials with these vectors, several patients developed leukemia and dysplasia of the bone marrow due to insertional mutagenesis [57, 58]. These adverse effects were partially reduced by the design of so-called self-inactivating vectors in which the genome sequences of the virus identified as responsible for mutagenesis, 3′LTR, are deleted. This idea for self-inactivating vectors had been already described in the literature by Yu et al. [59]. Another limitation of retroviral vectors is that they are recognized and inactivated by the complement system, which means that they are mainly used in ex vivo gene therapy.

Despite the mentioned limitations, and due to its high transfection efficiency, since 1989, the year in which the first clinical trial with gene therapy was launched, 514 clinical trials with retroviral vectors have been started (17.5% of the total of clinical trials with gene therapy) [54].

One of the most commonly used retroviruses in gene therapy is the murine leukemia virus (MLV), which has shown efficacy in different types of

immunodeficiency. In fact, recently the European Medicines Agency (EMA) has approved a drug based on ex vivo gene therapy with this retroviral vector for the treatment of patients with severe combined immunodeficiency due to adenosine deaminase (ADA-SCID) deficiency that cannot be treated with bone marrow transplant (Strimvelis; Orchard Therapeutics) [60].

4.1.2 Lentiviral Vectors

Lentiviruses also belong to the *Retroviridae* family. However, they have certain differential genes with respect to the rest of retroviruses that facilitate the entry of genetic material into the cell nucleus, so lentiviral vectors can also transfect nondividing cells. One of the most known and studied viruses of this subfamily is the human immunodeficiency virus (HIV).

The general structure of lentiviruses is the same as that described for retroviruses: two RNA strands included in a protein capsid, surrounded by a phospholipid envelope that includes different types of glycoproteins.

The genome of the lentiviruses (Fig. 8) shares with the retroviruses the *gag*, *pol*, and *env* genes, as well as the LTR, pbs, and ppt sequences. However, it presents other specific genes: *tat* genes (transcription regulators), *rev* genes (regulators of the expression of viral proteins), *vif* genes (necessary for the infection of different cell types), *vpr* genes (participate in the entrance to the nucleus), *vpu* genes (involved in the release of viral particles from infected cells), and *nef* genes (increase the infectivity of the virus).

The cycle of life is similar to that described for retroviral vectors, with the difference that lentiviruses present genes encoding nuclear localization signals that favor the entry of DNA (synthesized by the reverse transcription process) into the nucleus.

In order to use lentiviruses as gene delivery systems, the *tat* gene is removed, and the *gag* and *pol* genes are encoded on a different plasmid from that of the *rev* or *env* genes. The final vector results from three separate plasmids containing the necessary viral sequences for packaging [61]. In addition, the 3′LTR sequence of the viral genome may be deleted to generate self-inactivating (SIN) lentiviral vectors [62], as previously mentioned in the case of retroviral vectors.

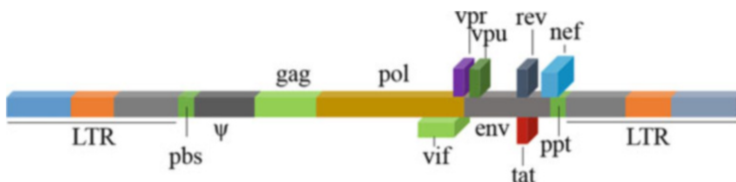


Fig. 8 General structure of lentiviral genome. *LTR* long terminal repeats, *pbs* first binding site, *ppt* poly-purine sequence, ψ packaging signal

Due to the tropism of lentiviruses and their ability to transfect cells that are not in division, the main application of lentiviral vectors is directed to introduce genetic material in vivo in cells of the central nervous system [63], for example, for the treatment of Parkinson's disease [64] or in cells of the retina [65] (suitable for the treatment of retinitis pigmentosa). Furthermore, lentiviral vectors efficiently transfect ex vivo cells of the hematopoietic system, which are difficult to transfect with other vectors. In fact, ex vivo gene therapy with lentiviral vectors to genetically modify CD34+ cells has been evaluated in more than 100 clinical trials in recent years for the treatment of monogenic diseases (i.e., β-thalassemia [66], X-linked adrenoleukodystrophy [67], metachromatic leukodystrophy [68], or Wiskott-Aldrich syndrome [69]), of different types of cancer [70], and of infectious diseases such as HIV infection [71].

4.1.3 Adenoviral Vectors

Adenoviruses encompass more than 50 different virus serotypes and are responsible for 5–10% of respiratory infections in children and adults. In gene therapy, serotypes 2 and 5 are the most used. Adenoviruses are non-enveloped viruses that consist of a double strand of DNA within an icosahedral capsid (Fig. 9).

The genome of an adenovirus (Fig. 9) has a size of approximately 35 kb. At the ends are the inverted terminal repeats (ITR), and close to the left ITR, the packing signal, ψ, is arranged. The numerous genes it contains differ in the early (*E*) and late (*L*) regions. The latter, responsible for the coding of structural proteins, are transcribed after replication of the viral genome, and the E regions are transcribed before synthesizing the viral DNA, since they give rise to regulatory proteins. These proteins alter the expression of host cell proteins that are necessary for DNA synthesis, intervene in viral replication, and also prevent the death of infected cells by blocking the apoptosis or avoiding recognition by the immune system.

Adenoviruses are endocytosized into the cell after binding to the CAR receptor (coxsackievirus and adenovirus receptor). The nucleocapsids are released into the cytoplasm, and with the help of cellular microtubules, they reach the nuclear membrane, and the genetic material is introduced to the nucleus of the cell through

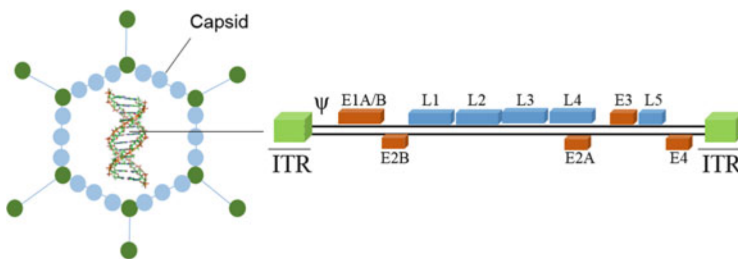


Fig. 9 General structure of adenoviruses and adenoviral genome. *ITR* inverted terminal repeats, ψ packaging signal

the nuclear pores. Replication then begins, and once all the components of the virus have been synthesized, they are assembled and released from the cell by cell lysis induced by the virus itself. During this process the adenoviral genome does not integrate into the genome of the host cell. This is one of the advantages of adenoviruses which makes them safer compared to other viral systems, although this also means that the viral life cycle is not adapted for long-term transgene expression.

To be used as vectors for gene therapy, E regions are deleted from the genome, and various levels of attenuation can be achieved by removal of different numbers of genes: only one E1B gene (first-generation vectors), the majority of early genes (second-generation vectors), and even full deletion of all genetic information of an adenovirus (so-called gutless vectors) [72]. The large size of the genome and the possibility to delete a major part of it provide high coding capacity for these vectors: 1–2 kb can be inserted in early generation vectors and up to 30 kb in gutless vectors. However, they need another helper virus that replicates normally and expresses all the proteins needed to assemble the adenoviral vector [73]. Despite its advantages, the total elimination of impurities from helper viruses is complicated and limits its clinical application. In fact, at high doses adenoviruses are toxic [74], and one of their main limitations is that they are very immunogenic [75], which decreases their effectiveness.

In spite of their limitations, the advantages of adenoviral vectors have meant that they have been evaluated in more than 500 clinical trials [76], most of them aimed at the treatment of cancer.

4.1.4 Adeno-Associated Viral (AAV) Vectors

Adeno-associated viruses (AAV) are small non-enveloped viruses with single-stranded DNA (Fig. 10). The genome has a size of about 4.7 kb and is composed of three regions, called rep, cap, and aap, flanked by the corresponding ITR. The rep region encodes nonstructural proteins involved in viral replication, packaging, and integration into the host genome, genes in the cap region encode the structural proteins of the capsid, and *aap* gene encodes the assembly-activating protein [77]. AAV vectors for clinical application are generated by replacing the *rep*, *cap*, and *aap* genes with the gene of interest.

The entry of AAV into the cell takes place by endocytosis after binding to the receptor-mediated cellular surface. Once the nucleocapsid escapes from the

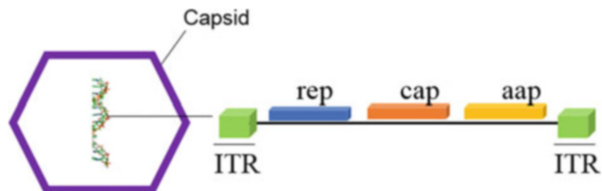


Fig. 10 General structure of AAV and AAV genome. *ITR* inverted terminal repeats

Table 2 AAV serotypes suitable for specific target tissues [79]

Serotype	Target tissue
AAV1	Nervous system, skeletal muscle
AAV2	Nervous system, kidney, photoreceptors, retinal pigment epithelium
AAV4	Nervous system, retinal pigment epithelium
AAV5	Nervous system, lungs, photoreceptors, retinal pigment epithelium
AAV6	Skeletal muscle, lungs
AAV7	Skeletal muscle
AAV8	Nervous system, photoreceptors, retinal pigment epithelial, liver, skeletal muscle, heart, pancreas
AAV9	Nervous system, heart, liver, skeletal muscle, lungs

AAV adeno-associated viruses

endosome and is transported to the nucleus, the viral genome is able to cross the nuclear membrane. In the nucleus a second strand of DNA, necessary for the replication of the virus, will be synthesized. In the presence of a helper virus (coinfection by an adenovirus or herpesvirus), the double-helix DNA generated can be integrated into the genome of the host cell, and replication of the virus will take place. In the absence of a helper virus, the AAV genome usually remains latent in the form of an episome. Replication of the viral genome and subsequent packaging results in the generation of viral particles that will escape from the host cell by lysis thereof [78].

These vectors are quite safe, can transfect cells with or without capacity of division, and provide long-term gene expression, up to 6 years. Another advantage of AAV is the possibility of selecting the most suitable serotype depending on the target tissue [79]. Table 2 shows the most suitable AAV serotypes for different tissues.

The main disadvantage of AAV is the limited size of the genetic material they can transport, which must not exceed 4.5 kb. However, viral vectors based on AAV have been evaluated in more than 180 clinical trials, most of them aimed at the treatment of monogenic diseases. In fact, a medicinal product called Luxturna and based on AAV2 have reached the market. Luxturna (voretigene neparvovec) is a gene transfer vector that employs an AAV2 as a delivery vehicle for the human retinal pigment epithelium 65 kDa protein (hRPE65) cDNA to the retina [80]. This medicine is indicated for the treatment of adult and pediatric patients with vision loss due to inherited retinal dystrophy caused by confirmed biallelic RPE65 mutations and who have sufficient viable retinal cells to express the protein and respond to the treatment.

4.1.5 Manufacturing of Viral Vectors

Viral vector production for clinical application requires viral propagation in suitable animal cell lines, viral recovery, concentration purification, and formulation [81]. To meet commercial and regulatory requirements, each process must be scalable and reproducible and must yield high virus titers. For large-scale manufacturing,

suspension-adapted cell lines cultured in bioreactors are more appropriate than adherent cells systems, conventionally used at laboratory scale [82]. Depending on the viral vector and the cell clone used, the most suitable bioreactor must be chosen [83].

The production of viral vectors may be carried out by transiently transfecting the producing cells with vector and helper or packaging plasmids or by generating stable producer cell lines. In the first case, culture cells are co-transfected with multiple plasmids, one containing the expression cassette for the transgene and other plasmids encoding regulatory and structural viral proteins. The main limitations of transient transfected cells are the amount of transfected cells achieved and the variability in transfection [84]. In addition, transient transfection results in contaminations of the final product due to excess plasmids [85] and residual transfection reagent. In the second case, cells are genetically modified, so that they contain, inserted in the cellular genome, the genes that encode the structural proteins necessary for the formation of viral particles containing the transgene of interest [86]. Processes using stable producer cell lines are easier to scale-up and result in less contaminated vectors, although some drawbacks have to be also considered: the gene products necessary to produce vectors are toxic to cells, each vector-produced cell line requires specific certifications as master cell bank, and, upon cell expansion, high-titer vectors are not always ensured [84].

One of the limitations of viral vector manufacturing is to purify a sufficient amount of viral particles even to start a clinical trial. The downstream processing or purification of viral vectors aims to eliminate contaminants, whether process or product-related. Process-related impurities derive from starting materials (residual DNA and residual host cell protein from each cell bank) or raw materials (culture reagents, purification reagents and equipment materials, helper viruses, and helper virus nucleic acid used in production), whereas product-related impurities include vectors with deleted, rearranged, hybrid, or mutated sequences. The ultimate goal of the downstream processing is to obtain a product with high purity, potency, and quality that can meet the guidelines of the FDA [87] and EMA [2] regulatory agencies. With this aim, different concentration methods have been developed: centrifugation, tangential flow filtration, ultrafiltration, polyethylene glycol precipitation, two-phase extraction, membrane filtration, liquid chromatography, or adsorption chromatography [88].

4.1.6 Quality Control of Viral Vectors

Another aspect to take into account are the quality controls that these vectors must overcome to ensure that each batch manufactured meets the specifications of purity, potency, safety, and identity and there are consistency and comparability between batches. This is a challenge given the high complexity of the viral systems due to the large number of protein subunits that make up the viral capsid and the composition of the lipid membrane present in enveloped viruses. In addition, it is necessary to develop specific analytical methods for each type of virus and even for each serotype. These methods can be divided into those that are similar to others already

validated for recombinant proteins and vaccines and those that are specific to each vector. The former include, for example, the analysis of impurities related to the production process, such as packaging cell proteins. Specific assays of viral vectors include the analysis of the activity of the resulting product of the therapeutic genetic material, as a measure of potency. It is also necessary to determine the impurities due to the presence of residual genetic material encapsulated in the vector. In any case, it must be considered that many of these methods developed to analyze the specific quality controls of viral vectors are not yet validated according to the standards established to license and market these products.

4.2 Non-viral Vectors

Non-viral systems can be defined as those physical or chemical methods that help in the process of transfer of exogenous genetic material to the cell, facilitating the entry and intracellular bioavailability thereof. Non-viral systems try to imitate the capacities of viruses as gene transfer vehicles, providing greater security from the point of view of biological risk and pathogenicity. However, reproducing with a non-viral system what a virus performs naturally is a great challenge that has led to the development of different strategies. The selection and design of the most appropriate non-viral system are conditioned by its efficacy and safety, by the tissue or target cell, and by the type of therapeutic genetic material [53].

4.2.1 Physical Methods

Physical methods are based on the application of physical forces to temporarily alter the permeability of the cell membrane, allowing the genetic material to cross the cytoplasmic membrane and reach the interior of the cell. It is important that there is a balance between the efficiency of cellular internalization and the damage exerted on the cell. These methods have been frequently evaluated as systems of administration of naked genetic material, without the need to formulate it or include it in a viral or non-viral vector, which gives them great simplicity. Nevertheless, physical methods are mainly evaluated and used in preclinical studies. Table 3 summarizes the main characteristics of physical delivery methods used in gene therapy [89].

4.2.2 Chemical Carriers

Chemical vectors are based on the use of different types of compounds capable of encapsulating or binding, electrostatically or covalently, the genetic material.

In order to develop a suitable non-viral delivery system, the selected vector must have the capacity to enter the cell and to overcome different barriers maintaining the stability of the nucleic acid throughout the entire transfection process. Once within the cell, it must guarantee the proper intracellular distribution of genetic material.

Table 3 Types of physical delivery systems in gene therapy and main features

Method	Advantages	Limitations
Needle injection Direct needle injection on a specific tissue	Simple Safe	Low efficiency Local inflammation
Hydrofection or hydrodynamic injection Intravascular injection of high volumes of a solution containing the nucleic acids	High efficacy in the liver	Hemodynamic changes
Microinjection Direct injection into host cell by microneedles	High efficiency	Cell by cell administration Time-consuming Need of specialist
Biolistic injection or gene gun Administration of metal microparticles at high velocity	Simple and fast Reproducibility	Low efficiency Low tissue penetration Cell damage High cost
Electroporation Application of electric pulses that open pores on cell membrane	Noninvasive Simple High efficiency Low cost Widely employed	Risk of tissue damage Surgery necessary to target internal organs
Sonoporation Application of ultrasounds (combined with microbubbles or nanocarriers) to permeabilize temporally cell membrane	Noninvasive Safe Targeting to specific tissues	Low reproducibility Tissue damage
Magnetofection Application of external magnetic fields combined with magnetic particles	Noninvasive Effective in primary cells (difficult to transfect)	Effective only on surface areas Mainly applied in vitro
Optofection Application of laser pulses combined with nucleic acid complexes or nanoparticles	Nucleic acids release from endosomes	Tissue damage Inflammation Restricted to single cells or small areas

Therefore, the main steps that these delivery systems have to overcome to reach the cytoplasm (in the case of RNAs) or the nucleus (in the case of DNAs) are the following: interaction with cell membrane, entry into the cytoplasm, intracellular distribution, and entry into the nucleus (Fig. 11). To date, different strategies for overcoming these limitations have been proposed, and the evolution of non-viral vector transfection has been significantly improved in recent years.

The first step in the genetic transfer process is the interaction between vectors and cell membranes. Cationic vectors interact electrostatically with the negative charge of the cell membrane surface, and the internalization process is started. Additionally, in order to enhance the interaction with specific cells, different ligands can be added to the vectors to improve binding to surface receptors [90, 91].

Once the vector has bound to the cell surface, it must penetrate into the cytoplasm. The internalization or entry into the cell can take place through two mechanisms: fusion with cell membrane or endocytosis. These two entry routes are not

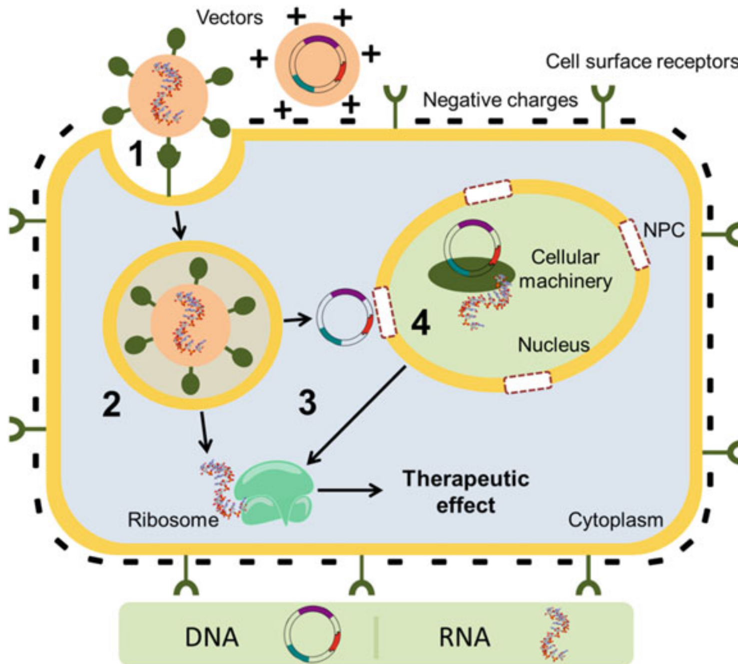


Fig. 11 Main stages during transfection process: (1) interaction with cell membrane, (2) entry into the cytoplasm, (3) intracellular distribution, and (4) entry into the nucleus. *NPC* nuclear pore complex

exclusive, and depending on the type of cell and vector, one or the other may predominate. However, in most cases vectors penetrate into the cells mainly through endocytic pathways. Endocytosis begins with the formation of a vesicle from the invagination of the plasma membrane, called an endosome. These endosomes fuse with lysosomes by creating endolysosomes, and their hydrolytic enzymes can degrade genetic material. Therefore, to achieve efficient gene transfer, it is necessary for the nucleic acid material to be protected from degradation by lysosomes to ensure release of nucleic acids into the cytoplasm. In fact, the endosomal escape represents an important barrier to achieve efficient transfection in the case of non-viral gene therapy [92].

In the case of DNA, once it is cytoplasm, it must be able to enter to the nucleus. However, the nuclear membrane is a selective barrier for macromolecules, such as DNA. The transport through this membrane is a highly regulated process, facilitated by a series of water channels of about 10 nm, called nuclear pore complexes (NPCs). The genetic material transported by non-viral vectors penetrates the nucleus through two main routes: NPCs or during cellular mitosis, when the nuclear membrane is temporally disrupted. The passage through the NPCs is carried out by means of an energy-dependent process that generally involves the recognition of specific nuclear localization signals (NLS) [93]. The NLS consist of one or more short sequences

of amino acids with positive charges containing arginines and lysines [94]. The formulation of DNA with compounds containing NLS is a strategy commonly used in non-viral gene therapy.

Chemical delivery systems or non-viral vectors are broadly categorized into inorganic, polymeric, lipidic, or peptidic particles. In many cases, the combination of some of different kinds of chemical compounds is used in order to improve their profile of efficiency, cellular specificity, and safety, giving rise to hybrid systems [53].

Inorganic Particles

Inorganic particles are nanostructured systems with different sizes, shapes, and porosity, designed to protect the genetic material from degradation and to escape from the reticuloendothelial system after its systemic administration. They can be composed of different elements, being used in gene therapy calcium phosphate [95], silica [96], gold [97], or magnetic compounds such as iron oxide [98].

These inorganic particles are of interest since they are easy to produce and ligands can be added to their surface that facilitate the union to the genetic material through electrostatic interactions. Cationic components are usually incorporated to the surface of the particle. An example of this type of system consists of combining iron oxide particles with PEI, which favors the condensation of nucleic acids, with polyethylene glycol (PEG), which favors the colloidal stability of the particles, and with cell penetration peptides, which favor cellular internalization [99]. In the case of gold particles, nucleic acids are previously thiolated to covalently bound to the delivery system [100].

Other types of inorganic materials used to develop inorganic particles that are showing encouraging results in vitro and in vivo in animal models are graphene [101] or fullerene [102]. However, it is still necessary to study in greater depth the long-term safety and the influence of functionalization, size, and shape in transfection efficiency to facilitate the clinical application of these newer compounds.

Polymeric Particles

The main component of these vectors is a cationic polymer that binds and condenses the genetic material, giving rise to the so-called polyplexes [103]. Cationic polymers bind by electrostatic interactions the negatively charged genetic material, so that the nucleic acid is adsorbed to the surface of the nanoparticulate system or is encapsulated in its interior. In addition, these systems allow the incorporation of different ligands that improve the transfection efficiency in the target tissue. In general, polymeric vectors are more stable than lipid vectors, and even in some cases, the progressive degradation of the polymer allows controlling the rate of release of the genetic material once it is inside the cell.

The polymers used in the preparation of non-viral vectors can be subdivided into synthetic and natural polymers (also called biopolymers).

The synthetic polymers most used in gene therapy are polyethyleneimine (PEI), the dendrimers [104], the polyesters (i.e., poly(lactic-co-glycolic) or PLGA) [105], or polymethacrylates [106]. Among them, PEI has been evaluated in various clinical trials for the treatment by local gene therapy of different types of cancer [107], but the high toxicity of this polymer has limited its application.

In the group of the biopolymers applied in gene therapy are polysaccharides, such as chitosan, cyclodextrins, alginate, pullulan, or dextran. Some of these polysaccharides are used by themselves as delivery systems, but most of them are generally used in combination with other non-viral vectors to improve their efficacy, safety, or biodistribution [90, 108, 109].

Lipidic Particles

Lipid-based systems are the most studied non-viral vectors at the clinical level. Up to 119 clinical trials have been documented, most of them in phases I and II, in which lipid vectors have been used as delivery systems for the genetic material. In most cases, vectors have been designed for the treatment of different types of cancer but also for the treatment of cardiovascular diseases, hepatitis C virus infection, or monogenic diseases such as cystic fibrosis [54]. Recently, a lipid-based siRNA delivery system called patisiran (Alnylam® Pharmaceuticals) has reached the market, as treatment of hereditary transthyretin-induced amyloidosis [35].

The main components of the lipid-based vectors are cationic lipids, formed by hydrophobic alkyl chains, linked through an intermediate binding structure to a polar group. The most used cationic lipids are 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP), 1,2-dimyristyloxypropyl-3-dimethyldhydroxyethylammonium bromide (DMRIE), or 3ß-[N-(N′, N′-dimethylaminoethane)-carbamoyl]-cholesterol (DC-cholesterol), although derivatives of these lipids are also being studied in order to improve their efficacy and safety [110]. Thanks to their cationic nature, these lipids are able to condense and protect the genetic material, as well as to bind to the negative charges of the cell membranes. The main limitations of non-viral vectors based on cationic lipids are the low efficacy in vivo due to the fact that they are not stable and that they undergo rapid clearance, as well as the possibility of generating inflammatory or anti-inflammatory responses.

Cationic lipids can be used by themselves to form complexes, known as lipoplexes, by mixing them directly with the negatively charged genetic material, but they are normally used to prepare colloidal systems that are then bound to the genetic material to obtain the lipoplexes. The preparation of these colloidal systems can involve other lipid components, which may improve the transfection efficiency of cationic lipids, such as the phospholipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), which has fusogenic function and facilitates endosomal escape, or polyethylene glycol (PEG), which forms a steric coating

that makes vectors more stable in vivo. The colloidal lipid systems used in gene therapy are liposomes, nanoemulsions, and solid lipid nanoparticles (SLNs) [92].

Nanoemulsions consist of a dispersion of an oil phase stabilized in an aqueous phase by means of a third component that acts as a surfactant, so that droplets of about 200 nm are formed. From the technological point of view, nanoemulsions are simple to manufacture, and they are very stable during storage. In spite of this, the application of cationic nanoemulsions in gene therapy is still quite limited [111].

SLNs are spherical particles in the range of nanometers, formed by a core composed of a solid lipid at room temperature surrounded by a layer of surfactants. In the case of SLNs designed to be applied in gene therapy, cationic lipids exert part of the surfactant effect and, in turn, confer positive charge to the surface of the particles. SLNs have shown efficacy as systems for administering different types of genetic material at preclinical level in vitro and in vivo, after their systemic or local administration, showing promising results especially in ocular pathologies [112–115], as well as in infectious diseases [37], lysosomal storage disorders [116], and various types of cancer [92].

Liposomes are spherical vesicles composed of one or more lipid bilayers surrounding an aqueous core, which show a size ranging from 20 nm to a few microns. Cationic liposomes are effective transfection systems in very varied types of cells in vitro and also in vivo after their local or systemic administration. In fact, in most clinical trials using lipid vectors, these are cationic liposomes.

Peptidic Particles

Some peptides are capable of condensing nucleic acids by themselves resulting in the formation of nanoparticulate systems. These include cationic peptides composed of short sequences of positively charged amino acids such as histidine, arginine, or lysine; in fact, poly-L-lysine is one of the peptide vectors with the highest transfection efficiency [117]. Proteins of natural origin, such as collagen or albumin [118], are also used as peptide vectors. In addition, it is very common to use peptides as ligands to functionalize some of the previously described polyplexes and lipoplexes. In this sense, peptides may be used to target non-viral vectors to a specific tissue (i.e., transferrin to target tumor tissue or hepatocytes [119] or RGD (arginine-glycine-aspartic acid) sequences to target specific tissues [120]). Another application of peptide as ligands is to improve their effectiveness by helping the genetic material to overcome some of the barriers of the transfection process: cell penetrating peptides [121] to improve cell entry, fusogenic peptides [122] to increase endosomal escape, or NLS [94] to entry into the nucleus.

Manufacturing and Quality Control of Non-viral Vectors

The production of nanoparticles for clinical gene therapy presents important hurdles, which are still hampering the translation from laboratory to patients. Non-viral

vectors are complex formulations that must be customized depending on the nucleic acid to be delivered, the variety of target diseases, and the administration route [107]. Due to their complexity, nanoparticulate systems show unique Chemistry, Manufacturing and Controls (CMC) challenges [123]. In fact, suitable methods for large-scale production of simple nanosystems, such as liposomes, have been developed [124]. However, when formulation becomes more complex, for example, with the addition of surface modification or ligands, the number of steps in the production process and the cost of the final product increase, and quality control is also more difficult [125].

Regarding quality attributes, parameters that must be especially considered because of their impact on biological yield are size, shape, surface charge, presence of ligands to provide effective targeting, surface modification with PEG, impurities associated to starting materials and the production process, and stability during manufacturing and long-term storage and upon administration [126]. Batch-to-batch variability of non-viral vectors can potentially led to changes in all these parameters. Therefore, small changes in manufacturing process variables (such as temperature, pH, time, agitation speed, quality of starting materials, etc.) can significantly affect the quality, efficacy, and safety of the final vector [127]. It is important to stablish procedures to assess nanotherapeutics not only at final steps but also at intermediate ones. Moreover, the application of concepts of quality by design (QbD) based on quality guidelines introduced by the International Conference on Harmonisation (ICH Q8, Q9, and Q10 [128]) has been proposed to address questions related to manufacturing processes and CMC complexities [123, 127]. The aim of QbD fundamentally aims at building quality and safety from the first design steps of the product [129]. This methodology intends for establishing a multidimensional design that defines process input requirements and operational ranges necessary to ensure that the product meets critical quality attributes. Designers of new nanotherapeutics will gain an understanding of these concepts and the role their preliminary data plays in preparing and positioning a potential nanoparticulate system for a gene therapy product development.

5 Applications of Gene Therapy

The first major clinical advance in the state of the field of gene therapy was in 1990, when the adenosine deaminase (ADA) gene was administered to a 4-year-old girl to treat the severe combined immunodeficiency (SCID) she suffered [130]. This clinical trial fostered the launching of additional studies, one of them in the year 2000 for patients with the X-linked form of SCID [131], which was an important landmark for gene therapy. On the one hand, it provided for the first time a demonstration of therapeutic effect of gene transfer for the treatment of a genetic disease. On the other hand, two of the patients developed T-cell leukemia as a result of insertional oncogenesis related to the retroviral vector used [57], which dampened the perspectives on gene therapy. Nonetheless, other potential hazards derived

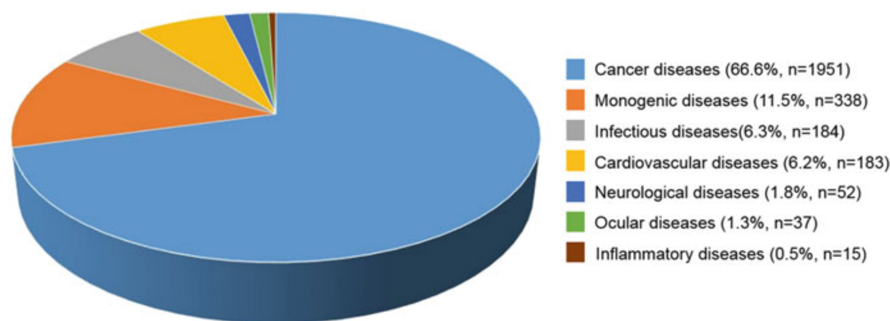


Fig. 12 Indications addressed by gene therapy clinical trials [54]

from the use of viral vectors to administer the genetic material were already known. In 1999, a systemic inflammatory response to the dosage of adenoviral vector administered into the right hepatic artery for the treatment of ornithine transcarbamylase (OTC) deficiency caused the death of Jesse Gelsinger, an 18-year-old male with partial OTC deficiency who participated in a pilot (safety) study of gene therapy [74].

Gene therapy has survived its previous failures, and it has emerged, thanks to the improvements of viral and non-viral vectors, the management of immune reactions, and the use of new mechanisms of action. Actually, a large number of clinical trials, almost 2,700 undertaken in 38 different countries, have been approved globally since 1989, and as can be seen in Fig. 12, most of them addressed cancer [54].

The extensive research activity in this field has not led to a significant number of gene therapy-based approvals. Since 2003, when Gencidine®, indicated for the treatment of head and neck squamous cell carcinoma, received approval in China as the first gene therapy medicinal product (GTMP) marketed worldwide [132], only 15 new products have been approved. However, GTMPs are becoming an emerging and expanding class of innovative medicinal products that can offer a more specific and causal/targeted treatment of many rare diseases, including rare cancers [133]. Gene therapy may be initially approved for patients who are lacking other therapeutic options, including conditions that in absence of treatment can cause disability or early death and conditions that require intensive and onerous maintenance therapy in form of enzyme or protein replacement. For these patients, gene therapy could offer long-term stabilization or improvement of their health, with the ultimate objective of obtaining a cure [134]. Table 4 shows nucleic acid-based products, including antisense oligonucleotides (ASOs) and gene-engineered cells, commercialized until present [135–137].

Apart from the five gene therapy products approved (Gencidine®, Oncorine®, Glybera®, Imlygic®, and Luxturna®), products based on ASOs, small interfering RNAs (siRNA), or aptamers have been also authorized, which have yet to exert a profound influence on the biopharma product landscape.

Kymriah®, Yescarta®, Zalmoxis®, and Strimvelis™ may be categorized as both cell and gene therapies. In all these cases, genetic modification is undertaken ex vivo

Table 4 Nucleic acid-based products approved

Product	Year/ agency (first approval)	Company	Indication/ administration route	Strategy/vector
Vitravene® (fomivirsen)	1998/ FDA	Isis Pharmaceuticals	CMV retinitis in AIDS patients/ intravitreal injection	In vivo – ASO
Gencidine®	2003/ SDFA	SiBiono GeneTech	Head and neck carcinoma/ intratumoral	In vivo – gene augmentation/ AD5
Macugen® (pegaptanib)	2004/ FDA	EyeTech	Wet form of AMD/intravitreal injection	In vivo – aptamer
Oncorine®	2005/ SDFA	Sunway Biotech	Nasopharyngeal cancer/local	In vivo – oncolytic virus/ AD5
Glybera® (alipogene tiparvovec)	2012/ EMA	UniQure	Lipoprotein lipase deficiency/ intramuscular	In vivo – gene augmentation/ AAV1
Kynamro® (mipomersen)	2013/ FDA	Kastle Therapeutics	Familial hypercholes-terolemia/subcutane-ous injection	In vivo – ASO
Imlygic® (Talimogene laherparepvec)	2015/ FDA	BioVex	Melanoma/local	In vivo – oncolitic virus/HSV-1
Spinraza® (nusinersen)	2016/ FDA	Biogen	Spinal muscular atro-phy/intrathecal	In vivo – ASO
Exondys 51® (eteplirsen)	2016/ FDA	Sarepta Therapeutics	Duchenne muscular dystrophy/intrave-nous infusion	In vivo – ASO
Zalmoxis® (nalotimagene carmaleucel)	2016/ EMA	MolMed	Haploidentical HSC transplant/intravenous infusion	Ex vivo – alloge-neic T cells genet-ically modified
Strimvelis™	2016/ EMA	Orchard Therapeutics	ADA-SCID/intrave-nous infusion	Ex vivo – autolo-gous CD34+ geneti-cally modified
Luxturna® (voretigene neparvovec)	2017/ FDA	Spark Therapeutics	Retinal dystrophy (RPE65)/subretinal	In vivo – gene augmentation/ AAV2
Kymriah® (tisagenlecleucel)	2017/ FDA	Novartis	ALL and DLBCL/ intravenous infusion	Ex vivo – autolo-gous CAR T cell
Yescarta® (axicabtagene ciloleucel)	2018/ FDA	Kite Pharma	DLBCL and PMBCL/ intravenous infusion	Ex vivo – autolo-gous CAR T cell
Tegsedi® (inotersen)	2018/ EMA	Akcea Therapeutics	hATTR/subcutaneous injection	In vivo – ASO

(continued)

Table 4 (continued)

Product	Year/ agency (first approval)	Company	Indication/ administration route	Strategy/vector
Onpattro® (patisiran)	2018/ FDA	Alnylam Pharmaceuticals	hATTR/intravenous infusion	In vivo – siRNA/ lipid nanoparticles
Zolgensma® (onasemnogene abeparvovec-xioi)	2019/ FDA	AveXis, Inc.	Spinal muscular atrophy/intravenous infusion	In vivo – gene augmentation/ AAV9

FDA Food and Drug Administration, *CMV* cytomegalovirus, *AIDS* acquired immunodeficiency syndrome, *ASO* antisense oligonucleotides, *SFDA* China State Food and Drug Administration, *AD5* adenovirus serotype 5, *AMD* age-related macular degeneration, *EMA* European Medicines Agency, *AAV1* adeno-associated virus serotype 1, *HSV-1* herpes simplex virus type 1, *HSCT* hematopoietic stem cell transplant, *ADA-SCID* severe combined immunodeficiency due to adenosine deaminase deficiency, *AAV2* adeno-associated virus serotype 1, *ALL* B-cell acute lymphoblastic leukemia, *DLBCL* diffuse large B-cell lymphoma, *PMBCL* primary mediastinal large B-cell lymphoma, *CAR* chimeric antigen receptor, *hATTR* hereditary transthyretin amyloidosis, *siRNA* small interfering ribonucleic acid

using a viral vector to achieve transduction, followed by infusion of the genetically modified cells into the patient. Kymriah®, Yescarta®, and Strimvelis™ use autologous cells, whereas Zalmoxis® uses allogeneic cells as a starting point. Strimvelis™ is a hematopoietic stem cell therapy, and the other three are T-cell therapies, being Kymriah® and Yescarta® the first chimeric antigen receptor (CAR) T-cell-based products. All four products have orphan status or target niche conditions and either are under additional monitoring or require further post-authorization safety studies.

Out of the total of GTMPs approvals, Vitravene® and Glybera® were withdrawn from market in 2002 and 2017, respectively. Moreover, the authorizations for use in the EU of Kynamro® and Exondys 51® were refused in 2012 and 2018, respectively.

A major challenge that faces most of the GTMPs is high development and production cost, which has led to pricing and reimbursement issues. A representative example is Glybera®, an adeno-associated virus serotype 1-based gene therapy for intramuscular administration in adult patients with familial lipoprotein lipase deficiency, a rare autosomal recessive disorder. Glybera® was commercialized in 2012 with a price of $1m per treatment, and it was pulled from market in 2017 despite being therapeutically successful [138].

As challenging as the generally high price is the developmental timeline of GTMPs that typically span two or even three decades from concept introduction to commercialization [138]. For instance, Kymriah®, the first CAR T-cell therapy, was approved by the FDA in 2017, after almost 30 years since the concept of redirecting T cells' potential to kill cancerous cells was introduced [139]. In the same way, in the case of Strimvelis™, it took nearly 15 years since the onset of the preclinical in vivo study [140] before its developer Fondazione Telethon (Italy) received orphan designation status from the European Commission in 2005. In this sense, there is already a similar precedent with the monoclonal antibodies; it took more than three

decades to get the commercialization of these products to become the primary drivers of the pharmaceutical market.

Besides the economic factors, other aspects have contributed to the low level of commercialization of GTMPs, such as the complexity of the technologies, difficulties in manufacturing processes, and regulatory barriers [138]. GTMPs face significant additional regulatory challenges when pursuing market approval due to the risks and concerns gene therapies which must be accounted during the regulatory process [141]. Moreover, the mismatch between the capacity of manufacturing vectors and the requirement of these emerging therapies is an important hurdle that is slowing down gene therapy progress [142].

Nevertheless, considering not only the intensive investigation performed but also the recent advances in the gene-editing field and T-cell-based therapies, GTMPs will undoubtedly have a substantial contribution on the biopharmaceutical market over the years to come. Furthermore, with several product candidates now undergoing regulatory review, it appears likely that clinicians will have increasing opportunities to generate their own assessments of gene therapy as a treatment modality [7].

5.1 Gene Therapy Medicinal Products for Cancer

Cancer diseases that have been targeted by gene therapy are primarily those that do not respond well to conventional treatment such as metastatic melanoma or glioblastoma. As mentioned above, the first gene therapy marketed was Gencidine®, approved in 2003 for the treatment of head and neck squamous cell carcinoma by the China State Food and Drug Administration (SFDA), although it is not available in the United States or Europe. Gencidine is a type 5 recombinant adenovirus, which has the E1 region replaced by a Rous sarcoma virus promoter linked with the human wild-type p53 gene and a poly (A) tail [143]. The tumor suppressor p53 and its target genes are essential regulators of cell cycle control and induction of apoptosis. The p53 signaling cascade modulates cell cycle and DNA repair to maintain the genetic integrity of cells. If irreparable DNA damages occur, p53 activates cellular apoptotic pathways to eliminate genetically damaged cells [144]. Gencidine®, administered by intratumoral injection, induces the expression of the tumor suppressor protein p53 causing growth arrest and apoptosis in tumor cells. However, the antitumor effects depend on the expression level of transduced p53 and on the integrity in p53-mediated cascades in the target tumors [145].

Another approach to address the treatment of cancer is the use of oncolytic viruses (OV) that selectively replicate in tumor cells without harming normal cells. Recombinant virus technology has allowed the development of conditionally replicating viruses, being Oncorine® (H101) the first OV marketed, which received approval in China in 2005 for treatment of nasopharyngeal cancer after intratumoral administration [146]. Oncorine® is a type 5 adenovirus with E1B-55KD and partial E3 deletion that cannot replicate in normal cells where p53 is active; therefore, it can selectively infect and kill tumor cells via the targeting of pro-apoptosis. The first

OV to gain approval by the FDA and EMA as an anticancer therapy was talimogene laherparepvec (Imlygic®), approved in 2015 for melanoma treatment. It is a modified type 1 herpes simplex virus (HSV-1) engineered to express human granulocyte-macrophage colony-stimulating factor (GM-CSF). The insertion of GM-CSF in place of both loci of the ICP34.5 gene, as well as by the deletion of the ICP47 gene, increased the selective replication within tumor cells, enhancing the tumor-specific immune response [147, 148]. The treatment is administered as a series of subcutaneous or intranodal injections over at least 6 months, and it has an estimated average cost of US$65,000 [134].

Targeting a sufficient number of cells, even when the vector could be injected into the tumors directly and repeatedly, represented a serious obstacle to achieving full efficacy. Furthermore, considering that metastasis is the source of mortality for most cancers, systemic gene therapy is of considerable interest, and nowadays it is available with the ex vivo infusion of genetically modified hematopoietic T cells. In this sense, genetically modified immune T cells represent a new class of therapeutics that has shown encouraging success for the treatment of some types of cancer. However, specialized manufacturing facilities and personal trained to conduct customized procedures for such therapies are vital to ensure accessibility and quality of care [134].

Zalmoxis® (nalotimagene carmaleucel) is an ex vivo GTMP approved by EMA in 2016 as adjunctive treatment in haploidentical hematopoietic stem cell transplantation (Haplo-HSCT) of adult patients with high-risk hematological malignancies. Haplo-HSCT can be associated with prolonged immunodeficiency posttransplantation, and Zalmoxis® aids immune reconstitution and reduces the risk of graft-versus-host disease [149]. This GTMP is based on allogenic somatic T cells genetically modified with a retroviral vector to express the herpes simplex thymidine kinase (HSV-TK) suicide gene and a truncated form of the human low-affinity nerve growth factor receptor (ΔLNGFR) genes (for identification of transduced cells). The expression of the HSV-TK gene allows the selective killing of T cells that have this suicide gene, upon administration of ganciclovir or valganciclovir, preventing further development if the patient develops graft-versus-host disease [150].

The most recent therapies approved by FDA and EMA against cancer are Kymriah® (tisagenlecleucel) and Yescarta® (axicabtagene ciloleucel), the first chimeric antigen receptor (CAR) T cell-based products, being both CD19-directed genetically modified autologous CAR T-cell immunotherapies. CARs consist of an antigen-binding domain, from either an immunoglobulin molecule or a T-cell receptor, fused to an intracellular signaling domain, from receptors such as CD28, OX40, and CD137, that mediates activation and costimulation to enhance T-cell function and persistence [47]. CARs recognize antigens independently of the major histocompatibility complex (MHC), which endows the CAR T cell with a fundamental antitumor advantage, because a major mechanism of immunoevasion by cancer is loss of MHC-associated antigen presentation by tumor cells. Another advantage is that CARs target nonprotein surface molecules, like carbohydrates and glycolipids. One limitation of current CAR T-cell strategies is that they require extracellular surface targets on the tumor cells [151].

CD19 is at present the most common CAR target; CD19 displays frequent and high-level expression in B-cell malignancies, it is required for normal B-cell development in humans, and it is not expressed outside of the B-cell lineage, which makes CD19 a nearly ideal target. For CAR T-cell therapy manufacture, T cells are isolated from the blood of the patient, activated, and then genetically engineered to express the CAR construct. T cells are modified by using a lentiviral or a retroviral vector for Kymriah® and Yescarta®, respectively. After ex vivo expansion of the CAR T cells, they are formulated into the final product for direct infusion [152]. However, CAR T-cell administration has been associated with serious systemic toxicities that often require intensive care and in some instances have caused patient deaths. To date, the most prevalent adverse effects following infusion of CAR T cells result from on-target T-cell activation, including cytokine release syndrome, macrophage activation syndrome, and tumor lysis syndrome [7].

Kymriah® and Yescarta® have a US list price of $475,000 and of $373,000, respectively [134]. Both in the United States and in EU, one of the indications of Kymriah® is the treatment of pediatric and young adult patients up to 25 years of age with B-cell acute lymphoblastic leukemia (ALL) that is refractory, in relapse posttransplant or in second or later relapse. The other indication is the treatment of adult patients with relapsed or refractory diffuse large B-cell lymphoma (DLBCL), after two or more lines of systemic therapy. Yescarta® is indicated for the treatment of adult patients with relapsed or refractory diffuse large B-cell lymphoma (DLBCL) and primary mediastinal large B-cell lymphoma (PMBCL), after two or more lines of systemic therapy (EMA).

5.2 Gene Therapy Medicinal Products for Other Applications

The resurgence of gene therapy in recent years for the treatment of genetic diseases is largely due to successes in trials that utilized both ex vivo strategies (for X-linked SCID and adrenoleukodystrophy) and in vivo approaches (Leber congenital amaurosis type 2 and hemophilia B) [7]. Gene therapies to treat rare disorders caused by single-gene mutations have made the most progress toward market availability. It has to be considered that in many of these diseases, there are few treatment options apart from supportive and symptomatic care. Development of gene therapies has also been influenced by ease of administration in target tissues, i.e., diseases of the eye and hematopoietic system.

Among the organs targeted by gene therapy, the eye has been at the forefront of translational gene therapy largely due to appropriate disease targets and its suitable anatomic features [153]. In fact, fomivirsen (Vitravene®) indicated for the treatment of cytomegalovirus retinitis (CMV) in patients with AIDS was the first therapeutic ASO approved by FDA in 1998. It was administered intraocularly, and its target was the mRNA that encoded the CMV immediate early (IE) 2 protein, which is required for viral replication. EMA also approved this product in 1999; however, Novartis stopped marketing the drug in 2002 in Europe and in 2006 in the United States [154].

Likewise, pegaptanib (Macugen®), indicated for the treatment of age-related macular degeneration, was the first therapeutic aptamer approved by FDA in 2004. Macugen® is an RNA aptamer for intravitreal administration that consists of 28 nucleotides that binds to 165 isoform of VEGF (vascular endothelial growth factor). Its anti-angiogenic effect not only stops the excessive growth of blood vessels but also prevents the formation of defective blood vessels [155].

Moreover, FDA and EMA approved Luxturna® in 2017 and 2018, respectively, for the treatment of adult and pediatric patients with vision loss due to inherited retinal dystrophy caused by confirmed biallelic *RPE65* mutations and who have sufficient viable retinal cells. Biallelic mutations in the *RPE65* gene, which encodes the all-trans-retinyl ester isomerase, in this gene, can be described as Leber congenital amaurosis type 2, retinitis pigmentosa type 20, early-onset retinal dystrophy, and other clinical labels for severe rod-mediated inherited retinal dystrophies, which all eventually progress to complete blindness [156]. Luxturna® was designated an orphan medicine by EMA for two forms of the disease, retinitis pigmentosa in 2015 and Leber's congenital amaurosis in 2012. Voretigene neparvovec (Luxturna®) consists of a recombinant adeno-associated virus serotype 2 vector carrying a functional *RPE65* gene. This gene augmentation therapy is given by bilateral subretinal injection and has a list price of US$425,000 (per eye treatment).

Ex vivo gene therapy approaches have mainly targeted hematopoietic system, being Strimvelis™ the first ex vivo GTMP approved for the treatment of an inherited disorder, adenosine deaminase deficiency-severe combined immunodeficiency (ADA-SCID). This recessive immune disorder is caused by mutations in the *ADA* gene and characterized by the absence of cellular and humoral immune function and a fatal outcome very early in life. Strimvelis™, with orphan designation and approved in 2016 by EMA under additional monitoring, saw the first clinical application on a single patient in March 2017. This hematopoietic stem cell therapy is indicated for the treatment of patients with ADA-SCID, for whom no suitable human leukocyte antigen (HLA)-matched related stem cell donor is available [134]. Patients receive CD34+ cells transduced with retroviral vector that encodes for the human ADA cDNA sequence. The genetically modified autologous CD34+ cells act by repopulating the hematopoietic system with cells that express active levels of the ADA enzyme. Strimvelis™, with a list price of €594,000, should be administered by intravenous infusion in a specialized transplant center. These ex vivo therapies require complex procedures and trained personnel to harvest, transduce, and reinfuse the hematopoietic target cells, and in the case of Strimvelis™, it is currently available only at a single center in Milan to where the patient and family are required to go for treatment [7].

Apart from gene therapy medicines, different nucleic acid-based products have also been approved for the treatment of diverse genetic diseases, including spinal muscular atrophy (SMA), Duchenne muscular dystrophy (DMD), familial hypercholesterolemia, and hereditary transthyretin amyloidosis (hATTR).

SMA is an autosomal recessive neurodegenerative rare disease that, in most cases, involves homozygous deletion of the survival of motor neuron 1 (*SMN1*) gene on chromosome 5q. As a consequence, patients suffer the deficiency of the survival of motor neuron (SNM) protein which plays a critical role in motor neuron

development. *SMN1* is one of two nearly identical genes that encode SMN; the other is survival of motor neuron 2 (*SMN2*). Infants with a more severe form of the disease (type 1 SMA) die before 2 years of age; later onset of the disease in infants is referred to as SMA2. FDA has approved in 2019 Zolgensma® (onasemnogene abeparvovec-xioi), a recombinant AAV9-based gene therapy designed to deliver a copy of the gene encoding the human SMN protein. Zolgensma® is administered as infusion, and it is indicated for the treatment of pediatric patients less than 2 years of age with SMA with biallelic mutations in the SMN1 gene. This product, with a price of $2.1 million, is the most expensive drug in the world.

Nusinersen (Spinraza®) is an ASO approved in 2016 by FDA and in 2017 by EMA (orphan medicine in 2012) for treating patients with SMA. Biogen has priced Spinraza® at $750,000 for the first year's treatment ($125,000 per injection) and $350,000 per year subsequently [154].

SMN protein is mainly produced from *SMN1*, whereas *SMN2* produces a small amount of full-length SMN protein. Typically, a higher number of copies of *SMN2* are associated with a less severe phenotype of the pathology. However, since the amount of protein formed is low, even multiple copies of *SMN2* do not fully stop the disease. The intron 7 in *SMN2* contains an intronic splicing silencer (termed ISS-N1) with binding sites for negative splicing factors (NSFs). Binding of these NSFs to intron 7 pre-mRNA precludes the recognition of exon 7 during the splicing process. The ASO nusinersen blocks the ISS-N1 site preventing the binding of the NSFs. As a result, Spinraza® administered via intrathecal injections modulates the splicing of the *SMN2* mRNA transcript to include exon 7, thereby increasing the production of full-length SMN functional protein [157].

DMD is a rare X-linked disease characterized by loss-of-function mutations in the *DMD* gene coding for dystrophin, which disrupt the reading frame of the dystrophin mRNA and cause the introduction of premature stop codons, leading to mRNA degradation and the loss of protein synthesis in striated muscle. It is a fatal disorder characterized by progressive muscle weakening and wasting, with boys losing ambulation by 12 years of age or earlier; death often occurs in the 20s, usually due to respiratory or cardiac complications [154, 158]. Eteplirsen (Exondys 51®) is a 30-nucleotide phosphorodiamidate morpholino oligomer and was approved as an ASO drug in 2016 by FDA, although authorization for use in the EU was refused by EMA in 2018. Eteplirsen promotes dystrophin production by restoring the translational reading frame of DMD through specific skipping of exon 51 in defective gene variants. The therapeutic strategy of antisense-mediated "exon skipping" is developed to force exon exclusion from mature mRNA of DMD with the purpose of restoring reading frame. Eteplirsen is suitable for 14% of DMD patients with *DMD* mutations, it is administered by intravenous injection, and it has a price of $300,000/patient/year [159].

Familial hypercholesterolemia is an autosomal dominant genetic condition resulting from mutations of the low-density lipoprotein cholesterol (LDL-C) receptor, apolipoprotein B (ApoB), or pro-protein convertase subtilisin/kexin 9 (PCSK9) [160]. Mipomersen (Kynamro®) is an orphan medicine approved in 2013 by FDA but with refused authorization for use in the EU by EMA in 2012. Mipomersen is a

single-stranded synthetic DNA ASO targeting ApoB-100, resulting in suppression of the hepatic production of the ApoB, total cholesterol, LDL-C, and non-high-density lipoprotein (HDL) cholesterol lipoproteins in rare genetic disorder patients with homozygous familial hypercholesterolemia. Kynamro® is available as a solution for injection under the skin. Due to the serious risk of liver toxicity, mipomersen is labeled a black box warning hepatotoxicity by FDA [159].

hATTR is a rare, autosomal dominantly inherited, progressively debilitating, and life-threatening disease. Misfolded transthyretin (TTR) proteins accumulate as amyloid deposits at multiple sites culminating in intractable peripheral sensorimotor neuropathy and, in many cases, autonomic neuropathy and/or cardiomyopathy. Recently, two different nucleic acid-based products have been approved for the treatment of hATTR, by using two different strategies [35]. Inotersen (Tegsedi®) is an ASO designed to suppress the expression of both wild-type and mutant forms of TTR. It has recently gained marketing authorization approval by EMA in 2018 (orphan designation in 2014) under additional monitoring for the treatment of stage 1 or stage 2 polyneuropathy in adult patients with hATTR and regulatory approval from the FDA for the treatment of the polyneuropathy of hATTR. It is available as a solution for injection under the skin in pre-filled syringes, and the recommended dose is one injection once a week [161]. Onpattro® (Patisiran) is a siRNA designed to target TTR to reduce the levels of both wild-type and mutant TTR. Patisiran is formulated in lipid nanoparticles that direct the siRNA to the liver, the primary site of TTR production. Patisiran, with the same indications that of inotersen, is available as a solution for infusion and received regulatory approval in 2018 from the FDA and EMA (orphan designation in 2011) [35].

6 Challenges of Gene Therapy

As has been commented in this chapter, gene therapy has still many challenges to be overcome: the science is complex, treatment is technically difficult, and the regulatory approval process is necessarily different to that for conventional therapies. Actually, it has been considered as the most complex "drugs" ever developed [47]. Efficacy, safety, and manufacturing issues are important challenges that must be faced. There are also difficult questions about cost, accessibility, and social justice that will need answers once the methods are shown to be effective and safe.

6.1 Efficacy Issues

The low efficacy, which may lead to treatment failure, is one of the most important challenges of gene therapy [162]. The development of new delivery systems with higher transduction rates and higher affinity to a specific cell or tissue is necessary to increase the number of products that reach clinical evaluation. Another

reason that may explain the low efficacy of a gene therapy product is the presence of endogenous natural antibodies against viral vectors or the transgene product. This is of particular importance because it prevents transduction and limits gene therapy product administration more than once.

6.2 Safety Issues

Potential immunogenicity and oncogenicity are the main challenges concerning safety. When a gene vector integrates into the host genome, there is potential for gene disruption and insertional mutagenesis giving with an increase in risk of tumor arising from the genetically modified cells. This occurs as a consequence of activation/upregulation of oncogenes or inactivation or downregulation of tumor/suppressing genes [162]. To overcome the potential oncogenicity of viral vectors, it is crucial to design new vectors that prevent the activation of oncogenic genes at integrations sites. The use of non-integrating vectors or highly targeted genomic integration at the desired chromosomal loci is also helpful.

Immunogenicity reactions may be due to both the viral vector and the transgene product due to the unpredictability of innate and antigen-dependent immune responses in humans [162]. One additional problem is that these responses are difficult to detect in animal models, as such effects arising in animals, who may have been given human product, are not indicative of whether such would occur in humans. Some alternatives to prevent immunogenicity include the administration of immunosuppressive agents prior or after the administration of the gene therapy product, the modification of the capsid proteins of the vector, and the elimination of viral genes.

6.3 Drug Development and Manufacture Issues

Because of its unique set of characteristics, the nonclinical development package of a gene therapy medicinal product is rather more complex than conventional medicinal products. One important difference with respect to a conventional drug product is that the approval of a gene therapy product must be based not only on data of the therapeutic transgene but also of data on the vector/delivery system.

Manufacturing of gene therapy products is an additional complexity factor. In the near future, there is a challenge for the development of manufacturing capacities, which must be sufficient to meet the coming demand, specifically the AAV production. In this sense, several companies of viral vector production are amplifying their manufacturing facilities to face the future increase demand for viral production [142].

6.4 Ethical Issues

Currently, at least in the Western countries, clinical use of gene therapy is limited to somatic cells for the treatment of a specific disease. As it has been explained above, germline gene therapy leads to hereditary modifications that pass on to subsequent generations, and therefore, it is the object of a heated discussion [163]. The recent announcement by a Chinese research of the birth of twins whose genomes were edited by CRISPR/Cas9 during in vitro fertilization has engendered broad condemnation for the premature clinical deployment of a still experimental biomedical area [164]. The organizing committee of "the Second International Summit on Human Genome Editing," held in Hong Kong in November 2018 under the auspices of the US National Academies of Science and Medicine, the UK Royal Society, and the Hong Kong Academy of Sciences, reiterated that "the scientific understanding and technical requirements for clinical practice remain too uncertain and the risks too great to permit clinical trials of germline editing at this time" [163].

The potential use of gene therapy for purposes other than diseases treatment is another important topic to be addressed. For instance, the application to eugenics, that is, the attempt to change or improve complex human traits related to a broader number of genes; such as, personality, intelligence, or character [162].

6.5 Affordability

Gene therapy-based medicines have a high cost of development, production, product storage, and transportation, which lead to very high prices. For instance, the price of Glybera was around 1,000,000 €; Strimvelis, 600,000 €; and Yescarta and Kymriah 300,000 and 400,000 €, respectively. Gene therapy medicinal products are expensive to develop and to manufacture, and sometimes, they are one-time treatments. These reasons, among others, may justify the high cost [164]. Affordability of novel innovative and high budget impact therapies has become an important topic in Europe, and so far, each country has come up with individual approaches to improve affordability [165]. For example, in the case of Zolgensma®, with an annualized cost of $425,000 per year for 5 years, the company proposes to payers to create 5-year outcomes-based agreements and novel pay-over-time options [166].

6.6 Intellectual Property Complexity

The complexity of the intellectual property "territories" that can surround a given gene therapy development also explains the slow progress of gene therapy [142]. In fact, a new gene therapy product under development may be conditioned by patents involving not only the therapeutic transgene itself but also its mechanism of

action, the non-viral or viral vector used as the delivery system, and the method for delivery of the nucleic acid therapy to the patient (e.g., the delivery devices, surgical techniques, and treatment protocols to be used).

Despite the numerous challenges, in the last years, important unified efforts by research and clinical scientists in academic, translational, and industry settings have resulted in tangible outcomes, with several marketing authorizations and approved commercial products. Initiatives for willingness to participate in clinical trials and equable access of patient population to somatic gene editing as a treatment option in clinical care are necessary to increase the opportunities to successful of gene therapy.

7 Conclusion

Gene therapy, regarded as one of the most promising and most active fields of medicine, is beginning to show encouraging results. Current therapies are primarily experimental with only a few gene therapy medicinal products on the market, although several product candidates are undergoing regulatory review.

The efforts and advances made in this area have led to the development of new therapeutic strategies to treat several disorders, many of them without currently available treatments. The remarkable basic, translational, and clinical research activity in gene therapy has been addressed mainly to cancer, but a significant number of clinical trials have also targeted a broad variety of diseases including monogenic, infectious, and cardiovascular diseases.

Nucleic acid-marketed products are based mainly on in vivo strategies. Initially gene augmentation was the main option, although ex vivo therapies and new ASOs seem to be major strategies at present. Moreover, the first product containing a siRNA has been already marketed. Recent strategies also include T-cell-based therapies, with two products marketed in 2018 for the treatment of hematological malignancies by immunotherapy, and the gene-editing tool CRISPR, whose rapid progress is boosting the development of new gene therapy-based medicines.

Despite the positive forecast that the nucleic acid-based products have on the biopharmaceutical market, different hurdles are slowing down their progress. The success of gene therapy may be compromised by two main challenges, the cost and reimbursement of the treatments, as well as the technological issues to ensure accessibility and quality of the treatments. The availability of specialized manufacturing personnel and facilities to conduct customized procedures and the progress in the manufacturing capacity of efficient and safe vectors to meet the upcoming demand of gene therapies are essential for the advance of this emerging therapy in the future.

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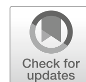
The Impact of Pharmacogenomics in Personalized Medicine



Dev Bukhsh Singh

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Abstract Recent advances in Pharmacogenomics have made it possible to understand the reasons behind the different response of a drug. Discovery of genetic variants and its association with the varying response of drug provide the basis for recommending a drug and its dose to an individual patient. Genetic makeup-based prescription, design, and implementation of therapy not only improve the outcome of treatments but also reduce the risk of toxicity and other adverse effects. A better understanding of individual variations and their effect on drug response, metabolism excretion, and toxicity will replace the trial-and-error approach of treatment. Evidence of the clinical utility of pharmacogenetics testing is only available for a few medications, and FDA labels only require pharmacogenetics testing for a small number of drugs. Although there is a great promise, there are not many examples

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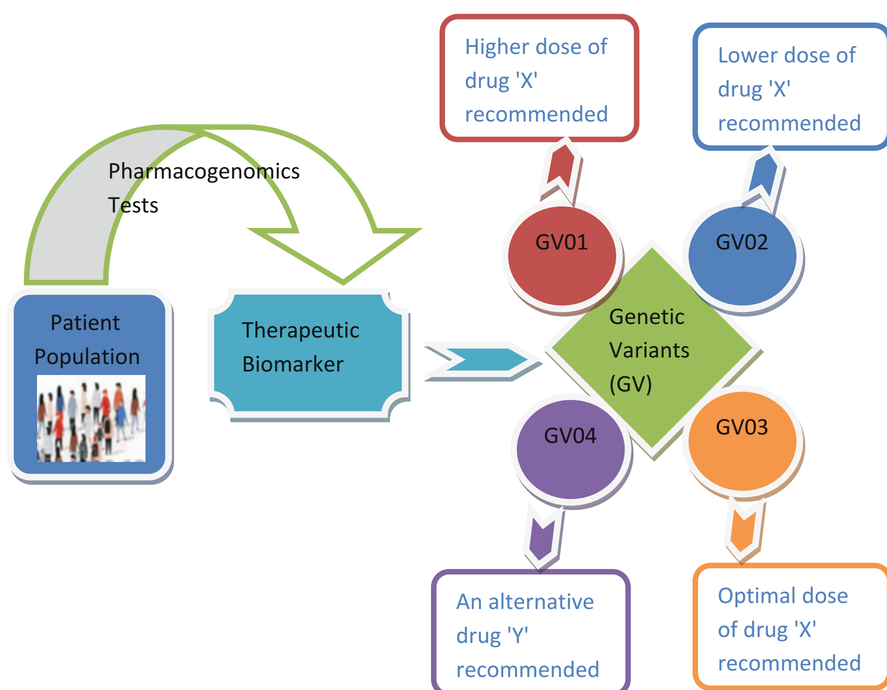
where Pharmacogenomics impacts clinical utility. Some genetic variants related to different diseases have been reported, and many have not been studied yet. The information related to the outcome of treatment with a particular drug and a genetic variant can be used to release a warning/label for the use of that drug. There are many limitations in the way of implementing the goal of personalized medicine. Future advances in the field of genomics, diagnosis approaches, data analysis, clinical decision-making, and sustainable business model for personalization of therapy can speed up the individualization of therapy based on genetic makeup.

Graphical Abstract



Keywords Clinical decision, Genetic makeup, Personalized medicine, Pharmacogenomics, Therapeutic response

1 Pharmacogenomics

Pharmacogenomics deals with the study of the genetic basis for varying response of drugs among individuals. It is an emerging and challenging field of therapy with a limited clinical utility and applicability. There are several factors such as environmental factors, age, weight, gender, and metabolism which affect the efficacy of a drug. The genetic makeup of the patient also decides the response drug of a drug

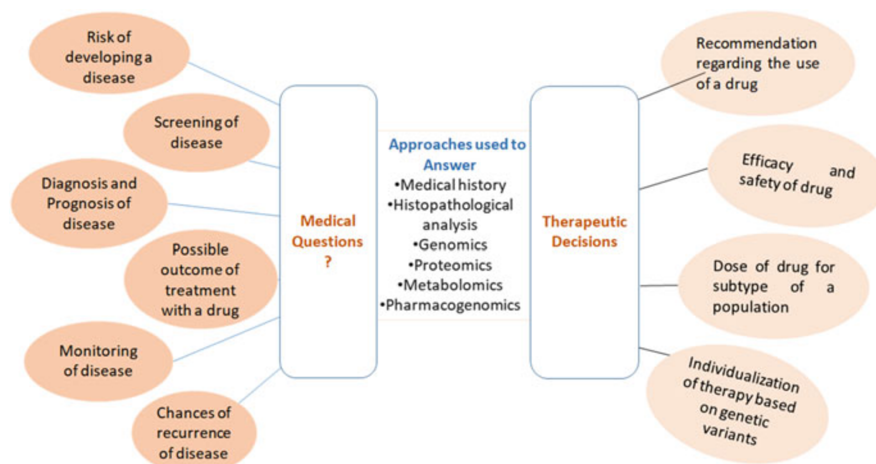


Fig. 1 Medical questions and use of omics and other approaches for better therapeutic decisions and individualization of therapy

[1]. There are many genetic variants associated with a disease. All genetic variants are not associated with the drug responses on treatment. Some genetic variants may be associated with the risk of developing a disease. The expression level of genes in different patients may be different. Patient with the higher expression level of a protein will produce a different response as compared to a patient with lower or no expression level of the same protein [2]. Many Pharmacogenomics tests have been developed for the treatment of some disease, but the clinical applicability of these tests is very limited.

Ethical issues related to the study and application of Pharmacogenomics should be addressed. There is a need to protect the confidentiality of patients, and all patients should have equal opportunity to get benefitted by personalized therapy. Pharmacogenomics can bring a significant improvement in the issues related to the safety and efficacy of the drug. Recent omics advances have made it possible to understand the significance of genetic variations on individual patient's response to a drug. Omics technologies have enabled us to answer the many medical questions and also promoted the personalization of therapies based on genetic makeup (Fig. 1). The long-term goal of Pharmacogenomics is to help medical practitioners in the diagnosis and prescription of a drug and its dosage, based on the patient's genetic makeup. The major objective of Pharmacogenomics is to study and catalog all the genetic and epigenetic variants that cause variation in drug response. Pharmacogenomics-related data, testing, and drug label are available for only some drugs. Pharmacogenomics-related study has been conducted for drugs used for the treatment of cancer, diabetes, depression, immunotherapy, anticonvulsant, anti-infective, cardiovascular, and psychotropic drugs, as well as for some other therapies.

Several deaths occur due to the adverse effect of the drug. Pharmacogenomics tests have reduced the cases of adverse drug reactions and also ignored the trial-and-

error approach of therapy. Thus, Pharmacogenomics approaches may lower down the cost of therapy for patients and physicians significantly. Currently, most of drug and dose prescriptions are based on age, weight, sex, lifestyle, and level of liver and kidney enzymes. Scientists can identify the genetic variation in a small subset of population which is associated with varying response to a drug [3]. The Food and Drug Administration (FDA) includes Pharmacogenomics information such as dosage guidelines, side effects, and efficacy of 150 drugs for patients with certain genetic makeup.

2 Factors Affecting Response of Drug

2.1 Genetic Polymorphism

Eighty-five percent of human diversity at Short Tandem Repeat (STR) and Restriction Fragment Length Polymorphism (RFLP) autosomal loci is the reason behind differences between individuals of the same population, whereas differences between individuals of the same continent account for 5–10% [4]. Variation in drug response is not the only result of a mutation in a single gene but also happens by the altered function of related genes. Variations in genes associated with pharmacokinetics and pharmacodynamics of drug may result in toxic, altered, or no response. These ADME-related variations decide the effective concentration of drug that reaches to drug target and its metabolism [5].

The information of genetic variants associated with cancer or other disease is very useful in early diagnosis of disease and also provides the basis for personalized therapy. Next-generation sequencing (NGS) and genome-wide association studies (GWAS) have made it possible to understand the genetic mechanisms involved in a disease and also promote the individualization of therapy based on genetic makeup. The GWAS have discovered many genetic variants related to different lethal diseases. These variants can be used for developing a biomarker for diagnosis and therapeutic categorization of a particular disease. The systematic cataloguing of genetic variants and related outcome on therapy provide the information of pathways and related enzymes and also explain the reason behind the varied response of treatment by different patients [6]. Genetic variants can provide better and accurate clinical decisions along with the family history of the disease. Rare genetic variants cannot be easily identified by GWAS, and they may have a significant effect on the risk of disease [7]. The 1000 genome projects have identified many genetic variants at lower frequencies [8]. The Human Genome Project has covered the sequencing of the entire human genome, including the 99.9% of the genome where all humans are identical in genetic makeup/composition [9]. The HapMap project has characterized the patterns of a DNA sequence within the 0.1% genome where a genetic variation exists among individuals. The HapMap has facilitated the development of some diagnostic tools and also guides the selection of drug target for the treatment of some

disease. Information's on polymorphism and related risk of occurrence of a disease can be used as an alert message for a patient.

2.2 Epigenetic and Other Factors

Epigenetic factors such as age, sex, liver and kidney function, lifestyle, previous disease, and adverse reaction are also decides the therapeutic response of a drug [10]. Old age patients have a high risk of adverse reaction due to the poor rate of physiology and metabolism. Gender-specific physiological differences such as pregnancy and breastfeeding also affect the outcome of treatment by a drug [11]. A significant difference between the level of different hormones between a male and female has been observed, which may cause a different response to a drug in male and female. Environmental chemicals, drugs, and natural compounds can alter the efficacy of the drug by drug-drug interaction or drug-herb interactions [12]. These factors can induce or inhibit drug-metabolizing enzymes and drug transporters.

The use of *Ginkgo biloba* (ginkgo) along with warfarin or aspirin results in bleeding and raises blood pressure, when used in combination with a thiazide diuretic [13]. The use of *Ginkgo biloba* (ginkgo) along with trazodone may cause coma in patients. Herb labels should also be available to avoid the use of a drug in combination with the herb. A drug-drug interaction (DDIs) involves pharmacokinetic or pharmacodynamic mechanisms which may affect the bioavailability, efficacy, and response of a drug. The pharmacokinetics and pharmacodynamics of many co-administered drugs are known, but the roles of co-administered herbs are not well explored due to a complex mixture of herbal extracts [14]. The pharmacokinetics and pharmacodynamics of drug-drug and herb-drug interactions cannot be ignored while prescribing a drug for therapy.

3 Pharmacogenomics Biomarker

Biomarkers include genetic or somatic gene variants, changes in expression level, functional irregularities, and chromosomal abnormality. Biomarkers may be used for diagnosis of diseases or assessment of therapeutic efficacy. Pharmacogenomics biomarker is used for prescribing the drug or its dose based on the level of biomarker and presence or absence of its variants. Some biomarker-related treatments of different diseases are available and can describe clinical response variability, risk of adverse drug reaction, a genotype-specific dose of a drug, mechanism of the drug, and polymorphic drug target. FDA-approved drugs for some disease and their biomarkers are listed in Table 1. It represents the drugs that can only be recommended to patients who carry a particular variant related to the biomarker. This can be very useful for prescribing safe and effective therapy based on the biomarker. Discovery of novel and potential biomarkers related to a disease can play a very important role in diagnosis, prescription, and personalization of therapy.

Table 1 List of Pharmacogenomics biomarkers used for the therapy of diseases [15]

Drug	Therapeutic area	Biomarker	Referenced subgroup	Outcome/efficacy
Abacavir	Infectious diseases (HIV)	HLA-B	HLA-B∗5701 allele carriers	High risk of immune-mediated hypersensitivity reaction and should not receive abacavir [16]
Afatinib (tyrosine kinase inhibitor)	Oncology	EGFR	EGFR exon 19 deletion or exon 21 substitution (L858R) positive	These mutants incur sensitivity to afatinib treatment [17]
Aripiprazole	Psychiatry	CYP2D6	CYP2D6 poor metabolizers	Half of the usual dose should be administered [18]
Carvedilol	Cardiology	CYP2D6	CYP2D6 poor metabolizers	High plasma concentrations of carvedilol, dose monitoring required [18]
Clobazam	Neurology	CYP2C19	CYP2C19 poor metabolizers	Avoid clobazam or start with a low dose (2.5 mg/day) [19]
Celecoxib	Rheumatology	CYP2C9	CYP2C9 poor metabolizers	Half/lower dose recommended [20]
Cisplatin	Oncology	TPMT	TPMT intermediate or poor metabolizers	Consider alternate drug or use lower dose to avoid ototoxicity [21]
Diazepam	Psychiatry	CYP2C19	CYP2C19 poor metabolizers	Consider lower dose to avoid prolonged sedation and unconsciousness [22]
Omeprazole	Gastroenterology	CYP2C19	CYP2C19 poor metabolizers	Lower the dose to avoid drug-drug interaction [23]

4 Pharmacogenomics Guidelines

Genotype-based dosing guideline has been published by clinical pharmacogenetics implementation consortium (CPIC), Dutch Pharmacogenetics Working Group (DPWG), or other organizations [24]. CPIC provides the supports for implementation of pharmacogenetics tests into clinical practice [25]. The DPWG is a multidisciplinary organization and includes the knowledge of experts such as pharmacists, pharmacologists, chemists, epidemiologists, and toxicologists [26]. The DPWG is also working for therapeutic (dose) recommendations based on pharmacogenetics data and guides the health practitioners and doctors in drug and dose prescription [27]. PGxOne™ provides the clinical Pharmacogenomics test and generates a relevant medical and clinical report which can guide the treatment of patients. PGxOne™ provides the dose-related guidelines for some drugs. [28] stated that “Justice demands that benefits of personalized medicine must be available to individuals of all racial and socioeconomic status” [28]. Peterson-Iyer [29]

has also suggested some recommendation for consideration in the policy of Pharmacogenomics [29].

5 Personalized Medicine

Personalized medicine is a therapeutic approach which utilizes the genetic and epigenetic information of an individual. The current trend of clinical therapy is to provide the medication to a particular patient based on its characteristics. Such personalization is achieved by the use of omics approaches which enable us to find the inter-individual variability at genomic, transcriptomic, proteomic, and metabolomic levels. The food and drug administration has approved some genomic companies for the screening of genetic health risks related to Alzheimer's disease and rare blood disorder. The current clinical treatment paradigm is the right drug, for the right patient, at the right time. Personalization of therapy requires data collection through diagnostic from patients, individualization of therapy based on analysis, and an appropriate and sustainable business model. Digital and mobile medical applications have played an important role in the characteristics of disease at the individual level. Many definitions of personalized medicine are available. Some definitions refer to medicine at an individual patient and while some consider its scope to a subpopulation. But, most examples of personalized medicine are not personalized. For example, a subgroup of women with early breast cancer and HER2 positive is suitable for treatment with trastuzumab. Personalized medicine is a subset of personalized health care. Personalized health care not only includes genomics but also considers the other information's which predicts the risk of disease and patients response to treatment.

Genetic variants associated with pharmacokinetics and pharmacodynamics of a drug decide the outcome or efficacy of treatment. For the application of Pharmacogenomics in cancer, the selection of a drug and target of drug both are important in deciding the response of treatment. It is important to decide whether a drug is based on the genetics of individuals or genetics of cancer cell or both. If a drug target to cancer cell, then the outcome of treatment depends on the genetics of individual as well also on the genetics of particular tumor cell.

A better understanding of heterogeneity that exists among individuals and diseases can help in implementing the goal of personalized medicine. The outcomes of the Human Genome Project have generated a lot of interest in the personalization of therapy by identifying the individual-level differences of diseases based on genetic makeup. Therapeutic decisions based on an individual's genetic profile and medical tests can be more accurate and efficacious in terms of response. The risk of disease and possible outcome of treatment can be estimated using information such as age, sex, genetic profile, expressions related to disease, medical tests, and history of the disease [30]. Disease screening tests and low-cost diagnostic tools can help in detection of fatal disease at an early stage, and these results can be utilized in making therapeutic decisions to improve the health status of a patient. Genomic information

greatly affects the dose and response of treatment. Dose efficacy and safety monitoring tests play a significant role in improving the status and cost-effectiveness of patient care.

As the result of omics effort, more than 10 million SNPs have been identified, and extensive studies on SNPs and related diseases have also been performed to find its role in clinical applications for Pharmacogenomics and personalized medicine [31]. There is a need to validate the biomarkers for patient stratification and dose selection. Clinical relevance, molecular mechanism, clinical evidence, and regulatory and clinical guidelines related to relevant SNPs can trigger the application of personalized medicine. Genomic informations are highly valuable in making a medical decision related to drug and dose. But, the overall therapeutic response is not only based on genomics test alone. Moreover, a better therapeutic response can be achieved by combining genomic test results with knowledge of age, sex, lifestyle, size, stage, nature, and origin of the disease. The nongenetic factors, such as environmental and clinical covariates, may provide important phenotypic information which can be used for better therapeutic decision [32]. In addition to CYP2C9 and VKORC1, the dose of warfarin therapy also depends on age, sex, body mass index, diet, and concomitant drug therapy.

5.1 Clinical Guidance for Personalization of Therapy

In previous decades, all patients with the disease were receiving the treatment with the same drug. But, now therapies have become more personalized. For example, breast cancer patients that produce estrogen receptor may be treated with the drug that targets the estrogen receptor. A significant fraction of patients (subtype) possessing a particular characteristic of the disease can be identified for treatment with a drug. This can be one of the ways to achieve the goal of personalized medicine. Different biomarkers related to a disease can be detected and quantified. Biomarkers can be used to divide the patients into different subtypes based on the susceptibility to disease and the outcome of treatment. Recent advances in technology have made it possible to sequence an entire genome of an individual and quantify all the proteins, metabolites, and microbiome in a tissue. Advance data analysis tools and algorithms can be used to mine the clinically significant biomarkers related to a disease. Regulatory guidelines related to the use of biomarkers as a diagnostic tool are needed, and it will help in the discovery and use of biomarkers in medical practice. Biomarker-based tests and related clinical guidance for the personalized treatment of some diseases are shown in Table 2. Advanced diagnostic approaches are allowing doctors to prescribe the drug and dose to patients based on their molecular profile [44]. There are several advanced diagnostic tests for different types of cancer which identifies the expression or mutation in genes related to the disease. These diagnostic tests are a key parameter for prescribing a drug to a patient. Personalized guidance regarding prevention of a disease can be given to a healthy person by analyzing his genome, proteome, metabolome, and microbiome.

Table 2 Some examples of personalized medicine and related clinical guidance

Therapy	Test and its description	Clinical guidance	Reference
Breast cancer	BRCA1: deleterious BRCA1 or BRCA2 mutants have a high risk of breast and ovarian cancer	Surveillance and chemo-prevention therapy	Redekop and Mladsi [30]
Breast cancer	HER2: tumors with HER2 overexpression	Use of Trastuzumab recommended	Redekop and Mladsi [30]
Epilepsy	HLA-B∗1502: HLA-B∗1502-positive patients show skin reactions on treatment with carbamazepine	Use of carbamazepine may be dangerous, choose an alternative drug	Redekop and Mladsi [30]
Atrial fibrillation	CYP2C9, VKORC1: dose of warfarin therapy partly dependent on CYP2C9 and VKORC1 genotypes	Dose of Warfarin	Redekop and Mladsi [30]
Hepatitis C	HCV RNA: measures the level of viral RNA after treatment with interferon alfa and ribavirin	Required duration of treatment	Redekop and Mladsi [30]
Antiplatelet medications	CYP2C19: clopidogrel is a prodrug that requires metabolic activation by CYP2C19	Use alternative antiplatelet agent other than clopidogrel for patients with decreased CYP2C19	Mousa et al. [33]
Immunosuppressive therapy	Thiopurine methyltransferase (TPMT): azathioprine is a prodrug converted to mercaptopurine that undergoes methylation to inactive metabolites by TPMT	Patients with decreased TPMT function have a higher risk for toxicity with azathioprine	Relling et al. [34]
Immunosuppressive therapy	CYP3A5: tacrolimus undergoes oxidative metabolism to inactive metabolites by CYP3A4/3A5 enzymes	Carriers of CYP3A5 alleles may require higher doses	Renders et al. [35]
Kidney transplants and invasive fungal infection	CYP2C19: metabolism of voriconazole occurs predominantly through CYP2C19	Use an alternative agent in case of CYP2C19 rapid or poor metabolizer	Guinea et al. [36]

(continued)

Table 2 (continued)

Therapy	Test and its description	Clinical guidance	Reference
Hyperlipidemia	SLCO1B1 SLCO1B1 is responsible for uptake of simvastatin from the blood to hepatocytes (metabolized). Simvastatin blood concentrations increases at reduced SLCO1B1 level	Use an alternative agent or lower dose in case of decreased SLCO1B1 activity	Armitage et al. [37]
Chronic myeloid leukemia (CML)	BCR and ABL gene are present on chromosome number 22 and 9, respectively. The BCR-ABL mutation occurs when pieces of BCR and ABL break off and switch places. BCR-ABL confirms the diagnosis of CML	Imatinib is a BCR-ABL tyrosine kinase inhibitor and can be used for treatment of BCR-ABL-based CML	Druker et al. [38]
Lung cancer	EML4-ALK fusion gene used for diagnosis of non-small cell lung cancer (NSCLC). Such types of cancer can be cured by targeting anaplastic lymphoma kinase (ALK) inhibitor	Crizotinib works only in cancer with overactive ALK	Kwak et al. [39]
Coronary artery disease	CYP2C19 converts clopidogrel into an active metabolite. Individuals who carry two nonfunctional copies of the CYP2C19 gene are poor metabolizers of clopidogrel	Use of clopidogrel may be toxic or less efficacious in poor metabolizer. Use another alternative agent/drug	Simon et al. [40]
Autoimmune encephalitis	CXCL13, aberrant expression of CXCL13, is linked to the development of autoimmune disorders	Antibodies targeting CXCL13-mediated signaling pathway	Leypoldt et al. [41]
Cystic fibrosis	G551D, G551D mutation in ATP-binding pockets abolishes ATP-dependent gating. It is one of the common mutation associated with cystic fibrosis	Ivacaftor recommended for the patients with the G551D mutation	Ramsey et al. [42]
Thrombosis	Factor V Leiden is a specific gene mutation that results in thrombophilia	Avoid prothrombotic drugs for factor V Leiden-positive patients	Vandenbroucke et al. [43]

Pharmacogenomics tests are responsible for drug metabolism, transport, and drug target. Genetic variations can increase the risk of toxicity or poor efficacy. Pharmacogenomics helps us to select the most suitable drug and dose to achieve a better therapeutic response. Several clinical recommendations for commonly used anticoagulants, antiplatelets, transplant medications, and other therapies are available [45]. Clinical decision-making depends on many factors such as variants in the patient or population and the quality of evidence, availability of testing and Pharmacogenomics data, pharmacokinetics and pharmacodynamics of drug, drug history, and drug-drug interactions. Service providers should also have a good knowledge of Pharmacogenomics resources to implement an accurate clinical decision. Pharmacogenomics mainly helps in the implementation of personalized medicine by predicting the patients who should receive a lower dose/higher dose or an alternative drug. The implementation of Pharmacogenomics is limited by challenges in clinical testing, data analysis, lack of education, and ethical, legal, and social implications. Despite the barriers to clinical Pharmacogenomics, several academic, medical, and community centers have initiated Pharmacogenomics implementation programs. Future advances in precision medicine and data sharing between health service providers and patients will help in achieving the goal of personalized medicine.

Imaging techniques can perform many diagnoses with good confidence and also avoids the patients to go for invasive testing and unnecessary surgery. Now computed tomography and ultrasonography provide better results with improved sensitivity and specificity in the diagnosis of many diseases [46]. Positron-emission tomography can detect metabolically active cancer that is not easily detectable with traditional imaging. A rich database of electronic health records can be a guide for clinical decision. In the future, software can be developed to identify patients with disease risk factors or to follow screening guidelines and also for drug selection and administration.

5.2 Impact of Pharmacogenomics on Personalized Medicine

5.2.1 Cancer

The concept of personalizing treatments provides the best possible direction for the treatment of many cancers. Different therapeutic strategies for the treatment of cancer are surgery, radiotherapy, chemotherapy, immunotherapy, hormone therapy, gene therapy, and dietary therapy. We can prefer therapies from a wide range of options based on the type, location, and stage of cancer. Current knowledge of cancer classification, predictive markers, and combination therapies holds the best possible avenues for cancer treatment in the future. The strategy is the most important issue in cancer therapy. As a result of pharmacogenomic advances, different therapeutic strategies for the treatment of various cancers have been adopted. Gene therapy approach has been used for the treatment of prostate cancer and as result necrosis observed at metastatic sites [47]. Personalized medicine is used

in cancer therapy for screening formulation in the epidermal growth factor receptor (EGFER) gene in lung adenocarcinoma.

Variants Associated with Risk of Cancer

Studies have shown an association between genetic variants of a gene and the risk of developing a disease. BRCA1, BRCA2, TP53, PTEN, STK11, CDH1, CHEK2, ATM, BRIP1, PALB2, RAD51C, RAD50, and NBN genes have been identified for breast cancer. Association of these genes with risk of disease has been further investigated. Three variants rs80358978 (Gly2508Ser), rs80359065 (Lys2729Asn), and rs11571653 (Met784Val) have been reported in the BRCA2 gene, and these variants have shown significant associations with risk of developing breast cancer [48]. Variants rs8176085, rs799923, rs8176173, and rs8176258 in the BRCA1 gene, one common variant in the CHEK2 gene (rs9620817), and one common variant in the PALB2 gene (rs13330119) are also associated with risk of this disease. Similarly, four genetic variants, Phe139Ser in SORD, Ala350Arg in KRT6A, Gly387Cys in SVEP1, and Gly144Arg in MRPL38, have shown significant association for risk of liver cancer [49]. These variants can be used for early detection of liver cancer and designing of relevant therapeutics for treatment. These variants only predict the risk of developing a particular type of cancer and may not be used for deciding the therapeutic outcome of treatment with a drug.

Nearly 100 genetic variants that signal an increased risk of colon cancer are available. Several combined genetic variants may be clinically relevant and can impact personalized screening. For breast cancer, 182 breast cancer susceptibility SNPs are available which can be used to understand the heritability of breast cancer [50]. Study of risk loci for breast cancer can be a guide for prevention, prognosis, and treatment of breast cancer. The polygenic risk score can be calculated to estimate individual risks for developing breast cancer. GWAS have identified more than 30 epithelial ovarian cancer risk loci which may involve genome editing to establish the cell-specific carcinogenic effects [51]. Studies have also identified susceptibility regions potentially shared between breast, ovarian, and prostate cancer. For example, in oral cancer, an association between TNF-α variant, rs361525, and risk of an oral precancerous lesion has been observed. An allelic variant of rs361525 SNP, located at a transcriptional factor-binding site of the promoter region of TNF-α, disturbs the expression of TNF-α [52]. Studies have shown that genetic alterations in immune system genes and genes with metastatic potential are associated with oral precancer and oral squamous cell carcinoma.

Variants Associated with Outcome of Treatment

Afatinib is a TKI inhibitor used for the treatment of patients with non-small-cell lung cancer (NSCLC) whose tumors are activated by EGFR [53]. It is also effective for NSCLC patients with EGFR-T790M mutation who are resistance to the TKIs

erlotinib and gefitinib. Afatinib has shown very good response among patients with tumors having HER2 or HER4 mutations [54]. HER2 mutations are rare; afatinib has shown high activity for NSCLC patients with HER2 mutation. Imatinib is a TKI inhibitor used for the treatment of CML. It has also been used for other BCR-ABL-, c-KIT-, and PDGFR-driven cancers. Imatinib is metabolized by CYP2C8 and CYP3A4 in vitro. CYP2C8 genotype affects the metabolism of imatinib and its systemic exposure [55]. But, no evidence regarding the metabolism of imatinib in CYP3A4 or CYP3A5 genotype is available. Many studies have been performed to find the association between genetic variants and the risk of developing particular cancer. Similarly, the varied response of the same drug has been noticed in different genotypes which may be affected by genes involved in pharmacokinetics and pharmacodynamics. FDA label regarding the clinical use of different therapeutics is available which may be taken into consideration while prescribing the drug and dose for the treatment.

Recent advances have made it possible to understand the mechanisms of the development and progression of cancer, diagnosis, and monitoring of cancer, clinical outcome, and risk of adverse reactions. The Cancer Genomics Analysis (TCGA) and the International Cancer Genomics Consortium (ICGC) projects have identified several genetic mutations responsible for various cancers. Liquid biopsy is carried out to obtain genomic information from cell-free DNA for screening, monitoring, and relapse/recurrence of cancer [56]. Recent advances have made it possible to detect and quantify biomarkers with high specificity and sensitivity. KRAS and BRAS are the most frequent mutation in human cancer, and analysis of these mutations might be an effective approach to screen cancers [57]. There are many mutations for which effective drugs are not available. Personalized biomarker selection can improve the response rate of targeted therapy for many cancers. A small subset of patients with a particular type of mutation can be targeted for the treatment with a drug. Germline variants related to ADMET are also important for personalization of therapy.

HLA genotypes may be used to predict the risk of drug-induced severe skin hypersensitivity and drug-induced liver injury. Different variants of HLA-B are used as a therapeutic biomarker for some diseases including cancer. Individuals with HLA-B∗15:02 or HLA-A∗31:01, HLAB∗57:01, and HLA-B∗58:01 variants have shown skin hypersensitivity for carbamazepine and abacavir drugs [58]. These side effects can be avoided by HLA genotyping of patients. Immuno-genomic analysis of cancer cells, such as quantification of infiltrated CD8+ T cells and their T cell receptor, can be useful for assessment of the immune response on therapy [59]. Chimeric antigen receptor T cell therapy and TCR-engineered T cell therapy have shown clinical effectiveness in hematological cancers.

5.2.2 Neurodegenerative Diseases

Use of novel CSF, blood-based, and neuroimaging biomarkers specific to particular diseases has played a very important role in the treatment of many neurodegenerative

diseases. The use of novel biomarkers for neurodegenerative diseases has improved the accuracy of diagnosis, prognosis, and the efficacy of therapy. Personalized therapy is suggested for Alzheimer's and Parkinson's diseases because these diseases are clinically heterogeneous and have strong genetic connections. Genetic risk for Alzheimer's disease was found related to the APOE4 gene. Homozygous individuals for APOEe4 allele have a 50% risk of developing Alzheimer's disease, while heterozygous individuals for the APOEe3/e4 genotype have a risk of 20–30% [60]. Mutation in other genes such as APP, PSEN1, and PSEN2 are also associated with early onset of Alzheimer's disease. High levels of neurogranin CSF is associated with progression to Alzheimer's disease and results in a synaptic loss. Blood-based biomarkers are very useful in the area of Alzheimer's disease progression. Neuroimaging allows for preclinical identification of individuals genetically susceptible to Alzheimer's disease.

Parkinson's disease is characterized by loss of dopaminergic neurons and loss of striatal dopamine signaling. GWAS have identified many genes and loci related to Parkinson's disease. Mutations in the genes SNCA, LRRK2, PINK1, DJ-1, and Parkin are associated with Parkinson's disease [61]. The activity of caspase enzymes is also associated with Parkinson's disease. Increased activity of Cas9 is associated with high cellular toxicity in Parkinson's disease and has been recommended as a therapeutic target for the treatment of Parkinson's disease [62]. CRISPR-Cas9 may also be used for gene therapy in Parkinson's disease. Immunoglobulin G exerts pro-inflammatory responses in the body and may be used as a unique biomarker for Parkinson's disease. Targeting ganglioside GM1 levels in patients of Parkinson's disease may also be a personalized therapeutic approach. Discovery of new biomarkers of Parkinson's disease might be more useful for the personalized therapy of Parkinson's disease.

Multiple sclerosis is an autoimmune disease which affects the function of the central nervous system. The allele HLA-DRB1 is associated with an increased risk of multiple sclerosis [63]. GWAS has identified other mutations which increase the risk of multiple sclerosis such as SNPs in interleukin-7 receptor an (IL7R) gene. The chemokine C-X-C motif ligand 13 (CXCL13) is used as a dual biomarker for prognosis and therapy monitoring. A high correlation was found between increased CXCL13 levels and multiple sclerosis [64]. Increased soluble CD163 levels in blood and CSF have been identified in multiple sclerosis patients, and soluble CD163 may also be used as a biomarker for multiple sclerosis [65].

Technological advances in Pharmacogenomics will ensure the clinical application of personalized medicine. Genomics, transcriptomics proteomics, metabolomics, and neuroimaging have to speed up the identification of novel biomarkers and genetic connection of diseases. GWAS, NGS, microarray data analysis, and identification of mRNA, miRNAs, metabolites, proteins, SNPs, copy-number variations, and other genetic alternations can be useful to assess the individualized risk levels for a patient and can also suggest a most effective therapeutic approach.

5.2.3 Thrombosis

Novel approaches are required to understand the genetic basis of thrombosis which can provide better and personalized therapy for thrombosis. Thrombosis is a complex disease caused by a combination of numerous factors such as environmental and genetic factors. Both plasma-based and genetic assays are important for assessing the risk of disease, but genetic factors are more informative in assessing platelet risk factors for thrombosis. Five genetic risk factors for venous thromboembolism are VTE, deficiencies of antithrombin, protein C, protein S factor V Leiden, and the G20210A prothrombin gene variant [66]. There is a lack of clinical data set related to thrombosis which limits the goal of personalized therapy. Clopidogrel is effective in platelet aggregation, but a subset of patients has shown no response to clopidogrel therapy. Varying response to warfarin therapy was noticed due to the effect of the gene variants of CYP2C9 and VCORC1 [67]. Treatment of thrombosis can be personalized by identifying genetic factors responsible for the development and recurrence of the disease. VCORC1 and CYP2C9 genotype-based strategies are very useful for initiating anticoagulant therapies. For CYP2C9 and VKORC1 genotypes, edoxaban produces better and safe response than warfarin [68]. CRISPR/Cas9 gene therapy and anti-microRNA approaches have also been used to treat thrombosis. More clinical trials on antithrombotic agents are required to collect and analyze the impact of genetic, clinical environmental, and dietary factors on response to therapy. Risk of thrombosis can be assessed by studying the effect of different genetic variants related to thrombosis.

5.2.4 Cardiovascular Diseases

Cardiovascular diseases are one the leading cause of death in developed countries. Patients with systemic lupus erythematosus and rheumatoid arthritis have increased risk of cardiovascular disease. Two new putative risk loci associated with increased risk for cardiovascular disease are identified [69]. An IL19 risk allele, rs17581834 (T), is associated with stroke/myocardial infarction in systemic lupus erythematosus and rheumatoid arthritis, while another SRP54-AS1 risk allele, rs799454(G), is associated with stroke/transient ischemic attack in systemic lupus erythematosus but not with rheumatoid arthritis. Several SNPs related to coronary artery disease have been identified, but their function is not clear yet. The risk allele of a common coronary artery disease-associated marker at the TOMM40/APOE locus was found to be associated with a lower level of high-sensitivity C-reactive protein [70]. GWAS have shown the association between coronary artery disease-associated risk variants and common inflammatory markers. Calprotectin, an inflammatory marker for of plaque instability, is used as a new biomarker of coronary artery disease.

Total 53 loci with significant effects in both coronary artery diseases were identified and at least 1 of low-density lipoprotein, high-density lipoprotein,

triglycerides, type 2 diabetes mellitus, C-reactive protein, systolic blood pressure, and type 1 diabetes mellitus [71]. These genetic loci implicate novel genetic mechanisms involved in coronary artery disease. These genetic loci may be used for earlier diagnosis, prevention, and individualization of coronary artery disease therapy.

5.2.5 Anesthesia

Succinylcholine, mivacurium, and other anesthetics are substrates for enzyme pseudocholinesterase. There are 70 different variants of pseudocholinesterase, and most common polymorphism is known as atypical which impairs the function of pseudocholinesterase [72]. An individual homozygous for atypical allele remains paralyzed for 2 h, while individuals who are heterozygous paralyzed for up to an hour. Similarly, the varying response of pseudocholinesterase inhibition has been reported for dibucaine. An individual with rare S-variant of pseudocholinesterase remains paralyzed for up to 8 h [73]. An individual with no or lower level of pseudocholinesterase may have an increased risk of toxicity. Codeine is a prodrug that is metabolized by CYP2D6 into the active metabolite, morphine. The dose of codeine may be recommended based on poor, intermediate, extensive, or rapid metabolizer which will depend on the expression of CYP2D6 gene [74]. Genes for the 5HT3B receptor, dopamine D2 receptor, the ABCB1 transporter, and CYP2D6 are associated with postoperative nausea and vomiting. Some polymorphisms related to these genes are associated with a high incidence of postoperative nausea and vomiting. ADRB1encodes for the β-1 adrenergic receptor, and certain polymorphism related to this gene has also been reported.

5.2.6 Type 2 Diabetes

Type 2 diabetes is one of the major causes of premature mortality. GWAS has identified hundreds of variants associated with type 2 diabetes which can be assessed for their clinical utility [75]. Some genetic variants related to type 2 diabetes are known that may have clinical significance in the prevention, personalized treatment of type 2 diabetes. TBC1D4 and nonsense variant (Arg684ter) have different prevalence in different population and may decide the risk of type 2 diabetes [76]. In Latinos population, a low-frequency missense variant (E508K) at HNF1A conveys a type 2 diabetes. These variants may be useful in the assessment of clinical utility and personalized therapy of type 2 diabetes [77]. Metformin is used for the treatment of diabetes treatment, and some cases of metformin intolerance have been found. Genetic variants at ATM and glucose transporter gene (SLC2A2) are associated with the glycemic response of metformin treatment [78].

5.2.7 Depression

Many genetic variants of cytochrome p450 (CYP450) system, relevant to the metabolism of many antidepressants, have been identified. However, all these variants are not providing the clinically significant information which can be utilized in decision-making [79]. Many efforts have been made to understand the impact of common genetic variation of CYP450 on treatment in depression. CYP450 2D6 (CYP2D6) is responsible for the oxidative metabolism of most of the antidepressants [80]. For CYP2D6, 60–85% of white individuals were found fast metabolizers, while some individuals with two disrupted copies of CYP2D6 were poor metabolizers. An effect of CYP450 on treatment with venlafaxine has been studied. Individuals who are less efficient in metabolizing venlafaxine will have lower blood concentrations of the active metabolite desvenlafaxine [81]. For many diseases, more clinical studies are required to take a better therapeutic decision regarding the recommendation of a drug and its dose. Other than CYP450 systems, the drug transport protein P-glycoprotein (ATP-binding cassette, subfamily B (MDR/TAP), member 1 (ABCB1)) was also studied due to its role in the efflux of drugs across the BBB [82].

5.2.8 Psychiatry

CYP has more than 90 known genetic variations and more than 60 alleles. Study of these CYP2D6 alleles may provide better clinical information related to treatment for psychiatry. CYP2D6 metabolizes many antipsychotics and antidepressants. The FDA has recommended the HLA-B∗1502 genotyping in Asians before prescribing carbamazepine to avoid toxic outcome [83]. A better understanding of the pharmacokinetics and pharmacodynamics of psychiatric drugs has allowed personalized prescription in clinical practice. Dosing recommendation of risperidone in psychiatric patients is based on the presence of CYP3A inducers and/or CYP inhibitors and CYP2D6 [84]. CYP2D6 has a higher affinity for risperidone and hydroxylating it to 9-hydroxyrisperidone. Poor metabolizer can be identified by CYP2D6 genotyping or by measuring the level of risperidone. Knowledge pharmacokinetic, pharmacodynamic, efficacy, safety, and adverse drug reaction are required to understand personalized prescription and its applications in psychiatry. The goal of personalized medicine can be achieved by applying the précised medical information or knowledge in therapeutic practices. AmpliChip CYP450 test for analysis of the CYP2D6 and CYP2C19 genes are available to detect poor and fast metabolizer [85]. Around 5% of the Caucasian population is CYP2D6 poor metabolizers for psychiatric drugs which may have some side effects. Metabolism of one drug depends on other drug taken which can inhibit or stimulate the metabolism of other. CYP450 isoenzymes (CYP1A2, CYP2C19, CYP2D6, CYP3A4) are involved in the metabolism of psychotropic drugs and may cause adverse drug reactions [86]. A concentration-to-dose ratio for prescribing clozapine and risperidone is known, and CYP

genotyping of patients can guide therapeutic dose monitoring. CYP1A2, CYP2B6, and CYP3A4 genotyping has a small utility from a clinical point of view, while CYP2C9 genotyping has no utility in psychiatry [87].

5.2.9 Hypertension

Variation in individual response on treatment with the same drug motivates us to look for genetic factors associated with this variation. Studies have shown the association between the response of blood pressure and specific gene polymorphism. Clinical data related to genetic polymorphism and therapeutic response can guide the way of personalized medicine for hypertension. Inhibitors of angiotensin-converting enzyme, β-blockers, angiotensin 2 blockers, and calcium channel blocker are well-studied inhibitors for the treatment of hypertension [88]. Effect of β1AR Arg389Gly polymorphism on responses of blood pressure to β-blocker therapy has been studied. On treatment with metoprolol, patients homozygous for Arg389 had shown a high reduction in diastolic blood pressure [89]. The β1-adrenergic receptor gene (ADRB1) encodes a 51.3 kDa protein with 477 amino acid residues. The β1-adrenergic receptor is present in the heart, controlling heart rate. Six SNPs located near the ADBR1 gene region are available in dbSNP. Out of six SNPs, two polymorphisms Ser49Gly (rs1801252) and Arg389Gly (rs1801253) have been studied well [90]. Studies have shown that siblings with Gly389 allele have a low diastolic blood pressure than those homozygous for Arg389.

Such types of knowledge related to polymorphism and outcome of treatment can be utilized while recommending the drug and dose to any hypertensive patient. More investigations should be performed to know the effect of other polymorphism on the outcome of treatment with a hypertensive drug. Studies have found that there is a genotype group that shows a less favorable response to β-blocker therapy. For adrenergic receptor polymorphism, the effect of drug bucindolol on different genotypes can be assessed [91]. This may help in finding the genotypes which show better or poor response on treatment with bucindolol. Knowledge of genetic factors that decides the response of a drug can enable us to choose the most suitable drug and dose for each patient based on genetic profile. Advances in "omics" technologies have made it possible to identify genetic markers that can be used to know the response of a particular drug and guide the way for individualized therapy [92]. A relationship between nephrosis (NPNS1) gene variants and response to angiotensin-receptor antagonist losartan has been found. Also, two other genes (ALDH1A3 and CLIC5) have shown their influence on blood pressure response to hydrochlorothiazide treatment. Many genetic polymorphisms that affect the response of treatment with hypertensive drugs such as enalapril, perindopril, lisinopril, metoprolol, atenolol, bisoprolol, losartan, and hydrochlorothiazide have been identified [93]. Some effective approaches for the personalized treatment of hypertension are aldosterone measurement, renin profiling, aldosterone-to-renin ratio, SNP and haplotype approach, and hemodynamic assessments.

5.3 Ethical Issues Related to Personalized Medicine

In hospitals and universities, numerous tests and kits are available to detect genetic diseases. But the accuracy of some kits and validity of some biomarkers related to disease have a low level of confidence. The ethics of personalized medicine became an issue when a healthy person has been reported to have a high risk of breast or ovarian cancer. Ethical issues are also associated with the use and storage of genetic information of an individual. Ethics related to personalized medicines are also affected by the health policies of a country [94]. The National Institutes of Health (NIH) is trying to develop the best way of treatment and also delivering clinical information to the health sector and patients. Many research organizations have confined their study and tests related to a specific genetic disorder in certain groups of people or region. Informed consent of a patient is required for the use of their body material to protect the privacy of an individual. There is a need to assure the protection of privacy of genetic data of an individual from the government or insurance companies.

6 Challenges and Future Opportunities

One of the important challenges in the way of personalized medicine is to manage the complexity associated classification of disease. A large number of new genetic alterations are being reported by next-generation sequencing. Association of some of these genetic alternations with a disease is well established, but the role of many mutations/alternation was not evaluated yet. Other alternations might have alone or combined effect on disease prognosis. Systematic storage, retrieval, and analysis platform are required to store the clinical data and also for decision-making. Better biomarkers of the disease can assist with detection and also can guide treatment. The financial incentives for designing of new diagnostics are required because a better diagnosis of the disease can improve the rate of cure. Advances in DNA sequencing have enabled studies of the microbiome which are present in our system. Microbiome studies have been considered for disease and may offer opportunities for personalized therapy.

Associations between a genetic variation and related outcome should be quantified. Implementation of Pharmacogenomics is based on these associations, and no more emphasis has been given on clinical validity and utility of the test. Pharmacogenetics tests should be adopted based on their clinical utility and cost-effectiveness. Pharmacogenomics-related data and tests are available only for some drugs, but still, this knowledge has not been potentially translated into clinical practice at a bigger scale. However, the clinical utility of Pharmacogenomics test is limited due to a gap between clinical validity and its application to patients through health-care providers. Pharmacogenomics-based CPIC dosing guidelines are available for some drugs. Primary care providers have no adequate guidelines on

how and when to Pharmacogenomics markers [95]. There exist a gap between evidence of association and the clinical utility of Pharmacogenomics tests, and there is need to overcome this challenge by exploring evidence from a health-care provider, patient, and policy perspective [96]. Well-defined steps of health care from clinical validity to clinical utility are required for successful implementation of Pharmacogenomics tests to patients. Many publications related to Pharmacogenomics reports only about association, but other pieces of evidence such as guideline development and coverage decisions are necessary for better implementation of Pharmacogenomics. Pharmacokinetics and pharmacodynamics of many drugs and its mechanism of interactions have been reported well, but its clinical utility is still very limited. The lack of clinical utility of a drug delays the application of Pharmacogenomics tests into health care [97].

Pharmacogenomics-based therapies will reduce the trial-and-error prescription and will also improve drug safety by avoiding adverse drug reaction. Pharmacogenomics will reduce the time and cost of a clinical trial. Genetic profiling will also help in recommending the use of a failed drug for a particular group of another population [98]. Availability of large scale of data related to the association of genetic variations with the disease across many countries will enable us to achieve the goal of personalized medicine. Study of genetic variations also speeds up the process of the clinical trial process by targeting the individuals for testing belonging to a specific population. The government and other funding agencies should give more incentive for adopting the practices related to personalized medicine. Faster, reliable and cost-effective approaches for sequencing, screening, and diagnosis of diseases should be developed which will bring the use of personalized medicine in real practice. In recent years, a large amount of knowledge-related genome, proteome, and metabolic pathways have been generated which enable us to use, monitor, and assess the effect of a drug on a patient's genotype. The government should take an initiative to frame policies related to the use of genetic material and individualization of therapy. Health-care centers, educational institutes, and hospitals may play important in promoting research and clinical practices of personalized medicine. It is expected that the cases of serious adverse drug reactions will reduce in the future with the implementation of Pharmacogenomics approaches for personalized therapy.

The cost of genetic tests and its availability to rural people are also a big challenge for many poor and developing countries. Governments will also have to think over the issues raised as a result of discriminations based on genotype. The pharmaceutical companies may take more interest in developing the drug for a genotype belonging to the rich group of the population rather than a disease or patient confined to a poor subgroup of the population. Pharmacogenomics has brought a big shift in the trend of therapies for many diseases; still, many patients are not in a position to take the benefit of pharmacogenomic knowledge in personalizing the therapy. Benefits of personalized medicine should be utilized without waiting for more discoveries related to Pharmacogenomics and biomarker. The personalized prescription can be given based on genetic, environmental, or personal factors that can affect the outcome of the treatment. The FDA has set many recommendations and guidelines to promote the use of Pharmacogenomics in the personalization of therapy. To

ensure the success of personalized medicine, some strategic recommendations have been suggested [99]. These recommendations are related to the discovery of more potential biomarkers, framing regulations for genetic privacy and confidentiality, regulatory incentives to pharmaceutical industries and equity of access to personalized medicine, standardization of genetic testing and documentation, regulation for private genetic testing firms, and better awareness about personalized medicine within the medical imaging community.

7 Conclusion

Pharmacogenomics has played a very important role in adopting the goal of personalized medicine for many diseases. It has greatly impacted the prescription of a drug and its dose for many diseases, but a small population of the world can enjoy the benefits of Pharmacogenomics. Personalized medicine offers the opportunity to identify patients for whom a drug can be both effective and safe. Efficient decision-making on clinical data and proper utilization of health-care resources can enable us to achieve the goal of personalized therapy. Pharmacogenomics also improves the process of drug development by focusing the companies to test the safety and efficacy of a drug in an individual or subgroup of a population. Pharmaceutical companies have to reduce the cost of personalized treatment so that poor patients can also utilize the benefit of pharmacogenomics. Medical imaging techniques have played a very important role in the diagnosis, prediction, and treatment of many diseases and can be very helpful in achieving the goal of personalized therapy.

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