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Research review paper

Mammalian cell culture for production of recombinant proteins: A review of the critical steps in their biomanufacturing

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ABSTRACT

The manufacturing of recombinant protein is traditionally undertaken in mammalian cell culture. Today, speed, cost and safety are the primary considerations for process improvements in both upstream and downstream manufacturing. Leaders in the biopharmaceutical industry are striving for continuous improvements to increase throughput, lower costs and produce safer more efficacious drugs. This can be achieved through advances in cell line engineering, process development of cell culture, development of chemically defined media and increased emphasis on product characterization. In the first part, this review provides a historical perspective on approved biotherapeutics by regulatory bodies which pave the way for next-generation products (including gene therapy). In the second part, it focuses on the application of *in vitro* and *in vivo* cell line engineering approaches, modern process development improvements including continuous manufacturing, recent developments in media formulation, and improvements in critical quality attribute determinations for products produced predominantly in mammalian cells.

1. General overview of biopharmaceuticals

1.1. Biopharmaceutical history, current market status and perspective

Biopharmaceuticals are prophylactic and therapeutic substances, inherently derived from biological sources, such as organs, tissues, microorganisms or animal cells (Kesik-Brodacka, 2018; Rader, 2008; Ryu et al., 2012). These large and complex biologically active macromolecules are produced using biotechnology methods and are generally used to diagnose, prevent, treat, and cure diseases and medical conditions. They include a diverse category of products such as vaccines, blood and blood components, allergenics, tissues, cellular or gene therapy products, and recombinant therapeutic proteins. Since the first biopharmaceutical, recombinant human insulin, approved by Foods and Drugs Administration (FDA) for therapeutic use in 1982, biopharmaceuticals have revolutionized the treatment of a wide range of diseases and are used increasingly in all branches of medicine (Kesik-Brodacka, 2018).

The global biopharmaceutical market was valued at $237.2 billion in 2018 and is projected to reach $389.0 billion by 2024, registering a staggering compound annual growth rate (CAGR) of 8.59% during the forecast period of 2019 to 2024. As far as therapeutics are concerned, oncology is forecast to remain the dominant therapy segment with projected worldwide sales reaching $233 billion by 2024, growing $129 billion in over 2017–2024 (Deloitte, 2020).

The growth of global biopharmaceutical market is driven by various factors, such as rapid advancements of our understanding of diseases and how they occur at cellular and molecular level, global population growth and widespread population aging, and the unmet medical needs of chronic diseases such as cancer and diabetes. Additionally, biopharmaceuticals offer high specificity and activity, fewer side effects, and the potential to cure disease rather than merely treating the symptoms. This has led significantly increased acceptance of biopharmaceutical products. Also contributing to the market growth is the rise in strategic collaborations among biopharmaceuticals companies and the increase in adoption of biopharmaceuticals globally (Ecker et al., 2015).

1.2. Antibodies as the main power horse of the biopharmaceutical industry

In recent years, the biopharmaceutical industry has experienced a significant growth in the production and approval of biopharmaceutical

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Table 1
Glossary of biopharmaceuticals approved by FDA (Jan 2014-Dec 2019)

Category	Biopharmaceuticals
Cytokines	Peginterferon beta-1a
Hormones	Insulin glargine and lixisenatide, Insulin degludec and liraglutide, Parathyroid hormone, Insulin degludec, Insulin human inhalation powder, Metreleptin
Growth factors	Antihemophilic factor glycopegylated, Antihemophilic factor PEGylated, Cenegermin, Coagulation Factor IX GlycoPEGylated, Antihemophilic Factor Single Chain, Coagulation factor Xa inactivated, Sargramostim, Coagulation Factor IX Albumin Fusion Protein, Antihemophilic Factor, recombinant Factor VIII, Coagulation Factor IX Fc Fusion Protein, Antihemophilic Factor Fc Fusion Protein, Antihemophilic Factor Porcine Sequence
Antibodies	Risankizumab, Caplacizumab, human Immune Globulin, Romosozumab, Trastuzumab and Hyaluronidase, Certolizumab pegol, Tildrakizumab, Rituximab, Fremanezumab, Lanadelumab, Ravulizumab, Emapalumab, Ibalizumab, Burosumab, Erenumab, Galcanezumab, Cemiplimab, Moxetumomab pasudotox, Mogamulizumab, Avelumab, Dupilumab, Brodalumab, Guselkumab, Inotuzumab ozogamicin, Emicizumab, Tocilizumab, Ocrelizumab, Human Rabies Immune Globulin, Sarilumab, Durvalumab, Ixekizumab, Lixisenatide, Bezlotoxumab, Obiltoxaximab, Daclizumab, Olaratumab, Atezolizumab, Adalimumab, Reslizumab, Alirocumab, Evolocumab, Secukinumab, Daratumumab, Idarucizumab, Elotuzumab, Necitumumab, Dinutuximab, Mepolizumab, Pembrolizumab, Nivolumab, Vedolizumab, Blinatumomab, Siltuximab, Immune Globulin Infusion 10% (Human) with Recombinant Human Hyaluronidase, Alemtuzumab, Bevacizumab, Ramucirumab, Polatuzumab Vedotin, Venetoclax Plus obinutuzumab, Avelumab Plus Axitinib, Pembrolizumab Plus lenvatinib, Brolucizumab, Crizanlizumab, Enfortumab Vedotin, Fam-trastuzumab deruxtecan, Luspatercept–aamt
Enzymes	Elapegademase, Calaspargase pegol, Pegvaliase, Cerliponase, Vestronidase alfa, Sebelipase alfa, Asfotase alfa, Elosulfase alfa
Inhibitors	PrabotulinumtoxinA, Human C1 Esterase Inhibitor, Aflibercept, C1 esterase inhibitor, RimabotulinumtoxinB
Vaccines	Dengue Tetravalent, Vaxelis[1], Hepatitis B Vaccine adjuvanted, Zoster vaccine adjuvanted, Cholera vaccine live oral, Meningococcal Group B Vaccine, Trivalent influenza vaccine, Smallpox and Monkeypox Vaccine
Cell therapy	Axicabtagene ciloleucel, Tisagenlecleucel
Gene therapy	Patisiran, Inotersen, Voretigene neparvovec, Nusinersen, Eteplirsen, Defibrotide Sodium, Talimogene Laherparepvec, Onasemnogene Abeparvovec, Givosiran, Golodirsen
Allergenics	Grastek[a], Oralair[a], Ragwitek[a]
Toxin	Tagraxofusp
Peptide	Semaglutide, abaloparatide, Albiglutide, Dulaglutide, liraglutide [rDNA origin], Bremelanotide

[a] For some biopharmaceuticals brand name is used when non-proprietary name is too long.

products, and typically 20–30 new products gain approval annually by the FDA. For instance, during the five year period between January 2014 and Dec 2019, the total number of biopharmaceutical products approved by FDA for human use has reached 129, and half of them (66) are monoclonal antibodies or conjugates as shown in Table 1. For complete information regarding brand name, target and therapeutic use, please refer to Supporting Information Table S1. It has become clear that the market of monoclonal antibodies has changed dramatically, and antibodies and their derivatives have emerged as the largest group of biopharmaceuticals when compared to other biological drug types (Grilo and Mantalaris, 2019).

In addition to an increase in spending on research and development for biological drugs, supportive government initiatives and demand for personalized medicines are contributing to the expectation that the global monoclonal antibody therapeutics market is projected to reach over $200 billion by year 2023 (Zion Market Research, 2018). They are currently utilized in therapies for cancer, inflammatory diseases, cardiovascular diseases, organ transplantations, infections, respiratory diseases, and ophthalmologic diseases (Ecker et al., 2015; Kesik-Brodacka, 2018). As illustrated in Fig. 1, the success of a monoclonal antibody discovery and development depends upon several aspects, including cell line development, upstream process, downstream process, antibody engineering, bioanalytics, delivery system and quality control. Many of these aspects translate to all biotherapeutic designs.

1.3. Accelerating the development of next generation biopharmaceuticals

Advances in biotechnology have opened the door to the design of new types of therapeutic drugs to fight disease and biopharmaceutical products have now become an integral part of the pharmaceutical industry. As a result, the portfolio and pipeline of biopharmaceuticals is becoming increasingly diverse, with many novel biologics being developed. As well as the rise of biosimilars (McCamish and Woollett, 2012), next-generation biopharmaceutical therapeutics have gained extensive attention due to the obvious benefits in higher bioavailability, increased half-life, lower immunogenicity and will eventually create added value over existing products (Moorkens et al., 2017). As demonstrated in Fig. 2, next generation therapeutics covers a variety of products, including monoclonal antibodies, cellular and gene therapy products, protein and bioconjugates, peptides and oligonucleotides, vaccines and microbiome therapeutics (Hanna et al., 2017; Kim, 2013; Lundin et al., 2015; Mimee et al., 2016; Ramsland et al., 2015; Stephanopoulos and Francis, 2011; Vlahos and Coghlan, 2005).

One promising emerging area is Chimeric Antigen Receptor (CAR) T-cell therapy, which is a form of immunotherapy involving genetic modification of patient's autologous T-cells via viral-based or nonviral based gene transfer methods to target specific cancer/autoimmune cells. It allows for the introduction of a high degree of tumour selectivity into adoptive cell transfer therapies (Androulla and Lefkothea, 2018; Filley et al., 2018). Two autologous CD 19-specific CAR T cell therapies (Yescarta TM and Kymriah TM, as shown in Table 1) were approved by FDA in 2017 and offered patients a new strategy to fight cancer.

Gene therapy is now a cutting-edge therapy with CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats), as an important tool for gene editing across various species. CRISPR/Cas9 can disrupt, delete, correct, replace or insert specific genes in the patient's DNA (Ceasar et al., 2016; Wang et al., 2019). The possibilities of gene therapy hold considerable promise in the treatment of cancer, cystic fibrosis, heart disease, diabetes, hemophilia and AIDS. In 2017, the first gene therapy, Luxturna TM, an adeno-associated virus vector-based product was approved by FDA for the treatment of vision loss due to confirmed biallelic RPE65-mediated inherited retinal disease.

As shown in Fig. 3, it has become clear that clinical trials in cell immunotherapy and gene therapy have been growing significantly since 2014. Cumulatively, there have been a total of 861 and 570 clinical trials in cell immunotherapy and gene therapy respectively as registered in ClinicalTrials.gov (correct in March 2020). Consequently, it is believed that the explosion in clinical trials along this pipeline will lead to significant growth in approval and production of novel next generation biopharmaceutical therapeutics.

1.4. Advances in biopharmaceutical analysis and regulatory quality requirement

Contaminants related to manufacturing and purification processes can significantly affect the safety and efficacy profiles of biopharmaceutical drugs. As discussed above, the biopharmaceutical industry has evolved significantly over the past three decades, the emerging of next generation therapeutics (Barry and Matter, 2006), integration of

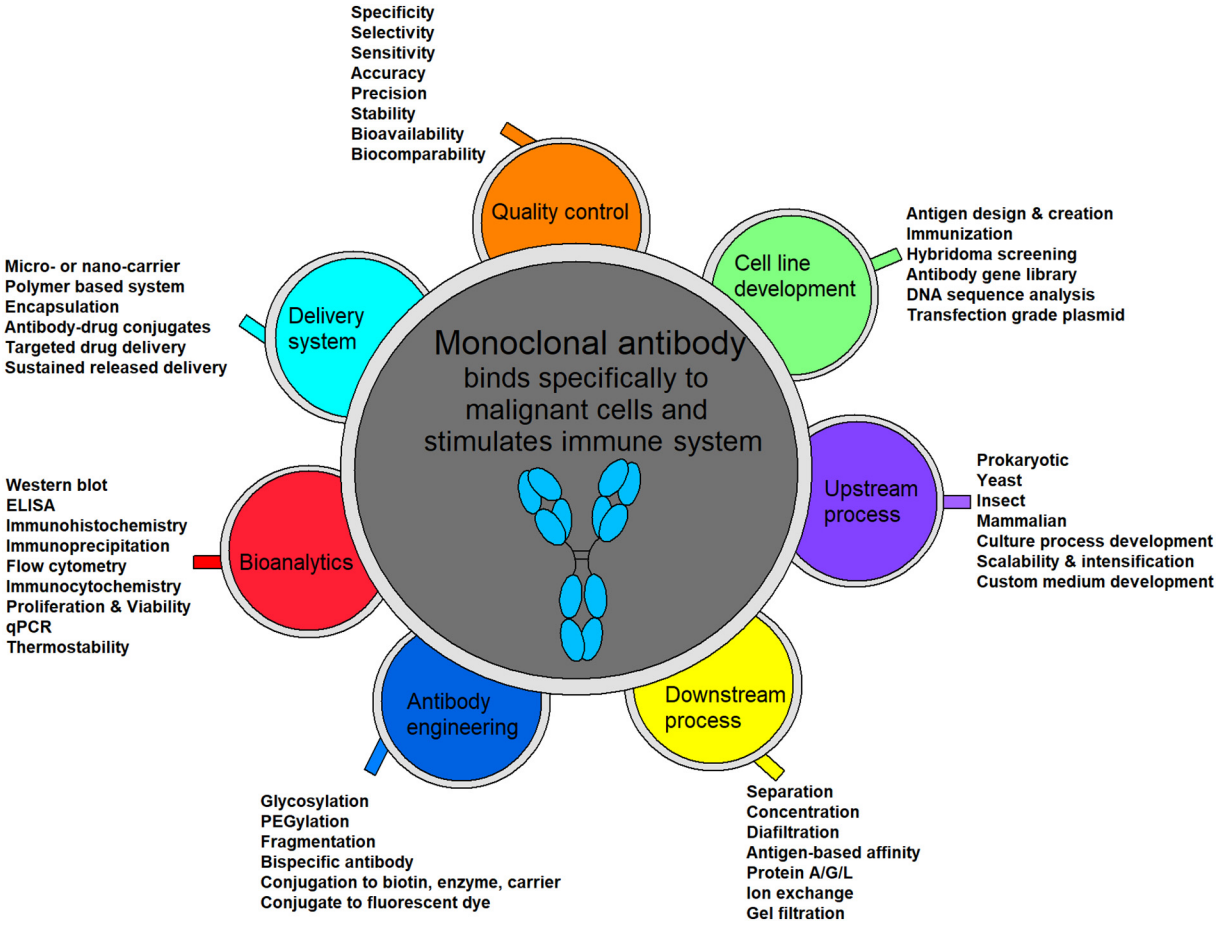

Fig. 1. Monoclonal antibody discovery and development.

continuous bioprocessing (Rathore et al., 2015), as well as the intrinsically complex post-translational modifications (PTMs) of the therapeutics (Walsh, 2010), are creating the need for timely, sensitive and accurate analytical techniques (Challener, 2016). A detailed characterization and comparability assessment of the complex and heterogeneous biopharmaceuticals mainly includes bioprocess impurities, metabolites, extractable and leachable, complete amino acid sequencing, molecular weight, conformation, stability, solubility, secretion, aggregation, heterogeneity, PTMs, protein isoforms, antibody-drug conjugates, degradants, disulphide linkages, affinity maturation, effector functions, drug metabolism and pharmacokinetics, antigenicity, and compatibility of excipients (Kim et al., 2005; Tsuchikama and An, 2018; Van Landuyt et al., 2018; Walsh, 2010; Zhong, 2018). The major critical safety concern relating to biopharmaceutical drug usage is immunogenicity, which can cause hypersensitivity responses, anaphylaxis, infusion reactions and a decreased efficacy (Kessler et al., 2006; Kuriakose et al., 2016; Ryu et al., 2012). Evaluating the immunogenicity of biopharmaceuticals is mandatory for regulatory approval (Pineda et al., 2016).

Currently, there are a variety of methods used throughout the biopharmaceutical development cycle (Fisher et al., 2016; Yu and Woodcock, 2015), such as immunoassays, real-time, quantitative polymerase chain reaction (qPCR) (Schmittgen et al., 2000), differential scanning fluorimetry, dynamic light scattering, high/ultra-performance liquid chromatography, mass spectrometry (MS) (Kaltashov et al., 2010; O'Flaherty et al., 2017; Rudewicz, 2013) and nuclear magnetic resonance (NMR) spectroscopy (Kiss et al., 2018; Wishart, 2013). The emerging and continued improvement of the new analytical technologies has advanced biopharmaceutical developments significantly.

As a result, the concepts of process analytical technology (PAT) (Rathore et al., 2010) and Quality by Design (QbD) (Sangshetti et al., 2017) are gradually introduced as part of the 'Pharmaceutical Current Good Manufacturing Practices (CGMPs) for the 21st Century-a Risk Based Approach' initiative, which play a key role in creation of a robust control and monitoring strategies for bioprocesses to ensure final product quality. New manufacturing, compliance, and quality regulations are likely to be required to keep up with the development of novel advanced therapies.

2. Expression systems for therapeutic proteins

While high yields of products are desired during biomanufacture, the structural quality of these molecules determines their therapeutic efficacy and safety. Therefore, beyond the efforts to drive the development of high yielding cell lines, the quality of PTMs, particularly glycosylation, has to be considered. The choice of expression system can have a profound impact on the type and degree of this glycosylation. As such, advances in cell engineering and an inherent knowledge of expression system characteristics have led to the design of proteins with defined structure and high yields.

The term expression system refers to the synthesis of recombinant proteins by a given cell and its transfected DNA vector. This process is based on the translation of the supplied DNA genetic information into a sequence of amino acids. Such information is enclosed in the messenger RNA (mRNA) and posteriorly delivered to ribosomes. Ribosomal RNA (rRNA) is part of the ribosomes structure and responsible to catalyse the link between two amino acids, which are carried by a transfer RNA (tRNA). This process results in the formation of a specific amino acid sequence characteristic of the protein (Clark and Pazdernik, 2013). After being translated, these molecules are subject to PTMs. The

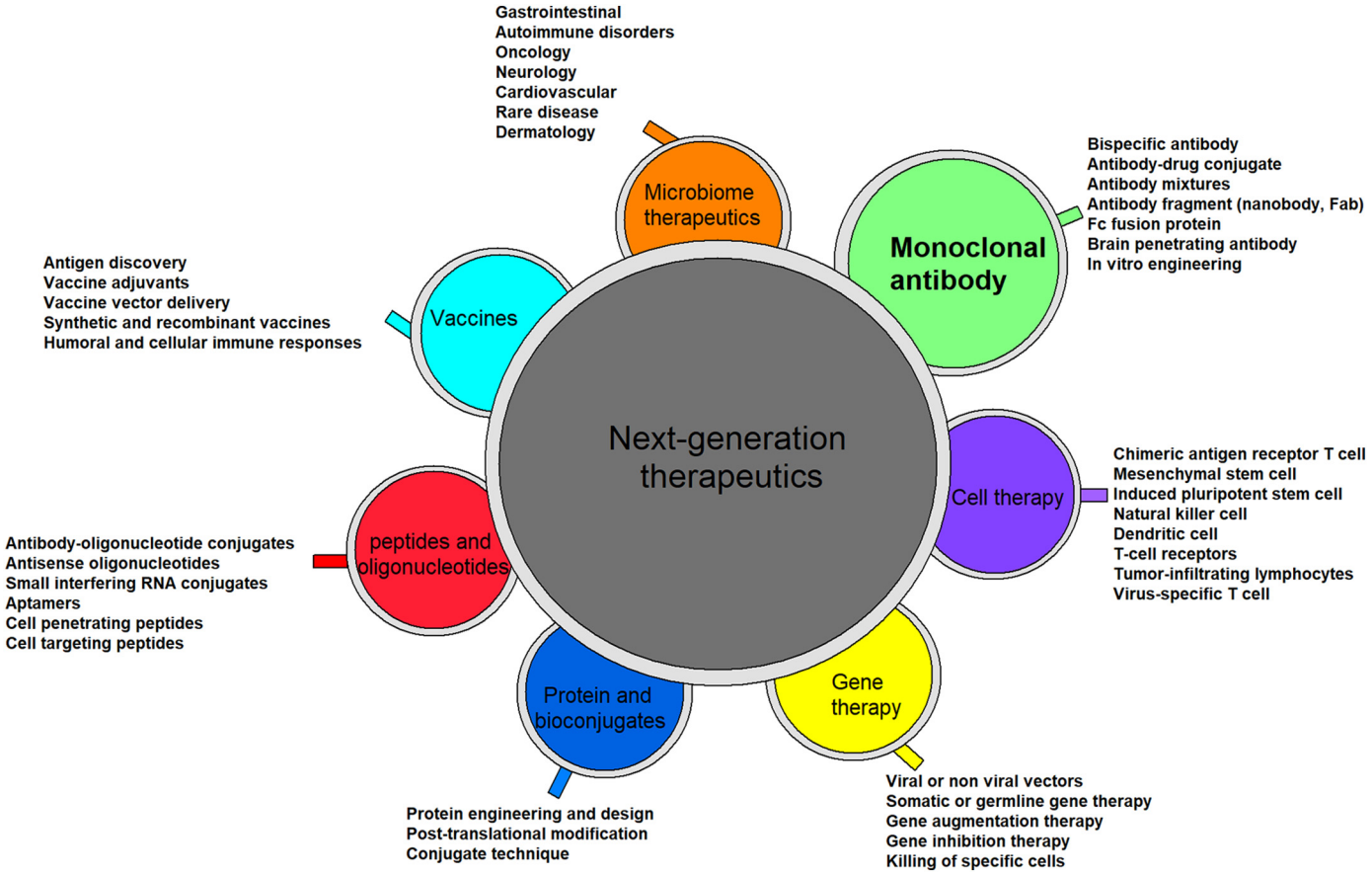

Fig. 2. Next generation therapeutics: trends in biotechnology.

frequency and complexity of PTMs can differ substantially between prokaryotes and nucleated eukaryotes, being much more extensive in the latter (Walsh et al., 2005).The biotherapeutic glycoforms influence protein function in various aspects, including immune-response, folding, aggregation, stability and transport (Rozov et al., 2018; Wang and Amin, 2014). The machinery necessary for human-like glycosylation is absent in prokaryotic cells, limiting their pharmaceutical use to express only simple proteins, such as insulin, hormones, interferon (Rozov et al., 2018), and non-glycosylated enzymes such as asparaginase and collagenase (Lalonde and Durocher, 2017). Although fungi, insect and plant cells perform glycosylation, the glycan structures are significantly different from those of human, a fact that threatens the onset of unwanted immunogenicity for therapeutics. Consequently, the majority of currently approved biotherapeutics are expressed in mammalian cell lines, most of which at least produce "human-like" glycosylation.

2.1. Vectors

The development of production cell lines starts with the construction of expression vectors. Vectors are autonomous, self-replicating DNA elements, into which a fragment of foreign DNA is introduced. Expression vectors are the vehicles that carry recombinant genes of interest into the host cell and in addition to the transgene, they usually consist of expression elements such as promoters, enhancers, multiple cloning sites (MSC), intron sequences, transcription terminators, selectable markers and DNA elements that modulate chromatin structure (Lund et al., 2014; Makrides, 1999). In addition, epigenetic elements, which optimize protein expression and mitigate the silencing effect, are frequently incorporated into the vector.

The most common locus control elements used are UCOE (ubiquitous chromatin opening elements), S/MAR (scaffold/matrix attachment region) and STAR (stabilizing and anti-repressor element) (Lalonde and Durocher, 2017; Durocher and Butler, 2009; Bandaranayake and Almo, 2014).

Selection of an appropriate vector and delivery method requires a multifaceted approach that takes into account the cell type, product quantity and safety, process economics, transition from pilot- to full scale, turnaround time and regulatory compliance. Vector delivery can be performed using reagent-based methods such as cationic lipid-based transfection, calcium-phosphate precipitation, diethylaminoethyl (DEAE)-dextran polyethylenimine and polymer- or dendrimer-mediated techniques; instrument-based methods such as electroporation and microinjection; and finally virus mediated methods such as adeno-associated viruses and lentiviruses (Kim and Eberwine, 2010). Non-viral delivery procedures have gained the advantage of regulatory clearance and are the most preferred methods for protein biomanufacturing (Nayerossadat et al., 2012).

The common principle of reagent-based transfection methods is that positively charged chemicals form complexes with negatively charged nucleic acids, which then are attracted to the negatively charged cell membrane. The complexes then pass inside the cell by endocytosis or phagocytosis. Calcium-phosphate precipitation and DEAE-dextran are the oldest methods of DNA delivery which, although very cost-effective, often suffer from low transfection efficiency (Mostaghaci et al., 2016) and high levels of cytotoxicity (Lalani and Misra, 2011). Calcium-phosphate method requires serum-based media and this constrains its use in biopharmaceutical manufacturing (Jordan and Wurm, 2004). Cationic polymers such as Polyethelyenimine (PEI) are widely used, cost effective, non-cytotoxic reagents that can be scaled up to hundreds of litres (Backliwal et al., 2008a; Hunter et al., 2019) and generate up to 100% transfection efficiencies (Geisse, 2009). Although PEI is

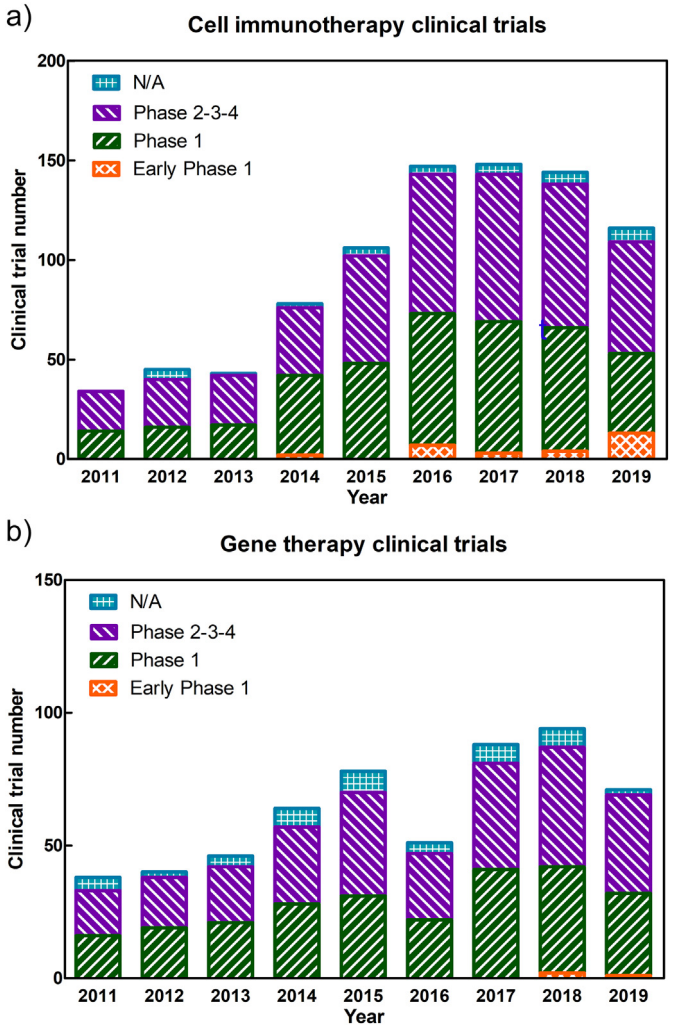

Fig. 3. Clinical trials registered in Clinicaltrials.org (January 2011 to December 2019).

incompatible with some growth media components (Hunter et al., 2019), large scale productions with HEK 293 and CHO cell platforms have been reported (Delafosse et al., 2016; Raymond et al., 2011; Thompson et al., 2012), with commercially available reagents such as jetPEI® frequently used by biopharmaceutical industries (Wong et al., 2010). Cationic lipid-based techniques belong to another popular category as they are easy to use, require minimum steps and can be scaled up. Proprietary cationic lipid formulations are commercially available from many vendors including Biorad, Polyplus transfection, Promega and ThermoFisher. They offer highly effective transfections, but have not been frequently used for large-scale due to their comparatively high cost. They are however the common choice at the early stages of research and development as they are highly efficient in culture dishes and are adaptable to high-throughput systems (Shen et al., 2005).

Electroporation (electric field–mediated permeabilization) is another popular technique for introducing foreign DNA into host cells through a brief electric pulse that generates temporary openings in the cell membrane (Longo et al., 2013). Under optimal conditions of electric field strength, pulse length, buffer conductivity, waveform and pulse number, this technique offers high transfection efficiency, increased viability, ease of use and wide cell line applicability without altering the biological structure or function or host cells. Electroporation is quite versatile in that it can be used to introduce other biological molecules such as exogenous proteins, mRNA or siRNA, and is suitable for both stable transformation and transient gene expression (Luft and Ketteler, 2015). Equipment to electroporate cells utilizes parallel plate electrodes separated by a fixed gap width in the form of electroporation cuvettes or microplates which limit the volume of cell samples processed. Microfluidic electropermeabilization approaches where cells experience electroporation while flowing through an electric field chamber are reported to offer the capacity for large-scale transfection with instruments such as the MaxCyte VLX® Scalable Transfection System claiming a turnout of up to 200 billion cells in a single 30 min electroporation flow-through run (Steger et al., 2015).

Contrary to chemical and physical transfections, viral delivery relies on viral mechanisms of cellular infection. These systems vary in their insert capacity depending on the type of virus or even the same virus serotype, and are usually applied for transient gene expression (Wurm, 2004; Colosimo et al., 2000). Replication-defective, recombinant viral vectors were the first enabling tools for efficient, nontoxic gene transfer into human somatic cells. Owing to their natural ability to invade cells and their exceptional gene delivery efficiency, viral delivery is currently the most frequently used method for in vivo gene therapy applications, with the leading platform being adeno-associated virus (AAV) vectors. AAVs are persistent viruses, induce low pathogenicity and toxicity, are available in many serotypes and contribute to long-term transgene expression through chromosomal integration (Daya and Berns, 2008). Other viral delivery systems include RNA and DNA viruses with either single-stranded (ss) or double-stranded (ds) genomes such as adenoviruses, alphaviruses, flaviviruses, herpes simplex viruses (HSV), rhabdoviruses, measles viruses, retroviruses, and lentiviruses. Selection of the appropriate vector is based on their packaging capacity of foreign DNA, toxicity, immunogenicity, lytic capacity, long- or short-term transgene expression and capability to infect non-dividing cells or replicating specifically in tumour cells (Lundstrom, 2018). Non-viral gene delivery has been overlooked in the past because of poor efficiency and transient expression of their transgenes, however advancements on physical transfection methods and delivery vehicles along with their low immunogenicity have rendered non-viral vectors attractive candidates for a new generation of gene transfer tools (Hardee et al., 2017).

Viral vectors on the other hand dominate the active gene therapy clinical trials worldwide (79% vs 21% of non-viral therapies), with the first gene therapy product receiving marketing authorization in Europe in 2012 (Hardee et al., 2017). Alipogene tiparvovec (Glybera®) is an adeno-associated virus serotype 1 (AAV1)-based gene therapy for the treatment of patients with lipoprotein lipase deficiency (Ferreira et al., 2014). Although viral vectors have been used on numerous occasions in cell culture for protein production, the disadvantages of virus gene transfer include more complex cloning strategies, biosafety concerns related to the bioprocess and health risks related to the possible presence of viral vector with the recombinant protein (Pham et al., 2006).

2.2. Mammalian expression systems

The vast majority of recombinant proteins on the market, around 70%, are produced by mammalian cells (Durocher and Butler, 2009; Lalonde and Durocher, 2017; Wurm, 2004). The high costs associated with culturing this expression system, challenging scale-up processing and risk of viral contamination (Rozov et al., 2018) are compensated by its ability to express proteins with complex PTMs. The general process of producing a recombinant protein in a mammalian expression system starts with transfection of a recombinant gene integrated in a vector into the chosen cell. The gene carries structural elements that regulates transcription. In order to select cells that successfully integrate plasmid into their genome, a selection system is also added, either, in the same or in a new plasmid. The most common selection markers used in mammalian cells are the glutamine synthetase (GS) and the dihydrofolate reductase (DHFR) genes, which only allow transformed cells to grow in a medium lacking glutamine and hypoxantine/thymidine, respectively. Following transfection and selective growth, single cells that survive are separated to generate a clonal population. After a

screening, the most productive and stable clones are selected and banks of cell lines are created for future protein production (Lalonde and Durocher, 2017; Wurm, 2004).

Mammalian expression systems can be used to produce proteins transiently or through stable cell lines. The choice between transient and stable expression systems affects the expression vector to be used. During transient gene expression (TGE) the vector never integrates into the genome of the cell and as result the transfected genes only express in a limited time span. TGE has been used for many years in laboratories for research purposes and only in the last decades has it been scaled-up and used for rapid screening of large numbers of antibodies or antibody-like molecules in order to identify promising candidates (Geisse, 2009; Gutiérrez-Granados et al., 2018). Alternatively, after TGE, milligrams to grams of protein can be harvested within 2–4 week, after which the cell lose the expression plasmid with passages and die (Andersen and Krummen, 2002; Lalonde and Durocher, 2017). For large-scale production stable cell lines are preferred, where the expression construct is integrated into the host genome, as they provide large amounts of proteins with consistent quality and regulatory familiarity. Following integration of the vector into the host cell genome, screening is performed to select stable transfected cell lines for production of proteins in moderate to large scale, i.e. stable expression. The establishment of stable clones can be lengthy i.e. take up to 12 months and requires a labour-intensive clonal selection process or complex and expensive laboratory infrastructure. Recent systems have dramatically reduced the timelines down to as little as 1–2 weeks by using novel approaches such as 3rd generation lentiviruses (Tandon et al., 2018).

CHO (Chinese Hamster ovary) cell lines are the workhorse for the production of many biopharmaceuticals, most of which are monoclonal antibodies. CHO cells are derived from Chinese hamster ovarian fibroblast cells. Since the original clone was generated, a number of CHO cell lines has been developed. These cells present many advantages such as capacity of growing in suspension, high yield production and ability to grow in serum free medium. Moreover, CHO cells are less susceptible to human virus infections, reducing biosafety risks. On the other hand, CHO cells can present some drawbacks. Although the glycans synthesised by these cells are generally characterised as "human-like", there are some non-human and potentially immunogenic structures, such as galactose-α(1,3)-galactose (α-gal) and *N*-glycolylneuraminic acid (Neu5Gc). Furthermore, CHO cells are unable to produce α(2-6)-sialic acid residues which are present in human glycoproteins (Bandaranayake and Almo, 2014; Dumont et al., 2015; Durocher and Butler, 2009; Lalonde and Durocher, 2017). The determination of the genome sequence of CHO-K1 has facilitated cell engineering of CHO and optimized its use as a platform for efficient protein expression (Xu et al., 2011). BHK (Baby Hamster Kidney) is another cell line derived from hamster. Their use is especially common for Factor VIIa, Factor VIII and vaccine production. Similar to CHO, these cells can express glycans with immunogenic terminal Neu5Gc and α-gal (Bandaranayake and Almo, 2014; Dumont et al., 2015; Durocher and Butler, 2009; Lalonde and Durocher, 2017).

NS0 and Sp2/0 are murine myeloma cells originating from tumour cells. Since these cell lines were developed they have been extensively used to produce commercial mAbs. The disadvantage of using these cell lines is that they can produce the immunogenic glycans α-gal and Neu5Gc in relatively high levels. (Lalonde and Durocher, 2017). Despite the inherent potential immunogenic potential of products from NS0 and Sp2/0 cell lines, a stringent clone screen can select the cells that produce a more desirable glycan profile. Moreover, manipulation of media components and upstream conditions can profoundly impact glycoform distribution (Goh and Ng, 2018; McCracken et al., 2014).

Contrary to hamster and mouse derived cells, human cell lines do not produce immunogenic glycans. On the other hand, they are susceptible to human viral infection, a reason why a series of viral inactivation is required when producing biotherapeutics from these and all cells (Dumont et al., 2015). HEK293 (Human Embryo Kidney 293) is a human cell line transfected with viral DNA. This is the most prominent human cell line used for protein expression and clearly produces human glycan profiles. Since its development, diverse variants of HEK293 have been generated, for example HKB11, used to produce factor VIII (Bandaranayake and Almo, 2014; Durocher and Butler, 2009). HT-1080 (human fibrosarcoma) is also a human cell line frequently used for protein expression. The line is derived from fibrosarcoma cells with an epithelial-like phenotype. It is known that epoetin delta produced by these cells has a more preferable glycan profile when compared to the same molecule produced by CHO cells, as the latter produces immunoglobulin G (IgG) with traces of the immunogenic Neu5Gc in the glycan profile(Dumont et al., 2015; Lalonde and Durocher, 2017). PER.C6, another cell line human-derived, was generated from human retinoblast transfected with E1 minigene, originally to produce adenovirus vector for vaccine development. However, the use of these cells has become popular for recombinant protein expression as they are able to grow in suspension at high density, and produce high titers of IgG (Bandaranayake and Almo, 2014; Dumont et al., 2015; Durocher and Butler, 2009). CAP (CEVEC's Amniocyte Production) are cells originate from human amniocytes transfected with an adenovirus type 5 E1 gene. The most well-known advantage of this system are the high yields and human-like glycosylation profiles of the proteins (Lalonde and Durocher, 2017; Bandaranayake and Almo, 2014). Lastly, HuH-7 (Human hepatoma) cells are a recently developed cell line also able to produce human-like glycoproteins, such as factor IX and recombinant FIX (Dumont et al., 2015).

Beyond cells, transgenic animals are also sources of biotherapeutic proteins. The development of genetically modified animals for this purpose has evolved exponentially since the 1920s, when insulin was extracted from pig pancreas. Almost a century later, in 2006, the first therapeutic for human use derived from a transgenic animal is approved in the European Union and later on (2009) in the United States. ATryn®, is a recombinant antithrombin produced in the milk of transgenic goats (Gavin et al., 2008). A second biopharmaceutical, approved in 2012 in the European Union and 2014 in the United States, under the trade name Ruconest®, is a recombinant human C1 esterase inhibitor protein this time produced in the milk of transgenic rabbits (Bertolini et al., 2016). In addition, egg white obtained from transgenic hens has been reported as a source of human interferon beta (Houdebine, 2009; Oishi et al., 2018; Walsh, 2014). Eggs are an attractive source for high level recombinant protein production as a single hen can lay up to 330 eggs in a year. Transgenic chickens have short generation times and can achieve increased reproduction rates via artificial insemination (Farzaneh et al., 2017). Two recently approved products from transgenic chicken eggs are a recombinant human lysosomal acid lipase (Kanuma) used to treat LAL-deficiency, which received approval by the FDA in 2015, and a recombinant human alpha-*N*-acetyl-glucosaminidase for the treatment of mucopolysaccharidosis, which received approval by the FDA in 2015 (Bertolini et al., 2016). Apart from simple proteins, monoclonal antibodies, including variants of currently available commercial products such as Trastuzumab, Adalimumab and Cetuximab, expressed in goat milk have been reported to exhibit glycosylation patterns that provide equivalent and even enhanced antibody function compared to their commercial alternatives (Bertolini et al., 2016).

Besides "pharmaceutical pharming", trangenic animals play an important role in the pre-clinical discovery of therapeutic proteins. Transgenic mice are the number one antibody discovery platform with seven out of the eleven monoclonal antibody drugs approved by the FDA between 2006 and 2011 deriving from transgenic mice. Humanized mouse platforms such as AlivaMab® Mouse, MeMo mouse, Kymouse and VelocImmune® Mice are currently licensed from the majority of pharmaceutical companies for antibody drug discovery at various sites around the world (Moran, 2013).

2.3. Non-mammalian expression systems

Although most of the recombinant glycoproteins approved by the FDA have been produced by mammalian cells, many factors have led to the development of alternative non-mammalian expression systems. Some of them, such as bacteria, yeast, insect and plant cells have proved to be efficient for high titres of protein expression, scalability and cost-effective production. In addition, these systems are not prone to mammalian pathogen infection. However, a common drawback of these heterologous expression systems is the lack of the required machinery to synthesize human-like PTM, such as glycosylation (Lalonde and Durocher, 2017; Yusibov et al., 2016). Nevertheless, if the desired therapeutic protein is small and does not require human-like glycosylation for its clinical function, then non-mammalian systems are the definite choice for cost-effective manufacture, a trend that can be seen across the industry when considering that the types of therapeutic proteins manufactured in microbial systems are mainly insulins, vaccine components and smaller cytokines like interferons (Walsh, 2014).

Bacterial systems are the most commonly used host cells for the expression of simple heterologous proteins (without PTMs), with around 30% of the approved therapeutic proteins being currently produced using a bacterial host (Overton, 2014). They are the most preferred because their growth requires cost-effective carbon sources, division of the cells is rapid, process scale-up is simple and produces high yields. On the other hand, proteins produced by this system often aggregate and are inactive due to the formation of inclusion bodies. Moreover, PTM in this system is also inefficient for production of more complex therapeutic proteins (Sahdev et al., 2008). *E. coli*, the first expression host used in biopharmaceutical manufacturing, is currently used as an expression system for large number of simple proteins such as the long lasting insulin-analog Lantus® (Linnebjerg et al., 2015), therapeutic enzymes such as the glucarpidase VORAXAZE® (Rattu et al., 2013) and peptide drugs such as the parathyroid hormone Preotact® (Möricke et al., 2011) and the granulocyte colony-stimulating factor Nivestim®(Gascon, 2012).

Similar to bacteria, yeast cells offer advantages such as expression of high yielding proteins and fast cell division. On the other hand, the high proportion of oligomannose glycan structures produced by these cells may result in faster clearance following therapeutic treatment and/or immunogenicity (Dumont et al., 2015). Recent advances in glycoengineering have enabled yeast to produce humanized sialylated glycoprotein (Hamilton et al., 2006), but human-like glycosylation profiles by these organisms are still difficult to produce. Therapeutic recombinant proteins obtained by heterologous expression in the baker's yeast *Saccharomyces cerevisiae* include insulin peptides, hepatitis vaccines, human serum albumin, and virus-like particles. *S. cerevisiae* is used, for example, for the production of the first FDA–approved cytokine sargramostim, (Waller, 2007) and for the GARDASIL®9 vaccine against Human papillomavirus infection (Marin et al., 2007). Another yeast system known for its ability to produce gram amounts of recombinant protein as secretory or intracellularly products is *Pichia pastoris*. Currently *P. pastoris* is used for the production of small proteins such as human insulin, human serum albumin and for a α-IL6 receptor single domain antibody fragment (Nanobody® ALX-0061)(Van et al., 2015)

The use of insect cell lines, such as Sf9, Sf21 and BTI 5B1-4, transfected with baculovirus is a well-known expression system able to produce recombinant protein with complex glycan structures. However, the platform produces high proportions of suspected immunogenic sugar structures such as oligomannose and paucimannose glycans. Another drawback is that the system is not able to produce sialylated glycan species. On the other hand, through the use of cell engineering, baculovirus-insect cells have become a powerful platform for vaccines and virus like particles production. Baculovirus/Sf9 cell production processes are increasingly adopted by biopharmaceutical companies such as Voyager Therapeutics and Biomarin, as they produce high yield of viral vectors per litre of culture, both for clinical or commercial scale gene therapy products. Biomarin's gene therapy for the treatment of Hemophilia A (Valoctocogene Roxaparvovec) is an AAV Vector-Mediated Gene Transfer of hFVIII produced in the Sf9/baculovirus insect cell system is on track for US and EU Regulatory Submission before the end of 2019 (Bunting et al., 2018).

Moreover, advances in baculovirus technology has led to the development of bacmam, a baculovirus vector containing a mammalian promoter able to express proteins in diverse mammalian cell types (Durocher and Butler, 2009; Kost et al., 2005; Lalonde and Durocher, 2017). Three insect-cell manufactured products that have been approved and released into the market are the trivalent influenza vaccine (RIV, Flublok™) manufactured with expresSF+® cells (Barr et al., 2018), an immunotherapy for treatment of prostate cancer (Provenge, sipuleucel-T) and a bivalent HPV vaccine (Cervarix, GSK), (Monie et al., 2008).

Similar to insect cells, plants do not produce sialylated glycans. In addition, immunogenic glycans α(1,3)-fructose and β(1,2)-xylose are synthetized by this system. However, genetically engineered plants able to produce glycans lacking such structures, have been used for production of FDA-approved recombinant proteins (Durocher and Butler, 2009; Lalonde and Durocher, 2017; Walsh, 2014). The first plant culture biopharmaceutical was approved by FDA in May 2012. Elelyso (Taliglucerase alfa) is an enzyme (acid β-glucosidase) produced in genetically engineered carrot cells, for treating type 1 Gaucher's disease (Grabowski et al., 2014).

Aiming to reduce the complexity and lengthy time of protein expression by cells, cell-free protein synthesis (SFPS) systems are emerging as an alternative platform to produce simple protein molecules, with potential future clinical application (Tran et al., 2018).

2.4. Expression systems overview

Each expression system has relative strengths and weaknesses relating to patient safety and therapeutic efficacy for recombinant protein production. Table 2 represents the reputed advantages and

Table 2
Reputed advantages and disadvantages of the main expression systems for therapeutic protein expression.

	Cell line	Advantages	Disadvantages
Mammalian	Human	Human PTMs, easily grown in suspension, serum-free growth	Susceptible to human viral contamination and capable of producing sialyl-Lewis^x
	CHO	Easily grown in suspension, serum-free growth, high yields, secreted proteins	High culture costs, challenging scale-up, risk of viral contamination, α-gal and Neu5Gc N-glycans, inability to produce a(2-6) sialic acids
	Murine		
Non-mammalian	Bacterial	Cost-effective growth, rapid cell division, simple scale-up, high yields of protein	Protein aggregation, formation of inclusion body, inefficient PTMs for therapeutic IgG
	Yeast	Fast cell division, high titres of protein, scalability and cost-effectiveness production, not prone to mammalian pathogens infection	High oligomannose production
	Plant		α1,3-fructose and β1,2-xylose *N*-glycans
	Insect		High oligomannose and paucimannose; unable to produce sialylated glycans

disadvantages of each system for mammalian and non-mammalian expression systems. Most notably, mammalian have more human-like PTMs but have a higher risk of viral contamination whereas non-mammalian expression systems have higher yields and are faster growing but may produce immunogenic epitopes potentially dangerous for patients. The pharmaceutical companies are consistently striving to improve any perceived weaknesses as well as focus on the higher throughput of their recombinant product (unless the drug has orphan status). As such, a balancing act is often needed to choose the best system for their particular needs.

2.5. Cell line engineering (in vivo) for defined therapeutics

Cell line engineering enables the addition or removal of genes that may affect the structure of synthesised proteins. The structures enable physiochemical properties that in turn may produce desirable therapeutic outcomes. Recent advances in genomics have enabled the design of expression systems with specific features. The process involves combining a cell with an engineered vector, followed by selection of the transfected cell lines. Selection systems are employed for this purpose and, even though other efficient systems, such as OSCAR™, have been developed, DHFR and GS genes remain the most popular (Lalonde and Durocher, 2017).

Resistance to antibiotic genes is an alternative selection system. It is also possible to insert genes that improve cell line production through diverse approaches, such as genes to express growth factors, to control the cell cycle, anti-apoptotic genes and proto-oncogenes. Recombinases have been developed to target integration of transgenes in the active euchromatin region, aiming at higher expression. The use of new nucleases technology, such as CRISPR/Cas9, has emerged as a potent tool to facilitate the transgene insertion at hotspots of the host genome, and consequently, execute higher expression of therapeutics (Lalonde and Durocher, 2017; Wurm, 2004).

Therapeutic activity of mAbs is often related to an effector function such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) or antibody-dependent cellular phagocytosis (ADCP), each of which is associated with a binding interaction that depends upon the glycan structure. Cell engineering allows the knock-in or knock-out of genes expressing enzymes involved in the glycosylation pathway that controls these glycan structures. For example, knock-out of fucosyltransferase FUT8 gene in CHO cells has proved to be efficient for production of afucosylated antibodies, which presents a dramatically higher ADCC activity (Yamane-Ohnuki et al., 2004). Moreover, co-expression of ST6Gal1 and β4GalT1 enables production of highly sialylated IgG. This way it is possible to rationally design features on the glycoprotein to enhance its immune function (Durocher and Butler, 2009; Lalonde and Durocher, 2017).

2.6. In vitro glycoengineering

The structure of *N*-glycans attached to the Fc portion of recombinant proteins is of major interest for therapeutic applications. Such structure has tremendous impact on the function and safety of the protein. It is known that mAbs have increased ADCC activity when the level of fucosylation is reduced (Umaña et al., 1999; Yamane-Ohnuki et al., 2004). Increased galactosylation has correlated with increased ADCP and ADCC under certain circumstances (Chung et al., 2014; Thomann et al., 2016). Higher galactosylation can also increase CDC (Peschke et al., 2017). Furthermore, mAbs with higher proportions of oligomannose glycans are cleared faster from the organism (reduced half-life), which can reduce IgG therapeutic effect. Therefore, diverse strategies have been developed to control IgG glycan profiles produced by expression systems (Wang and Amin, 2014). Cell engineering, as discussed above, optimization of media and in vitro glycoengineering are the main strategies to control glycosylation in mammalian cells.

In vitro glycoengineering (IVGE) requires the use of enzymes (glycosyl transferases and glycosyl hydrolases) and respective substrates involved in the glycosylation pathway. The greatest advantage of this method is its efficiency to produce single and homogeneous glycoforms. Therefore, *in vitro* glycoengineering is a valuable tool to generate a library of *N*-glycosylated mAbs for enabling specific functions *in vivo* and *in vitro,* for the development of next-generation mAbs. Moreover, the increased availability of new enzymes and substrates can open a new avenue for the use of IVGE on an industrial scale, delivering biotherapeutics with improved efficacy, safety and cost-effectiveness, once it can be integrated to the downstream processing. (Li et al., 2017; Tayi and Butler, 2018; Tayi and Butler, 2015; Wang and Amin, 2014). At a research and development level, this strategy provides many advantages over genetic approaches. For example, this strategy allows scientists to provide a library of glycoengineered variants which can be subsequently tested for desirable structure and function whereby the entire workflow for the starting material (mAb) does not need to be adapted. Selecting the most efficacious glycovariant would then be used to direct a chosen path for large-scale manufacturing.

3. Process development for mammalian cell culture

3.1. Cell culture background

Mammalian cells such as CHO are the most common choice in the production of recombinant protein therapies. Product consistency using these expression systems is dependent upon a controlled culture based upon parameter ranges defined by the design space of the process. Mammalian cells which have been exposed to variable growth conditions may fail to reach desired densities or their productivity may be significantly reduced. Critical quality attribute changes including protein misfolding, variations in amino acid sequences or alterations in the glycosylation pattern can all occur with changes to culture parameters (Ivarsson et al., 2014). The mode in which CHO cells are grown will therefore greatly impact their growth and productivity. When moving towards a continuous upstream process, cell must remain stable and maintain productivity over the lifespan of the culture. Advancements in cell line engineering can improve cell stability and may improve productivity. An example is the overexpression of the bcl-2/bcl-xL genes which can impart apoptosis resistance in high density cultures (Krampe and Al-Rubeai, 2010). Furthermore, the choice in cell culture media will impact the performance of cells (Palm and Thompson, 2017).

3.2. Cultivation methods

Mammalian cells are typically grown in suspension for industrial applications. The originator recombinant therapies such as Trastuzumab®, Infliximab® and Etanercept® which were licenced at the turn of the century were mostly suspension cultures grown in large stirred tank reactors (Chu and Robinson, 2001). Cells were grown in batch systems using poorly defined media, which yielded low titres compared to today's enhanced processes. Fed-batch cultures are now in routine use, prolonging the cell viability by delivering optimised feeds on specifically chosen days (Bibila and Robinson, 1995). A representation of a fed-batch culture is presented in Fig. 4. High glucose feeds extend the period of high cell viability beyond that of a batch process, up to two weeks. Expected titres from a fed-batch culture remains around 1-5 grams per litre, however yields reaching 10 g/L of antibody have been described (Birch and Racher, 2006; Huang et al., 2010).

Perfusion cultures advance this concept by feeding fresh media at a constant rate which is expressed as reactor volumes per day (RV/day), whilst retaining cells within the system (Fig. 5). Perfusion cultures are the primary enabler for continuous manufacturing once integrated with a multi-column downstream setup.

Shifting towards a continuous platform has advantages; reducing the residence time of the antibody in the culture results in a more

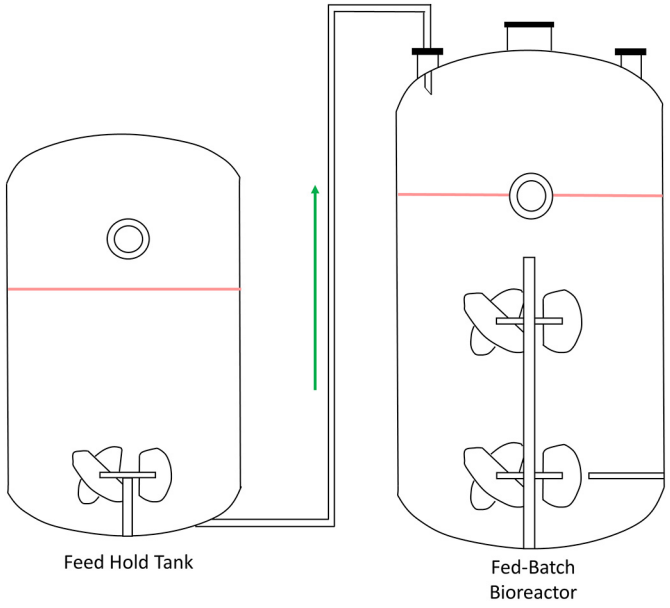

Fig. 4. A representation of a batch (left) and fed-batch culture (right). Batch cultures are favoured during scale-up steps in a bioprocess, whereas batch cultures are employed during the final upstream step. A feed source is added to the production bioreactor on pre-determined days to prolong the viability of the culture beyond that of a batch system.

uniform product quality profile. Proteases and secreted enzymes in the culture broth will interact with the glycan structure on the antibody, potentially reducing the efficacy of the product. The scale of integrated continuous processes reduces the scale of operation of these systems, therefore single-use technologies become a good candidate for use instead of stainless steel equipment (Klutz et al., 2015). In order to facilitate the advancement in continuous upstream technologies, there must exist a purification train that can handle the culture volumes that are been produced (Zydney, 2016). Multi-column skids that can be switched to prevent product losses through overloading of resin exist, which in turn returns better resin utilisation when compared to traditional batch loading cycles. Virus inactivation in continuous systems remains a challenge however, especially for viral filtration steps where the potential for filter fouling is high.

Many of the traditional fed-batch bioreactor design concepts can be applied to perfusion cultures. Inputs for control of critical process parameters and a robust feedback loop must be present. Examples of critical process parameters that will have an impact on product quality include temperature, dissolved oxygen content, pH and agitation (del-Val et al., 2010). The complexity of a perfusion reactor is increased however when we consider the recirculation of cells. The method of cell separation must not be prone to fouling/shearing or else the efficiency of the perfusion system may be lost over time. An increased emphasis must also be placed on monitoring cell viability and density values. If the viability of the culture does not remain constant then productivity can be severely impacted. Chemostat cultures maintain a constant culture volume over an indefinite amount of time. The system is kept in steady-state by setting a media flow in-rate that matches the removal rate of cells and spent media. Nutrient availability governs the specific growth rate; cells will maintain a density where there are enough nutrients to support growth but none in excess.

Notwithstanding the challenges currently facing downstream continuous processing, the genetic instability of cells over a period of time is therefore the new limiting factor when determining culture duration (Kim et al., 2011). The replenishment of fresh nutrients and the removal of cellular by-products such as ammonia and lactate allow for a more consistent product quality profiles in chemostat cultures. To increase product titre values however, the cell density must be increased beyond what is possible in a chemostat culture. A "closed" system is preferred, where cells are retained within the system whereas spent media is removed. Perfusion cultures build upon the chemostat principle by retaining cells that would otherwise be lost through bleeds through the use of a hollow fibre membrane. Cell densities exceeding $6x10^7$ cells/mL are commonly reported (Chotteau et al., 2014; Karst et al., 2017; Xu and Chen, 2016). The residence time of the product in culture is reduced significantly, this is especially important for fragile products which may be prone to degradation. In the case of mAbs where the residence time is not as crucial, perfusion rates as low as 0.5 RV/D are typical.

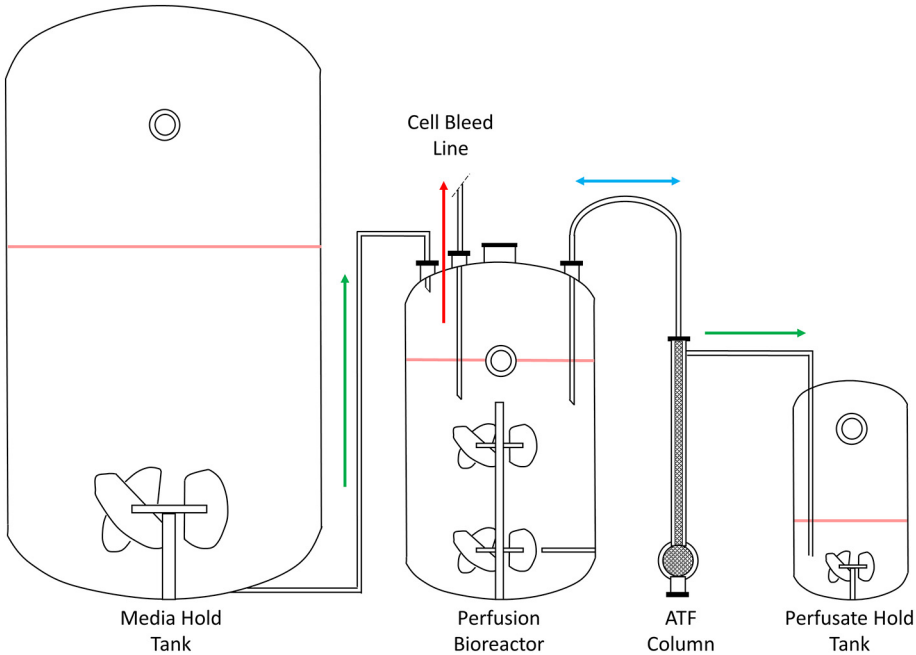

Fig. 5. A representation of a perfusion system. To produce a similar quantity of antibody in comparison to batch and fed batch models, the required perfusion bioreactor is much smaller. However, a large media hold vessel is needed to supply perfusion media at the required rate over the duration of the culture.

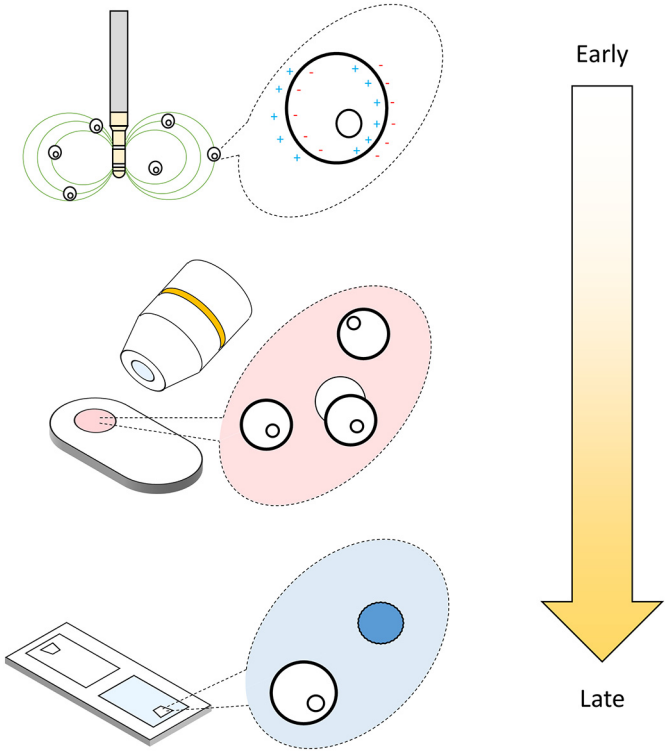

Fig. 6. Methods of analysing cell culture viability. (Top) In-line capacitance probes measure the bulk capacitance of the biomass in a cell culture. Live cells will build a charge on their outer membrane as the ions within the cell migrate towards the poles of the probe. When the cell has become compromised, this ability to hold charge in the cell is lost. (Middle) Optical systems can image cells in real time, allowing for classification of physical cell parameters such as the circularity and size of cells. Cells which have entered apoptosis will exhibit different physical attributes in comparison to a healthy specimen. (Bottom) Trypan Blue exclusion is the current industry standard. Cells which have been dyed blue have a compromised outer membrane which allows the Trypan Blue to penetrate. A digital counter is then used to count both the live and dead cell populations in a sample.

There exist a few different methods for implementing a perfusion system (Voisard et al., 2003). The most common method of separating cells in a perfusion setup is through the use of porous membranes. Spin filters were previously used for perfusion applications, however they have lost popularity due to scale up difficulties associated with filter fouling (Deo et al., 1996). The authors observed that expected versus actual flow rates across the filter were not consistent as the duration of the culture increased, and the pore size of the filter had to be increased due to fouling, resulting in excessive product loss. To reduce the potential of filter fouling, a self-clearing membrane was devised. Examples of membrane systems include tangential filtration flow (TFF) and alternating tangential flow (ATF) (Karst et al., 2016). Both methods work on a similar principle; culture broth is fed tangentially onto a membrane of a defined pore size. The pore size of the membrane allows media to flow through (filtrate) whereas cells (retentate) continue to pass along the feed stream. The retentate stream is fed back to the bioreactor whereas the filtrate is captured offline. The flow of the culture across the membrane tangentially reduces the potential for filter fouling. In TFF applications, the feed stream travels in a single direction across the membrane. ATF systems include a pump that can create a bidirectional flow across the membrane, further reducing the potential for filter fouling. Evidence suggests that TFF systems are prone to retaining significant levels of produced mAb (up to 50%) when compared to an identical ATF system (Clincke et al., 2013). Both systems are scalable with proven success.

3.3. Scale up/scale down considerations

The scaling of cell cultures is a delicate process that must be performed sequentially over a number of passages. Mammalian cells produce growth factors which activate metabolic pathways such as glucose and amino acid transporters (Palm and Thompson, 2017). In the absence of sufficient growth factors due to a non-optimised scale up system, cell proliferation may be reduced. The challenges associated will process scaling are further heightened when considering that they are often cell line and process specific. A high throughput experimental screening design should be developed to analyse process variables. In essence, the cells should be grown and passaged in conditions that are uniform throughout all steps in the scale-up train. However, equipment design and volume changes will have an effect on how these parameters are controlled (Nienow, 2006).

Although the focus of industry is to increase the scale of processes to meet market demands, there is also a justification for scaling-down processes to allow for the use of disposable technologies. 20,000 L stainless steel production bioreactors are common in single product manufacturing facilities, whereas agile single use technologies are often 10-fold smaller in scale and allow for greater flexibility (Jacquemart et al., 2016). Fed-batch processing in stainless steel was used as a platform for the originator drug products, but they are inefficient in terms of their productivity output. Furthermore to cope with increased flow rates, downstream columns are oversized to ensure no loss in product. Single use bioreactors allow for a more flexible process, considering multi-product facilities.

3.4. Process monitoring

Next generation manufacturing aims to automate or eliminate the need for offline sampling through the use of robust process analytic technologies (PAT). This data should be collected in real time and to identify process variability to a high degree of accuracy (Read et al., 2010). The control strategy that is in place should be robust enough to maintain all critical process parameters within their characterised ranges. Current dissolved oxygen, pH and temperature probes are capable of continuous monitoring with a high degree of sensitivity. Routine offline sampling is typically sufficient for monitoring cell health and productivity in batch cultures but this can prove problematic in perfusion when bleeding of the bioreactor is dependent on cell density values. Non-optimised cell bleeds can lead to excessive product loss or increased media losses, therefore an online sensor capable of measuring cell biomass or density in order to feed back into the process control loop would be desirable.

Cell measurements are a fundamental aspect of cell culture that is performed at development and commercial scale. An overview of measurement techniques are provided in Fig. 6. The viability and density of cells is a key indicator as to how the process is performing. Trypan blue dye exclusion testing is a widely accepted standard for the analysis of cell density and viability (Cadena-Herrera et al., 2015). Trypan blue is excluded from cells which have an intact membrane; loss of membrane integrity is an indicator of cell death, resulting in the inclusion of the dye. Automated cell counters can distinguish live and dead cell populations based on this principle. Basic standardised protocols are available in the literature to perform a trypan blue exclusion test (Strober, 2015). Due to the destructive nature of adding dye to cells, offline samples of the culture must be taken to perform counts. The loss of membrane integrity is a late stage apoptotic event.

The measurement of cell capacitance to measure bulk biomass has shown comparable results to trypan blue exclusion during the exponential growth of cells (Braasch et al., 2013). Biomass probes work on the principle of measuring electrical capacitance (C). Cell cytoplasm is highly conductive due to the high salt and ion concentration. The cell membrane is non-conductive, therefore the cells act as small capacitors. When an electric field is applied, the positively charged ions will

migrate in the direction of the electric field whereas the negatively charged ions will flow in the opposite direction. The ions within the cell cytoplasm will travel until they reach the membrane, which acts as an insulator. A charge separation is formed within the cell; the greater number of cells results in a higher capacitance reading (Carvell and Dowd, 2006). The frequency of the electric field will have an effect on cell capacitance; capacitance readings will drop dramatically if ions cannot reach the cell membrane due to high frequency values. It is important to optimise the frequency at which measurements are taken. The critical frequency (fc) is the frequency at which $\Delta C = 0.5$. A frequency sweep is performed, and the readings are taken at a suitable frequency that provides a stable capacitance reading. The use of a commercially available biomass probe has been cited in literature (Karst et al., 2017), with additional emphasis on their application of controlling the cell bleed from a perfusion process.

Given the multiple methods of examining the health of cell cultures, we must examine the best methods used throughout academic and industrial applications. At what phase of apoptosis should we consider a cell as no longer viable? The late stage of apoptosis is the current indicator that is routinely analysed through the use of trypan blue exclusion, however the use of novel techniques may provide an earlier reading that the culture is losing its viability. Furthermore, optical systems have reached the market that will capture images of cells in real time which will allow for in-depth analysis of morphological shifts.

4. Cell culture medium development

Culture medium is a crucial part of cell culture which can simply be defined as a liquid or gel that supports the growth of cells and provide them with suitable amount of growth factors, vitamins, minerals, glucose, and amino acids. Basically, culture media are classified into natural and synthetic media (Arora, 2013). The natural media rely on the presence of the naturally occurring biological materials including biological fluid such as serum, human placental cord serum, and amniotic fluid. The second types of natural medium includes tissue extracts such as hepatic, spleen, tumours, bone marrow, the bovine embryo, and chick embryo extracts. Plasma clots and coagulants can be another form of natural media. The main shortcomings are the complex and undefined nature of these media which makes the outcomes poorly reproducible (Morgan et al., 1950). On the other hand, the synthetic or the artificial media provide a fruitful avenue for research and development.

Essentially, media are sub-categorized into four groups. Serum containing medium is the one of the most frequently used culture media which is based on using fetal bovine (calf) serum (FBS) as the sole or main added component to basal media. Generally, serum from small animals is superior over the adults due to the fact that it contains less γ-globulin content which induces less antibody interactions and thus less negative impact on cell growth (Astori et al., 2016). Serum provides a plethora of bioactive components including proteins, vitamins, minerals, growth factors and hormones that supply cells with all the essential nutrients as well as boost cell proliferation and improve specific cells function (Gstraunthaler et al., 2013). Furthermore, it contains several carrier mediators such as albumin and transferrin which deliver a variety of vitamins, hormones, and lipophilic molecules into the cells. In addition, serum can improve cells' anchor and adhesion to substrates by the pivotal role of fibronectin. The presence of protease inhibitors and some metals such as calcium, iron, zinc, and magnesium protect cells against proteolysis. Serum is a viscosity enhancer that can protect cells against shear stress, particularly in suspension culture. It also acts as a buffering agent (Arora, 2013; Yang and Xiong, 2012). However, the main disadvantages of serum include lack of reproducibility due to batch to batch variation. This variation is due to the fact that serum contains ill-defined amounts of bioactive components including growth factors, trace elements, hormones, and proliferation-driving transcription factors. These components are quite variable among different batches of serum which can cause inconsistent cell growth, as well as, altering cellular response to chemicals. Subsequently, the comparison among different research groups and laboratories are unreliable (Dimasi, 2011). Serum represents an important source of contamination caused by microbes with such bacteria like mycoplasma, viruses, prions, fungi, and yeasts, as well as endotoxins (Doucet et al., 2005; Urbano and Urbano, 2007). In addition, some of FBS components can be metabolically incorporated into the culture cells and expressed as surface-linked molecules such as antibodies or receptors which adversely affects the interaction and response of cells to surrounding environments (Bauman et al., 2018). The other important drawback of serum is the ethical considerations due to the inhuman and harsh way to collect the serum from calves via cardiac puncture which is associated with suffering and pain. Reports refer that more than a million calves are sacrificed each year to get 500,000 litres of FBS (Jochems et al., 2002; Tekkatte et al., 2011). Economically, serum costs and demands have been constantly rising despite the shift of industrial companies to produce serum free medium for recombinant protein production (Brindley et al., 2012). Logistically, the production cost of serum has also increased due to the implementation of the good manufacturing practice (GMP) on the infrastructure and animal breading facilities. As an approximate 3 calves are needed to get 1 L of serum, breeding animals for serum production is not a cost efficient business for farmers (Bauman et al., 2018). Although serum is well-known to have a growth stimulatory components, some components in serum can interact with other media additives forming highly toxic compounds. For instance, polyamine oxidase can interact with added polyamines such as spermine and spermidine forming highly toxic poly-spermine (Yang and Xiong, 2012).

The second type is culture media without serum which are further classified into serum-free media, animal-derived components free media, protein-free media, and chemically-defined media. While serum-free media refer to the use of supplements other than serum such as discrete large proteins from plant or animal tissues, they contain several undefined components, for instance, animal or plant hydrolysates. The animal-derived components free media are free from any animal or human components, instead they contain catalysts, nutrient broth, enzymes, plant hydrolysates and cell-culture derived recombinant proteins such as hormones, growth factors, and cytokines (Grillberger et al., 2009; Karnieli et al., 2017). Protein free media contain only hydrolysates or digested proteins or low-molecular weight proteins such as insulin. These media, in addition to supporting cell growth and productivity, facilitate the downstream processing of protein isolation and purification. However, they may contain some poorly identified components such as lipids, therefore they are not deemed as chemically-defined media (Karnieli et al., 2017; Valk et al., 2010). Chemically-defined media contain chemically and structurally defined organic or inorganic components. They also contain recombinant proteins such as hormones, growth factors and cytokines that are manufactured by a specific cell line or produced in bacteria or yeast. The main advantages are good reproducibility, no ethical issues, and defined composition. For this reason, many biopharmaceutical companies are now shifting from their existing manufacturing processes with animal derived products to chemically defined media. However, the main drawbacks are the potentially higher costs, cell-type specificity and need for longer periods of cellular adaptation (Bauman et al., 2018).

4.1. New approaches in culture media developments

Several novel approaches have been introduced into the field of culture media optimization, including multi "omics" technologies such as proteomic, genomic, epigenomic, metabolomic, transcriptomic and glycomics that enable better understanding of cell metabolism and provide novel insight into the molecular mechanisms which may help in developing strategies to improve cellular production machinery (Stolfa et al., 2018). A study by Schaub *et al* compared the gene

expression of mAb-producing cell line under two different media formulations. The study showed that different media formulations of lipid can affect gene expression causing high or low antibody titres. Furthermore, based on the transcriptomic data, optimization of lipid metabolism resulted in a significant improvement of the differential gene expression and therefore, increased antibody titre by 20% (Schaub et al., 2010). The use of a proteomic approach to optimise media composition plays an integral role in minimising the accumulation of toxic byproducts and growth inhibitors. Some studies used high performance liquid chromatography (HPLC) combined with mass spectrometry (MS) to characterise media components, intracellular and extracellular metabolites of amino acids, and growth-related pathways that detrimentally affected cells proliferation and productivity (Chong et al., 2009; Chong et al., 2011; Constantin et al., 2007; Selvarasu et al., 2012). Another study used nuclear magnetic resonance (NMR) and MS to qualitatively and quantitatively analyze the toxic metabolites that were accumulated in spite of well controlled lactate, NH_3, and osmolality levels (Emwas et al., 2019). The use of such powerful -omics techniques necessitate a careful titration of certain monoacids including aromatic amino acids such as phenylalanine, tyrosine, tryptophan, as well as methionine, leucine, serine, threonine, and glycine which were found to produce toxic end by-product, therefore, they should be added at a level matches the rate of consumption (Mulukutla et al., 2017).

As the accumulation of toxic metabolites and depletion of nutrients induce the activation of signalling pathways such as autophagy or apoptotic cell death, addition of autophagy inhibitors (Baek et al., 2016; Kim et al., 2013) or apoptosis inhibitors (Butler, 2005; Tintó et al., 2002; Zanghi et al., 2000) can improve cell longevity. However, these inhibitors are quite expensive and add more cost for the overall media formulation costs. The newer approach involves adding growth factors such as Insulin, IGF − 1, and LongR3 which have been found to counteract apoptosis, thus improve cells growth and productivity (Adamson and Walum, 2007; Morris and Schmid, 2000).

The addition of certain chemicals such as histone deacetylase inhibitors, namely, valproic acid and sodium butyrate to the culture media can improve cell growth via making the growth genes more accessible for transcription as well as, improve cell productivity due to the fact they can arrest cells at G1 phase and thus increase the population of cells at the productive phase (Backliwal et al., 2008b; Chen et al., 2011; Mimura et al., 2001; Yang et al., 2014). However, a careful titration of concentration and timing is required for those chemicals as they can induce detrimental effects to the overall cell growth and productivity (Ritacco et al., 2018).

Recently, some feeding strategies have successfully improved the overall growth rate and antibody titre by manipulating the amount of nutrients consumed by cells and thus reducing the overall inhibitory metabolites and waste products. For instance, implementation of a glucose feeding pump called HIPDOG (Hi-End pH-Controlled Delivery of Glucose) was shown to significantly increase cells proliferation, specific and overall antibody productivity (Hiller et al., 2017). Increased lactate consumption by the cells and the subsequent rise of the media pH activates the pump and delivers more glucose to the media, which is then consumed by cells and lactate is produced thus re-balancing the pH (Gagnon et al., 2011). This technique was successfully applied for the hybrid perfusion and fed-batch process in which the rate of perfusion is regulated by pH (Hiller et al., 2017). Another study provided a fruitful avenue for dynamic feeding strategy based on adding optimised medium at a specific rate calculated based on online capacitance cell density measurement or glucose monitoring which was associated with a substantial increase of antibody production (Lu et al., 2013). Other researchers have shown that the use of lactate and pyruvate along with fully optimised media formulation can markedly minimize the accumulation of toxic CO_2 and NH_3, as well as synergistically increase the population of growing cells and subsequently, their antibody productivity (Li et al., 2012).

One of the newer approaches of culture media development involves the addition of nucleotides such as deoxyuridine to fed-batch culture of CHO cells to maximise IgG production. Furthermore, a synergistic improvement of cell productivity was reported following the addition of pyrimidine nucleosides including deoxycytidine, deoxyuridine, and thymidine to the fed-batch culture with subsequent increase of cell productivity to more than 9g/L of mAb producing CHO cells and more than 4g/L of Fab fragment concentration in a fed-batch culture containing Fab fragment expressing CHO cells during 16 days of culture (Takagi et al., 2017).

5. Critical quality attributes

The International Conference for Harmonisation (ICH) defines critical quality attributes (CQAs) as *a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality*. CQAs have been identified both for biological drugs and the process that have been used to manufacture them (Yu et al., 2014).

The heterogeneity of biopharmaceuticals has already been discussed at length in the literature (Sandra et al., 2014; Tejwani et al., 2018). Originally, the identification and characterisation of CQAs has provided a standardised approach to ensuring that each batch of drug had the same efficacy as previous batches. However, as many patents on biopharmaceutical drugs have expired and biosimilars have entered the market, the analysis of CQAs arguably has become even more important. Biosimilars closely mimic the original drug product and can offer more affordable treatment for patients. The analysis of CQAs is the first step in demonstrating the biosimilar is safe for consumer use and shows equivalent (or better) efficacy (Gaughan, 2016).

The required testing to assess CQAs for biological drugs has been broken down into three main areas: analytical testing (glycosylation, aggregation, fragmentation), binding assays (e.g. enzyme-linked immunosorbent assay (ELISA)), and cell based potency tests. Analytical testing has also been used to evaluate process CQAs. Any media buffer components or supplements must be tested to confirm purity and the absence of any contaminants. In addition, host cell proteins must be removed from the culture media. The ICH has produced guidelines that relate to identification and testing CQAs (European Medicines Agency, 2011).

5.1. Glycosylation

As mentioned above, glycosylation has been identified as CQA for biopharmaceutical drugs. The glycosylation profile has the ability to alter the stability, half-life, bioactivity and other physiochemical properties of the drug (Zhang et al., 2016). Glycosylation of a protein produced by a mammalian cell involves PTMs. The process in which glycans are attached is complex and involves a host of cellular machinery, this process has been described elsewhere in the literature (Tejwani et al., 2018; Dang et al., 2019). Glycan heterogeneity can be attributed to many factors: cellular processes in the endoplasmic reticulum and golgi apparatus, availability of substrates, and enzymatic functions such as glycosyl transferases and glycosyl hydrolyazes. Another reason is that glycosylation is also affected by the conditions in which the cells are cultured (Hossler et al., 2017). Differences in media composition, pH, agitation, and media supplementation have been shown to alter the glycan profile (Sha et al., 2016). Mammalian cells, such as CHO cells are used to produce therapeutic proteins that have human-like glycans. However, they also have the ability to produce non-human glycans that would have a negative immunogenic effect to human such as αGal epitope (Ramm et al., 2015) and NGNA (*N*-glycoyl neuranimic acid).

The variation discussed above relates to variation of the glycan pattern at a specific site; this is termed micro-heterogeneity. Macro-heterogeneity is used to describe variation of the location of the glycosylation and also the number of glycans present on the protein

(Zhang et al., 2016). Given the inherent heterogeneity in glycan profiles and the risk of producing non-human glycans, the glycan profile must be accurately determined and warrants its designation as a CQA.

5.2. Challenges of modern therapeutics

As stated previously, biopharmaceutical drugs are complex proteins; and as such evaluating CQAs presents a significant challenge. In addition to heterogeneity, the molecular weight of the drugs can span 3 orders of magnitude, for example insulin has a molecular weight of approx. 5 kDa while mAbs can have a molecular weight in excess of 100 kDa. Traditional techniques used for small molecule drugs have limited application to their biopharmaceutical counterparts.

NMR has been used extensively for the structural elucidation of small molecules but the large molecular weights of modern biopharmaceutical drugs exceed the capabilities of traditional NMR. However, modern approaches using solid phase NMR may open up more avenues in the future. One limitation of NMR is it only provide the average signal across the entire protein. Conventional 1D NMR would not detect areas that have been folded incorrectly or contaminants in low concentrations. 2D NMR can be used to determine primary, secondary, tertiary and quaternary structures of therapeutic proteins (Sauve et al., 2008). Typical experiments include heteronuclear ^{1}H, ^{15}N- or ^{1}H, ^{13}C correlated NMR (Brinson et al., 2019; Sauve et al., 2008).

In addition to ensuring that any therapeutic protein produced matches the appropriate CQAs, care must be taken to confirm that in-process impurities in the final drug formulation are below the accepted limits. An example of typical contaminants arising from biopharmaceutical production are host cell proteins (HCP)(Gilgunn and Bones, 2018). The presence of HCP in biopharmaceutical batches can reduce product purity, which is another CQA. Interestingly, neither the American or European regulatory guidelines do not specify what an acceptable level of HCPs are, although industry strive for total HCP value of less than 100 ppm in the final drug product (Vanderlaan et al., 2018). Currently the gold standard for HCP detection is ELISA, although this test does not provide information on specific HCPs (Tscheliessnig, et al., 2013); alternatively LC-MS may be used. This approach has several advantages including, specific identification of HCP, high sensitivity, and quantitative results. However, development of methods can require significant experience, and the cost of an LC-MS instrument may be prohibitive for smaller labs (Thompson et al., 2014).

Analysis of CQAs must be conducted before the drug enters Phase I clinical study. The cell line must be fully evaluated to ensure that mutations have not occurred during routine cell culture. The cell line must also be sterile and free from mycoplasmic contamination.

5.3. Recent trends in techniques

As the assessment of CQAs can increase batch release times, recent trends in techniques have attempted to shorten analysis times (Alekseychyk et al., 2017). This has been accomplished in several ways: improvement of instrumentation, development of high-throughput sample preparation methods.

Reversed-phase and HILIC (Hydrophilic Interaction Chromatography) chromatography separations have been used extensively for analysis of biopharmaceuticals (Ikegami, 2019; Stockmann et al., 2015). They have been applied across each CQA from determining the sequence of amino acids in the protein to assessing the glycan profile. In a standard instrument set up, samples are analysed by only one stationary phase at a time. 2D-LC has gone some way to improve this by "heart-cutting" sections of the chromatogram and diverting them to a second, orthogonal stationary phase (Sandra et al., 2017).

The development of high-throughput methods has also received increased attention in an effort to cut analysis time, particularly for *N*-glycan analysis (Varadi et al., 2014). Typically, glycans are analysed by enzymatic cleavage from the mAb before being derivatised by 2-aminobenzamide (2-AB) to increase detectability by fluorescence detection or mass spectrometry. One approach that has recently been developed is the parallel analysis of transferrin and sample preparation of its associated *N*-linked glycans on a microfluidic compact disc platform with subsequent analysis by MALDI-MS (Quaranta et al., 2016). Samples loaded on a single CD can be processed in 3.5 hours, and showed good reproducibility with %RSD less than 15%. In the future miniaturised techniques like these could be used as a complementary analysis to the traditional LC based approach.

6. Conclusion

Advances in manufacturing of recombinant proteins have moved at a swift pace over the past decade. Mammalian cells still remain the most popular choice at present, in part due to historical reasons as biopharmaceutical companies seek to harness/reemploy existing facilities, processes from other products and also due to the fact that their glycosylation closely mimics that of human glycosylation. Glycosylation of a recombinant protein is tightly linked to biological function and can impact on its safety and efficacy. If however, new advances using other expression systems can mimic human glycosylation or generate favourable glycosylation for therapeutic efficacy, there may be dramatic shift in the landscape. Notwithstanding the advances in cell line engineering, development of chemically defined media and product characterization, one of the most prominent trends in biomanufacturing of mammalian cells is the shift towards continuous bioprocessing to enhance product throughput, process speed and minimize costs. Continuous upstream processing has been well adapted by industries and is now well established in many biopharmaceutical industries in their research and development sites and is incrementally being introduced into their manufacturing sites. However downstream continuous culture remains the bottleneck and warrants some more time and cost investments. Lastly, the bioprocesses for next generation therapeutics including fusion proteins, bispecifics and cellular and gene therapy products will all benefit from current advances in mammalian cell production and will also present new unique challenges to overcome in the future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biotechadv.2020.107552.

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