Cell culture processes for biopharmaceutical manufacturing

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Recombinant proteins manufactured using animal cell culture processes comprise a significant fraction of biopharmaceuticals. With the expiry of patents on this class of therapeutics, there is also a significant interest in manufacture of biosimilar versions of such therapeutics. This article provides a birds-eye view of upstream process development for animal cell culture processes, with a focus on advances pertinent to the development of processes for biosimilars.

Keywords: Biopharmaceutical, CHO, cell line development, glycosylation, monoclonal antibody.

Introduction

IN 2015, six of the top 10 drugs with sales of USD 59 billion were recombinant protein biopharmaceuticals manufactured in animal cells[1]. Expiry of patents on biopharmaceuticals along with increasing clarity on regulatory pathway for clearance of biosimilar versions in Europe and USA has generated significant interest in the manufacturing of such recombinant protein therapeutics world-wide. Unlike small molecule pharmaceuticals, recombinant protein therapeutics are larger, more complex molecules which cannot be chemically synthesized. As an illustration of the difference in size, aspirin has 21 atoms with a molecular weight of 180 Da, while a monoclonal antibody, currently the fastest growing class of recombinant protein therapeutics, has ~20,000 atoms with a molecular weight of ~150,000 Da. Manufacturing these therapeutics requires harnessing the synthetic capability of living cells and is largely carried out in prokaryotic cells like *E. coli*, or in eukaryotic cells like animal cells. A significant factor governing the choice of the type of cell used to produce a particular protein is the capability of cell type to perform any required post-translational modifications (PTMs). PTMs are chemical modifications of a protein, which can widen the range of functionality of the protein. PTMs observed in context of therapeutic proteins include glycosylation, carboxylation, hydroxylation, sulphation, amidation, etc. with glycosylation being the most common modification[2,3]. Proteins requiring PTMs like glycosylation for their therapeutic effect need to be expressed in animal cells, since *E. coli* and yeast are unable to provide appropriate PTM. There have been some efforts to engineer yeast to provide human-like glycosylation profiles, but there are no glycoproteins yet in the market using such a platform[4]. Animal cells remain the predominant platform to manufacture recombinant glycoprotein therapeutics.

Upstream process development for manufacturing recombinant proteins in animal cells comprises several steps outlined in Figure 1. Briefly, it starts with the selection of appropriate host cell line, which may be engineered to incorporate desirable features for production. During stable cell line development for production, the host cell is transfected with transgene encoding the protein of interest. Of the millions of cells transfected, selection and screening of single cell clones is carried out to identify the best possible clone with suitable growth and productivity attributes and showing stable expression of protein over a period of 2–3 months. Medium and process conditions are optimized for the identified cell line to increase culture longevity and productivity of the cell line. Biosimilars development may have an added goal to achieve a product quality attribute similar to the innovator. Production has historically been carried out in stainless steel stirred tank reactors, but advances in single use bioreactors have provided the option of utilizing single use technology with reduced capital cost. This article aims at providing a birds' eyeview of upstream process development for animal cell culture processes, with a focus on advances relevant to the development of processes for biosimilars. An important area especially for biosimilar manufacturing not covered in this review is the application of high resolution analytical techniques to compare biosimilar products to innovator molecules[5].

Host cells and host cell engineering

Hamster and mouse cell lines have been widely used industrially as host cells for recombinant protein production and include Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, mouse myeloma (NS0) and hybridoma (SP2-0), with CHO cells being the most commonly used[6]. Aspects such as PTM on recombinant protein and host cell impurity profile can differ depending on the host cell species. For example, nature of glycosylation of a recombinant glycoprotein can vary with the host

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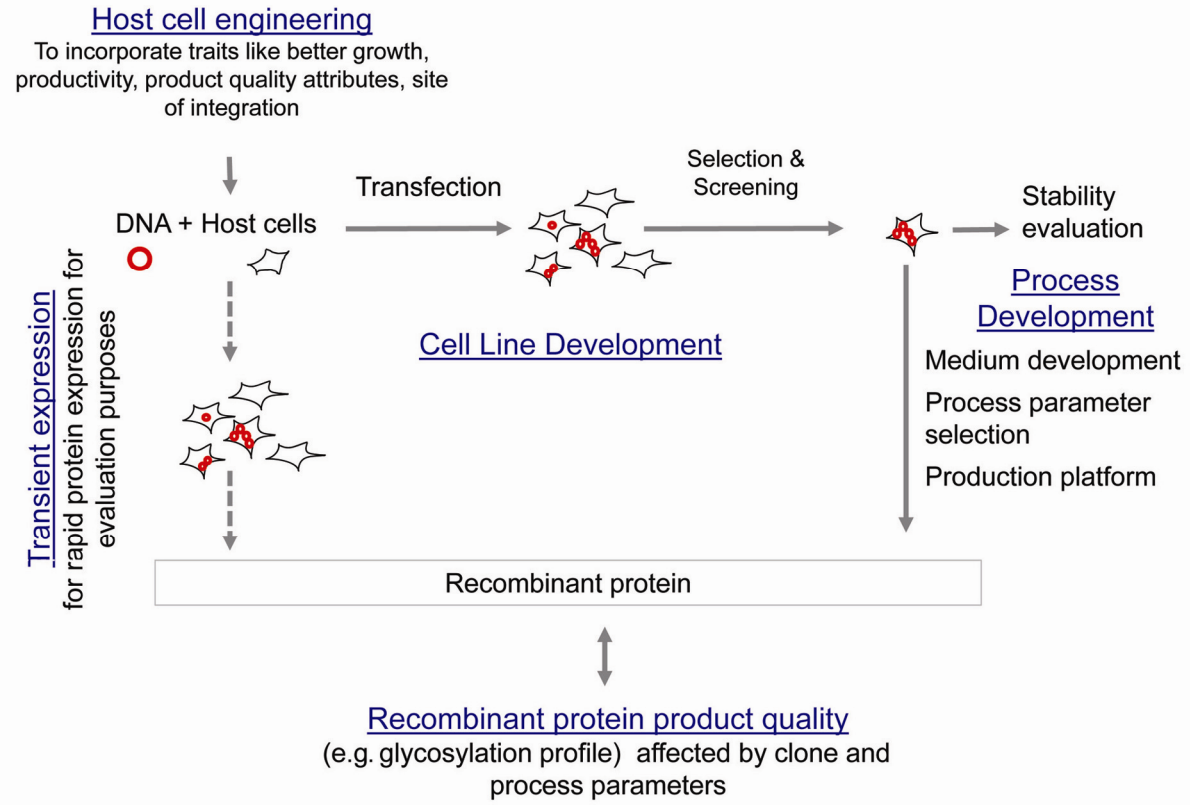

Figure 1. Steps during upstream process development for manufacturing recombinant proteins in animal cells.

cell type. Recombinant glycoproteins produced in non-human hosts can have glycans that are antigenic in humans[7]. Two glycan epitopes that can elicit immunological reactions are Gal(α1,3)Gal residues and N-glycolylneuraminic acid[8]. The enzymes responsible for their addition are expressed to a higher level in mouse cells compared to hamster cells, making the risk of an antigenic response higher in mouse cell line derived therapeutics compared to CHO. Human cell lines have not been widely used for manufacturing, though they can provide the advantage of human glycoform. One of the issues for this may be the regulatory hurdles due to lack of species barrier allowing easier transfer of adventitious agents[9], though it has been argued that infection of human cells with human pathogenic agents will result in detectable pathogenic effect in cells whereas the infection may remain undetected in other cells[10]. Human cell lines with documented history such as Per.C6 are being developed commercially as hosts.

For biosimilar manufacturing, using the same host cell species as the innovator may perhaps decrease risks associated with unintended changes in clinical performance of therapeutics due to change in protein quality from different host species, though using the same host cell line does not guarantee, that the final biosimilar product will be identical to the innovator. The large percentage of therapeutics manufactured in CHO provides an opportunity to biosimilar manufacturers for establishment of superior CHO host cell platforms by host cell engineering. Optimized host cell could include features such as better growth, no cell aggregation, more efficient metabolism like reduced lactate production, increased tolerance to culture insults by incorporation of anti-apoptosis genes and desirable changes in nutritional requirements such as serum independence. For a review on cell engineering strategies for bioprocessing, the reader is referred to[11]. These characteristics could be obtained by targeted engineering of relevant genes where mechanistic information is available. For example, downregulation of lactate dehydrogenase[12] or overexpression of pyruvate carboxylase[13] can reduce lactate production, while overexpression of anti-apoptosis genes can increase productivity and resistance to apoptosis[14]. In the absence of mechanistic knowledge of cause of a phenotype, desirable features can be incorporated by adapting cells to the desired conditions or by screening cell clones to isolate clone with desired characteristics[15–17]. High-throughput omics data can contribute to providing better understanding of the mechanistic causes of cellular phenotypes desired for bioprocessing which can further help in such targeted interventions, though this may be difficult for those phenotypes which are a result of multiple alterations. A recent example is the identification of two genes *Igfbp4* and *AqpI* whose silencing resulted in faster

adaptation of cells to suspension conditions[18]. The host cell can also be engineered to achieve desired product quality attributes. For example, knocking out Fut8 or FX genes can achieve fully afucosylated glycoforms[19]. FX knockout cell line can be supplementated with fucose to express antibodies with desired ratio of fucosylated to afucosylated glycans[20]. Host cells may also be engineered to reduce some host cell proteins susceptible to be retained as impurity during downstream processing which may have an adverse impact on the product during processing or storage[21]. Another avenue for improving host cell line as discussed in the later sections, is the identification and tagging, if necessary, of an optimal site of integration in host cell genome to reliably target the transgene to that location[22]. Development of such optimized host cells with desirable features can potentially result in faster development of high productivity processes.

Host cells are transfected with plasmid carrying a transgene, encoding the protein of interest, to develop a stable cell line expressing recombinant protein. In addition to the transgene, plasmid carries other biological elements required for its expression such as promoters, enhancers, poly adenylation sequences, any addition of introns, selection marker and DNA elements that modulate chromatin structure[23,24]. There is a significant time investment of 2–6 months required for the development of a stable cell line precluding the use of stable cell lines for evaluation of various biological elements. Transient gene expression can help to rapidly express recombinant protein for initial evaluation of the effect of plasmid elements and host cells on the quality of recombinant therapeutic prior to establishing a stable cell line[25], and can be scaled up to rapidly producing material for pre-clinical evaluation.

Transient expression for rapid evaluation of expression systems

Transient gene expression (TGE) involves transfection of host cells with a plasmid vector carrying the transgene followed by expression of recombinant protein by pool of transfected cells for a period of 1–2 weeks. It thus provides a rapid way to produce sufficient proteins for initial analytical and toxicology studies. Suspension-adapted human embryonic kidney (HEK) 293 and CHO cells have been used widely for TGE. Yields from transient expression are much lower than those that are obtained from a stable cell line. To generate sufficient protein, TGE can be carried out on a large scale, and scales up to 100 L in stirred tank reactors[26] and 20 L in single use WAVE™ bioreactors[27] have been reported.

Transfection is a critical step in TGE as it governs the fraction of non-productive cells which have not received plasmid and also the amount of plasmid delivered to the transfected cells. Several transfection agents amenable to use on a large scale such as calcium phosphate, cationic lipids and cationic polymers like polyethyleneimine, have been used for TGE (ref. 28). Of these, based on cost and efficiency, linear polyethyleneimines (PEI) have been more widely used for large scale TGE[29]. Transfections can be carried out at a high cell density to increase TGE yields as seen by 250 mg/l antibody yields from TGE in CHO cells transfected at 4×10^6 cells/ml (ref. 30). Large scale transfections require producing and purifying large amounts of plasmid DNA in *E. coli*. Efforts to decrease plasmid requirement have used non-linear relationship between amount of DNA delivered to the nucleus and extracellular DNA concentration, along with saturation kinetics of transgene expression as a function of intranuclear plasmid availability[31,32]. We have showed that the supplementation of plasmid DNA with non-coding DNA to increase extracellular DNA concentration, increases the transfection efficiency and TGE yield per unit plasmid[33]. Such an increase was shown to be accompanied by an increase in transgene mRNA levels[34].

After the initial delivery of plasmid to the cell, plasmid copy numbers continuously decrease due to dilution by cell division resulting in decrease in protein expression. The use of episomal systems from viruses such as EBNA1/Ori-P from Epstein Barr virus which allows plasmid replication and hence maintenance of plasmid copy number increases TGE yields[35]. Another successful approach to increase yield is subjecting the culture to mild hypothermia post transfection which slows down cell growth[36]. In summary, advances in TGE have showed that this approach can be used at large scale and can rapidly provide sufficient protein for pre-clinical testing, as well as for initial assessment of expression systems. However, commercial production requires a cell line stably expressing the product over a long period of time.

Stable cell line development

Development of a cell line stably expressing the recombinant protein involves transfection of transgene into the host cells, selection of cells with transgene integrated into the host cell genome, gene amplification if necessary and screening of a large number of single cell clones to identify the best available clone.

Transfection systems for stable cell line development are similar to those used for TGE, as discussed earlier. Selection involves several strategies; use of metabolism-gene mutants such as nutritional auxotrophs have been common in CHO and NS0 cells to help select clones with high transgene expression levels. Dihydrofolate reductase (DHFR) mutant isolated in CHO cells allows for screening and amplification of transfected gene using methotrexate (MTX)[37]. DHFR is required for formation of tetrahydrofolate and its absence necessitates the

supplementation of hypoxanthine and thymidine to the medium to enable DNA synthesis. DHFR gene is co-transfected with transgene and selection is carried out in the absence of hypoxanthine and thymidine. To further increase selection for cells expressing large amounts of DHFR, cells are treated with MTX, which binds and inhibits DHFR. Thus with increasing MTX concentration, only those cells expressing large amounts of DHFR and higher expression of transgene by association[38], can proliferate. Another selection and amplification system is glutamine synthetase (GS) selection with methionine sulfoximine (which inhibits GS) based amplification[39,40]. It is commonly used in GS – NS0 cells lacking GS activity that are unable to grow under glutamine deprivation. GS expression in such cells allows for selection of transfected cells under glutamine starvation. An additional advantage of GS selection is the continued selection pressure in the absence of glutamine in medium, and lower accumulation of waste metabolite NH_4^+ in the culture. CHO cells have an endogeneous functional GS gene. To improve GS selection in CHO, endogeneous GS gene was knocked-out which increased the productivity achieved in CHO from GS-MSX selection-amplification[41]. Improvements to the basic selection strategy have been reported, such as attenuation of selection marker to reduce its activity thus requiring greater expression of the marker, and by association with the transgene, to survive the selection pressure[42].

Despite the use of such selection strategies, there is a large heterogeneity in growth and productivity characteristics of single cell clones derived after transfection. Indeed, phenotypic drift occurs even in untransfected 'clonal' cell populations. Barnes *et al.*[43] carried out three rounds of limited dilution cloning of parental untransfected NS0 cells and showed that it was not possible to obtain phenotypically similar cell lines even after three rounds of cloning, though the variation in growth reduced with cloning steps. In MTX-amplified cell lines, amplification caused chromosomal rearrangements resulting in variability among clones in terms of growth, productivity and stability[44]. One possible cause of heterogeneity in recombinant protein expression could be variation in the site of random integration of transgene into the host cell genome resulting in varying levels of transcription depending on whether the integration happens in a transcriptionally active or inactive part of the genome. Strategies to target transgene to optimal locations in the host genome to support high levels of transcription and to prevent gene silencing, is an active area of research.

Integration-site effects

Lee *et al.*[45] showed that the extent of variation in productivity of clones reduced when transgene was targeted to specific locus in the genome as compared to random integration. (However, random integration resulted in some clones having higher productivity than that achieved in clones obtained by targeted integration.) Gene silencing, which can contribute to the decrease in recombinant protein expression levels over time in some unstable clones through mechanisms like histone modification and CpG methylation, could also be affected by the site of integration[46]. Uncertainty associated with integration site can be reduced by directed integration of transgene at a specific genomic location on the host genome.

Optimal genomic loci for targeting a transgene need to be identified empirically. Identification of genomic site is carried out by integration of a reporter gene such as green fluorescent protein followed by the identification of highest expressing clone. Such sites can be tagged by introduction of specific DNA sequences identified by site specific recombinases with the reporter gene[47]. Expression of recombinase protein then allows homologous recombination mediated integration of transgene at the tagged location. The Cre-loxP system[48] and the FLP/FRT system[49] are examples of site specific recombinases used for targeted transgene integration. If the reporter protein is not a secreted protein, then this method does not guarantee that the identified clone is the one most suitable for high expression of secreted proteins. Development of artificial nucleases like zinc finger nucleases (ZFN), transcription-activator like effector nucleases (TALEN) and the most recent CRISPR/Cas nucleases, comprising of a DNA binding domain coupled to a nonspecific nuclease domain have enabled sequence-based targeting of the gene of interest to a pre-identified genomic location and have done away with the need for 'tagging' the genomic location[50]. More recently developed CRISPR/Cas system is the simplest among the three nucleases. These nucleases allow the introduction of single or double stranded breaks at a specified location thus providing targeted genome engineering opportunities including the ability to rapidly knock-out genes. Though intellectual property issues for CRISPR/Cas technology may make it less accessible for biomanufacturing, it has an immense potential in research related to engineering of better cells.

An alternative approach that has been used to overcome the effect of chromosomal position on transgene transcription is the use of DNA elements that modulate chromatin structure, such as chromatin insulators, locus control regions, scaffold attachment regions, matrix attachment regions, or ubiquitous chromatin opening elements[51]. These sequences are incorporated into the vector carrying transgene. Incorporation of such DNA sequences along with transgene reduces the effect of the surrounding chromatin structure on transgene expression, thus reducing the effect of gene integration site. However, irrespective of the methodology, transfection leads to single cells with varying levels of expression and varying growth characteristics, and screening is required to

identify the best set of cells. Growth of single cells results in 'clones', and the screening process is known as 'clone screening'.

Strategies for high and medium throughput clone screening

Successful secretion of a recombinant protein needs adequate transcription of transgene, translation of protein, post translational modification and secretion through the secretory pathway comprising several cellular organelles, viz. endoplasmic reticulum (ER), golgi and secretory vesicles. These steps require availability of appropriate substrates, energy and maintenance of appropriate redox balance. Any differences in these steps will affect the productivity of the clone and may contribute to heterogeneity in growth and productivity. This necessitates empirical screening of clones to identify a high producing cell clone.

The first high throughput stage traditionally has been limited dilution cloning during which single cells are deposited into individual wells and the non- or low-performing clones are discarded based on analytical measurement of product concentration in the supernatant. Robotic technology can be used to increase throughput at this stage. Alternatively, fluorescently labelled antibodies to recombinant protein can be used to measure productivity of clones[52]. Single cells are labelled through binding of antibody to the membrane associated recombinant protein, and fluorescence-activated cell sorting (FACS) is used to separate small percentage of clones with high secretion rates[53]. Another method involves suspending single cells in a semi solid medium to enable accumulation of secreted protein in the vicinity of the clone followed by quantification of that protein using a fluorescently labelled antibody[54]. High producing clones can be identified based on fluorescence detection and expanded. Such methods allow automated screening of a large number of clones to identify the small subset of high producers. Since the present industry regulatory authorities demand assurance of 'clonality' of the production cell line, this first screening step may need to be appropriately validated to assure clonality[55].

Once the initial high throughput screening step discards the low producers, clones are typically screened at higher culture volumes like, in shake flasks or Tube-Spin® tubes. These culture platforms have an obvious limitation of not being able to control parameters like pH and dissolved oxygen (DO) concentration which are controlled in bioreactors, though single use sensors have been used in shake flasks to monitor these parameters[56]. These parameters are known to affect cellular metabolism and a lack of their control at the screening stage can increase uncertainty associated with the behaviour of the selected clone upon its scale-up to bioreactors. To systematically study this, Porter *et al.*[57,58] selected 30 clones at different levels of productivity and experimentally evaluated their performance at different stages of traditional screening process, viz. in 96-well plates, 24-well plates, shake flasks in batch and in fed batch mode in bioreactors to understand the relative ranking of these clones at different screening stages. They showed that clone ranking during screening in fed batch mode in shake flasks is not identical to their ranking in pH controlled fed batch bioreactors. To help understand the effect of lack of pH control on the outcome of screening process, we developed a mathematical model to simulate changing culture pH due to lactate formation in animal cell cultures, along with incorporation of the effect of pH on cell metabolism and growth as reported in the literature[59]. Such models can help guide the design of screening assays; for e.g., this model suggested that culture duration for screening assay has greater effect on the outcome of the assay. Here optimal durations differ based on the ability or inability of clones to consume lactate.

Concern over the lack of control of process parameters during screening led to significant interest in the use of microbioreactors to enable assessment of clones under controlled pH and DO conditions for better screening outcomes[60,61]. Simcell was among the first microbioreactor to be commercially available with culture volumes of less than a millilitre[62]. Later microbioreactor platforms, several of which are now available commercially, were developed to culture larger culture volumes in the millilitres range, which may also enable expression of greater amounts of product for better characterization[63]. Microbioreactors essentially comprise single use sensors and automated liquid handling for control and feeding, and have higher running and capital cost associated with their use. An alternative approach being explored for in situ feeding and pH control is through *in situ* diffusion of nutrient and base through a carrier such as a hydrogel. Use of hydrogels has been reported for controlled delivery of growth factors to animal cells[64]. Continuous delivery of glucose and sodium carbonate using silicone elastomer discs has been reported for *in situ* release to microbial systems over a period of one or two days to achieve nutrient feeding and pH control respectively[65,66]. Unlike microbial cultures which run for short durations of 24–48 h, animal cells in fed batch are cultured for 7–15 days. Our group is exploring the use of *in situ* delivery of base and nutrients through hydrogels to enable fed batch culture of animal cells in shake flasks to increase the quality of data from shake flask screening[67,68]. We mainly show that the release rate of base through these hydrogels increases with decreasing pH providing a rudimentary feedback for pH management[68,69]. Further development of such hydrogel based in situ delivery techniques can potentially reduce manual intervention and improve the quality of fed batch culture data obtained through ubiquitous screening platforms like shake flasks.

Upstream process development

Commercial cell culture processes are run either in fed batch or perfusion mode of operation[70], with fed batch currently being more widely used. In fed batch mode, cell density and culture longevity are increased by appropriate feeding of nutrients during culture. In perfusion mode a high cell density is attained by continuous feeding of medium and withdrawal of culture supernatant containing the product, which also reduces the residence time of product in the bioreactor. In general, fed batch is thought of as a more robust process with lower failure rates than perfusion, while perfusion processes though more complex to operate, result in higher productivity and require smaller bioreactors. A perspective on the pros and cons of the two modes of operation is presented[71]. Modelling frameworks have been developed to compare fed batch and perfusion process performance in terms of their economics and environmental impact while including different probabilities of failure of these processes[72,73]. Such analyses can help choose the right mode of operation during facility design.

Once a clone is selected, significant improvement in productivity can be obtained by development of medium formulation suitable for that particular clone. A substantial improvement from batch culture volumetric productivity of 50–100 mg/l in 1980s to the current fed batch volumetric productivities of 1–5 g/l has been achieved by improvements in medium and bioprocesses. Screening of basal medium composition, nutrient additives in the feed medium for fed batch culture and physical parameters like pH and temperature, including assessment of temperature shift to mild hypothermia which is subsequent to initial growth phase, identifies the best set of design parameters for that clone to improve volumetric productivity. Such medium and process development efforts are important and have also been reviewed[74,75]. In the recent past, guidance from US-FDA has resulted in a push towards better characterization of processes to identify operating parameter space within which the performance of the process is robust in terms of productivity and product quality[76]. An example for the effect of changes in process operating parameters on product performance is through variation in glycosylation pattern of glycoproteins. A better understanding of such effects will help better design robust processes to achieve a specified glycoform, which is of interest to biosimilar industry.

Advances in controlling glycoform profile of glycoproteins

Unlike DNA replication, mRNA transcription and protein translation, glycosylation is not a template-driven process. Glycans are oligosaccharides either attached to the N-terminal of an Asparagine at a consensus sequence Asn-X-Ser/Thr (N-linked glycosylation) or on the O atom of Serine (O-linked glycosylation). N-linked glycosylation is more widely studied form of glycosylation and of importance to the largest class of biopharmaceuticals, viz. monoclonal antibodies. Different N-glycan structures can attach at the same site on different molecules of the protein (microheterogeneity) or at different glycan attachment sites on the same protein (macroheterogeneity). Though a large number of N-glycan structures are possible, only a few are observed on recombinant antibodies. Figure 2 shows an example of multiple glycans attached to a commercial antibody therapeutic along with a fraction of each glycan i.e. the glycoform profile[77]. Glycans structures are important since they affect many biological properties of the glycoprotein such as, its efficacy and in vivo clearance[78,79]. Batch-to-batch variation in glycoform is tightly controlled, though some changes in glycoform have been reported in innovator molecules, possibly due to regulator-approved changes in the manufacturing process[80].

Glycan heterogeneity is established during their assembly in the protein secretion pathway in ER and golgi. Except for glycosidase and mannosidase responsible for trimming of glucose and mannose residues in the initial steps, all glycosylation enzymes are transferases involved in attachment of sugar residues onto a growing glycan structure using a nucleotide-sugar (NSD) molecule as a donor of the sugar. Some of the intermediate glycans are substrates for multiple transferases while some enzymes are able to act on more than one glycan structure. Various parameters such as relative activities of different enzymes, processing time in ER and golgi prior to secretion of protein, availability of NSDs and transport of NSDs into ER and golgi can affect the glycan structures. Glycan profile can hence vary from clone to clone. Unlike the case of manufacture of generic pharmaceuticals, cell line developed by innovator is not available to biosimilar manufacturer. Manufacturing a biosimilar glycoprotein may thus require establishing a process mimicking the glycoform profile of the innovator using a different cell clone.

Targeted genetic engineering is one approach to influence glycoform and has been reported to increase sialic acid content by strategies such as overexpression of sialyltransferase[81], CMP-sialic acid transporter[82] or down-regulation of sialidase[83]. Genetic engineering strategies such as knocking out *fut8* gene have also been used to produce afucosylated glycoform[19]. Cell culture medium additives have been shown to influence glycoform[84]. For a given clone, glycoform can also be sensitive to culture conditions such as pH, DO concentration, temperature, availability of nutrients such as glucose, galactose and amino acids and availability of trace elements such as Mn^{2+} which is known to be required for the activity of some glycosyltransferases. Knowledge of the effect of changes in process parameters, macro-nutrient

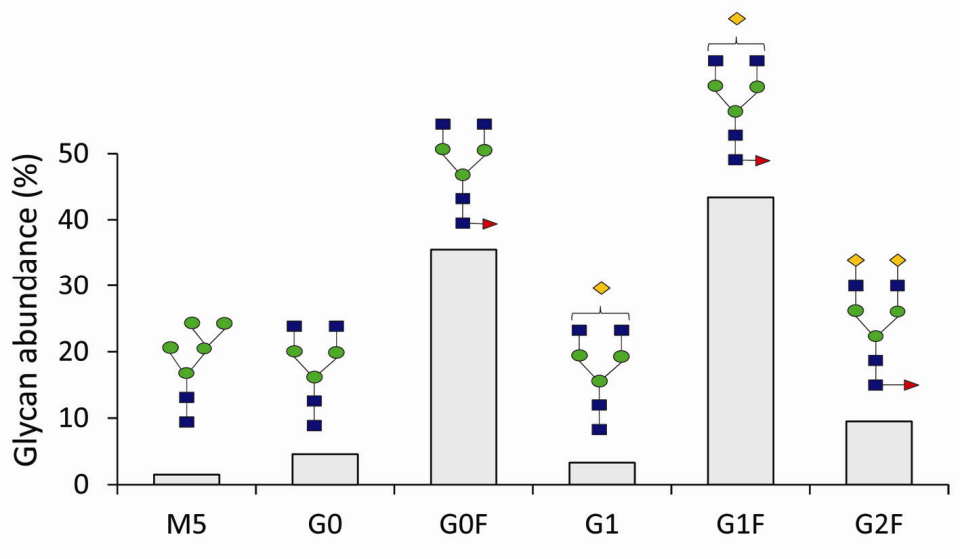

Figure 2. Glycan fractions on an exemplary therapeutic antibody[77]. Glycans with less than 1% abundance are not shown. Monosaccharide legend – Blue squares, N-acetylglucosamine; Green circle, Mannose; Red triangle, fucose; Orange diamond, galactose.

availability and micro-nutrient concentrations on glycoform profile can help to leverage these parameters to mimic a glycoform. There is now a significant amount of literature on the effect of culture parameters such as pH, DO concentration, temperature and availability of nutrients such as glucose, amino acids and trace metals on glycan profiles of recombinant glycoproteins[85–87]. One of the important conclusions from such studies is that there is no consistent response of these parameters on glycoform profile. This is probably because the effect of each component depends on other variables; for e.g., an increase in enzyme activity may not result in the desired change in glycoform if the nucleotide sugar substrate for that enzyme is limiting or vice versa. Also, change in these parameters can affect other aspects of metabolism such as accumulation of waste metabolites like NH_4^+ which in turn are known to affect glycosylation.

One of the widely reported nutrient supplementation strategy is the addition of Mn^{2+}, uridine and galactose which together appear to increase galactosylation of glycans. Among all the nucleotide sugar donors, availability of UDP-Gal is reported to be the most limiting[88], and supplementation of culture with uridine and galactose can increase intracellular UDP-Gal levels. Mn^{2+} is required for the activity of galactosyltransferase, and is supplemented if its availability in the medium is limiting or if the culture results in reduced intracellular Mn^{2+} (ref. 89). Improvement in either one of the two aspects alone may or may not be sufficient[90] since supplementation of all, i.e. uridine, galactose and Mn^{2+} which targets both the availability of NSD substrate and enzymatic activity, usually achieves better galactosylation[91]. At the same time, it is important to remember that nutrient and trace element supplementation can also affect other aspects of cellular metabolism and cause unintended effects. Mn^{2+} supplementation has been reported to both increase and decrease high mannose glycans[92,93]. We found that Mn^{2+} supplementation substantially increases high mannose glycoforms when glucose is absent or limiting, but not when glucose is available in abundance[94]. Such studies exemplify the contextual nature of effects of these supplements on galactosylation making the design of supplements to target a particular glycoform, still an empirical approach.

Use of mathematical models for glycosylation process has the potential to help better understand the effect of various parameters on glycoform in order to predict suitable conditions required to achieve a given glycoform based on some basal measurements for a specific clone. There, several models have been proposed by incorporating varying levels of complexity[95–98]. Coupling of models of glycosylation with models of cell growth and metabolism[99] is necessary to understand effects on glycosylation which are modulated by changes in availability of the nucleotide sugar donor. Such models are being developed[100], and advances in this area will help further our understanding of glycosylation for bioprocessing applications. In future, incorporation of clone specific information in these models might help in prediction of clone-specific effects of these parameters on glycoform, reducing the experimentation required to design a process targeting a specific glycoform from a given clone.

Once a clone has been identified, the focus shifts to manufacturing. A range of technologies is available depending on the scale. Some of the newer technologies are reviewed in the next section.

Single-use technologies for upstream manufacturing

Commercial manufacturing of biopharmaceuticals has been historically carried out in stainless steel bioreactors which require significant capital cost upfront and establishment and validation of protocols for cleaning and sterilization of equipment between runs. There has been a surge of interest in the recent past in the use of single-use reactors for upstream processing which result in faster installation and commissioning and reduce the effort associated with cleaning and sterilization operations. Shukla and Gottschalk[101] enumerate several market factors driving this surge such as emphasis on production cost, flexible, multiproduct manufacturing facilities, biosimilars, multiple and smaller manufacturing plants collocated with markets and increasing number of low-volume biopharmaceutical products. Improvements seen over the last couple of decades in cell culture productivity have also reduced the size of bioreactors from 10,000 to 25,000 L scale stainless steel reactors for fed batch cultures, which may also make these processes more suitable for single-use bioreactors.

Single-use bioreactors comprising simplistically of plastic bags as reactor vessels are available commercially from several vendors up to scales of 2000 L (refs 101, 102). The first among these was the WAVE bioreactor[103] where mixing takes place by virtue of a rocking motion resulting in formation of 'waves' within the reactor. Orbitally shaken single-use bioreactors use rotating motion of the vessel to provide mixing and gas transfer. The most widely used stirred-tank bioreactor is employed in single-use systems by mounting plastic bags placed inside a cylindrical shell to support the bag. The mixing is carried out by an agitator bringing the working principle close to that of the traditional stainless steel bioreactors. Higher self-containment of such systems can provide further benefits in terms of operational ease and infrastructure requirements. Some of the key technical issues with the use of single-use bioreactors are the possibility of some media components being sequestered by binding to plastic bags[104,105], or of leachables from the plastic bags adversely affecting cells[106]. These interactions depend on the composition of cell culture medium and bioreactor material, and should be evaluated for the specific system.

A rapidly growing therapeutic area which can potentially benefit from developments in the upstream cell culture manufacturing is T-cell immunotherapy of cancer[107]. This requires the harvesting of T-cells from a patient, their manipulation and expansion *in vitro*, followed by transferring expanded cell product back to the patient. This is an example of large number of 'products' at small volumes at its extreme, where every patient's T-cells are different products which require separate containment, making the use of some form of reasonably self-contained single-use manufacturing technology the only possible manufacturing strategy for such type of therapy[108]. The knowledge gained and advances in robust upstream processes using single-use devices can be directly applied to manufacturing related issues for such therapeutics.

Conclusion

Recombinant proteins form a significant part of overall therapeutic molecules. There have been significant advances in upstream processes for manufacture of such therapeutics since the commercialization of early drugs in this class. Developments in genome targeting technologies now enable precise targeting of genetic manipulations such as transgene integration or gene deletion which can help in faster development of processes with high productivity. Advances in high throughput screening technologies further help in identification of high productivity clones. Despite the long history of manufacturing using animal cell cultures, there are still unresolved issues such as the demonstration of clonality of the production cell line. Biosimilar manufacturing poses additional technical challenges such as mimicking the glycoform of the innovator molecule. Better understanding of genetic engineering and process engineering strategies to influence glycoform will continue to contribute to the development of processes for biosimilars. The use of single-use bioreactors which were not available at the time the first such drugs were developed can lower the capital cost requirement. Indeed, entire manufacturing trains comprising of single-use technologies might enable mobile manufacturing units for recombinant protein production.

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