Glycobiology vol. 19 no. 9 pp. 936–949, 2009
doi:10.1093/glycob/cwp079
Advance Access publication on June 3, 2009

REVIEW

Optimal and consistent protein glycosylation in mammalian cell culture

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Received on April 6, 2009; revised on June 1, 2009; accepted on June 1, 2009

In the biopharmaceutical industry, mammalian cell culture systems, especially Chinese hamster ovary (CHO) cells, are predominantly used for the production of therapeutic glycoproteins. Glycosylation is a critical protein quality attribute that can modulate the efficacy of a commercial therapeutic glycoprotein. Obtaining a consistent glycoform profile in production is desired due to regulatory concerns because a molecule can be defined by its carbohydrate structures. An optimal profile may involve a spectrum of product glycans that confers a desired therapeutic efficacy, or a homogeneous glycoform profile that can be systemically screened for. Studies have shown some degree of protein glycosylation control in mammalian cell culture, through cellular, media, and process effects. Studies upon our own bioprocesses to produce fusion proteins and monoclonal antibodies have shown an intricate relationship between these variables and the resulting protein quality. Glycosylation optimization will improve therapeutic efficacy and is an ongoing goal for researchers in academia and industry alike. This review will focus on the advancements made in glycosylation control in a manufacturing process, as well as the next steps in understanding and controlling protein glycosylation.

Keywords: bioprocessing/mammalian cell culture/protein glycosylation/protein therapeutics/protein quality

Introduction

Protein glycosylation is of paramount importance to the efficacy and manufacturing of therapeutic glycoproteins. Mammalian cell expression systems are the preferred method for the commercial production of these glycoproteins because their innate protein processing machinery, including that of protein glycosylation, closely resembles that in human.

Glycosylation of proteins takes on the form of oligosaccharides attached to either the side chain of asparagine (*N*-linked) or serine/threonine (*O*-linked) with the former being the most prominent (Warren 1993; Helenius and Aebi 2001; Sinclair and Elliott 2005). Glycans have a very prominent role toward affecting therapeutic efficacy and determining the in vivo half-life (Elliott et al. 2003). It is due to both of these features that the glycoform profile of a therapeutic glycoprotein must be extensively characterized in order to meet regulatory agency demands (FDA 1996).

In this review, we summarize the salient features of protein glycosylation control from a bioprocess perspective. Through our own process development and characterization efforts with fusion proteins such as Orencia™ and Belatacept and monoclonal antibody Ipilimumab, we have gained an appreciation over the past, present, and future state of the control of this very important metabolic pathway. Table I lists glycosylated therapeutics currently on the market, which is the impetus for understanding their metabolic control. Although CHO cells are the most prevalent for producing glycoprotein therapeutics, other cell lines such as human embryonic kidney cells (HEK) (Durocher et al. 2007), baby hamster kidney (BHK), mouse myeloma (NS0), and human retinal cells (PERC.6) (Jones et al. 2003; Wurm 2004; Petricciani and Sheets 2008) are being developed as high producing cell lines.

There have been numerous reports on the effects of cell type, culture process, and culture media toward the resulting glycoform profile. This review will summarize these efforts, as well as new attempts toward achieving uniform, consistent, and/or optimal glycosylation. These efforts share a common need for a more fundamental understanding of the metabolic pathway, which is the next logical progression from the current paradigm of glycosylation maintenance. The ultimate goal would be to take the lessons learned from previous studies, combined with new technologies, to transform a well-characterized cell line toward one that can control the glycosylation profile. In order to truly incorporate process quality by design (QbD) into the final glycoprotein product (Rathore and Winkle 2009), more fundamental studies on the dependence of glycosylation on process conditions and cellular physiology will be required to gain physiological insight. The information gathered from such research together with clinical studies will continue to enhance our capability toward generating highly efficacious glycoprotein drugs.

Protein glycosylation and product quality

Metabolic pathway

The addition of oligosaccharides onto a glycoprotein is a complex metabolic pathway, characterized by the en bloc transfer of polysaccharide chains, as well as the step-wise addition and removal of individual monosaccharides. The number of pathways traversed is dependent on reaction site accessibility, the

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Table I. Glycosylated therapeutics approved by the FDA and/or EMEA

Product	Class	Mode of action	Indication	Cell line	Company
Aranesp (Darbepoetin alfa)	Erythropoiesis stimulating protein	Regulates red blood cell production	Anemia	CHO	Amgen
Arcalyst (Rilonacept)	IL-1 Trap	Binds IL-1β to prevent the interaction to cell surface receptors	Cryoporin-associated periodic syndromes	CHO	Regeneron
Avastin (Bevacizumab)	rMab	Binds to the vascular endothelial growth factor (VEGF) to inhibit angiogenesis	Colorectal cancer	CHO	Genentech
Avonex (Interferon β−1a)	Interferon	Binds to type I interferon receptors to activate two Jak tyrosine kinases	Multiple sclerosis (MS)	CHO	Biogen Idec
Cerezyme (Imiglucerase)	Enzyme	Engineered to have mannose-terminated oligosaccharide chains that are recognized by endocytic carbohydrate receptors on macrophages. Catalyzes hydrolysis of glycolipid glucocerebroside to glucose and ceramide in those macrophages which accumulate lipids in Gaucher disease	Gaucher Disease	CHO	Genzyme
Elaprase (Iduronate sulfatase)	Enzyme	Hydrolyzes the 2-sulfate esters of terminal iduronate sulfate residues from the glycosaminoglycans dermatan sulfate and heparan sulfate in the lysosomes of various cell types	Hunter syndrome	HT-1080	Shire
Enbrel (Etanercept)	Fusion protein	Mimics inhibitory effects of naturally occurring soluble TNF receptors to reduce inflammatory response	Rheumatoid arthritis	CHO	Amgen
Epogen (Epoetin alfa)	Erythropoiesis stimulating protein	Recombinant human erythropoietin interacts with erythropoietin (EPO) receptors to stimulate production of red blood cells from bone marrow stem cells	Anemia	CHO	Amgen, Kirin
Erbitux (Cetuximab)	rMab	Binds to the extracellular domain of the epidermal growth factor (EGFR) preventing activation of EGFR to impair cell growth and proliferation	Colorectal cancer	SP2/0	Imclone/BMS
Herceptin (Trastuzumab)	rMab	Binds to HER2+ tumor cells, blocks downstream HER2 signaling to inhibit proliferation of cells	Breast cancer	CHO	Genentech
Wellferon (Interferon α)	Cytokine	Binds to type I interferon receptors (IFNAR1 and IFNAR2c) which, upon dimerization, activate two Jak (Janus kinase) tyrosine kinases (Jak1 and Tyk2). Upregulates expression of MHC I proteins	Chronic hepatitis C	Human lympho-blastoid	Wellcome
Mircera (Methoxy polyethylene glycol-epoetin beta)	Erythropoiesis stimulating protein	Recombinant human erythropoietin interacts with erythropoietin (EPO) receptors to stimulate production of red blood cells from bone marrow stem cells	Anemia	CHO	Hoffmann-La Roche
Myozyme (Alglucosidase alfa)	Enzyme	Recombinant acid a-glucosidase (GAA) to replace GAA deficiencies	Pompe disease	CHO	Genzyme
Naglazyme (Galsulfase)	Enzyme	Recombinant form of polymorphic human enzyme *N*-acetylgalactosamine 4-sulfatase which catabolizes glycosaminoglycans (GAG)	Mucopolysaccharidosis VI (MPS VI)	CHO	Biomarin
NeoRecormon (Epoetin beta)	Erythropoiesis stimulating protein	Recombinant human erythropoietin interacts with erythropoietin (EPO) receptors to stimulate production of red blood cells from bone marrow stem cells	Anemia	CHO	Roche
Orencia (Abatacept)	Fusion Protein	CTLA4-Ig acts as a selective modulator of the costimulatory signal required for full T-cell activation	Rheumatoid arthritis	CHO	Bristol-Myers Squibb
Procrit/Eprex (Epoetin alfa)	Erythropoiesis stimulating protein	Recombinant human erythropoietin interacts with erythropoietin (EPO) receptors to stimulate production of red blood cells from bone marrow stem cells	Anemia	CHO	Johnson & Johnson, Schering-Plough
Rebif (Interferon β-1a)	Cytokine	Immunomodulation through the induction of cell membrane components of the major histocompatibility complex	Multiple sclerosis (MS)	CHO	Merck Serono
Remicade (Infliximab)	rMab	Binds to TNF and inhibits TNF action	Crohn's disease & Rheumatoid arthritis	SP2/0	Centocor/Johnson & Johnson
Rituxan (Rituximab)	rMab	Binds to the cluster of differentiation 20 (CD20) which is expressed on B-cells. Fc portion mediates ADCC and CDC	Non-Hodgkins lymphoma	CHO	Genentech (IDEC)

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Table I. (Continued)

Product	Class	Mode of action	Indication	Cell line	Company
Soliris (Eculizumab)	rMab	Binds to the complement protein C5, inhibiting terminal complement mediated intravascular hemolysis	Paroxysmal nocturnal hemoglobinuria	CHO	Alexion
Synagis (Palivizumab)	rMab	Targets an epitope in the A antigenic site of the F protein of respiratory syncytial virus (RSV)	Respiratory syncytial virus	NS0	MedImmune
TNKase (Tenecteplase) (tPA)	Enzyme	Recombinant fibrin-specific plasminogen activator	Acute myocardial infarction	CHO	Genetech
Tysabri (Natalizumab)	rMab	Binds to α4 integrin of adhesion molecule VLA-4 and sterically inhibits binding of VLA-4 to VCAM-1	Multiple sclerosis (MS)	CHO	Biogen Idec
Vectibix (Panitumumab)	rMab	Binds to EGFR	Colorectal carcinoma	CHO	Amgen
Zenapax (Daclizumab)	rMab	Binds to the α subunit (p55 α, CD25, or Tac subunit) of human IL-2 receptor expressed on the surface of activated lymphocytes	Acute organ rejection	SP2/0	Hoffmann-La Roche

enzymatic substrate specificities, as well as spatial localization of the various enzymes and nucleotide-sugar substrates that are necessary for the reactions to proceed in a particular order (Krambeck and Betenbaugh 2005; Butler 2006).

Whereas *N*-linked glycosylation initiates primarily in the ER of mammalian cells, *O*-linked glycosylation has been shown to initiate in either the ER or Golgi apparatus. Typical *N*- and *O*-glycan structures with their symbolic monosaccharide representation and nomenclature are shown in Figure 1.

There exists the potential for a diverse array of product glycans on each glycoprotein molecule as demonstrated in the KEGG GLYCAN database (Hashimoto et al. 2006). In most industrial bioprocesses, this large repertoire of glycans is typically not seen on recombinant glycoproteins expressed by mammalian cells such as CHO and NS0, suggesting that there may be pathway constraints. Control of this metabolic pathway can be manifested through a variety of different conditions, including the cell lines, bioprocess, and cell culture media used for protein production.

Monosaccharide and oligosaccharide roles in protein therapeutic efficacy

N- and *O*-linked glycans have been shown to have a large effect on the immunogenicity, efficacy, solubility, and half-life of commercial biologics (Lis and Sharon 1993; Van den Steen et al. 1998; Lowe and Marth 2003). Individual monosaccharides on *N*- and *O*-glycan structures can help determine a glycoprotein's therapeutic efficacy by their immunological and pharmacokinetic impact (Sethuraman and Stadheim 2006).

N-Linked Glycosylation. Figure 2 is a schematic of *N*-linked glycosylation for a protein therapeutic in a CHO cell culture. The terminal *N*-acetylneuraminic acid (NANA) content in a glycoprotein has been shown in many cases to be intricately linked to circulatory half-life, and thus is a typical marker for product quality assessment in protein therapeutics due to its proven role toward affecting circulatory half-life. Exceptions have been shown with monoclonal IgG_1 antibodies, where there was no significant difference in the clearance rate of molecules carrying different amounts of sialic acid (Millward et al. 2008). However, quite the opposite has been historically shown with non-antibody therapeutics. Asialylated glycoproteins can be selectively cleared by the asialoglycoprotein receptor (ASGPR) found in the liver (Fukuda et al. 1989; Stockert 1995). Erythropoietin (EPO) has been documented in the literature to be very sensitive toward its sialylation status and the resulting pharmacokinetics (PK) (Delorme et al. 1992; Walsh and Jefferis 2006). The plasma half-life of recombinant human EPO administered intravenously to rodents has been documented to be 5–6 h, as opposed to <2 min in the case of desialylated EPO (Erbayraktar et al. 2003). In another study, recombinant bovine acetylcholinesterase (AChE) was in vitro modified via exoglycosidases and sialyltransferases to facilitate better end-capping with sialic acid to maximize residence time (Kronman et al. 2000).

N-Glycolylneuraminic acid (NGNA) is a derivative of NANA that is not typically found in adult humans because it is an oncofetal antigen (Muchmore et al. 1989) but is present in CHO and NS0 cultures (Noguchi et al. 1995; Baker et al. 2001). The choice of mammalian expression host is thus very important toward determining the final glycoform profile. Recombinant proteins such as EPO contain low levels of NGNA (i.e., 1% of total sialic acids) that elicits a negligible immunogenic response; however, higher NGNA levels (e.g., fetuin contains 7% NGNA) have been verified to elicit an immune response (Noguchi et al. 1995). High NGNA levels on a chimeric CT4-IgG fusion protein have also been shown to cause rapid removal of the glycoprotein from circulation (Flesher et al. 1995). The ratio of NANA to NGNA is variable and dependent on cell culture conditions (see Figures 2 and 3) and cell line (Raju et al. 2000; Baker et al. 2001).

The importance of galactose (Gal) monosaccharides attached to *N*-glycans is frequently associated with its impact on NANA levels. Exposure of β(1,4)-linked Gal on *N*-glycans is associated with cognate recognition with ASGPR (Van Den Hamer et al. 1970). Although it has been reported that terminal galactosylation positively impacts the ability of Rituximab to lyse CD20 expressing cells, even the authors contend this to be a solitary experience (Jefferis 2005). The workhorses most often used in mammalian cell culture processes (i.e., cells derived from some hamster sources and all murine sources) have the potential to also add sugars that are not normally found, or found at low levels, in circulating human IgG. α(1,3)-Linked Gal attached to

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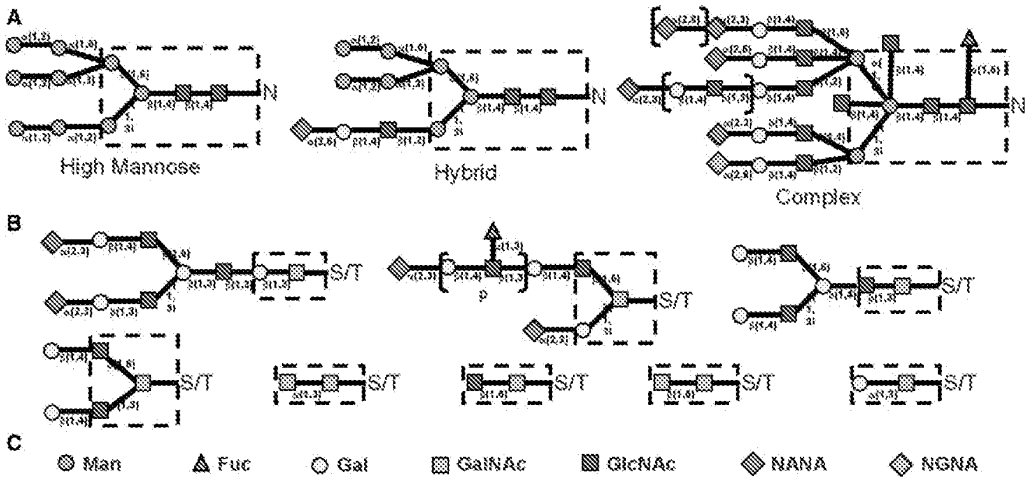

Fig. 1. In contrast to *N*-glycans, which have a common core pentasaccharide structure, *O*-glycans have up to eight different core structures that make their study and experimental measurement more difficult. (**A**) Representative structures of the three classes of *N*-linked glycans showing both monosaccharide composition and bond linkage information (note: the number of potential structures is too large to represent symbolically, and the glycans shown are merely representative of the types of structures within each category). (**B**) Representative structures of the eight core classes of *O*-linked glycans. (**C**) Symbolic representation of the monosaccharides commonly observed on *N*- and *O*-glycans (note: symbolic nomenclature is consistent with the guidelines established by the Nomenclature Committee of the Consortium for Functional Glycomics).

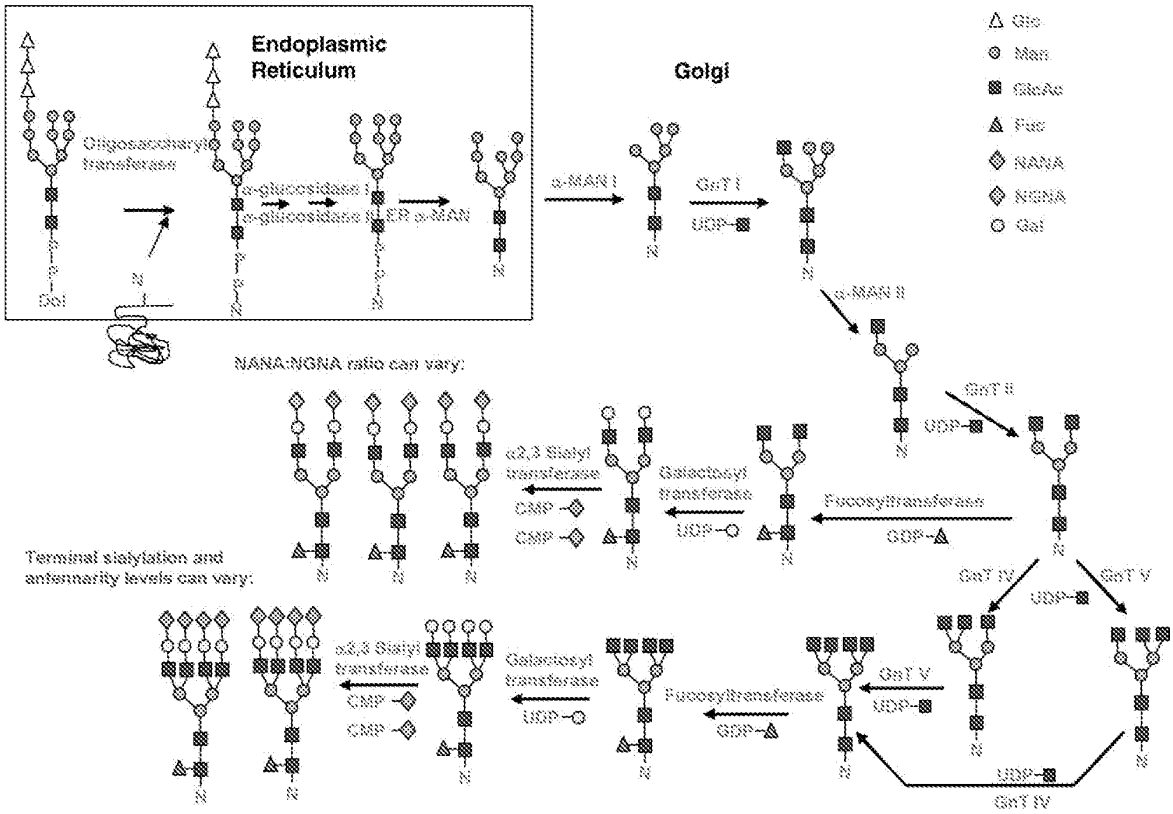

Fig. 2. Schematic view of the glycosylation pathway in a CHO cell line. The precursor $Glc_3Man_9GlcNAc_2$ is transferred from the dolichol-phosphate (D-P-P) donor to an asaparagine on the nascent protein in the endoplasmic reticulum. The final glycoprotein forms can be variable depending on the level of sialylation and terminal sugars. The level of antennarity and sialylation will be dependent on cell culture conditions and expression levels of each enzyme.

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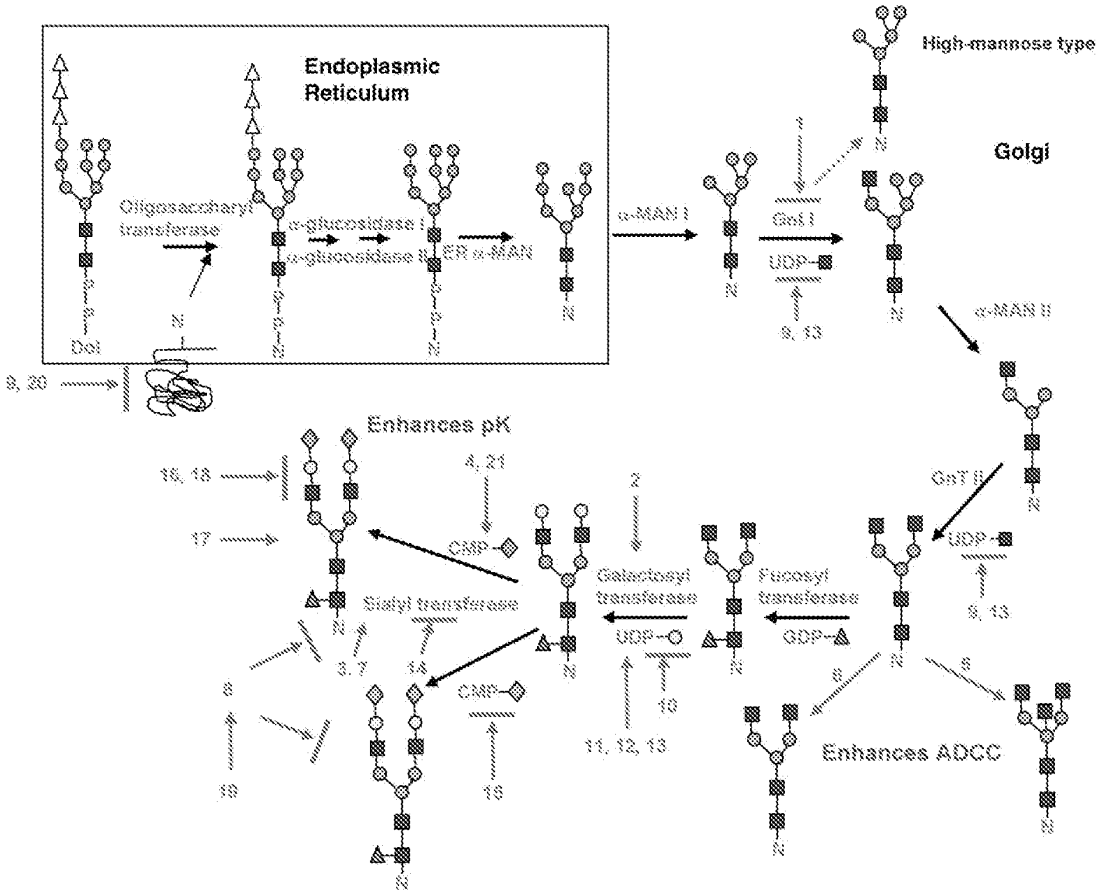

Fig. 3. Schematic view of control methods in the glycosylation pathway in a CHO cell line. Both molecular controls (mutants, knockouts, loss or gain-of-function) and cell culture parameters can modulate the final form for a glycoprotein. The following are known to modulate the glycoform: (1) Gnt $I^{-/-}$ Lec1 Mutant, (2) β(1.4)-GalT +, (3) α(2,3) Sialyltransferase +, (4) CMP-sialic acid transporter +, (5) GnT III+ Mutant, (6) $Fut8^{-/-}$ Mutant, (7) antisense RNA sialidase, (8) Sialidase activity, (9) low glucose/gln (<1 mM), (10) Low DO, (11) pH range, (12) manganese supplement, (13) sodium butyrate, (14) ammonia, (15) high pCO_2 (>100 mmHg), (16) DMSO, (17) glycerol, (18) low temperature (30–32°C), (19) low cell viability, (20) shear stress, and (21) *N*-acetylmannosamine.

N-glycans is an example known to be immunogenic (Jefferis 2005), and approximately 1% of circulating IgG molecules in normal human serum comprise these glycans (Galili et al. 1984).

Both mannose (Man) and *N*-acetylglucosamine (GlcNAc) can also modulate the efficacy and PK of a glycoprotein therapeutic. The circulating mannose receptor binds Man and GlcNAc via natural killer (NK) and macrophage cells which recognize them as being foreign (Rademacher 1993). Similarly, glycoproteins with terminal mannose or terminal GlcNAc are cleared through the reticulo-endothelial system, which relies on several receptors with high affinity for both Man and GlcNAc (Maynard and Baenziger 1981). In humans and cynomolgus monkeys, glycoproteins with terminal GlcNAc were selectively cleared from circulation through the mannose receptor (Jones et al. 2007).

N-Acetylglucosamine also plays a principal role in determining *N*-glycan antennarity and the resulting level of terminal glycosylation. In EPO, it was specifically determined that tetrasialylated tetra-antennary *N*-glycans increased in vivo bioactivity, rather than the ratio of tetraantennary to biantennary *N*-glycans (Yuen et al. 2003). Lenercept, a tumor necrosis factor alpha (TNF) antagonist, was extensively characterized and found to have human pharmacokinetic variability solely correlated to terminal GlcNAc levels and not with terminal galactose, NANA or NGNA (Keck et al. 2008). The antennarity of a glycoform can vary dependent on the activity and presence of GlcNAc transferases (Figure 2).

In contrast, hypermannosylation or hybrid mannose structures on hybrid-type glycans can potentially elicit immune recognition responses. In some cases, this immune recognition is desired for targeted drug delivery. Cerezyme, for example, is a recombinant enzyme used to treat Gaucher disease. The molecule has exposed Man sugars, which facilitates macrophage recognition via mannose receptors on their cell surfaces (Walsh and Jefferis 2006). Corresponding enzyme molecules without this modification are quickly removed from circulation by hepatocytes (Furbish et al. 1978).

The presence of α(1,6)-Fuc attached to *N*-glycans has been shown to be important toward antibody functions, including F_c receptor binding and antibody-dependent cell cytotoxicity (ADCC). To evaluate the role of fucosylated oligosaccharides

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toward IgG function, researchers utilized the Lec13 mutant CHO cell line expressing human IgG1 deficient in its ability to add Fuc (Shields et al. 2002). Binding of the nonfucosylated IgG1 to human FcçRIII A was improved up to 50-fold.

O-Glycosylation. *O*-Linked glycosylation is more difficult to predict due to lack of consensus recognition sequences; however, neural network approaches have been developed to better predict mucin-type *O*-linked sites (Julenius et al. 2005). Most reports to date document the role of *O*-glycans in the glycoproteins' binding capability or the masking of its peptide backbone. The formation of *O*-linked glycans in herpes simplex virus type 1 glycoprotein gC-1 is modulated by the presence of *N*-linked glycans and may also interfere with the glycoprotein's epitope binding domain, hence affecting its binding capability (Biller et al. 2000). In another study (Hermeling et al. 2004), it was found that patients treated with CHO-derived rhGMCF did not develop antibodies to the glycoprotein therapeutic with both *N*- and *O*-linked glycans. Only when the *O*-glycans were cleaved off, did antibodies develop, which the authors attributed to recognition of the peptide backbone, whose antigenic sites were being masked by the *O*-glycans. In an example of glycosylation engineering, it was demonstrated that long-acting follicle stimulating hormone (FSH) analogs with more *O*-linked glycosylation sites had less bioactivity than those analogs with *N*-linked glycosylation (Weenen et al. 2004). How *O*-glycans can affect glycoprotein therapeutic efficacy, as well as potential immunogenicity, is still an area that requires further investigation.

Effect of Oligosaccharides. The collective effect of glycosylation on the properties of recombinant glycoproteins is the culmination of each of its glycosylation sites, which may have varying, or even opposing influences depending on its glycosylation status. In one example, three recombinant monoclonal antibodies (rMAbs) with specificity for α(1,6) dextran and differing only in potential *N*-glycosylation sites in the CDR of the V_H region had a 10- to 50-fold higher affinity for antigen when glycosylated on Asn 54 and Asn 58 compared with that of the aglycosylated forms (Coloma et al. 1999; Gala and Morrison 2004).

Recombinantly expressed tissue plasminogen activator (tPA) has been shown to be variably glycosylated through macroheterogeneity. It has been classified into two types as a result: type I with all three glycosylation sites occupied and type II for two glycosylation sites occupied (Bennett 1983). Type I molecules have been shown to display reduced fibrin binding and clot lysis activity (Einarsson et al. 1985; Wittwer et al. 1989), and the glycosylation status of one site (N_{184}) has been shown to affect plasma clearance rates (Beebe and Aronson 1988; Cole et al. 1993). Introducing additional *N*-linked glycosylation sites in EPO improved its efficacy and catabolic half-life (Egrie and Browne 2001).

Predicting how macroheterogeneity and/or microheterogeneity will affect function and affinity requires development of better in vitro models and structure–activity relationships for glycoprotein therapeutics. In industry, predicting the manufacturing impact on macroheterogeneity and microheterogeneity is desired from a safety and regulatory perspective.

Protein glycosylation and regulatory considerations

Protein glycosylation typically leads to a diverse array of product glycans due to multiple factors. The question of native versus foreign glycosylation in humans is a contentious subject because it is not totally clear what the native glycosylation state is since it can vary so frequently between and even within each species. This is especially true in IgG, the backbone molecule of most therapeutic monoclonal antibodies (Rademacher 1993). Even with knowledge of human glycoform profiles for a particular glycoprotein, matching those attributes on the recombinant biologic is not trivial. The choice of the host cell expression system has a primary role toward the resulting protein glycosylation form (Jenkins et al. 1996; Sethuraman and Stadheim 2006).

Regulatory agencies around the world require biopharmaceutical companies to both characterize and maintain product quality attributes (including glycosylation) within defined acceptance limits. These limits typically cover a range of values in the individual glycoform profile. The US FDA, the European Medicines Evaluation Agency (EMEA), and the International Conference on Harmonization (ICH) provide documents for guidance on expectations and requirements for the production of therapeutic proteins commercially (FDA 1996; EMEA 1999).

In biopharmaceutical manufacturing, the acceptance limits are based on what the cell line, upstream cell culture, harvest operation and downstream purification can support, and on the known glycoform species, and efficacy data from animal and/or human PK. The acceptance range in glycoform profile can vary depending on the specific protein therapeutic and is considered intellectual property of the biopharmaceutical manufacturer. A universal set of acceptance limits does not exist, but what is universal is the idea of comparability, and the fact that the process must provide for it for batch release of the material. Changes in production methods of a biological product requires extensive comparability analysis to ensure that changes did not occur on the product that affect safety, identity, purity, or efficacy (Schaffner et al. 1995). In addition to biochemical comparability data, monkey or human PK bridging studies may be required to demonstrate clinical comparability for a protein therapeutic.

This is a challenge for biopharmaceutical companies where a cell line change, process changes and/or change of manufacturing site requires extensive comparability studies to prove that the molecule remains the same (Woodcock et al. 2007). This was most recently highlighted in the case of Genzyme's recombinant enzyme Myozyme (News 2008). Genzyme was unable to demonstrate by FDA standards that Myozyme had the same carbohydrate structure when transferring the manufacturing process from the 160-L to 2000-L bioreactor scale, underlining how critical it is to understand what affects the glycosylation profile.

Comparability has been described as being a pragmatic evaluation (FDA 1996; Walsh and Jefferis 2006). The FDA has specified regulations for reporting requirements of manufacturing changes for biologics, with pre-approved "Comparability Protocols" (CP). The CP defines the predetermined acceptance criteria for evaluating the effect(s) of changes to the manufacturing process on the protein product (Schenerman et al. 1999). Outside the USA, other regulatory agencies have similar requirements.

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Protein glycosylation control

There have been numerous strides toward understanding and improving protein quality control, including a better understanding of molecular engineering and cell culture processes on the resulting glycosylation. The various mechanisms of glycosylation control are schematically depicted in Figure 3 and described in corresponding Tables II and III for an *N*-linked glycosylation pathway typical of CHO cells.

Documented reports have shown both direct and inverse relationships between that of protein synthesis rates and the resulting protein glycosylation. Lowering the protein synthesis rate by cycloheximide improved the glycosylation site occupancy of recombinant prolactin produced by C127 murine cells (Shelikoff et al. 1994). However, studies on tPA synthesis in CHO cells suggest that the protein synthesis rate has little effect on protein glycosylation (Bulleid et al. 1992). Myeloid HL-60 cells incubated at 21°C to decrease glycoprotein flux had a 100% increase in *N*-acetyllactosamine repeats in lysosomal membrane glycoproteins, suggesting that the increased residence time in the Golgi facilitated a more fully glycosylated structure (Wang et al. 1991).

Cell line

Understanding how a cell line can affect glycosylation is critical in designing new protein therapeutics. As can be seen in Table I, the predominant cell line used in commercial manufacture of glycosylated protein therapeutics is CHO with a few exceptions using mouse myeloma SP2/0, human HT-1080, human lymphoblastoid, and NS0. The IgG-Fc glycoform profile of chimeric and humanized rMAbs produced by CHO, NSO, or Sp2/0 cells can vary inter and intra-species and are dependent on the mode of production and culture conditions (Raju et al. 2000). Suspension adapted CHO cells expressing human β-interferon have been shown to produce a similar glycoform profile as CHO cells attached to Cytopore microcarriers (Spearman et al. 2005). In addition, mammalian cells have a variable capacity to glycosylate proteins due to a different complement of functionally expressed enzymes which can also vary by tissue within the same organism.

Expression levels of specific glycosylation genes can also vary. α(2,6) SiaT expression levels have been found to vary 50- to 100-fold in various rat tissues (Lee et al. 1989), but cell lines such as CHO and BHK lack a functional α(2,6) SiaT and make exclusively α(2,3)-linked NANA. CHO cells such as CHO-K1 and DUKX-B11 have also inactivated the gene for α(1,3) GalT and also make low levels of NGNA (Jenkins et al. 1996).

Manufacturing mode

The three most common cell culture production modes are batch, fed-batch, and perfusion. The production method can have a pronounced effect on the resulting glycoform profile (Kunkel et al. 2000). Protein glycosylation profiles of a CHO DG44 cell line producing the reporter protein-secreted alkaline phosphatase (SEAP) were compared between unamplified cells lines cultured in different operating modes (batch, repeated fed-batch, semi-continuous perfusion), as well as different levels of gene amplification (parental line versus MTX amplified producer line) in batch mode (Lipscomb et al. 2005). The glycoform profile from the amplified cell line exhibited less mannosylated glycans, as well as less overall sialylation, although these differences were less than 10% of the total profile. Overall sialylation was increased in the perfusion mode cultures compared to fed-batch mode, the slower growing cells in perfusion mode facilitated a more fully glycosylated protein compared to the fed-batch mode where cells grew faster. There is debate within the biotech industry on which mode, perfusion or fed-batch, is best in terms of cost and development time; however, the decision may depend more on each individual glycoprotein.

Process strategies

Researchers investigated the effects of various bioreactor control parameters on the resulting Epo-Fc glycoprotein expressed in CHO cells (Trummer et al. 2006). It was found that the resulting sialic acid ratio (i.e., moles of NANA to mole of glycoprotein) had a maximum (~13) around pH 7.0, which decreased if the pH was higher or lower. The dissolved oxygen (DO) level that provided for the maximum ratio was 50% of air saturation. Similarly, the sialic acid ratio decreased from 14 to 8 when the culture temperature was brought down from 37°C to 30°C. This process mapping is a fine example of range finding efforts that are pivotal toward process understanding and effective process control of protein glycosylation.

Table II and Figure 3 summarize the key process variables that affect glycosylation. Dissolved oxygen levels are monitored continuously throughout a mammalian cell culture process and have been shown to affect protein glycosylation (see #10 in Figure 3). Hypoxia in bioreactor culture has shown mixed effects on the resulting protein glycosylation. For example, only minor changes were seen in tPA glycosylation of CHO cells (Lin et al. 1993), but significant sialylation changes were seen in CHO-derived FSH (Chotigeat et al. 1994). A murine hybridoma expressing an IgG1 glycoprotein had a decrease in galactosylation as the DO level was reduced from 100% to 50% to 10% (Kunkel et al. 1998). The reason for these decreases in terminal galactosylation at low DO levels has been suggested to be potentially due to a decreased UDP-Gal level, or transport into the Golgi apparatus (Butler 2006).

Ammonium ions are a cellular waste product, generally toxic to cells, which accumulates in cell culture media principally as a result of glutamine and asparagine metabolism. Ammonium chloride causes an increase in intracellular pH and since terminal glycosylation occurs in the acidic distal regions of the Golgi complex, an increase in intracellular pH may correlate to a decrease in terminal sialylation (Thorens and Vassalli 1986) (see #14 in Figure 3).

Bioreactor pH has been found to affect protein glycosylation (see #11 in Figure 3). The culture pH of a hybridoma cell line has been shown to affect the resulting galactosylation and sialylation of the monoclonal antibody (Muthing et al. 2003). The highest levels of agalacto and monogalacto complex *N*-glycans were measured at pH 7.2 and pH 6.9, and the highest digalacto-complex-type glycans were measured at pH 7.4 in HEPES buffered cultures. The latter condition also facilitated the highest NANA/NGNA ratio, compared to any of the pH experiments. The proportion of acidic isoforms of EPO increased with decreasing culture pH with an optimal range of 6.8–7.2 favoring sialylation (Yoon et al. 2005). Interestingly, even though higher pH and higher buffered conditions facilitated a higher NANA content in the above experiment, the opposite was found with polysialic acid attached to neural cell adhesion molecules

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Table II. Effect of cell culture process variables and media on glycosylation

Figure 3 legend	Variable	Effect on glycosylation	References
9	Low glucose/glutamine concentration	Glycosylation of monoclonal antibodies by human hybridomas in batch culture IFN-γ produced by CHO cell perfusion culture	(Hayter et al. 1992, 1993; Tachibana et al. 1994)
10	Dissolved oxygen (DO)	Variable effect on glycosylation that is cell line specific and/or protein specific, typically 10–100% DO will allow consistent glycosylation	(Butler 2006; Chotigeat et al. 1994; Restelli et al. 2006)
11	Bioreactor pH	Galactosylation, sialylation, microheterogeneity vary with pH setpoints (6.8–7.8)	(Borys et al. 1993; Muthing et al. 2003)
12	Manganese (Mn)	Mn modulates the glycosylation profile and lack of Mn can specifically inhibit *O*-linked glycosylation	(Crowell et al. 2007; Kaufman et al. 1994)
13	Sodium butyrate	Protein specific changes when using sodium butyrate	(Chotigeat et al. 1994; Rodriguez et al. 2005; Sung et al. 2004)
14	Ammonia	High concentrations prevent terminal glycosylation, ideally should be minimized to < 2 mM in culture	(Andersen and Goochee 1995; Borys et al. 1994; Thorens and Vassalli 1986; Yang and Butler 2000, 2002)
15	pCO_2	Polysialylation decreases with increasing pCO_2	(Zanghi et al. 1999)
		A decrease in NGNA when pCO_2 is increased	(Kimura and Miller 1997)
16	DMSO	Dimethylsulfoxide (DMSO) decreases sialylation	(Rodriguez et al. 2005)
17	Glycerol	Glycerol enhances sialylation	(Rodriguez et al. 2005)
18	Temperature	Low temperatures (30°C) causes a decrease in sialic acid	(Kaufmann et al. 1999; Trummer et al. 2006)
		Lower temperatures do not reduce sialidase activity	(Clark et al. 2004)
		Shift from 37°C to 33°C can maintain glycosylation and productivity	(Bollati-Fogolin et al. 2005; Yoon et al. 2003)
19	Harvest criteria – cell viability	Extracellular sialidase and glycosidases decrease sialic acid, cell viability can be a critical factor in final sialic acid levels	(Gramer and Goochee 1993)
20	Shear stress	High shear minimizes site occupancy in a glycoprotein	(Senger and Karim 2003)
21	Nucleotide-sugare content	*N*-Acetylmannosamine supplementation improved sialylation of IFN-γ but did not change for TIMP-1 although CMP-sialic acid pools increased in both cases	(Baker et al. 2001; Gu and Wang 1998)
n/a	Glycine betaine	Glycine betaine inhibits polysialylation at osmolalities <435 mmHg but protects polysialylation at higher osmolalities	(Schmelzer and Miller 2002)
n/a	Culture system and operation	Perfusion increases sialylation over fed-batch	(Lipscomb et al. 2005)
		Stirred tank system increases cell death which resulted in a decrease in sialylation	(Goldman et al. 1998)
n/a	pH and temperature	Shifting pH to <7.0 while reducing temperature maintains productivity and sialylation	(Yoon et al. 2003)

Table III. Cellular and protein engineering effects on glycosylation

Figure 3 legend	Variable	Effect on glycosylation	References
1	GnT I	Knockout creates high-mannose-type IgG1 antibody that had reduced ADCC and shorter half-life.	(Kanda et al. 2007)
2	β(1,4)-GalT	Reduction in terminal GlcNAc	(Weikert et al. 1999)
3	α(2,3)-SiaT	Resulted in sialylation of >/= 90% of available branches	(Weikert et al. 1999)
4	Sugar-nucleotide transporter	Overexpression of CMP-sialic acid transporter (CMP-SAT) resulted in 4–16% increase in site sialylation of IFN-gamma	(Wong et al. 2006)
5	GnT III	IgG1 antibody created with bisecting GlcNAc; ADCC activity increased 10- to 20-fold. 15- to 20-fold improvement in ADCC Increase in GlcNAc-bisected hybrid-type glycans	(Davies et al. 2001; Ferrara et al. 2006; Umana et al. 1999)
6	α(1,6)-FucT	50% decrease in fucosylated antibody; 100× higher ADCC response; no change in cell growth profile IgG1 antibody same binding activity	(Mori et al. 2007, 2004)
n/a	α(1,3)-FucT	Activation of endogenous α(1,3)-FucT	(Potvin et al. 1990)
7	siRNA Sialidase	Sialidase antisense RNA expression decreased extracellular sialidase activity; higher NANA content on DNase glycoprotein	(Ferrari et al. 1998)
8	Sialidase	Extracellular sialidase will reduce sialylation	(Gramer and Goochee 1993, 1994)

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(NCAM) expressed on recombinant CHO cell surfaces (Zanghi et al. 1999).

Reducing temperature can increase overall protein production by prolonging cell viability (Moore et al. 1997) which should in principle improve glycosylation. Cell viability is critical because extracellular glycosidases can accumulate in the medium and step-wise remove monosaccharides from the glycan (Gramer and Goochee 1993; Gramer et al. 1995) (see #19 in Figure 3). Temperature shifts in a production bioreactor can increase volumetric product titers while maintaining glycoform quality (Yoon et al. 2003; Clark et al. 2004; Bollati-Fogolin et al. 2005). In contrast, EPO-Fc had a decrease in sialylation by 20% and 40% when reducing the temperature to 33°C and 30°C (Trummer et al. 2006). In this specific case, a reduced temperature of 30°C showed a correlation between increased specific productivity and decreasing levels of sialylation. It is still unknown whether higher productivity correlates to the expression rate, thereby, reducing the intracellular processing time for glycosylation and causing an increase in less sialylated protein population. High-cell viability (less sialidase activity) in conjunction with high cell productivity (shorter residence time) may diminish the overall effect on sialylation.

Shear stress in a bioreactor culture has been reported to be an important engineering variable for the resulting glycoform profile (see #20 in Figure 3). By manipulating agitation speeds and the resulting shear stress, it was found that maximum levels of damaging shear were required to minimize the extent of tPA Asn184 site occupancy, which was attributed to a decreased residence time of tPA in the ER (Senger and Karim 2003). This can be an important consideration during the scale-up or transfer of an existing process to a different facility. Monitoring the effects of shear differences on protein glycosylation during the transition between production modes (e.g., from perfusion to fed-batch) is also important for ensuring product comparability.

Cell culture media

The effect of media on glycosylation is summarized in Table II and Figure 3. Mammalian cell culture media is typically a mixture of 50–100 different chemically defined components, as well as a handful of undefined components that are sometimes supplemented. These undefined components include peptones, yeast extracts, plant hydrolysates, and serum. The literature has reported the effects of some of these undefined components on protein glycosylation (Gawlitzek et al. 1995; Gu et al. 1997). A monoclonal IgG1 produced by mouse hybridoma in serum-free media had higher levels of terminal NANA and Gal compared to cultures with serum, whereas terminal Gal was higher from CHO cells cultured in media with serum (Patel et al. 1992). Currently, serum and animal-derived components are avoided in the biopharmaceutical industry due to regulatory and safety issues (Geigert 2004).

Defined media components such as glucose, glutamine, and galactose can also play a role in glycosylation control. Glucose-limited continuous cultures of CHO cells allowed for the production of IFN-γ without glycosylation (Hayter et al. 1992). CHO fed-batch cultures producing IFN-γ found that below 0.1 mM glutamine and 0.7 mM glucose (Wong et al. 2005) led to decreased sialylation profiles and an increase in hybrid and high mannose type glycans. In contrast, under low glucose and glutamine concentrations, continuous cultures of BHK-21 cells expressing a human IgG-IL2 fusion protein showed no differences in the oligosaccharide profile compared to a nonnutrient limited culture (Cruz et al. 2000). Lower glycan occupancy levels may be related to a decreased intracellular UDP-GlcNAc pool in glucose- and glutamine-limited cultures (Nyberg et al. 1999). Galactose feeding can help facilitate a more fully galactosylated *N*-glycan profile (Andersen 2004).

Frequently, small molecules are included in the culture media to induce a desired cellular behavior, such as high specific productivity. Sodium butyrate has been well documented in the literature for increasing specific productivity, as well as decreasing growth rates by upregulating apoptosis mechanisms and modulating sialylation (Chotigeat et al. 1994; Santell et al. 1999; Rodriguez et al. 2005). CHO DUKX-B11 cell lines in shake-flask culture under varying levels of sodium butyrate had an overall decrease in product quality through increased microheterogeneity, reduced sialylation, and decreased in vivo activity (Sung et al. 2004). Indeed, the pleiotropic effects of sodium butyrate on the gene and protein expression level of mammalian cells have been well documented (Yee et al. 2007).

Amino acid supplementation is critical for mammalian cell growth and productivity, but the exact concentrations delivered can cause changes in the glycosylation profile. Supplementing additional amino acids (cysteine, isoleucine, leucine, tryptophan, valine, asparagine, aspartic acid, and glutamate) that had been depleted in early culture caused an increase in the lower sialylated fraction of recombinant human erythropoietin (rHuEPO) (Crowell et al. 2007). In the same study, the authors demonstrated that the addition of manganese to the cell cultures increased galactosylation which in turn facilitated an increase in *O*- and *N*-linked glycosylation. CMP-sialic acid synthetase activity is also dependent on metal ions for activity, and in particular, manganese gives optimal activity at neutral pH (Higa and Paulson 1985).

Lipid supplements and carriers (dolichol) have been shown to improve *N*-glycan site occupancy of IFN-γ (Jenkins et al. 1994; Castro et al. 1995) in addition to sugar nucleotides (Kochanowski et al. 2008). Nucleotide-sugar precursors modulate intracellular nucleotide-sugar pools and the resulting sialylation and antennarity levels. CHO-K1 cells secreting EPO incubated with greater than 10 mM glucosamine decreased sialylation on tetrasialylated glycans by 41%, and the proportion of tetraantennary glycans by 37%, and with less than 30 mM ammonia decreased tetrasialylated glycans by 73%, and the proportion of tetraantennary glycans by 57% (Yang and Butler 2002). The intracellular pool of UDP-GlcNAc was at least a 2-fold higher level upon the addition of ammonia or glucosamine. GS-CHO and GS-NS0 host cells producing the tissue inhibitor of metalloproteinases 1 (TIMP-1) in spinner cultures were supplemented with 10 mM glucosamine and 2 mM uridine, as well as 20 mM *N*-acetylmannosamine in the hopes of increasing the intracellular pools of UDP-*N*-acetylhexosamine (i.e., UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine), and CMP-sialic acid (Baker et al. 2001). The glucosamine/uridine supplemented cultures did increase *N*-acetylhexosamine levels which increased the antennarity of the measured *N*-glycans from CHO, but not in NS0 cells. This increase was also accompanied by a decrease in sialylation. The addition of *N*-acetylmannosamine increased intracellular CMP-sialic acid levels, but did not affect the sialylation on the resulting TIMP-1 from either CHO or NS0. In contrast, researchers

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(Gu and Wang 1998) supplemented CHO cell culture expressing IFN-γ with 20 mM *N*-acetylmannosamine (a sialic acid precursor) and found an increase in sialylation on one *N*-glycan site. This supplementation increased the intracellular levels of CMP-sialic acid by almost 30-fold.

Cellular engineering strategies

A number of strategies have been attempted to modify the protein glycosylation pathway (see Table III and #1–7 in Figure 3). β(1,4) GalT and α(2,3) SiaT were overexpressed in CHO cells secreting a TNFR-IgG fusion protein or a modified tPA. The overexpression caused significant reduction in GlcNAc-terminated *N*-glycans and an increase in terminal sialylation, which resulted in a longer PK residence time in a rabbit model (Weikert et al. 1999). There are numerous other cases in the literature for the overexpression of *N*-glycan biosynthetic enzymes (Zhang et al. 1998; Schlenke et al. 1999) as well as nucleotide-sugar transporters within the Golgi (Wong et al. 2006).

ADCC activity has been correlated to both Fuc attachment on *N*-glycans and GnTIII expression. GnTIII was cloned and expressed in a DG44 CHO cell line (which normally does not express functional GnTIII) producing the glycoprotein mouse/human α-CD20 IgG1 antibody (Davies et al. 2001). This produced bisecting GlcNAc on *N*-glycans located on the Fc region which improved its efficacy by eliciting ADCC. GnTIII was also engineered into CHO cells which facilitated a 15- to 20-fold improvement in ADCC for an anti-neuroblastoma antibody (Umana et al. 1999). Biowa Inc. utilized gene knock-down technology to remove α(1,6)-linked Fuc sugars from *N*-glycans in a CHO cell line to create Potelligent® cells that produce fucose-free antibodies with 50 to 100 times enhanced ADCC activity with the same growth and productivity as in the parental cell line. Interestingly, it has been shown that the effectiveness of a nonfucosylated antibody in eliciting a greater ADCC response is both dependent on glycosylation sites on FcγRIIIa (Shibata-Koyama et al. 2009), as well as the recruited effector cell type (Peipp et al. 2008).

The above studies all attempted to introduce more of a particular enzyme or eliminate enzyme function through cellular engineering; however, these studies merely controlled absolute expression levels. There have also been reports and reviews highlighting various structural requirements for Golgi-specific enzyme localization (Grabenhorst and Conradt 1999). Researchers have attempted to control localization of these enzymes to enhance the glycosylation machinery at key protein processing organelles. For example, changing the localization of GnTIII within the Golgi apparatus led to an increase in GlcNAc-bisected hybrid-type glycans (Ferrara et al. 2006).

Identification of glycosylation gene knockouts in mutant CHO cell lines has greatly increased our understanding of the metabolic pathway and generation of individual glycan structures (Stanley 1989; Raju et al. 1995; Raju and Stanley 1996). By using siRNA against FUT8, mRNA expression was knocked down to 20% of basal levels in a CHO DG44 cell line expressing antibody in serum-free fed-batch culture, with a 60% ratio of defucosylated antibody and a 100 times higher ADCC response, but with little change in cell growth profiles (Mori et al. 2004). Researchers used homologous recombination to knockout both FUT8 alleles in a CHO DG44 cell line producing chimeric anti-CD20 IgG_1 and showed the same binding activity, but a 100 times enhanced ADCC response (Yamane-Ohnuki et al. 2004). Researches have expressed sialidase antisense RNA in CHO cells, facilitating a decrease in extracellular sialidase activity and a higher NANA content on DNase (Ferrari et al. 1998).

Protein engineering strategies

Inserting additional *N*-linked sites has been proven to be clinically beneficial for recombinant glycoproteins. Two new *N*-linked glycosylation sites were incorporated into recombinant human erythropoietin (rhEPO) via site-directed mutagenesis into the polypeptide chain, creating darbopoetin alfa, which substantially increased in vivo activity and PK (Elliott et al. 2003; Elliott, Chang, et al. 2004; Elliott, Egrie, et al. 2004). In another study (Perlman et al. 2003), protein engineering was applied to FSH by introducing additional *N*-linked glycosylation sites into the molecule through structure-aided, site-directed mutagenesis within the FSH molecule and by the addition of *N*-terminal extensions. The resulting molecule (FSH1208) was found to have a 3- to 4-fold increased serum half-life, compared with wild-type recombinant FSH. This strategy of incorporating new *N*-linked sequons to improve activity and PK has also been successful with antibody fragments (Stork et al. 2008). Although we can design proteins with increased glycosylation, understanding how effective the cell machinery is in producing these recombinant proteins is critical in bridging the gap between characterization and production.

Discussion: Future considerations for glycosylation control

The above section highlighted process, media, cellular, and protein engineering strategies that have been documented to exert some control over the protein glycosylation pathway. Mammalian cell lines such as CHO, BHK, and PerC.6 process recombinant glycoproteins in a manner similar to humans. Efforts to exert control over protein glycosylation have revealed success stories with a particular cell line expressing that a particular protein does not always carry over to others. Understanding these differences may require a more in-depth physiological understanding of the mammalian cell responses to a particular set of conditions, as well as the detailed glycoprotein characterization. Achieving uniform and/or consistent protein glycosylation may be characterized by a certain set of gene/protein expression levels, as well as spatial localization of the enzymes and nucleotide-sugar substrates. There may be a set of physiological requirements that need to be met within a particular clone for these goals to be achieved that can then be subsequently screened for. Once this could be identified, it would serve as a model for future bioprocesses to duplicate. Product quality screening should be taken into consideration in the early cell clone selection and evaluation process, in addition to both cell growth and productivity (Walsh and Jefferis 2006).

The impact of protein quality on therapeutic efficacy of recombinant glycoproteins has had far-reaching effects to the point that the economics of these processes is largely shaped by it. Clearly, any improvements toward the control of this important biochemical pathway will have far-reaching influences on industry, and the use of mammalian cells as the workhorse expression systems in general. However, the question remains as to what an optimal glycoform profile should be targeted for, and this must be addressed on an individual glycoprotein basis.

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The optimal glycoform at each glycosylation site of a protein for beneficial efficacy and PK is typically not identified due to time constraints and/or the inability to produce purified drug substance with a particular type of glycosylation. Glycoform mapping studies with respect to circulatory clearance should be investigated so that an optimal glycoform profile can be defined and targeted for in process development, better enabling QbD approaches required by the FDA. Since the final glycoform profile is likely to comprise a mixture of *N*- and *O*-glycan structures, an objective way of quantifying the overall efficacy or PK is to define a weighted average over all the individual structures (Cumming 1991) based on the total mole fraction of each glycan species, and its overall intrinsic contribution toward efficacy or PK. Characterizing glycans on protein molecules with respect to their efficacy and PK contributions could allow for the quantitative interpretation of glycosylation profiles and define what is optimal on a case-by-case basis. Such an analysis would also be amenable toward the functional analysis of macroheterogeneity, or on a glycosylation site-by-site basis.

The scientific studies reviewed in the previous section all attempted to analyze only one component of the protein glycosylation pathway, which is more likely simultaneously dependent on the interplay of many components toward the determination of the final glycoform profile (see Figure 3). Glycomics in a relatively new –omics that has helped increase the analytical throughput, and the amount of relevant physiological information (Shriver et al. 2004; Zaia 2008). To fully implement QbD in existing and future bioprocesses, having the necessary PK and analytical data to relate to glycoforms will be helpful for product quality control. High-throughput analysis using databases of glycans chains in combination with HPLC methods will improve upon the analytical tools available to researchers (Sheridan 2007). It will also be critical to better define and refine approaches to accurately measure glycans profiles and heterogeneities for the establishment of glycoproteomics (Geyer H and Geyer R 2006).

Visual tools have been developed to understand the various pathways that can be traversed toward *N*- and *O*-glycan biosynthesis and enzymatic substrate specificities (Hossler et al. 2006). Computational studies have shown that glycan homogeneity and uniformity could be achieved only as a result of both enzyme overexpression and preferential localization of appropriate enzymes to the various Golgi compartments (Hossler et al. 2007). Further understanding of enzyme localization mechanisms may be necessary. Other glycomodulation attempts have removed all context of the cellular processing machinery altogether, and instead used in vitro glycosylation of glycoproteins as an effective method to increase the extent of terminal glycosylation (Raju et al. 2001). Cellular engineering is a proven method for modulating protein glycoform profiles and should be implemented for early-stage processes that are amenable to parental cell line modifications.

Mammalian cells have a proven role toward supporting the quantities and qualities of therapeutics that are required. It is for these reasons that mammalian cells will continue to play a dominant role in the production of glycoprotein therapeutics. Ultimately, efforts in optimizing cell physiology for protein therapeutic production need to be guided by a thorough understanding of the optimal glycoform profile targeted for each glycoprotein. The literature has reported the PK responses toward particular glycoforms, and the responses toward individual sugars, but more such studies are necessary. Ideally, the targeting of specific glycoforms needs to be pursued on an individual glycoprotein basis determined principally by a thorough understanding of the glycoprotein's in vivo function.

Acknowledgements

The authors wish to acknowledge the Process Sciences and Process Analytical Sciences groups within Technical Operations of Bristol-Myers Squibb & Co., for their perspectives and help toward the writing of this review. We would also like to thank Dr. Mark A. Lehrman for his useful critique of the manuscript.

Conflict of interest statement

All authors were employed by Bristol-Myers Squibb & Co., a biopharmaceutical company which produces glycosylated therapeutics, at the time this article was written.

Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; ASGPR, asialoglycoprotein receptor; DO, dissolved oxygen; EMEA, European Medicines Agency; EPO, erythropoietin; FDA, United States Food and Drug Administration; FSH, follicle stimulating hormone; Fuc, fucose; FucT, glycoprotein 6-α-L-fucosyltransferase; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GalT, β-*N*-acetylglucosaminylglycopeptide β-1,4-galactosyltransferase; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GnTI, α-1,3-mannosyl-glycoprotein 2-β-*N*-acetylglucosaminyltransferase; GnTII, α-1,6-mannosyl-glycoprotein 2-β-*N*-acetylglucosaminyltransferase; GnTIII, α-1,4-mannosyl-glycoprotein 4-β-*N*-acetylglucosaminyltransferase; GnTIV, α-1,3-mannosyl-glycoprotein 4-β-*N*-acetylglucosaminyltransferase; GnTV, α-1,6-mannosyl-glycoprotein 4-β-*N*-acetylglucosaminyltransferase; ICH, International Conference on Harmonization; Man, mannose; MTX, methotrexate; n/a, not applicable; NANA, *N*-acetylneuraminic acid; NGNA, *N*-glycolylneuraminic acid; PK, pharmacokinetics; QbD, quality by design; rMAbs, recombinant monoclonal antibodies; SiaT, β-Galactoside α-2,3/6-sialyltransferase; tPA, tissue plasminogen activator; UDP, uridine diphosphate.

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