

Protein Glycosylation Control in Mammalian Cell Culture: Past Precedents and Contemporary Prospects

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Abstract Protein glycosylation is a post-translational modification of paramount importance for the function, immunogenicity, and efficacy of recombinant glycoprotein therapeutics. Within the repertoire of post-translational modifications, glycosylation stands out as having the most significant proven role towards affecting pharmacokinetics and protein physiochemical characteristics. In mammalian cell culture, the understanding and controllability of the glycosylation metabolic pathway has achieved numerous successes. However, there is still much that we do not know about the regulation of the pathway. One of the frequent conclusions regarding protein glycosylation control is that it needs to be studied on a case-by-case basis since there are often conflicting results with respect to a control variable and the resulting glycosylation. In attempts to obtain a more multivariate interpretation of these potentially controlling variables, gene expression analysis and systems biology have been used to study protein glycosylation in mammalian cell culture. Gene expression analysis has provided information on how glycosylation pathway genes both respond to culture environmental cues, and potentially facilitate changes in the final glycoform profile. Systems biology has allowed researchers to model the pathway as well-defined, inter-connected systems, allowing for the *in silico* testing of pathway parameters that would be difficult to test experimentally. Both approaches have facilitated a macroscopic and microscopic perspective on protein glycosylation control. These tools have and will continue to enhance our understanding and capability of producing optimal glycoform profiles on a consistent basis.

Keywords Genomics · Mammalian cell culture · Protein glycosylation · Systems biology

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
BHK	Baby hamster kidney cells

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CDC	Complement-dependent cytotoxicity
CHO	Chinese hamster ovary cells
CMP-NeuAc	Cytosine monophosphate-N-acetylneuraminic acid
DO	Dissolved oxygen
EPO	Erythropoietin
ER	Endoplasmic reticulum
Fuc	Fucose
FucT	Glycoprotein 6- α -L-fucosyltransferase
Gal	Galactose
GalNAc	N-Acetylgalactosamine
GalT	β -N-Acetylglucosaminyl glycopeptide β -1,4-galactosyltransferase
GDP-Fuc	Guanidine diphosphate-fucose
GlcNAc	N-Acetylglucosamine
GnT I	α -1,3-Mannosyl-glycoprotein 2- β -N-acetylglucosaminyltransferase
GnT II	α -1,6-Mannosyl-glycoprotein 2- β -N-acetylglucosaminyltransferase
GnT III	β -1,4-Mannosyl-glycoprotein 4- β -N-acetylglucosaminyltransferase
GnT IV	α -1,3-Mannosyl-glycoprotein 4- β -N-acetylglucosaminyltransferase
GnT V	α -1,6-Mannosyl-glycoprotein 4- β -N-acetylglucosaminyltransferase
GS	Glutamine synthetase
IFN- γ	Interferon-gamma
Man	Mannose
Man I	Mannosyl-oligosaccharide 1,2- α -mannosidase
Man II	Mannosyl-oligosaccharide 1,3-1,6- α -mannosidase
ManNAc	N-Acetylmannosamine
NeuAc	N-Acetylneuraminic acid (sialic acid)
NeuGc	N-Glycolylneuraminic acid
PK	Pharmacokinetics
PCA	Principal component analysis
tPA	Tissue plasminogen activator
SiaT	β -Galactoside α -2,3/6-sialyltransferase
UDP-Gal	Uridine diphosphate-galactose
UDP-GlcNAc	Uridine diphosphate-N-acetylglucosamine
UPR	Unfolded protein response

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1 Introduction

It has been deduced that more than half of all catalogued proteins are actually glycoproteins, with varying levels of glycosylation characterization [1]. Due to their ubiquity in nature and in recombinant protein expression, protein glycosylation is a post-translational modification that is of paramount interest to the biopharmaceutical industry. Glycosylation has been proven to affect various physiochemical properties of glycoprotein therapeutics, including protein folding [2], solubility [3], binding [4], and stability [5]. The contributing factors that help shape the final glycoform profile have been reviewed [6], and include the cell expression system, the glycoprotein structure itself, the activity and specificity of enzymes involved in the metabolic pathway, as well as the various cell culture environmental variables. Understanding the dependence of the final glycoform profile on these parameters is of ongoing interest to the biopharmaceutical industry.

Due to the nature of the metabolic pathway and its inherent dependence on a multitude of factors, there is typically a diverse array of asparagine linked (N-) and serine/threonine linked (O-) glycans across the various glycosylation sites on the protein product. The heterogeneous glycan structures that are observed at a particular glycosylation site on different glycoprotein molecules is termed microheterogeneity, whereas the differences in glycan site-occupancy at different glycosylation sites across different glycoprotein molecules is termed macroheterogeneity. Microheterogeneity exists for a variety of reasons, with reports showing a direct relationship to cell culture conditions, and to the particular cell line. Macroheterogeneity also exists for a variety of reasons, including protein folding. Since hundreds of different N- and O-glycan species have been described to date, numerous nomenclature systems have been devised to facilitate better classification and communication. Though no completely standard naming convention has been adopted, a common convention is highlighted in Butler et al. [7] as well as the convention established by the Consortium for Functional Glycomics [8] (Fig. 1). In order to make the pathway more computer-representation-friendly, there have been efforts towards development of an XML notation for glycan structures as well [9,10].

An extensive survey of the various glycan structures, their structural and functional roles on the molecule, and the biological processes they modulate has

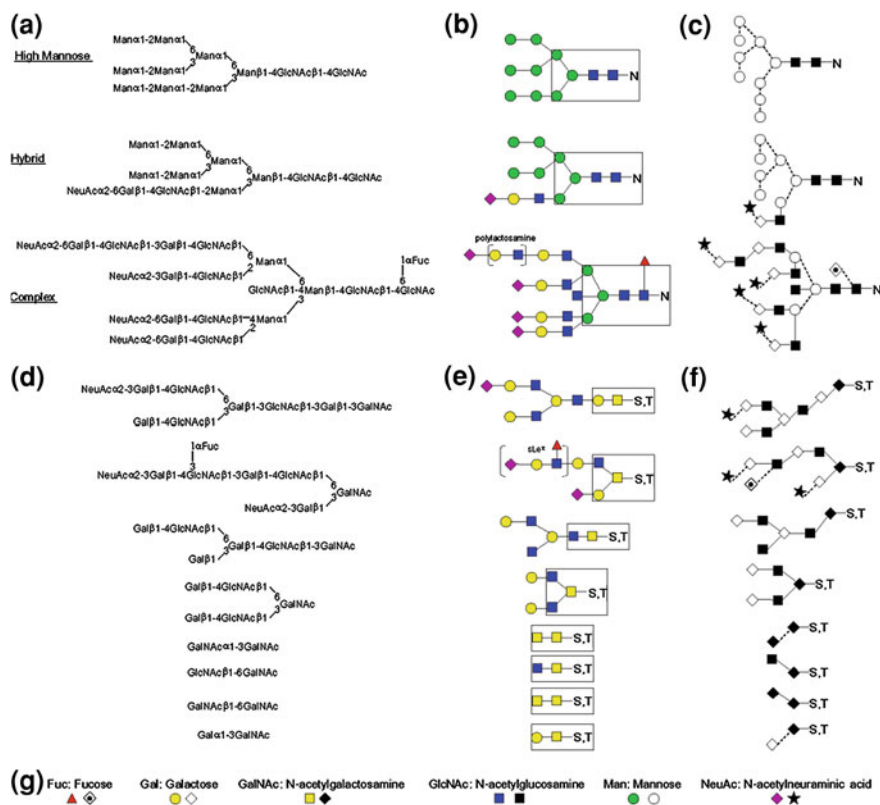


Fig. 1 Comparison of commonly used N- and O-glycan abbreviated nomenclature. **a** Consortium for functional glycomics (CFG) text nomenclature for N-glycans; principal types of N-glycans are shown for representative purposes. **b** CFG symbolic nomenclature for N-glycans; common core pentasaccharide structure are shown in boxes. **c** Symbolic nomenclature of N-glycans consistent with Butler et al. [7]. **d** CFG text nomenclature for O-glycans; eight principal types of O-glycans are shown for representative purposes. **e** CFG symbolic nomenclature for O-glycans; eight different core structures are shown in boxes. **f** Symbolic nomenclature of O-glycans consistent with Butler et al. [7]. **g** Monosaccharide symbol legend

been documented [11,12]. The presence of individual N- and O-glycan structures has been shown to have important roles in the immune system [13], and can affect clearance rate [14] and antigenicity [15] of the glycoprotein therapeutic. For example, the heavy chains of monoclonal antibodies typically contain at least one N-linked glycosylation site, which facilitates two N-glycans per fully assembled antibody molecule. These glycans have been shown to be essential for both interactions with Fc receptors (FcR) and their accompanying effector functions, including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [16,17].

Despite a typically diverse glycoform profile, there is no fundamentally ideal glycoform profile ubiquitous for all glycoproteins. Some glycans have been found

to be more ideal depending upon the function of the glycoprotein they are attached to. In other cases, there is a relative insensitivity towards the particular glycan on the resulting glycoprotein function [18]. Thus, there is sufficient evidence in the literature and other sources to necessitate at least the evaluation of this potential effect and for biopharmaceutical companies to adequately characterize the expressed glycoform profile to ensure it lies within the assigned acceptability limits. Typically, a consistent spectrum of product glycans is targeted in industry, since in most cases it is not possible to fully characterize or predict the *in vivo* effect of every particular glycosylation pattern [19]. To achieve consistency during manufacturing, all efforts in upstream and downstream process development are controlled as tightly as possible.

Since there is a relatively large repertoire of product glycans, controlling their relative amounts in production cell culture often proves difficult to understand. Reports of glycosylation control have centered on either the cell culture process environment, or at the genetic level through overexpression or suppression of key glycosylation enzymes. Two additional tools that have been applied recently towards the understanding and control of protein glycosylation include genomics and systems biology. The gene expression studies conducted to date have highlighted the diverse responses towards cell culture environments which have helped elucidate the regulation of this important metabolic pathway. Systems biology has facilitated our understanding of the protein glycosylation pathway as a system with direct and indirect relationships whose ultimate controllability is dependent upon our understanding of these relationships, and their inherently complex nature. Through continual use of these tools, a more thorough understanding of the regulation and control of protein glycosylation should continue to be realized.

2 Metabolic Pathway

Protein glycosylation is characterized by two main forms depending on the amino acid the oligosaccharides are added onto, including asparagine-linked glycans (N-glycans) and serine/threonine-linked glycans (O-glycans). The processing of N- and O-glycans occurs through a series of monosaccharide removal and chain elongation reactions within the endoplasmic reticulum (ER) and Golgi. This sequential series of reactions forms a metabolic pathway that is linear in some stages, in that each reactant produces only one glycan product. In other stages the pathway is branched, in which a glycan reactant is capable of producing multiple glycan products (Fig. 2). As a result, the glycan processing pathways can diverge at multiple processing steps, which eventually become increasingly convergent as the terminal glycan processing steps (galactosylation and sialylation) are met.

N-glycosylation is initiated by the *en bloc* transfer through an oligosaccharyl-transferase enzyme (OST) from a pre-formed 9-mannose glycan attached to dolichol on asparagine residues of glycoprotein molecules with an Asn-X-Thr/Ser (where X represents any amino acid but proline). This processing step occurs

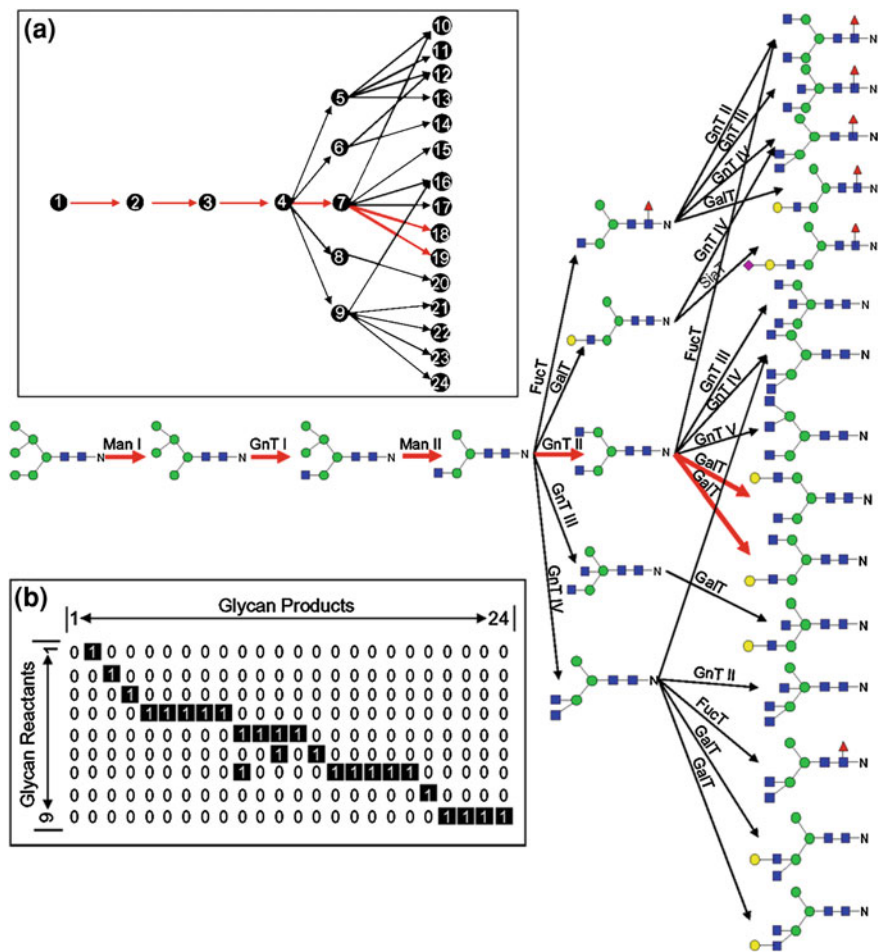


Fig. 2 A portion of the N-glycan biosynthetic pathway with hypothetical preferential pathway highlighted in red. Subset A: Metabolic network representation of the glycosylation pathway highlighting a systems level perspective with arbitrary glycan numbering. Subset B: Adjacency matrix representation of glycosylation network; allowed reactions between reactant and product N-glycans are assigned a value of 1

shortly around the time of initial protein folding in the lumen of the ER. A trimer of glucose sugars on this N-glycan are removed in a step-wise fashion, while concomitantly binding to the calnexin and calreticulin lectins, which ensure proper protein folding and serve as a quality control step within the ER before transport to the Golgi [20]. Should improper folding or attenuated removal of these glucose monosaccharides exist, a signal is provided that directs the glycoprotein towards proteasomal degradation. The high-fidelity mechanism of glycan processing that exists inside the ER ensures the N-glycans are approximately uniform. However, a similar mechanism does not exist inside the Golgi. After some initial mannose

removal reactions in the ER, the N-glycans are transported to the Golgi apparatus where a series of additional mannose removal steps precedes a highly diverse series of step-wise additions of a small number of monosaccharides that facilitates a potentially large number of product glycan structures. The innate substrate specificity of the enzymes towards their substrate glycans, as well as the relative concentrations of enzymes and nucleotide-sugars across the various cisternae of the Golgi, control the final glycoform profile. The diverse array of contributing factors towards protein glycosylation have been documented [6]. As a result of differences in the extent of Golgi processing, three N-glycan types have been categorized. High mannose glycans are the least processed, complex type glycans are the most processed, and hybrid type glycans are intermediately processed with structural attributes of both high mannose and complex glycans (Fig. 1a–c).

In contrast to N-glycosylation, O-glycosylation begins with the transfer of a single monosaccharide (GalNAc), compared to a pre-formed oligosaccharide attached to dolichol. GalNAc is transferred from its cognate sugar-nucleotide UDP-GalNAc onto the hydroxyl group of Ser or Thr residues of glycoproteins. They are then further modified by sialic acid, fucose, galactose, xylose, and/or polylactosamine additions (Fig. 1a–c). The cellular compartment in which the pathway is initiated has been found to be either the ER or Golgi [21]. In contrast to N-glycans, which have a common core pentasaccharide structure, O-glycans have up to eight different core structures which make their study and experimental measurement more difficult (Fig. 1d–f). O-glycans have also been shown to have an intricate role in the physiochemical properties of glycoproteins. A good overview of the metabolic pathway and the effects of O-glycans on proteins can be found in Van den Steen et al. [22].

The monosaccharide source for addition onto N- and O-glycans comes from a common pool of nucleotide-sugars transported into the lumen of the ER and Golgi organelles either from de-novo biosynthesis, or from the recycling of monosaccharides through proteins degraded in the lysosome. All nucleotide-sugars are generated in the cytosol, except CMP-sialic acid which is generated in the nucleus. An excellent review highlighting the details of nucleotide-sugar transporters is given in Ishida and Kawakita [23].

3 Established Methods for Protein Glycosylation Control

3.1 *Expression Host*

Glycosylation varies frequently between and even within each species. This is especially true in immunoglobulin G (IgG), which is the backbone molecule of most of today's monoclonal antibodies [24]. A comparative study of IgG between various species (human, rhesus, dog, cow, guinea pig, sheep, goat, horse, rat, mouse, rabbit, cat, and chicken) revealed differences in neutral oligosaccharides

and NeuAc/NeuGc content, as well as isomer differences, suggesting not only differences in glycan processing, but also enzymatic specificities [25]. Even with knowledge of human glycoform profiles for a particular glycoprotein, matching those attributes on the recombinantly expressed biologic is not trivial. Human serum erythropoietin (EPO) has been shown to be significantly different from recombinantly expressed EPO [26]. It is due to these potential differences that the choice of host cell expression system is very important, and one of the principal reasons for the biopharmaceutical industry's focus on a few established mammalian cell lines. The choice of host cell expression system and the resulting protein glycosylation has been extensively reviewed [27, 28].

Some monosaccharides present on N- and O-glycans from mammalian cells are undesirable. Human cells, for example, do not normally express $\alpha(1, 3)\text{GalT}$ due to a frameshift mutation [29], and are able to mount an immune response to the attached $\alpha(1, 3)\text{Gal}$. Numerous mouse expression systems, including hybridomas producing humanized antibodies, do add them on secreted glycoproteins. However, other commonly used expression systems, such as NS0 and CHO cells, do not add this monosaccharide and as a result there is usually not a problem with the expressed proteins [28]. N-Glycolylneuraminic acid (NeuGc) is another monosaccharide that is generally undesirable for recombinant therapeutic glycoproteins. Adult human cells typically have terminal NeuAc on their glycans. However, other mammals sometimes have terminal NeuGc, which differs from NeuAc by the incorporation of one additional hydroxyl group through the action of the CMP-NeuAc hydroxylase enzyme. Thus recombinant proteins expressed by rodent species, including mouse and hamster, can have terminal NeuGc within the glycoform profile. NeuGc has been detected on the glycans attached to glycoproteins derived from NS0 [30], as well as CHO [31].

Frequently the choice of mammalian expression host has an effect on the resulting glycoform profile due to a different complement of functionally expressed protein glycosylation enzymes. For example, $\alpha(2, 6)\text{SiaT}$ activity varies 50–100-fold in various rat tissues [32], but cell lines such as CHO and BHK both lack a functional $\alpha(2, 6)\text{SiaT}$, and make exclusively $\alpha(2,3)$ linked NeuAc. In another example, CHO cells do not typically have detectable levels of GnT III, while human and other mammalian cell lines do [33].

Mammalian cells glycosylate proteins that are on average more consistent with humans than non-mammalian cells. Some of the more common non-mammalian host cell expression systems for recombinant glycoproteins include insect, plant, and yeast systems. These cells all have been shown to glycosylate expressed proteins; however, in the majority of the cases their glycoform profiles are either different from that observed in human, or are not optimal. Insect cell lines have been used as host cells for glycoproteins, including monoclonal antibodies. However, the glycoproteins expressed are typically hybrid, high-mannose, or paucimannose glycans [34]. Some insect cell lines have also been shown to add non-human $\alpha(1,3)\text{Fuc}$, which is potentially immunogenic. Plant cell lines have also been used as expression systems for glycoproteins, but typically lack Gal and NeuAc, and instead add xylose and $\alpha(1,3)\text{Fuc}$. Yeast expression systems have

become more common for glycoproteins, especially *Pichia pastoris* because of robust expression as well as scalable fermentation. However, yeast systems also hypermannosylate the expressed proteins. As a result of this, efforts have been made to successfully humanize glycosylation in these cells, as well as to incorporate human glycosylation characteristics, including sialylation [35]. Although there have been a few very successful attempts to “humanize” glycosylation patterns in these cells, in the majority of the cases mammalian cell lines are still the preferred host expression system towards the production of recombinant glycoprotein therapeutics with human-like glycosylation characteristics.

3.2 Cell Culture Process Conditions

The effect of cell culture environmental conditions on the resulting protein glycosylation has been well-documented. Different culture conditions frequently facilitate differences in the resulting protein glycosylation. The choice of bioreactor production mode, process control variables, as well as culture media and respective nutrient levels have all been documented to elicit an effect on the resulting protein glycosylation. Previous reviews have described in detail these potential effectors of glycoform profiles [19].

Different production bioreactor modes and vessels have been shown to cause differences in protein glycosylation using the same cell line and culture media. In Kunkel et al. [36], even with the same cell line expressed under the same process conditions, but with different bioreactor controller platforms, differences were observed in the final glycoform profile. In another study it was shown that with CHO-derived human IFN- γ cultured in a perfused, fluidized-bed bioreactor attached to macroporous microcarriers there was an increase in the proportion of tri- and tetrasialylated N-glycans, along with a decrease in monosialylated and neutral N-glycans after 210 h in culture [37]. In contrast, cells that were cultured in a stirred tank bioreactor showed similar sialylation time profiles until late-stage, where a decrease in overall sialylation was observed as the cells began to die, potentially due to extracellular degradation. In another study the three main glycoforms from a cytokine fusion protein were expressed from BHK cells, and showed subtle differences across different culture platforms, and at different times in a fixed bed bioreactor with continuous perfusion [38].

Process variables that are typically monitored and/or controlled during the course of a typical mammalian cell culture process have been documented to have a major role in modulating the final glycoform profile. Dissolved oxygen (DO) levels, bioreactor pH, and culture temperature are three process parameters commonly controlled in any cell culture process. All three variables have been shown to have an appreciable effect on protein glycosylation in at least some instances [19]. Butler [6] provides a very good review of the relationship between process control variables and the resulting protein glycosylation. The results published to date all point to the absolute necessity of mapping the acceptable

operating ranges (AORs) for each of these variables to ensure glycosylation consistency in a good manufacturing practice production environment.

Dissolved oxygen levels have a proven role in modulating glycoform profiles. In one study, it was reported that high dissolved oxygen levels were correlated with a higher NeuAc content on recombinant follicle stimulating hormone (FSH) [39]. In another study, low levels of DO caused little change in the glycosylation of tPA from CHO cells [40]; however, a change was observed in the case of the glycosylation of an IgG1 antibody in mouse hybridomas which demonstrated decreased galactosylation with decreases in DO levels [41]. The above results suggest that DO does play a role in glycosylation, but the effect is likely cell- and protein-specific, and relationships need to be investigated on a case-by-case basis.

Process temperature has also shown mixed results with respect to protein glycosylation. Shifts in temperature setpoints during production bioreactor cultures is a common practice for increasing final product titers. In numerous studies, temperature shifts have been shown to preserve product quality compared to non-shifted cultures. A fourfold increase in specific productivity was elicited by a temperature shift from 37 C to 33 C from CHO-derived EPO [42]. The sialic acid profiles from both process conditions were in fact comparable, with at most a few percent differences in composition. Reducing the culture temperature from 37 C to 33 C in a CHO cell line expressing human granulocyte macrophage colony stimulating factor saw little change in protein glycosylation and terminal sialylation, but did enhance specific productivity [43]. Interestingly, even the glycosidase enzymes between temperature-shifted and non-temperature-shifted cultures were reported to be similar. A temperature shift from 37 C to 34 C over an 8-day time period caused intracellular sialidase activity to spike immediately after inoculation for both cultures, followed by a decay over time that was roughly similar to that of a non-temperature-shifted culture [44]. Despite these results, other researchers have reported differences in the final glycoform profile on shifting temperature. In one investigation, EPO-Fc expressed by CHO cells facilitated a 20–40% decrease in sialylation with a shift to a lower temperature [45]. In yet another study, it was found that a temperature shift at a later stage of a CHO cell culture facilitated a 59% decrease in NeuGc levels compared to when the temperature shift occurred earlier [31]. These results suggest that the commonly used temperature shift strategy to enhance productivity is generally a safe practice from a protein glycosylation perspective, but glycoform profile changes have been reported in at least some instances.

pH is also an important process control variable with a proven role in controlling glycoform profiles. In one study, different pH levels were shown to affect the final glycoform profile of a monoclonal antibody in hybridoma cultures [46]. At lower pH levels (6.9 and 7.2), relatively higher levels of agalacto and monogalacto complex type N-glycans were measured, compared to higher pH levels (7.4) which facilitated the highest degree of galactosylation as well as the highest NeuAc/NeuGc ratio. Typically, base is added into a production bioreactor to control pH. In one study it was found that the base type can have an important role on the resulting protein glycosylation [31]. In CHO cell cultures expressing

glycoprotein B1 it was found that using sodium hydroxide as the base instead of sodium carbonate facilitated a 33% decrease in NeuGc levels.

The harvest criteria for a production bioprocess is frequently determined by cell culture viability, which is monitored throughout the duration of the culture. The fractional amount of non-viable cells is an important process variable because of the proven role of extracellular glycosidases which can accumulate in the media from lysed cells, and step-wise remove monosaccharides from the glycan. Prominent amongst these is sialidase, which has been shown to accumulate in cell culture media, and is active at neutral pH [47, 48]. Other glycosidases found to date include β -galactosidase, β -hexosaminidase, and fucosidase found in 293, NS0, and hybridoma cells. Interestingly, the sialidase activity found in cultures of these other cells is much lower than that found in CHO cell culture [49].

Metabolic waste products have been shown to affect the resulting protein glycosylation in mammalian cells. In particular, the effect of ammonia on the resulting glycosylation has been well documented. Ammonium chloride has been shown to prevent terminal glycosylation of immunoglobulins in plasma cells without affecting secretion [50]. Ammonia has also been reported to decrease terminal sialylation in both N- and O-glycans. For example, ammonium ion concentrations above 2 mM resulted in reduced $\alpha(2, 6)$ NeuAc attached to O-glycans on granulocyte colony stimulating factor (GCSF) produced by recombinant CHO cells [51], and excess ammonia supplemented to CHO cells expressing EPO also caused a decrease in terminal sialylation [52]. The extent of glycosylation has also been shown to decrease over time in batch culture [53], potentially due to the lack of sufficient nutrient availability in late-stage culture, or the accumulation of toxic metabolites such as ammonia. Indeed, this was proven by Borys et al. [54], where increased ammonium ions reduced the extent of recombinant placental lactogen N-glycosylation in CHO cells. The reason for these effects of ammonia is not completely known, but it has been observed that ammonium chloride causes an increase in intracellular pH [55].

3.3 Cell Culture Media

Cell culture “core” media components (glucose, trace metals, and amino acids) have been shown to modulate the resulting protein glycosylation profile. The glucose concentration in culture, in particular, has been shown to affect the glycosylation of monoclonal antibodies produced by human hybridomas in batch culture and of IFN- γ produced by CHO cells in continuous culture [56–58]. Studies have also shown mixed results with respect to low glucose levels and the resulting impact on glycosylation. There was reported to be little change observed with continuous cultures of BHK-21 cells producing a recombinant IgG-IL2 fusion protein, where nutrient limitations (glucose, glutamine) caused microheterogeneity to be largely unchanged [59]. However, in another report it was found that the fraction of fully processed N-glycans attached to CHO-expressed IFN- γ was higher

during culture periods of glucose excess [56]. A potential reason for these changes in protein glycosylation as a result of low nutrient levels may be explained through another study where glucose- and glutamine-limited CHO cell cultures led to a significantly lower intracellular pool of UDP-GlcNAc and UDP-GalNAc [60].

Metal ions have also been shown to have an important role towards the resulting protein glycoform profile. Many glycosylation enzymes are dependent on metal ions for activity. Manganese in particular has been shown to have a prominent role in increasing galactosylation [61]. CMP-sialic acid synthetase (the enzyme responsible for CMP-sialic acid biosynthesis) has been shown to be dependent on metal ions for activity, including manganese which facilitated optimal activity at neutral pH, and magnesium which facilitated optimal activity at pH 9.5. Interestingly, lower activity levels for this enzyme were demonstrated with the addition of copper, and no effect on activity levels were observed upon the addition of iron, zinc, and strontium, suggesting that the increase in activity is likely to be specific [62].

Amino acids have also been shown to affect the resulting protein glycosylation profile. One study showed how the supplementation of key amino acids (cysteine, isoleucine, leucine, tryptophan, valine, asparagines, aspartate, and glutamate) facilitated an increase in a lower sialylated fraction of recombinant EPO expressed in CHO [61]. Whether or not this was due to the resulting increase in productivity elicited as a result of the amino acid addition which exceeded the reactive capability of the glycosylation enzymes remains to be seen.

Commonly added media supplements have also been shown to modulate the resulting protein glycosylation. Although serum is used less nowadays in production of biologics, researchers have evaluated the effect of fetal calf serum on the oligosaccharide profile on IL-Mu6 glycoproteins secreted by BHK cells in both suspension and microcarrier cultures [63]. Decreases in sialylation levels in both cultures upon the incorporation of 2% serum were observed, as well as large changes in the relative amounts of complex type N-glycans. In another study, researchers supplemented CHO batch and fed-batch cultures expressing IFN- γ with Primatone RL tissue hydrolysate and found decreased sialylation at each of the two glycosylation sites [64]. There have been a few case studies that have explored the effect of sodium butyrate addition on the resulting glycosylation. In one study, CHO cells expressing tPA were cultured under conditions of decreased growth rate by supplementing with sodium butyrate and/or quinidine which resulted in a glycoform profile with increased site occupancy of Asn-184 [65]. In another report, it was found that the addition of sodium butyrate decreased the NeuGc levels on glycoprotein B1 expressed by CHO cells by 50–62% [31].

The addition of nucleotide-sugar precursors to cell culture media is an established method of modulating the final glycoform profile. In one published study, it was found that supplementing ManNAc into a CHO cell culture expressing IFN- γ resulted in an increase in sialylation on one N-glycan site associated with a 30-fold increase in the intracellular levels of CMP-sialic acid [66]. In another study, Baker et al. [30] supplemented glucosamine, uridine, and ManNAc to GS-CHO and GS-NS0 cells expressing tissue inhibitor of metalloproteinases 1 (TIMP-1).

The glucosamine- and uridine-supplemented cultures increased N-glycan antennarity in CHO, but not NS0. Sialylation also decreased in this case. In the case of ManNAc addition, there was no change in the resulting sialylation in either CHO or NS0 despite an increase in intracellular CMP-sialic acid levels. In another study, researchers investigated the effect of intracellular glycosylation on various feeding strategies of nucleotide-sugars in shake flask cultures of CHO cells expressing IFN- γ [67]. The addition of galactose $\pm$ uridine, glucosamine $\pm$ uridine, and ManNAc $\pm$ cytidine enabled a 12%, 28%, and 32% increase in sialylation, respectively, compared to untreated control cultures. These results were partially attributable to the $\sim$ 20-fold increase in UDP-hexose, 6–15-fold increase in UDP-HexNAc, and 30–120-fold increase in CMP-NeuAc observed between the above conditions. An overall conclusion made by the researchers was that the addition of nucleotide-sugar precursors to the cultures had a prolonged effect on the resulting increased levels of intracellular nucleotide-sugars with the resulting behavior observed up to 48 h post-supplementation.

The collective information of the effects of cell culture media components and supplements on the resulting protein glycosylation suggests that studies need to be conducted on an individual glycoprotein and process basis to ascertain any potential role in glycomodulation. This had led some to attempt to incorporate high-throughput screening technologies to study the effect of these cell culture conditions, as well as the incorporation of high-throughput glycan measurements via lectin arrays [68], and high-throughput HPLC [69].

3.4 Cell Line Engineering and Selection

Innate differences in glycoprotein processing inherent in the use of different mammalian cell lines is typically associated with a different repertoire of active glycosylation enzymes inside the ER and Golgi. In addition, these enzymes have overlapping specificities for multiple glycans as potential substrates. Relative gene expression amongst the glycosyltransferases and glycosidases are furthermore not similar for all mammalian cells. Thus, potential changes in the glycoform profile, either through biosynthesis or degradation, are expected, and are dependent upon the relative enzyme amounts directly or peripherally related to protein glycosylation. Researchers have attempted to utilize this by increasing or decreasing the gene expression profile of various genes through cell line engineering. To date there has been a considerable amount of work on the cell line engineering of individual protein glycosylation enzymes. The information gleaned from these studies suggests that glycosylation of proteins expressed in mammalian cell lines does typically respond to changes in gene expression of glycosylation enzymes and nucleotide-sugar transporters.

There have been many successful attempts at modulating the protein glycosylation profile through the genetic manipulation of individual protein glycosylation genes. These attempts have included the introduction of new active enzymes,

as well as the targeted increase and decrease in enzyme expression. Many research attempts have targeted maximizing NeuAc content, through either maximizing the addition onto N-glycans, or precluding its removal. A high NeuAc, low NeuGc content on glycoproteins is frequently targeted, since it has been found to promote a longer circulatory half-life [70]. In Weikert et al. [71], $\alpha(2, 3)$ SiaT was overexpressed in CHO cells expressing a TNFR-IgG fusion protein and tPA resulting in a significant increase in NeuAc content, and a significant decrease in N-glycan microheterogeneity. $\alpha(2, 6)$ SiaT is not typically expressed in hamster cells such as CHO and BHK; however, researchers have been successful in stably transfecting this enzyme into both cell types, with the EPO protein having $\alpha(2, 6)$ NeuAc content [72, 73]. In Ferrari et al. [74], researchers took the opposite approach and attempted to maximize sialic acid content by reducing its removal. The researchers used antisense RNA technology to significantly knock-down expression of sialidase in CHO cells expressing Dnase, which resulted in a higher sialic acid content since its removal was attenuated. Another strategy towards increasing NeuAc content is to increase the supply of CMP-NeuAc, the nucleotide-sugar donor for NeuAc addition onto N-glycans. In Wong et al. [75], researchers reported the transfection of a hamster CMP-NeuAc transporter. Transfection and overexpression of the transporter in a CHO cell line producing recombinant human IFN- γ resulted in single clones that had 2–20-fold increase in total CMP-NeuAc transporter expression at the transcript level and 1.8–2.8-fold increase in CMP-SiaT at the protein level when compared to untransfected CHO IFN- γ -expressing cells. The overexpression of this transporter facilitated a 4–16% increase in site sialylation of IFN- γ . As mentioned previously, NeuGc is the sialic acid type that is generally undesirable within the final glycoform profile. Cell line engineering studies have also targeted the enzyme responsible for its formation. In one report, CHO cells were transfected with antisense CMP-sialic acid hydroxylase, which resulted in an 80% reduction in hydroxylase activity [76]. The targeted maximization of NeuAc and minimization of NeuGc will likely be of continued interest in the years ahead.

Additional protein N- and O-glycosylation enzymes have been successfully overexpressed or knocked-down in mammalian cell lines. GnT III adds a bisecting GlcNAc on N-glycans (Fig. 2) in many mammals, but not CHO [33]. Umana et al. [77] successfully introduced this enzyme in CHO cells expressing a chimeric IgG₁ antibody. At various levels of expression, the researchers found that the enzyme could support a final glycoform profile with approximately 50% bisected, complex-type N-glycans. Additional studies have also shown the successful introduction of this gene into CHO cells expressing an IgG [78]. Other notable cell line glycoengineering studies include Mori et al. [79], who used siRNA against FucT VIII in CHO cells, which facilitated an increase in ADCC, and Potvin et al. [80], who transfected $\alpha(1, 3)$ FucT into CHO and found activation of the previously silent host enzyme which supplemented the activity of the newly transfected enzyme. The targeted cell line glycoengineering of mammalian cell lines has indeed had many success stories.

Mammalian cell lines with targeted enzyme knock-outs as well as mutations that allow for non-functional protein glycosylation genes have been reported in the literature. In particular, there have been numerous reports on the isolation of CHO mutants defective in various components of the protein glycosylation pathway whose characterization has significantly increased our knowledge of the protein glycosylation pathway in general, and in CHO cells in particular [25]. In Yamane-Ohnuki et al. [81], a FucT VIII CHO knockout was generated, resulting in a significant decrease in fucose content and a significantly higher ADCC. In another report, researchers reported a CHO mutant line, MAR-11, which was selected using lectin affinity for decreased levels of cell surface sialic acid relative to wild-type CHO cells [82]. These particular cells expressed a truncated version of the CMP-NeuAc transporter which facilitated recombinantly expressed IFN- γ devoid of sialic acid.

The introduction, suppression, or knockout of protein glycosylation genes is a proven method for protein glycosylation control in mammalian cells. The studies to date have shown an appreciable sensitivity of the final glycoform profile to the corresponding gene expression levels of the enzymes and nucleotide-sugar transporters involved. However, the genetic perturbation of these enzymes on an individual basis does underscore the need for better understanding of the regulation of the pathway, how it can be optimized further, and also to better answer the degree to which the pathway can be further controlled. Genomics studies on protein glycosylation have been attempted, and the information gleaned from these studies has begun to provide a more comprehensive perspective of the pathway, its regulation, and the cellular response to different culture environments.

4 Contemporary Prospects for Understanding and Enhancing Protein Glycosylation Control

4.1 Genomics

One central theme throughout the established methods for protein glycosylation control is that these control points often have mixed results, and that a case-by-case study is generally required for understanding glycosylation control. In addition, most of these studies are univariate in nature, in that they have targeted one process variable, media component, or protein glycosylation enzyme at a time. Through the advent of genomics and large-scale gene expression analysis tools, researchers have been able to take a more multivariate approach to the understanding of the protein glycosylation pathway, and its relationship to other biological pathways. These results highlight some interesting conclusions that have in fact advanced our understanding of the protein glycosylation pathway, its relationship to other metabolic pathways, and its relative sensitivity to the systems investigated. The information generated through these case studies has begun to

permit the transition of the current status of protein glycosylation control from a purely heuristic exercise to that of better process and cellular understanding.

The effect of elevated metabolic waste products on the resulting protein glycosylation gene expression has been evaluated. In Chen and Harcum [83], the effect of elevated ammonia was evaluated via quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) on 12 genes in CHO cells. In general, the authors found that numerous cytosol- and ER-localized genes associated with early glycosylation steps were insensitive to ammonia levels, in contrast to the later glycan processing steps in the Golgi which were sensitive. Specifically, the authors found that $\alpha(1, 3)$ FucT and sialidase did not change over time with elevated ammonium levels. In contrast, the UDP-Gal transporter, $\beta(1,4)$ GalT, and $\alpha(2, 3)$ SiaT genes were found to have lower expression over time in those cultures with elevated ammonium levels. CMP-NeuAc transporter and UDP-glucose pyrophosphorylase were found to be sensitive towards ammonium levels, but not culture time. Only one gene, the UDP-Gal transporter, was found to be expressed at a higher level in the higher ammonium cell culture condition. Since the gene expression profile for $\alpha(2, 3)$ SiaT was significantly lower in the elevated ammonium-treated cultures, and the CMP-NeuAc transporter profile was modestly lower, the authors concluded that this effect on gene expression elicited by elevated ammonium is at least partially responsible for the commonly observed decrease in NeuAc content under elevated ammonium. Sialidase levels were not significantly different regardless of ammonium levels in the culture media, suggesting that NeuAc addition was attenuated, and there was no elevated NeuAc removal from the tPA glycoprotein analyzed. This case study is a fine example of how glycosylation gene expression information complements the understanding of protein glycosylation control.

The effect of cell culture media components and supplement levels has also been evaluated on glycosylation gene expression. As mentioned previously, sodium butyrate is a commonly added supplement to target increases in product titer levels, as it is a known modulator of chromatin structure and has been extensively studied for its role in increasing cellular specific productivity [84, 85]. In an interesting study, researchers added sodium butyrate to both hybridoma and CHO cell cultures and measured the gene expression relative to control cultures via mouse and CHO cDNA microarrays [86]. The addition of sodium butyrate was shown to cause differential expression of a modest number of protein glycosylation genes in both the hybridoma and CHO cell cultures. These results suggest that commonly used cell culture media supplements can have a significant role in modulating the protein glycosylation gene expression profile. Although differential expression was observed, it is uncertain whether the glycoform profiles from these cultures changed. However, it was shown in other reports that the addition of sodium butyrate does not appreciably change the glycoform profile of expressed proteins [87].

In another study highlighting the effect of culture media conditions on gene expression levels, Korke et al. [88] cultured hybridoma cells in continuous bioreactor cultures in the presence of controlled low glucose levels which facilitated

low lactate production. Using a mouse cDNA and oligonucleotide microarray, the researchers evaluated the gene expression profiles at different metabolic states. In particular, they reported no differentially expressed protein glycosylation genes in the low-lactate-producing cells. In another study, researchers cultured primary fibroblasts with tunicamycin as well as glucose-limited cultures and measured the resulting relative gene expression [89]. The authors found a much higher fold change in expression (>2 -fold) with cells cultured with tunicamycin compared to glucose-limited cultures. Hence, in these two particular cases it appears that low nutrient levels do not cause a significant change in protein glycosylation gene expression. The effect of cell culture media conditions on the resulting protein glycosylation gene expression profiles will likely be of continued interest in the years ahead.

The supplementation of nucleotide-sugar precursors in cell culture media is an established method for protein glycosylation control. Researchers have utilized a microarray with glycosylation-pathway-specific genes and qRT-PCR to analyze 79 protein glycosylation-related genes to study in-depth the effect on intracellular glycosylation of feeding various nucleotide-sugars in shake flask cultures of CHO cells expressing IFN- γ [67]. This analysis enabled both an intracellular and extracellular perspective on protein glycosylation control. Gene expression results indicated a significant upregulation of genes involved in CMP-NeuAc biosynthesis for the cultures supplemented with galactose and uridine. In each of the conditions evaluated, however, there was no significant change in the expression of the nucleotide-sugar transporters. Similar to previously discussed studies, genes involved in early glycan processing were not significantly changed in the various cultures; however, those involved in glycan branching and terminal processing (galactosylation and sialylation) were. GnT II and GnT IV were upregulated for those cultures with glucosamine $\pm$ uridine and N-acetylmannosamine $\pm$ cytidine. Lysosomal sialidase and cytosolic sialidase were upregulated for the glucosamine $\pm$ uridine and N-acetylmannosamine $\pm$ cytidine supplemented cultures. Another interesting result from this study was that the addition of the nucleotide-sugar precursors had a consistent response in the downregulation of protein-folding genes, including chaperones (BiP), as well as calnexin, calreticulin, and protein disulfide isomerase. Hence, the relationship between protein glycosylation and the other protein-processing steps in the secretory pathway is indeed a connected one.

In an investigation evaluating the effect of bioreactor culture time on the resulting protein glycosylation, researchers targeted 24 N-glycosylation genes for expression analysis through qRT-PCR during the exponential, stationary, and death phases of a fed-batch culture of CHO cells expressing IFN- γ [90]. The genes investigated included those involved in N-glycan chain extension, glycan branching, terminal sialylation, nucleotide-sugar synthesis and transport, and N-glycan degradation. The researchers found that 21 of the 24 genes evaluated were either up- or downregulated throughout the time course of the experiment. It was observed that as the culture progressed there was a decreased expression of CMP-NeuAc biosynthesis genes (UDP-GlcNAc-2-epimerase), as well as increased expression of sialidases which correlated to the decrease in sialic acid measured on

the secreted product ($\sim 13\%$ increase in asialo glycan structures). Sialyltransferase gene expression increased in expression throughout the culture, indicating that abrogated biosynthesis of CMP-sialic acid, as well as the increase in sialic acid removal, were more likely the reason for the decrease in sialic acid attached to the protein. Nucleotide-sugar transporter gene expression did not significantly change over time. Biantennary glycans were found to increase over time at the expense of tri- and tetra-antennary glycans, which correlated to the relatively higher GnT II expression measured. The authors further analyzed the gene expression of low-glutamine controlled CHO fed-batch bioreactor cultures and found that GnT V was upregulated and GnT IV was downregulated, which was consistent with the N-glycan measurements which reflected a decrease in tetra-antennary glycans, and an increase in tri-antennary glycans. At low glutamine levels there was also a reduction in sialylation levels, which was consistent with the measured decrease in expression of UDP-GlcNAc-2 epimerase (a ManNAc biosynthesis gene required for CMP-sialic acid biosynthesis), as well as $\alpha(2, 3)$ SiaT genes. The above results highlight the more in-depth understanding afforded through the use of microarrays and gene expression applications in general, since the relative expression results and the resulting glycosylation results were in alignment in this particular study.

Maintaining high cell-specific productivity conditions is certainly the goal of any production bioreactor. A supporting goal, however, is to ensure that this does not come at the expense of product quality. A case study on the effect of 11 different GS-NS0 cell clones operating at different IgG productivity levels on the resulting differential gene expression has been recently described [91]. In this study, differential expression was evaluated using an oligonucleotide mouse microarray. The extensive statistical analysis revealed significant differential expression of numerous genes involved with post-translational protein processing as a direct result of the different productivity levels. It would be interesting to see what if any glycoform profile differences were observed amongst the cell lines investigated. Whether the glycosylation machinery compensates for increases in specific productivity with concomitant increases in expression, or alternatively the fraction of non-fully processed N- and O-glycans increases as a result of expression and/or enzyme activity not compensating fast enough, remains an open question. However, reports have shown both direct and inverse relationships between protein synthesis rates and the resulting protein glycosylation. In one study, lowering the protein synthesis rate by cycloheximide improved the glycan site occupancy of recombinant prolactin from C127 cells [92]. Similarly, decreasing culture temperature to 21 C in HL-60 cells to decrease secretory pathway activity resulted in a 30–50% increase in the presence of N-acetylglucosamine repeats [93]. However studies on tPA synthesis in CHO cells suggest that the protein synthesis rate has little effect on protein glycosylation [94]. In a study using BHK and CHO cells producing glycoproteins, similar glycan profiles were observed even after a 200-fold increase in productivity [95].

The glycosylation gene expression results from cell culture studies to date suggest that the terminal glycan-processing enzymes are more sensitive to culture environment changes compared to earlier glycan-processing enzymes.

Comelli et al. [96] probed the relative gene expression profile of glycosylation-related genes using mammalian tissues and have confirmed this. The researchers developed an oligonucleotide microarray with 436 genes to focus on glycosylation-related genes, including human and murine glycosyltransferases, nucleotide sugar transporters, nucleotide synthesis, glycosidases, proteoglycans, and glycan-binding proteins. The relative expression profiles of nine murine tissues were investigated. The biosynthetic enzymes responsible for the core regions of N- and O-linked oligosaccharides were ubiquitously expressed in the tissues evaluated; however, the enzymes for the addition of terminal sugars were expressed in a more tissue-specific manner. These enzymes included families of sialyltransferases and fucosyltransferases. These gene expression results show that most of the diversity at the gene expression level is more typically associated with the terminal sugar additions, that is, those that are localized in the Golgi, rather than at the initial stages of protein glycosylation in the ER.

How a mammalian cell line responds to changes in glycosylation or protein production rates is not fully understood. However, in yeast the cellular response has been shown to involve internal cellular signaling networks that may provide clues for mammalian cells. Several genomics studies have been reported for the characterization of the cellular response to abrogation of glycosylation reactions [89, 97] or analyzing mutants partially defective for N-linked glycosylation [98]. In the latter case, a good example can be seen in Cullen et al. [99]. In this study the researchers used a yeast glycosylation mutant with a partial loss of function of PMI40, an enzyme required for conversion of fructose-6-phosphate to mannose 6-phosphate (an early step in protein glycosylation). The major class of differentially expressed genes could be considered part of the starvation response. These included genes involved in the tricarboxylic acid and glyoxylate cycles, and protein and amino acid biosynthesis. Expression profiling generated from this study demonstrated that genes encoding structural proteins of two mitogen-activated (MAP) kinase pathways, and most of the genes that play a role in the unfolded protein response (UPR), were also induced in response to this glycosylation defect. Whether such a coordinated cellular response occurs in mammalian cell lines is not clear at this time. Also not clear is what occurs when the glycosylation rate is enhanced, rather than attenuated as the above references all documented.

In another study with yeast, researchers studied the unfolded protein response (UPR), which was found to have a direct interaction with the protein glycosylation pathway [97]. The UPR is activated from the accumulation of unfolded proteins in the ER, and the UPR facilitates changes which allows the cell to alleviate the stress. The authors found multiple targets in protein glycosylation that changed as a result. These included 17 genes in the ER and six genes in the Golgi, in addition to numerous other genes involved in protein transport. The genes upregulated as a response by the UPR included those involved in core oligosaccharide synthesis, glycosyltransferases, glycosylphosphatidylinositol anchoring, and O-linked glycosylation, as well as vesicle trafficking in both anterograde and retrograde fashion. It is uncertain whether a similar response in mammalian cells

would exhibit these changes on the gene expression level as a result of a corresponding change in the UPR. But it does show that the glycosylation pathway is readily changed at the gene expression level as a result of other glycoprotein-processing steps in secretory pathway organelles.

The protein glycosylation pathway has a proven role in interacting with numerous other metabolic pathways. In a case study using yeast, Klebl et al. [98] studied a mutant deficient in the synthesis of the lipid-linked oligosaccharide precursor (the initiation step for protein glycosylation). Their results indicated that this deficiency and the resulting perturbation of the N-glycosylation network caused a dramatic reprogramming of various signaling and metabolic networks. Some of these included genes involved in the mitogen-activated protein kinase (MAPK) cascades, as well as phosphate, amino acid, carbohydrate, mitochondrial, and ATP metabolism. This genomics study helped elucidate the protein targets with which glycosylation interacts in peripheral networks, as well as revealing the metabolic and cellular signaling and regulatory elements with which the pathway has an interaction. More such studies are needed in mammalian cell lines to understand better the intricate relationship between the protein glycosylation pathway and other closely related pathways.

4.2 Systems Biology

The complex relationship between cell, process, and metabolic pathway parameters shaping the final glycoform profile suggests that a more systems-level perspective is required to achieve a better understanding of glycosylation control. As discussed earlier, the protein glycosylation pathways are highly complex, with multiple routes possible towards the generation of each of the measured product glycans. With additional glycan site-occupancy variability on the protein, and an intricate relationship with nucleotide-sugar biosynthesis pathways, one can certainly appreciate the complexity of the system. The particular pathway(s) traversed in generating a given glycoform profile is dependent on the relative concentrations of the enzymes involved, the glycan accessibility on the protein to the various enzymes, the spatial localization of these enzymes across the ER and Golgi compartments, the relative nucleotide-sugar concentrations, the substrate specificity for each of the reactant glycans and their subsequent glycan products, as well as the many other factors that have been shown to have some interaction with the protein N- and O-glycosylation pathways. The above sections highlight some of the studies that have identified the cell culture process variables which modulate protein glycosylation, as well as the attempts to control the pathway into a more idealized state.

The interpretation of how these parameters help modulate micro- and macro-heterogeneity is difficult and indirect. Understanding how they interact with the variables that have a direct role on the pathway is especially important. In addition, for many of these successful attempts at modulating the glycosylation pathway, there

are typically similar studies that have shown little to no effect on the resulting glycosylation. As a result of the very interactive and case-by-case nature of these variables, the non-linearity in the response and the complexity of the pathway itself has led some to incorporate a more mathematical interpretation of the protein glycosylation pathway and its relationship to the final glycoform profile. Attempts have been made towards the systematic understanding of protein N- and O-glycosylation with systems biology approaches and metabolic modeling. Numerous systems biology approaches have been attempted to understand the protein N- and O-glycosylation pathways. Though not typically viewed as such, protein glycosylation has begun to be looked at from a more network-based perspective (Fig. 2a, b) that has typically been associated with other models of metabolism such as central energy metabolism [100]. The information gleaned from these case studies has reinforced our awareness of the pathway complexity, and has helped increase our understanding of glycoform profile sensitivity to the various controlling parameters.

Some of the mathematical and statistical tools that are commonly used in systems biology include metabolic kinetic models, sensitivity analysis, numerical optimization, and principal component analysis. Sensitivity analysis evaluates the measured or calculated response of a particular parameter as a result of a change in an interacting variable. This change can be large or small, though small changes are typically targeted. Numerical optimization attempts to determine the set of target conditions that best allow for convergence of the response to a specified criteria. In this analysis, an objective function of the various control variables is defined in which the optimization routine attempts to find a local minimum or maximum. Also required is an initial first guess at a particular set of target conditions, since multiple convergent values are possible. Principal component analysis (PCA) provides a quantitative measure of determining the relative importance of control parameters towards a particular measured response. In this procedure, the factors important for a response are correlated into a smaller number of uncorrelated variables that account for the variability in the response data. These mathematical and statistical tools have been used for the analysis of metabolic pathways including the protein N- and O-glycosylation pathways.

In an early systems biology approach to protein glycosylation, Umana and Bailey [101] developed a metabolic model of N-glycan microheterogeneity for 33 different N-glycans, including high-mannose, hybrid, and complex-type oligosaccharides. Using kinetic parameters obtained from the literature, the authors were able to simulate the effect of perturbing the various model parameters on the resulting glycoform profile. Included in this model were four discretely localized Golgi compartments, differential glycosylation enzyme localization across these four compartments, and intra-Golgi transport. The model was essentially a reaction-transport system, where the ten Golgi protein glycosylation enzymes must react upon the substrate glycan(s) to form the subsequent product glycan(s) before transport to the next compartment occurs. Simulating the effects of increasing specific productivity, the model predicted decreases in tri-antennary glycans, and at sufficiently high levels decreases in bi-antennary glycans and increases in non-fully processed high-mannose-type glycans. The reason for this is intuitive in that

without concomitant increases in the levels of these enzymes to compensate for the increase in protein traversing the system, a decrease in the degree of glycan processing is to be expected. The researchers also simulated the effect of over-expressing the GnT III enzyme, which due to its overlap in glycan substrate specificity led to the preferential channeling of glycan fluxes towards bisected-hybrid-type glycans and away from bisected-complex-type glycans. The formation of bisected-complex glycans could be rescued through the mislocalization of GnT III, or overexpression of GnT II and Man II so as to change the sequence of enzymatic reactions to ensure the complex-type glycans are not restricted from being formed. The model developed by the researchers provided an early glimpse of the detailed glycosylation pathway understanding that could be achieved through the use of systems biology.

Later work expanded this model by incorporating additional reactions involving galactosylation, fucosylation, sialylation, and the incorporation of N-acetylglucosamine repeats (see Fig. 1b), as well as the consideration of nucleotide-sugars in the Golgi compartments [102]. Additional N-glycan substrate specificities were included in this model. The final system considered 7,565 N-glycans involved in 22,871 enzymatic reactions. The researchers successfully applied nonlinear optimization in their model for the estimation of enzyme concentrations requisite for the matching of the predicted N-glycan profile with that of published data from recombinant human TPO [103]. Simulating the effect of increasing specific productivity, the researchers saw a steady decline in the degree of sialylation. The researchers later applied their model to the prediction of enzyme concentrations and UDP-GlcNAc levels needed for returning the predicted N-glycan profile for a four-fold increase in specific productivity back to the glycoform profile observed with control levels of specific productivity. Information gleaned from use of the model supports its utility as a guidance tool for better understanding of glycosylation, as well as an experimental guide for re-directing intracellular glycan fluxes.

Hossler et al. [104] studied the protein N-glycosylation pathway in the Golgi, and constructed two different metabolic models to describe the potential biosynthesis of 341 N-glycans. One model described the Golgi maturation view of intra-Golgi transport, and the other described the vesicular transport view. Which of these two views is the most valid has been debated in the literature, with studies providing evidence for both [105,106]. In the Golgi maturation model, the Golgi cisternae have been described as relatively mobile and mature as they traverse geographically within the cell along with their protein contents. In the vesicular transport model, the cisternae are conceived as being relatively stationary, while Golgi vesicles transport the protein contents in both directions through anterograde and retrograde transport. Mathematically, these two systems were analyzed using a system of 341 nonlinear ordinary differential equations which were solved simultaneously for each of the reactant and product glycans. Reported glycan substrate specificities for each of the ten enzymes considered were incorporated into the respective models, as were the additional variables of preferential enzyme localization across the Golgi cisternae [107], nucleotide-sugar levels, and

intra-Golgi transport. The metabolic fluxes through the pathway and the glycan distribution were visualized directly on the pathway itself through the GlycoVis visualization program [108], previously written for the purposes of analyzing glycan biosynthetic fluxes, as well as deducing preferential glycan biosynthetic pathway specificities (Fig. 3).

Simulations using the Golgi maturation model revealed that the resulting terminally processed glycan profile was particularly sensitive to a few key enzymes, especially GalT which, due to the addition of Gal at particular key branching steps, could have potentially dramatic effects on the resulting N-glycan profile. The use of nonlinear optimization revealed that, although many terminally processed glycans could be predicted to be formed in significantly high abundance, not all of them could do so. That is, glycan uniformity could be achieved for some glycans, but not all, due to the nature of the metabolic pathway and the location of key branching points in the network. Controlling the order of monosaccharide addition is thus an important variable in determining the resulting glycoform profile. In addition, it was also found that, on incorporating a multi-compartmental system in this Golgi maturation model, the appearance of early and intermediately processed N-glycans was observed in the final glycoform profile. These results agree well with reports from the literature where the appearance of high-mannose-type glycans is frequently reported [109].

Simulations using the vesicular transport model revealed a wider spectrum of intermediately processed N-glycans, rather than an over-representation of a few glycans in abundance. This suggested to the authors that a purely vesicular transport system as simulated using the chosen model parameters cannot completely predict experimentally measured glycan profiles without the incorporation of additional model variables, including perhaps imperfect mixing of the enzymes in each of the Golgi compartments, or selective transport perhaps through some form of sorting mechanism. The results also pointed to the Golgi maturation model as having a higher likelihood for modeling intra-Golgi transport, or perhaps a hybrid mechanism involving both maturation and vesicular transport, a mechanism whose possibility has been discussed elsewhere [110]. However, both models did highlight a shared importance of the particular order of monosaccharide addition in determining the final N-glycan profile. Using the compartmentalized Golgi maturation model, glycan uniformity could be generated for each of the terminally processed N-glycans (i.e., those not capable of reacting further). This could only be achieved by mislocalizing the glycosylation enzymes so as to ensure the correct sequence of processing occurred, to prevent enzymatic glycan substrate specificities from channeling glycan fluxes away from the desired product N-glycans. Glycosylation enzyme mislocalization has been documented in the literature, suggesting that this glycoengineering strategy is a viable means of glycomodulation [111].

In another systems biology approach to the study of microheterogeneity, researchers derived an equation for the analysis of the degree of sialylation on a generalized glycoprotein [112]. Assuming Michaelis–Menten-type kinetics for a sialyltransferase enzyme with glycan substrate, the authors found that at

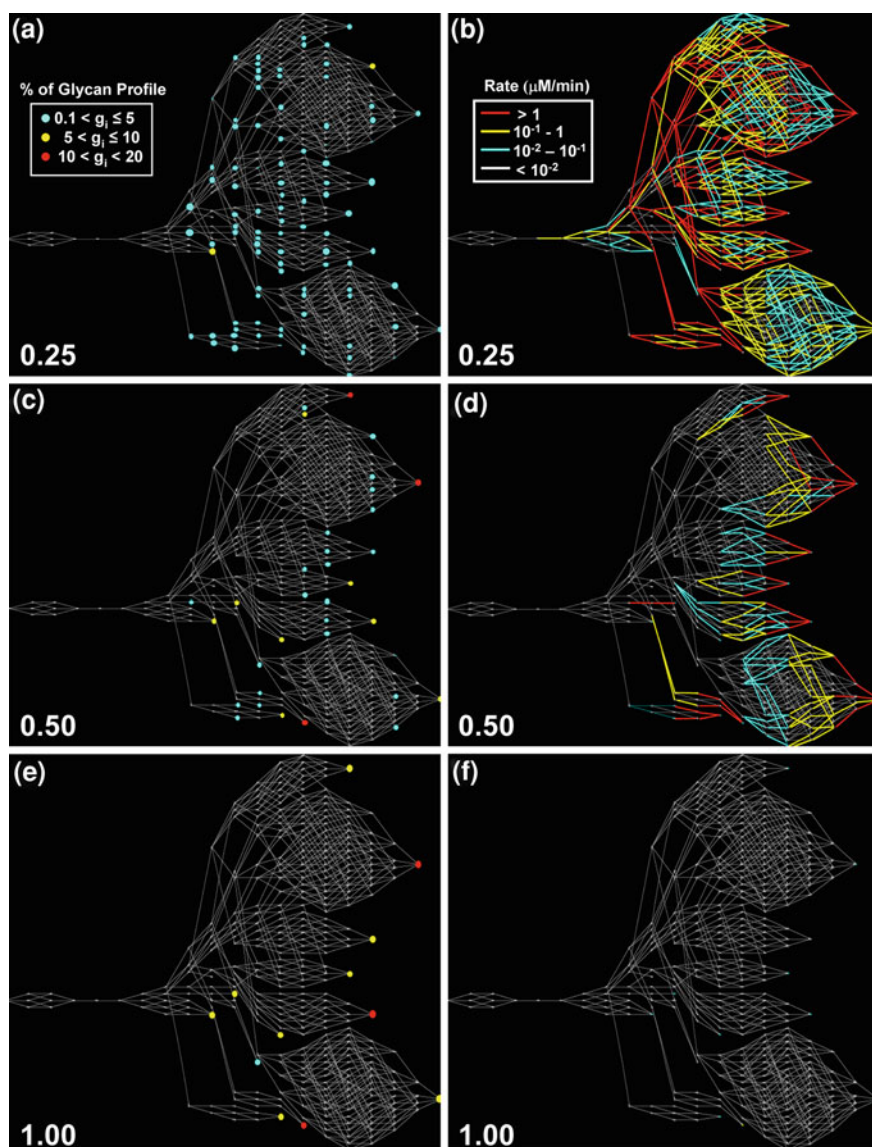


Fig. 3 *In silico* mapping of the glycoform profile (a, c, e) and glycan reaction rates (b, d, f) through the protein N-glycosylation pathway in a simulated Golgi maturation model. Numbers reflect normalized distance across a Golgi organelle at 25% (a, b), 50% (c, d), and 100% (e, f) traversal. [reprinted from Hossler et al. [104], originally published under the license terms of The Public Library of Science (PLOS) and the Creative Commons Attribution License (CCAL)]

sufficiently high concentrations of glycans, the amount of sialylation was dependent on the enzyme concentration, enzymatic kinetic parameters, and the protein residence time within the reaction compartment. However, under non-saturation

conditions of glycan substrate, the degree of sialylation was actually independent of the amount of protein in the trans-Golgi network (TGN), and the reactant glycans attached to it. Although this study concentrated on sialylation alone, these mathematical results do point out to us a potential reason for the strong dependence of the final glycoform profile on the many cell culture parameters reported in the aforementioned sections. In a typical production bioprocess using a relatively high-expressing cell line, the recombinant protein is either likely to be transported through the secretory pathway relatively fast, the amount of glycoprotein being transported is relatively high, or a combination of both. In each of these scenarios it is expected that the cumulative glycan throughput is in an elevated state, in which case the strong dependence on proven pathway control parameters, such as relative enzyme amounts, is consistent with this study.

Macroheterogeneity of N-glycans has also been modeled using a systems approach [113]. In this particular model the initiation of protein glycosylation in the ER in the timeframe and space immediately after translation in the ER was analyzed. The mathematical framework established provides some interesting insights into the origins of glycan site-occupancy and macroheterogeneity. The central hypothesis of this work is that the initial glycosylation event catalyzed by the oligosaccharyltransferase (OST) enzyme takes place in a very defined region in space in the ER lumen, which also competes with other processing events, including protein folding. The translocation into the ER lumen proceeds at the same rate as protein translation, which is modeled as a linear velocity parameter. By incorporating a mass balance, the authors were able to derive various dimensionless groups which describe their system as a function of the glycosylation event, as well as the effects of incorporating protein folding. The main conclusion from this work was that due to the competing nature of the first initial glycosylation event, and the protein folding that occurs in the ER of mammalian cells, there is an optimal mRNA elongation rate one could expect to observe for achieving maximum site occupancy. Though no study testing this experimentally could be found in the literature, the modeling results do suggest that specific productivity can indeed have a role in helping determine the glycan site occupancy.

The O-glycan biosynthetic pathway has also been examined through a systems-level approach. In Liu et al. [114], researchers analyzed the formation of sialyl Lewis-X (sLe^x) structures (see Fig. 1e) on P-selectin glycoprotein ligand-1 (PSGL-1), which has 71 potential O-glycan sites. Though not typically seen on glycoproteins constitutively secreted from mammalian cells, this glycan attached to the cell surface of leukocytes does have prominent biological roles, and the approach and results from this study are nonetheless instructive in the analysis of O-glycan pathways in general. The researchers' goal was to combine various modeling and analysis methods for the *in silico* determination of enzymatic reaction rate constants which could be compared to experimental measurements for comparison purposes. Lysates from human leukocyte (HL-60) cells were used as the source material for the experimentally measured rate constants. Five glycosyltransferases were included in their model, as well as the known product glycans that had been reported elsewhere to generate a model of the diverse

pathways potentially traversed from an initial reactant glycan to give rise to the spectrum of product glycans. Subset pathways were subsequently focused on, due to their more prominent role connecting the substrate and known product glycans. Focusing on these subset pathways, the researchers applied a nonlinear optimization approach for the determination of the rate constants which facilitated the optimal agreement between the predicted versus experimental O-glycan profile. The various subset pathways which facilitated close agreement to the final profile were analyzed using hierarchical clustering followed by principal component analysis (PCA) to assign relative importances to the individual network reactions. Assessing the relative importance of each of the individual glycosylation reactions facilitated the determination of the most likely pathway traversed for the two sialyl Lewis-X structures of interest. Sensitivity analysis was also used to find that final sLe^x levels had a direct relationship with the expression of one particular sialyltransferase (α (2,3)ST3Gal-IV), and an inverse relationship with that of another (α (2,3)ST3Gal-I/II). Through this very detailed and systematic approach to O-glycan biosynthesis, the authors were able to conclude that the final O-glycan profile is determined principally through individual enzyme activity, as well as competition of the enzymes for the O-glycan substrates.

In a complementary case study, researchers combined gene expression measurements with those of glycosyltransferase activity in an attempt to predict the resulting O-glycoform of PSGL-1 expressed by HL-60 cells and neutrophils on their cell surface [115]. Although distinct differences were observed by the researchers in the enzyme activity between neutrophils and HL-60 cells, overall there was a relatively close agreement between measured enzyme activities and the corresponding enzyme gene expression (although the agreement was not strictly linear). Differences observed between activity and expression results were hypothesized to be due to gene expression changes manifesting rather quickly compared to the intracellular enzyme levels, which have a more pronounced turnover time. Whether or not protein glycosylation enzymes in mammalian cells have slow intracellular turnover times in general remains to be seen, however. The authors further found that enzyme activity measurements were more directly correlated with the resulting O-glycan profile than with gene expression measurements. For more accurate prediction of the final O-glycan profile utilizing gene expression data, additional information including preferential enzyme localization in the Golgi and additional glycan substrate specificities would need to be considered. This conclusion certainly points to the validity and utility of adopting systems-level perspectives in predicting final glycoform profiles.

5 Discussion of Protein Glycosylation Control

Protein glycosylation is a fascinating pathway comprising both divergent and convergent pathways characterized by a relatively small number of enzymes, but with a potentially larger number of substrate and product glycans. The fact that not

all these glycans are measured in a typical cell culture process suggests that a great deal of control is manifested. There have been various reports in the literature of researchers utilizing these controlling mechanisms for glycomodulation, either directly through cell line engineering, or indirectly by controlling cell culture process and media conditions. Many cell culture process and media conditions have shown mixed results on glycosylation, with specific results sometimes being observed, and sometimes not. These results suggest that understanding the effect of cell culture process and media conditions requires a case-by-case analysis. Attempts to control protein glycosylation through cell line engineering have also had many successes. The N- and O-glycoform profiles from CHO cells appear to be particularly responsive to these attempts. Many enzymes have been over-expressed, silenced, or knocked-out completely in CHO and other industrially relevant mammalian cell lines. The cumulative results of these studies indicates that in most cases the targeted genetic manipulation of these cells does indeed have the expected effect on the resulting N- and O-glycan profiles. Thus cell line engineering remains a very viable means of attempting glycomodulation in cell culture bioprocesses. It is likely that the effect of cell culture media components and process conditions on protein glycosylation, as well as cell line engineering, will be of interest to the biopharmaceutical industry for some time to come. Despite the limitations in understanding from a glycosylation pathway perspective, the reason for these potential changes has become more apparent.

The proven sensitivity of the protein glycosylation pathway to the various parameters mentioned in this review highlights the difficulty in controlling this pathway. The dependence of the pathway on cell culture process conditions, relative enzyme and nucleotide sugar expression levels, and the expression host is well-established. What is not understood as well in many cases, however, is the reason for these changes at the glycosylation pathway level. Through the use of tools such as genomics and systems biology, there is now even more appreciation for the complexity of protein glycosylation. Gene expression analysis and systems biology have provided additional information on the understanding of the pathway as a whole, and its relationship to other pathways, in particular protein secretion.

Gene expression results have shown that the pathway is particularly sensitive to the expression of the terminal processing enzymes, including galactosylation and sialylation, as well as the nucleotide-sugar transporters responsible for nucleotide-sugar import into the ER and Golgi organelles. Additional gene expression results have shown that the resulting measured glycoform profile does not always correspond exactly to the relative gene expression levels. This further suggests that the enzyme expression and relative gene expression amounts and activities in the ER and Golgi need to be investigated in parallel to provide a more comprehensive understanding.

Systems biology studies of N- and O-glycan micro- and macroheterogeneity have attempted to incorporate these various pathway considerations and have revealed much about the protein glycosylation pathway as a result. *In-silico* prediction of preferential pathway specificities needed for the prediction of glycoform profiles that match those of experimentally measured glycans has shown that there

is significant pathway redundancy even for the theoretical prediction of uniform protein glycosylation. Enzyme concentration levels are necessary but not sufficient for achieving uniform glycosylation for some terminally processed glycans. In these particular cases, controlling the order of monosaccharide addition onto an N- or O-glycan is required through the preferential localization of enzymes across the Golgi compartments. The application of mathematical and statistical tools, including optimization, PCA, and clustering have also permitted the theoretical prediction of pathway specificities for the formation of experimentally measured glycan profiles. Indeed, these systems-level approaches have allowed for the theoretical analysis of the pathway requirements for producing uniform and/or desired glycoform profiles. The use of these tools will continue to supplement and guide experimental efforts at glycomodulation in the future.

The aforementioned case studies of genomics and system biology on protein glycosylation in mammalian cells have certainly increased our awareness of the complexity of the protein glycosylation pathway. We do not yet have the capability to fine-tune at will this pathway in mammalian cells. With time and continued incorporation of these relatively newer tools in mammalian cell culture, that capability, as well as a clearer picture of protein glycosylation control, should continue to emerge. Indeed these approaches will continue to contribute towards our ultimate goal of producing optimal glycoproteins on a consistent basis in mammalian cell culture.

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