

Chapter 2

Glycoengineering of CHO Cells to Improve Product Quality

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Abstract

Chinese hamster ovary (CHO) cells represent the predominant platform in biopharmaceutical industry for the production of recombinant biotherapeutic proteins, especially glycoproteins. These glycoproteins include oligosaccharide or glycan attachments that represent one of the principal components dictating product quality. Especially important are the N-glycan attachments present on many recombinant glycoproteins of commercial interest. Furthermore, altering the glycan composition can be used to modulate the production quality of a recombinant biotherapeutic from CHO and other mammalian hosts. This review first describes the glycosylation network in mammalian cells and compares the glycosylation patterns between CHO and human cells. Next genetic strategies used in CHO cells to modulate the sialylation patterns through overexpression of sialyltransferases and other glycosyltransferases are summarized. In addition, other approaches to alter sialylation including manipulation of sialic acid biosynthetic pathways and inhibition of sialidases are described. Finally, this review also covers other strategies such as the glycosylation site insertion and manipulation of glycan heterogeneity to produce desired glycoforms for diverse biotechnology applications.

Key words Chinese hamster ovary (CHO), N-linked glycosylation, Glycoengineering, Sialylation, Glycosylation site insertion, Heterogeneity

1 Introduction

Therapeutic glycoproteins represent a rapidly growing segment of the biopharmaceutical industry with total sales of many tens of billion dollars annually [1]. These products include several protein classes such as enzymes, hormones, cytokines, growth factors, clotting factors, as well as monoclonal antibodies and Ig-Fc-Fusion proteins [2–4]. The increasing demand of biotherapeutics for the treatments of diseases, such as cancer, immune disorders, infectious diseases, genetic disorders, and ailments such as Alzheimer's and Parkinson's, are the main drivers for the development of glycoprotein therapeutics [1].

Glycosylation is a critical posttranslational modification found on most of these biotherapeutics. What is unique about glycosylation compared to other posttranslational processing events is the

structural variety and functional diversity present, in which the glycosylation can vary widely even for a single protein and also from organism to organism. Glycosylation characteristics can play a major role in modulating a protein's stability, folding, targeting/trafficking, immunogenicity, biological activity, and especially circulatory half-life [5]. Oligosaccharides are attached cotranslationally through glycosidic linkages on specific asparagine (N-linked) or serine/threonine (O-linked) residues. While N-glycans are the most common modification on biotherapeutics including monoclonal antibodies and will be the focus of the current review, several therapeutic glycoproteins such as erythropoietin (EPO) and etanercept (Enbrel) also include O-glycan modifications [6]. N-glycans are linked to the Asn of the Asn-X-Ser/Thr consensus sequence in which X denotes any amino acid except proline [7]. A consensus sequence for O-linked glycosylation has yet to be identified [5]. Given its non-template-driven nature, heterogeneity of glycosylation arises both from variations in glycosylation site occupancy and in the diversity of final glycan structures attached to glycoproteins emerging from the cellular secretory compartments. As a result of the stochastic nature of interactions between enzymes and oligosaccharide substrates and the variety of enzymes that can act on any one glycan substrate, a wide range of different glycans are generated for most proteins as these polypeptides traverse through the endoplasmic reticulum (ER) and various Golgi compartments [8, 9].

In particular, the N-linked glycosylation pathway in mammalian cells involves a highly complex and interconnected reaction network catalyzed by glycosidases and glycosyltransferases contained within different compartments of the ER and Golgi apparatus, depicted in the schematic of Fig. 1. The biosynthesis of mammalian N-glycans initiates at the cytoplasmic face of the ER membrane with the transfer of GlcNAc-P from UDP-GlcNAc to the dolichol phosphate (Dol-P) lipid carrier to generate dolichol pyrophosphate N-acetylglucosamine (Dol-P-P-GlcNAc) [10]. Then 14 sugars are sequentially added to Dol-P-P-GlcNAc to form an oligosaccharide precursor ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) [10]. Next, oligosaccharyltransferase (OST) selects Asn-X-Ser/Thr sequons in a nascent polypeptide and proceeds with an en bloc transfer of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ to the side chain amide of asparagine and releasing Dol-P-P in the process [11]. The glucose residues on the precursor are sequentially trimmed by ER α -glucosidase I and II to form monoglucosylated glycan, which is a key intermediate in the ER lectin chaperones calnexin/calreticulin-associated glycoprotein folding control cycle [12]. Once correctly folded, the precursor is trimmed by ER α -mannosidase I to yield $\text{Man}_8\text{GlcNAc}_2$ -protein before exiting ER. After translocation into the cis-Golgi, the $\text{Man}_8\text{GlcNAc}_2$ glycoform is further trimmed by Golgi α -mannosidases I to give $\text{Man}_5\text{GlcNAc}_2$, a key intermediate along the pathway to form hybrid and complex N-glycans and sometimes found as a final glycan product.

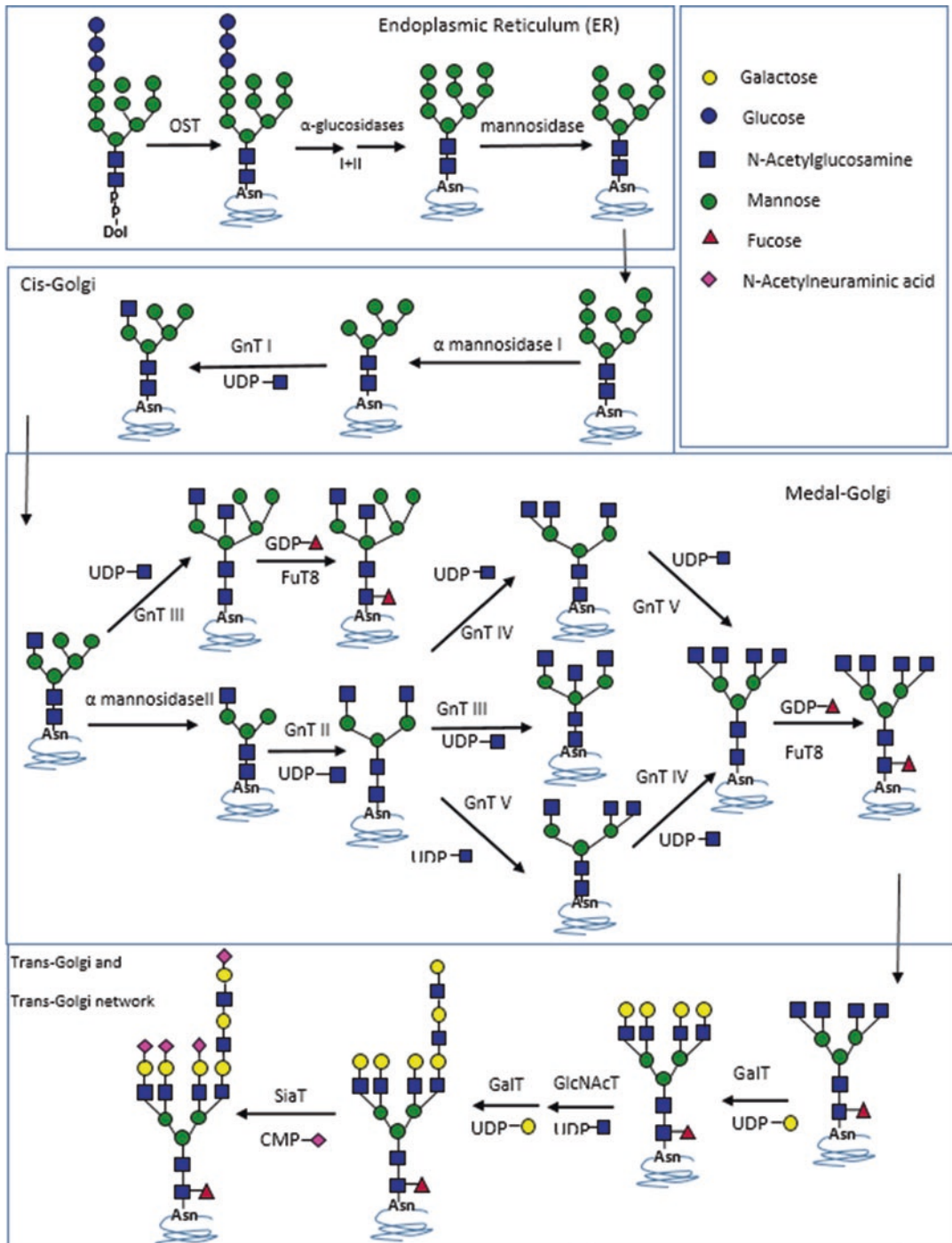


Fig. 1 Schematic of N-glycosylation biosynthesis pathway in CHO cell

As shown in Fig.1, biosynthesis of hybrid and complex N-glycans begins in the medial-Golgi by the action of an *N*-acetylglucosaminyltransferase (GnT-I or Mgat1), which adds a GlcNAc to $\text{Man}_5\text{GlcNAc}_2$ [10]. Then the majority of N-glycans are trimmed by Golgi α -mannosidase II removing two mannoses from $\text{GlcNAcMan}_5\text{GlcNAc}_2$ to yield $\text{GlcNAcMan}_3\text{GlcNAc}_2$. Hybrid N-glycans result when a structure such as $\text{GlcNAcMan}_3\text{GlcNAc}_2$ either undergoes no further extension or trimming to remove exposed mannose residues resulting in structures with one or two terminal Man residue. In addition, sometimes another GlcNAc can be added to the innermost Man group by the enzyme β 1,4-*N*-acetylglucosaminyltransferase III (GnT-III or Mgat3) in the medial Golgi, resulting in bisecting GlcNAc structures that can also alter the capacity for other downstream enzymes to act on the glycan structure.

Next, the enzyme β -1,2-*N*-acetylglucosaminyltransferase II (GnT-II or Mgat2) adds a GlcNAc to the $\text{GlcNAcMan}_3\text{GlcNAc}_2$ structure to generate the glycan product $\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$, which is the precursor for all multiantennary complex N-glycans. Tri- and tetra-antennary branches can be achieved by adding GlcNAc at α (1,3)-mannose site by *N*-acetylglucosaminyltransferase IV (GnT-IV or Mgat4) and at α (1,6)-mannose site by *N*-acetylglucosaminyltransferase V (GnT-V or Mgat 5). Additional modifications of complex and hybrid N-glycans can occur in the trans-Golgi and include the addition of core α (1,6)-fucose to the GlcNAc adjacent to Asn at the N-glycan sites by α -(1,6)-fucosyltransferase and the branch elongation by the addition of a β -linked galactose residue to GlcNAc by galactosyltransferase to produce $\text{Gal}\beta$ 1-4GlcNAc, or poly-acetyl-lactosamine (poly-LacNAc) sequences. Finally, these terminal Gal residues can serve as acceptors for several sialyltransferases, leading to even more complex structures [10].

Chinese hamster ovary (CHO) cells are widely used for production of many commercial and clinical biopharmaceuticals due to their capacity to produce glycoforms that are, with exceptions, accepted by the human immune system [2, 13]. Alternative mammalian cell lines also used in the production of biopharmaceuticals include baby hamster kidney (BHK21), murine myeloma and hybridoma cell lines (NS0 and Sp2/0), and, to a lesser extent, human host cell lines such as human embryonic kidney (HEK293) and human retinal cells (PER. C6) [2, 3, 14].

Two nonhuman glycans—terminal $\text{Gal}\alpha$ 1,3-Gal linkages (α -Gal) and N-glycolylneuraminic acid (Neu5Gc) residues—exist in nonhuman mammalian cells and could elicit adverse immunogenic reactions in humans [2, 15]. Mouse cells have an α 1,3-galactosyltransferase enzyme that produces glycans containing the α -Gal linkage [16]. The second potential immunogenic epitope Neu5Gc is common in all non-primate mammalian cells [2] due to the presence of the enzyme, *N*-acetylneuraminic acid

hydroxylase, which converts CMP-Neu5Ac to CMP-Neu5Gc in all mammals other than old-world primates [17]. Furthermore, the presence of a circulating polyclonal anti-Neu5Gc antibody response has been detected in humans [2, 15]. In contrast to the alpha-Gal epitope, Neu5Gc can even be taken up from the media as a metabolite by all mammalian cells, including human cells, and then metabolically incorporated onto cell surface glycoconjugates. While all mammalian cells have the potential for immunogenic epitopes, mouse myeloma cells (NS0 and Sp2/0) tend to express higher levels of both of these epitopes compared to hamster (CHO and BHK), making recombinant products from murine cells a higher likelihood for being immunogenic in humans. This potential immunogenicity can be especially concerning when the therapeutic glycoproteins are administered repeatedly in large doses for chronic diseases [17–19].

Even without these two nonhuman immunogenic epitopes, the glycosylation patterns of proteins expressed in CHO and human cell lines are likely to differ [20]. CHO cells typically do not express *N*-acetylglucosaminyltransferase III (GnT-III) and thus lack bisecting GlcNAc residues in their glycoforms, which can impact antibody efficacy [21]. Human cells contain GnT III and can produce glycans with bisecting GlcNAc, while antibodies produced in mouse myeloma cells also contain a fraction of glycans with bisecting GlcNAc residues [22].

The glycosylation of biotherapeutics has been identified as a critical quality attribute [23] because each biotherapeutic requires defining glycosylation characteristics to maintain consistent quality parameters such as solubility, thermal stability, protease resistance [24], aggregation [2, 3], serum half-life [25], immunogenicity [5], and efficacy [26]. Thus, in order to tailor the glycosylation structures produced by CHO cells, a number of researchers have undertaken metabolic glycoengineering strategies to alter the final glycan profiles and distribution in CHO. In this review, we will document recombinant protein N-linked glycoengineering studies in CHO cells and evaluate the impact on the N-glycosylation patterns attached to proteins used across the biotechnology industry. Given the diversity of structures possible, this review will focus on glycoengineering primarily for non-antibody motifs and briefly discuss the glycoengineering approaches in antibodies.

2 Glycoengineering Strategies in CHO Cells

2.1 Sialylation

Among the numerous sugar moieties found in glycans, the terminal sialic acid (Neu5Ac) is considered particularly important for the lifespan of glycosylated proteins. As an electro-negatively charged acidic 9-carbon moiety, sialic acid is α -glycosidically linked on the C3- or the C6-hydroxyl group of the terminal galactose in humans,

through the action of $\alpha 2,3$ -sialyltransferases (ST3) or the $\alpha 2,6$ -sialyltransferase-1 (ST6) [27–29]. Terminal sialic acid residues can alter protein properties including biological activity and in vivo circulatory half-life. Serving as a biological mask, the distal sialic acid can shield galactose residues that when exposed prompt a fast removal of the protein from blood circulation due to the endocytosis-mediated uptake by asialoglycoprotein receptors on hepatocytes [29–31]. Therefore, in mammalian cells, it is generally desirable to maximize the distal sialic acid content of a glycoprotein to ensure its quality and consistency as an effective therapeutic [12].

However, the sialic acid content of glycoproteins expressed in CHO cells can sometimes be incomplete, which is due to two opposing cellular processes. The first process consists of two steps—the biosynthesis of cytidine monophospho-sialic acid (CMP-SA) substrate and the transfer of sialic acid from this substrate onto a glycan catalyzed by a sialyltransferase. The second process is the extracellular removal of sialic acid by sialidase cleavage [32]. Both these pathways are targets for genetic engineering. Hence, in the next sections, we discuss genetic manipulation of the sialylation process, and divide it into three parts: genetic engineering of sialylation pathways, overexpression of *N*-acetylglucosaminyltransferase (GnT) genes, and inhibition of sialidase activity and present a table to summarize the achievements of glycoengineering to improve protein sialylation (Table 1).

2.1.1 Genetic Engineering of Sialylation Pathways

Genetic engineering of sialyltransferase enzymes is probably the most straightforward method to alter sialylation content in terms of modifying the oligosaccharide biosynthesis reaction networks. Sialyltransferases are ultimately responsible for introducing a Neu5Ac residue to the penultimate galactose residue.

Six β -galactoside $\alpha 2,3$ -sialyltransferases (ST3GAL1–6) and two β -galactoside $\alpha 2,6$ -sialyltransferases (ST6GAL1–2) are responsible for forming these terminal sialic acids in mammalian cells. Human glycoproteins bear sialic acid residues in both $\alpha 2,3$ - and $\alpha 2,6$ -linkages, whereas only $\alpha 2,3$ -terminal sialic acids are found in glycoproteins from CHO and BHK cells. A report from our group revealed that three genes from the $\alpha 2,3$ -sialyltransferase family (ST3GAL3, ST3GAL4, and ST3GAL6) are responsible for $\alpha 2,3$ -sialylation in CHO cells using siRNA knockdown approaches, among which ST3GAL4 plays the critical role in dictating glycoprotein $\alpha 2,3$ -sialylation [33]. ST6GAL1 appears to prefer the Gal $\beta 1$ -4GlcNAc disaccharide sequence linked to a protein, whereas ST6GAL2 shows a preference for free disaccharide Gal $\beta 1$ -4GlcNAc substrate in humans [34].

The overexpression of heterologous $\alpha 2,6$ -sialyltransferase with or without recombinant $\alpha 2,3$ -sialyltransferase serves to introduce linkages similar to those found in human cells and has been adapted to elevate the amounts of sialic acid on recombinant proteins [29]. Since the first introduction of ST6GAL1 in CHO cells

Table 1
Summary of Glycoengineering strategies in CHO cells to improve protein sialylation

Enzyme name	Enzyme function	Increase biotherapeutic properties	Target protein	Reference
ST6GAL1 (+)	α 2,6-sialyltransferase 1	Capping Gal residues with α 2,6 sialic acid	t-PA EPO INF- γ IgG	[36] [37] [38–40, 61] [38, 39]
ST3GAL4 (+)	α 2,3-sialyltransferase 4	Capping Gal residues with α 2,3 sialic acid	EPO	[43, 46]
β 1,4GALT1 (+)	β 1,4-galactosyltransferase 1	Adding Gal to GlcNAc	EPO	[43]
CMP-SAS (+)	CMP-sialic acid synthase	Synthesize the CMP-sialic acid in the nucleus	EPO	[46, 54, 55]
GNE/ MNK (+)	UDP-GlcNAc 2-epimerase/ ManNAc kinase	Epimerization of GlcNAc to MANNAc/ phosphorylation of ManNAc	EPO	[46]
CMP-SAT (+)	CMP-sialic acid transporter	Transport CMP-SA from Cytosol to Golgi	INF- γ EPO	[55, 56]
GnT-IV (+) GnT-V (+)	α 1,3-d-mannoside β -1,4- <i>N</i> -acetylglucosaminyltransferase α -1,6-d-mannoside β -1,6- <i>N</i> -acetylglucosaminyltransferase	Adding GlcNAc to α 1-3 mannose residue Adding GlcNAc to α 1-6 mannose residue	INF- γ EPO	[59–61]

(continued)

Table 1
(continued)

Enzyme name	Enzyme function	Increase biotherapeutic properties	Target protein	Reference
GnT-I (+)	N-acetylglucosaminyltransferase I	Transfer UDP-GlcNAc to the terminal α -1,3-linked Mannose	EPO	[63–65]
Neu 3 (-)	Neuraminidase 3 (membrane sialidase)	Release of sialic acid from the Galactose residue	INF- γ	[76]
Bcl-x _L (+)	An anti-apoptotic member of the Bcl-2 family	Anti-apoptosis protein prevents cell death	Fc-fusion	[80]
30Kc19 (+)	Cell-penetrating protein	Anti-apoptosis	EPO	[82, 83]

Note: “+” indicates overexpression or introcution, “-” indicates knockdown or inhibition

in 1989 [35], rat or human ST6GAL1-expressing CHO and BHK cells were successively generated and tested for various therapeutic glycoproteins production [36, 37]. Expressing rat ST6GAL1 in a recombinant human tissue plasminogen activator (tPA)-expressing CHO cell line significantly increased the α 2,6 sialylation level [36]. A similar protocol was applied to modify recombinant human interferon- γ (IFN- γ) and tissue-inhibitor of metalloproteinases-1 in CHO and human erythropoietin (EPO) in BHK-21A cells. Analysis of the IFN- γ showed about 40% content of α 2,6-linked sialic acid for engineered CHO expressing recombinant ST6GAL1 when compared to non-detectable levels of α 2,6 sialic acid for wild-type IFN- γ produced by CHO cells [38]. In all cases, a mixture of α 2,6- and α 2,3-linked sialic acids was observed [38–40]. These findings indicate a competition between the endogenous α 2,3 sialyltransferase and exogenous α 2,6 sialyltransferase for the same sialic acid donors and acceptors [41].

Meanwhile, the step prior to sialylation for N-glycans typically involves the addition of galactose onto the branched N-glycan chains (*see* Fig. 1) and insufficient or inconsistent galactosylation can also result in an unsatisfactory sialylation level [42]. Thus, over-expressing both the human β 1,4-galactosyltransferase (β 1,4-GalT) and α 2,3-sialyltransferase (α 2,3-ST) was applied in the synthesis of glycoprotein products with a greater and consistent proportion of fully sialylated N-glycans [42]. The resulting oligosaccharides showed greater homogeneity compared with control cell lines, in which $\geq 90\%$ of available branches were capped with sialic acid [42]. Compared to α 2,3-ST expression alone, co-expression of β 1,4-GalT and α 2,3-ST in a CHO-producing EPO cell line achieved a higher sialic acid content and more trisialylated glycans [43].

Aside from sialyltransferase level, the availability of nucleotide sugar substrates and the transport of them into the Golgi can also affect the extent of protein sialylation [3]. The biosynthesis of sialic acid in mammalian cells takes place in the cytosol, and then is completed in the nucleus followed by the transport into the Golgi, as shown in Fig. 2. In eukaryotes, uridine diphosphate (UDP)-GlcNAc is initially epimerized to N-acetylmannosamine (ManNAc) by UDP-GlcNAc 2-epimerase (GNE), and ManNAc is phosphorylated to ManNAc-6-phosphate by ManNAc kinase (MNK). These two enzymes are integrated into a single bifunctional enzyme (encoded by GNE/MNK) [44–46]. As a rate-limiting enzyme, GNE is regulated by feedback inhibition of the level of cytoplasmic free CMP-Neu5Ac. A genetic disease called “sialuria” arises from the absence of feedback regulation of this enzyme, leading to excessive synthesis of free sialic acid, which is accumulated in cytoplasm and secreted into urine [47, 48].

Previous studies on the sialic acid pathway have enlightened researchers about new approaches to increase sialylation of therapeutic proteins. As a direct precursor of sialic acid, N-acetylmannosamine

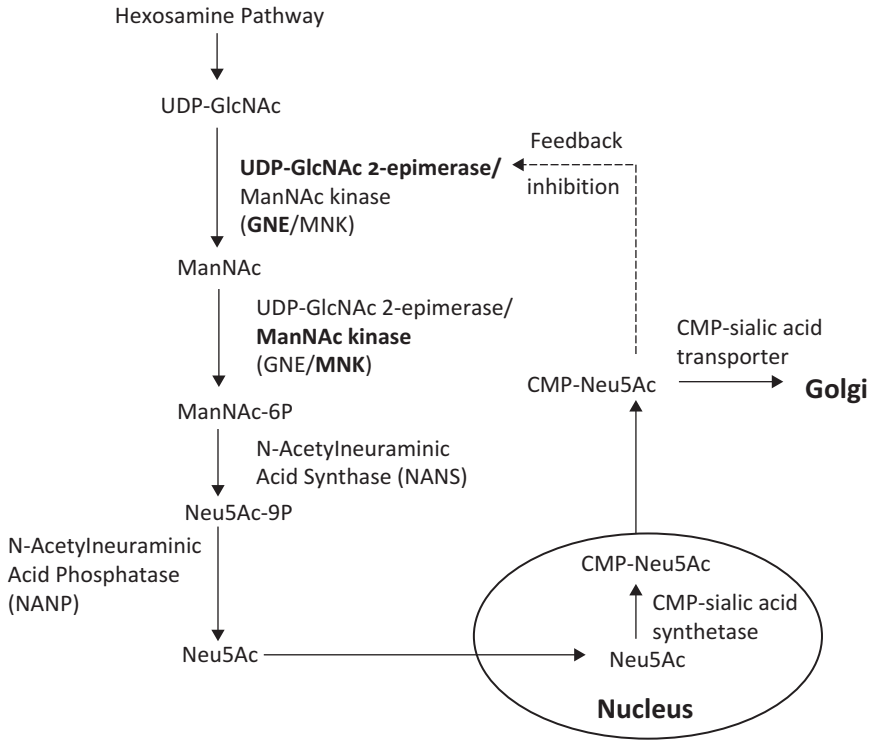


Fig. 2 Schematic representation of sialic acid biosynthesis pathway in mammalian cells

(ManNAc) supplementation has long been investigated for its effect on sialylation. Numerous reports demonstrated that ManNAc supplementation can significantly increase CMP-sialic acid in the intracellular pool up to 12-fold, but only improve protein sialylation to a very limited extent of about 10–20% increase [49–53]. A similar issue exists with the overexpression of CMP-SAS and sialuria-mutated GNE/MNK in the biosynthesis pathway, which can increase sialylation to a limited extent [46, 54, 55]. All these approaches can increase the intracellular pool of CMP-sialic acid, but the next step—transporting CMP-sialic acid to the Golgi is hampered by the inefficiency of the CMP-sialic acid transporter (CMP-SAT) on the Golgi membrane, thereby causing the reduced availability of CMP-sialic acid substrate for sialylation. Previous researchers overexpressed CMP-SAT alone in CHO IFN- γ cell line and resulted in a 4–16% increase in site sialylation of IFN- γ [56]. In addition, several groups have implemented combinatorial engineering to apply multiple genes in the pathway to improve sialic acid content in the intracellular pool and the availability of sialic acid substrates in the Golgi. One group overexpressed α 2,3-ST, CMP-SAS, and CMP-SAT in a CHO recombinant EPO cell line, and a corresponding increase in the sialylation was observed compared to the co-expression of α 2,3-ST and CMP-SAS [55]. Another group introduced a sialuria-mutated rat GNE/

MNK, Chinese hamster CMP-SAT, and human $\alpha 2,3$ -sialyltransferase ($\alpha 2,3$ -ST) simultaneously into recombinant human EPO-producing CHO cells and found the sialic acid content of rhEPO produced from engineered cells was 43% higher than that of control cells. The fraction of tetra-sialylated glycans for rhEPO produced from engineered cells increased $\sim 32\%$, and fraction of asialo- and mono-sialylated glycans decreased $\sim 50\%$ compared with controls [46].

2.1.2 Overexpression of *N*-acetylglucosaminyltransferase (GnT) Genes

Overexpression of branching genes can also be applied to increase sialylation acceptor sites. For human proteins, bi-, tri-, and tetra-antennary structures are produced with complex-type N-glycans consisting predominantly of the disaccharide Gal $\beta 1,4$ -GlcNAc capped by a terminal sialic acid. As illustrated in Fig. 1, tri- and tetra-antennary complex N-glycans are controlled by UDP-*N*-acetylglucosamine: α -1,3-d-mannoside β -1,4-*N*-acetylglucosaminyltransferase (GnT-IV or Mgat4) and UDP-*N*-acetylglucosamine: α -1,6-d-mannoside β -1,6-*N*-acetylglucosaminyltransferase (GnT-V or Mgat5). These branched structures are associated with various biological functions, including cellular proliferation, cell surface signaling [21], cancer metastasis, and regulation of T-cell activation [57] and also affect therapeutic proteins' clearance rate by the glomeruli of the kidneys [58]. In one study, only a small fraction of glycoproteins produced in a CHO cell line contained the GlcNAc $\beta 1$ -6 branch product of GnT-V [59]. Thus, more extensive modifications to glycoform distribution can occur if genetic modulations are introduced in the branching pathway of N-oligosaccharide biosynthesis, thereby potentially increasing the number of sialylation acceptor sites.

In order to tailor the multi-antennary glycoforms of recombinant proteins, overexpression of GnT-IV and V was used in CHO cells producing human IFN- γ and EPO cell lines [59, 60]. In both cases, tri- and tetra-antennary sugar chains increased significantly, representing $\geq 50\%$ of the total sugar chains and almost all N-glycans were in tri- or tetra-antennary glycoforms [60]. At the same time, an increase in poly-*N*-acetyllactosamine (Gal $\beta 1$ -4GlcNAc $\beta 1$ -3) was also observed [59, 60]. However, the increase in sialic acid content was not equal to the increase of available sialylation acceptor sites because of incomplete sialylation. In another study, mouse ST3 and/or rat ST6 were introduced into CHO IFN- γ cell lines stably transfected with GnT-V, reaching 61.2% sialylation in $\alpha 2,3$ - and $\alpha 2,6$ -linked sialic acid content [61]. Furthermore, the coordinated overexpression of GnT-IV, GnT-V, and ST6GAL1 genes in a CHO EPO cell line by our group enhanced sialic acid content approximately 45% compared to control CHO EPO expressing cells [60].

Another approach to enhance sialylation is to restore missing functions in CHO-deficient mutants. For example, GnT I-deficient mutants are generated either through the lectin *Ricinus communis agglutinin I* (RCA-I) selection or by genetic modulations. Treating

the CHO cells with the cytotoxic lectin *Ricinus communis agglutinin I* (RCA-I) was designed to select mutants with defects in the N-glycosylation pathway upstream of galactose addition as this lectin was reported to be specific for terminal β 1,4-linked galactose [62]. Unexpectedly, genetic analysis of RCA-I-resistant CHO mutants showed that they are all the same type of mutants with genetic mutations in the GnT-I gene [63], similar to Stanley's Lec1 mutant. A plausible explanation is that RCA-I is not specific for terminal β 1,4-linked galactose but possibly binds many glycan structures except for $\text{Man}_5\text{GlcNAc}_2$ [64]. Without functional GnT-I, the cells fail to transfer *N*-acetylglucosamine to $\text{Man}_5\text{GlcNAc}_2$ glycan (Fig. 1). Surprisingly, the restoration of functional GnT-I in these mutants led to an increase in the sialylation of recombinant proteins both in transient expression and in stably transfected clones [63]. While the molecular mechanism for this phenomenon remains unknown [65], recombinant EPO generated in this RCA-I mutant line displayed 30% greater sialylation compared with the control EPO producing CHO clone cultured under the same conditions [66]. Moreover, HPAEC-PAD and MALDI-TOF MS analyses showed that EPO produced by the GnT I-restored CHO-GnT I-deficient cells also contained a higher content of tri- and tetra-antennary glycans [66].

2.1.3 Inhibition of Sialidase Activity

Any attempt to maximize sialic acid content of a therapeutic protein should also consider the sialidase activity because the glycoprotein is subject to desialylation and degradation during prolonged cell culture [29, 67]. Sialidases (neuraminidases, *N*-acylneuraminosyl glycohydrolases, EC 3.2.1.18) are exoglycosidases catalyzing the hydrolytic removal of sialic acid from sialoglycoconjugates (glycoproteins, polysaccharides, gangliosides) [68]. The resulting asialoglycoprotein product would then be rapidly cleared from the plasma by asialoglycoprotein receptors in the liver. There are four sialidases (Neu 1–4) identified in human, mouse, rat, and CHO cells and their activity has been localized to different subcellular compartments: Neu1 is located in the lysosome, Neu2 is a cytosolic protein, Neu3 is located in the plasma membrane, and Neu4 is a second lysosomal sialidase [67–69]. The functions of these sialidases vary in part due to different substrate specificities and subcellular locations [29]. These sialidases can be crucial to various biological functions such as growth control and differentiation, tumorigenesis, T-cell activation and immune cell interactions, neuronal differentiation, and genetic defects [68, 70–73]. Therefore, in mammalian cells, it is often desirable to lower the cellular sialidase activity to ensure product quality and consistency for secreted biotherapeutic glycoproteins.

In mammalian cell culture, some extracellular sialidase originates from the cytosol of the CHO cells and is released to the cell culture supernatant as a result of cell lysis [74]. Gene manipulation techniques can be applied to inhibit the sialidase's activity in CHO

cells and prevent the enzyme from being released into the culture medium. When gene expression of CHO Neu2 was knocked down to 40% by homologous recombination or RNA interference (RNAi), the sialic acid content of the recombinant glycoprotein was improved but only when cells were in the death phase [67, 75]. In another study, CHO cells overexpressing recombinant human interferon gamma (IFN- γ) were treated using short interfering RNA (siRNA) and short-hairpin RNA (shRNA) to reduce expression of the Neu1 and Neu3 sialidase genes [76]. By knocking down expression of Neu3, a 98% reduction in Neu3 sialidase activity was achieved in CHO cells. Accordingly, the sialic acid content on recombinant IFN- γ was found to be increased 33% and 26% for samples from the cell stationary phase and death phase, respectively, as compared to corresponding controls [76]. Interestingly, when using the same siRNA technique to knock down both genes individually, Neu3 (located in the plasma membrane) knockdown almost silenced sialidase activity, while Neu2 (located in the cytoplasm) knockdown only reduced sialidase activity to 40%. Unlike Neu2 knockdown effects that acted exclusively in the death phase, protein sialylation was enhanced in the whole cell process after knocking down Neu3 expression, suggesting different mechanisms of protein sialylation regulation by Neu2 and Neu3 [29].

In addition to silencing the genes for sialidases, other approaches have focused on inhibiting glycan degradation. Bcl-x_L, an antiapoptotic protein that inhibits the release of proapoptotic molecules from mitochondria, is well documented for its role in extending culture longevity by suppressing apoptotic cell death and improved glycoprotein production [77–79]. Overexpression of Bcl-x_L can enhance the sialylation of glycoproteins produced from CHO cell lines by reducing cell lysis and delaying the extracellular accumulation of sialidase activity during prolonged cell culture [80]. Likewise, the investigation of anti-apoptotic components of silkworm hemolymph revealed *Bombyx mori* 30Kc19 gene expression can also enhance recombinant protein production and sialylation in CHO [81, 82]. Stable expression of the 30Kc19 gene in a CHO cell line producing recombinant human EPO increased the EPO production and sialylation by 102.6% and 87.1%, respectively [82]. Moreover, with the introduction of 30Kc19 gene the host suspension cells produced recombinant human EPO with more complex glycan structures and a larger content of sialic acid and fucose [83]. The 30Kc19 protein is able to maintain the activity of glycotransferases involved in the glycosylation process [83].

2.2 Introduction of Additional Glycosylation Sites

In addition to modifying the oligosaccharide structures at specific glycan sites (microheterogeneity), glycoengineering can also be applied to control the glycan site occupancy of a target protein (macroheterogeneity) by altering the N-glycan consensus sequence

as well as the number and position of the glycosylation sites on the nascent peptide chain using site-directed mutagenesis.

Asn-X-Ser/Thr, where X is any amino acid except proline, is the preferred N-glycan consensus sequence of choice [84]. The presence of Pro at the X position completely blocked the glycosylation at that site, while Glu, Trp, Asp, and Leu showed inefficient glycosylation [7, 84–87]. Moreover, the sequon of Asn-X-Thr is more likely to be glycosylated than Asn-X-Ser. Studies on rabies virus glycoprotein (RGP) showed that using site-directed mutagenesis on an Asn-X-Ser sequon at Asn37 site, to substitute of Thr for Ser at position 39 dramatically increased core glycosylation efficiency of Asn37 in both membrane-anchored and secreted forms of RGP; whereas substitution of Cys for Ser blocked the core glycosylation [88]. Thus, the glycosylation of the target protein was enhanced when threonine was present instead of serine at the hydroxy position of N-glycan sites [84]. In addition to which amino acid is at the X position and whether the hydroxy amino acid in the sequence is serine or threonine, the efficiency of core glycosylation on the asparagine residue at the consensus sequence is also dependent on the accessibility of the consensus sequon for the active site of the OST complex and proper transfer of the oligosaccharide moiety from its lipid-linked carrier [84].

Based on previous studies of N-glycan consensus sequences, introduction of additional N-glycan target sites into desired positions on the protein backbone by genetic mutation has been used to create glycoproteins with enhanced levels of glycosylation and consequently sialylation, leading to extended serum half-life and improved in vivo activity [29, 89, 90]. These manipulations have produced hyperglycosylated recombinant protein analogues for use as biotherapeutics. For example, through the selection of several dozen analogues of recombinant EPO containing one or two amino acid mutations, two additional oligosaccharide-attachment sites at asparagine residues 30 and 88 have been incorporated into EPO, creating darbepoetin alfa (also called novel erythropoiesis stimulating protein, NESP), with a total of five N-linked oligosaccharides, a threefold longer serum half-life, increased in vivo potency and pharmacokinetics (PK), and less frequency of administration to obtain the same biological response [89, 90]. In another study, additional N-linked glycosylation sites have been added to the follicle-stimulating hormone (FSH) molecule through N-terminal extensions. The resulting FSH1208 variant was found to have a three- to four-fold increased serum half-life compared with wild-type recombinant FSH [91]. Introduction of N-glycosylation sequons onto the flanking linker and a C-terminal extension on a recombinant antibody has also been shown to yield prolonged circulation time [92]. However, in producing rHuACHE from HEK-293 (HEK) cells, the decisive factor in determining the clearance rate was related to the number of N-glycan termini which are not occupied by sialic

acid residues, rather than the absolute amount of N-glycan units [93]. Thus, the N-glycosylation load, terminal N-glycan sialylation, and subunit oligomerization act together in determining the ultimate residence time of a biotherapeutic [94]. These results clearly suggest that a multifactorial mechanism is involved and that multiple factors exert their influence in a hierarchical manner on protein clearance. Terminal N-glycan sialylation is the governing factor in this hierarchy, since totally desialylated forms of AChE are cleared rapidly, and equally as well, from the circulation within minutes, regardless of their oligomerization state and their number of appended N-glycans [95, 96]. In this case, increasing the number of N-glycans on the enzyme surface resulted in an increase in the number of terminal Gal residues, which serve as highly potent clearance epitopes. Thus, for glycosylation mutants of rHuAChE produced in the HEK cell system, addition of N-glycan sites had a clear adverse pharmacokinetic effect, owing to the increase of pharmacokinetically unfavorable uncapped glycan termini [96].

2.3 Heterogeneity of Glycans

Another issue with N-glycans of therapeutic proteins is the generation of heterogeneous glycoforms, which present challenges in protein purification, product consistency, and lot-to-lot reproducibility, resulting in variable therapeutic efficacy. This diversity can sometimes adversely affect drug potency and pharmacokinetics [97, 98]. However, N-glycans can also be crucial for protein folding, so these difficulties cannot necessarily be overcome by removing N-glycosylation sites [99]. Heterogeneity is attributed to the lack of 100% efficiency for each step in mammalian N-glycan biosynthesis due to variability in enzyme levels, substrate concentration, intracellular location, and the competition of different enzymes for the same substrates [99].

In order to provide homogenous glycoforms, Zhang et al. conducted a comprehensive Zinc-finger nuclease knockout screen of 19 glycosyltransferase genes and identified the key genes that control decisive steps in N-glycosylation in CHO [100]. The authors stacked knockouts of GnT-IV-A/GnT-IV-B/GnT-V to produce almost homogenous biantennary N-glycans [100]. Subsequently, the introduction of ST6Gal-I in CHO ST3Gal4/6 knockout cells produced a normal range of N-glycans with only α 2,6-sialylation, and when combined with a GnT-IV-A/GnT-IV-B/GnT-V knockout, homogenous biantennary N-glycans capped by α 2,6-sialic acid residues were generated [100].

3 Conclusion

This review has highlighted the role of glycosylation as a critical quality attribute in biotherapeutic production, and more importantly how these glycans can be manipulated in CHO expression

systems through cell engineering, as summarized in Table 1. Mammalian cell lines such as CHO can produce valuable recombinant proteins that can be accepted by humans as therapeutics. However, subtle differences between glycosylation in human and other mammals exist and understanding these differences requires knowledge of the physiological characteristics of each cell type. Moreover, these differences can help to direct efforts toward glycan reengineering to make a wider selection of glycan moieties in CHO cells. Efforts to exert control over protein glycosylation in CHO cells have been demonstrated through several success stories for maximizing terminal sialylation such as overexpression of sialyltransferases and other glycosyltransferases, inhibition of sialidases, and manipulation of nucleotide sugar substrate levels. The advent of advanced technologies such as CRISPR Cas9, TALE nucleases, RNA interference tools, as well as the combination of next generation of sequencing with systems biotechnology will further facilitate the enhancements in cell glycosylation processing. These tools will enable cell engineers to make even more highly refined and targeted modifications to the processing capability of these cells to meet the demand for diverse and highly effective biotherapeutic glycoproteins for future health care needs.

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