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N-Glycosylation of IgG and IgG-Like Recombinant Therapeutic Proteins: Why Is It Important and How Can We Control It?

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Abstract

Regulatory bodies worldwide consider *N*-glycosylation to be a critical quality attribute for immunoglobulin G (IgG) and IgG-like therapeutics. This consideration is due to the importance of posttranslational modifications in determining the efficacy, safety, and pharmacokinetic properties of biologics. Given its critical role in protein therapeutic production, we review *N*-glycosylation beginning with an overview of the myriad interactions of *N*-glycans with other biological factors. We examine the mechanism and drivers for *N*-glycosylation during biotherapeutic production and the several competing factors that impact glycan formation, including the abundance of precursor nucleotide sugars, transporters, glycosidases, glycosyltransferases, and process conditions. We explore the role of these factors with a focus on the analytical approaches used to characterize glycosylation and associated processes, followed by the current state of advanced glycosylation modeling techniques. This combination of disciplines allows for a deeper understanding of *N*-glycosylation and will lead to more rational glycan control.

INTRODUCTION

CH2 domain: IgG domain on the heavy chain, site for complement and Fc receptor binding

Bispecific: engineered antibody that contains two (or more) different antigen-binding sites

Fc-fusion protein: protein that contains an Fc domain combined with another peptide (ligand, peptidic antigen)

Antibody–drug conjugate: an antibody combined with an active drug or cytotoxic molecule via a chemical linker

N-glycosylation refers to the cotranslational covalent addition of an oligosaccharide moiety to a relevant asparagine (Asn) side chain of secreted and membrane glycoproteins. Approximately 50% of the proteins encoded within the human genome include the consensus sequence for *N*-glycosylation. It is appropriately classified as a critical quality attribute during manufacturing (1, 2) owing to its potential to influence biological activity, serum half-life, and efficacy of protein therapeutics (3). *N*-glycosylation can also affect solubility, thermal stability, protease resistance, and aggregation of these proteins (4). Sometimes, glycan removal can detrimentally impact protein secretion, thermostability, and activity, such as in the case of erythropoietin. However, glycan removal may also enhance enzyme activity, such as for ribonuclease A and tissue plasminogen activator (5), thus highlighting their complex biological functions.

Monoclonal antibodies (mAbs) continue to dominate the biopharmaceutical market, representing more than 50% of new product approvals since 2015 (6). Of the five major classes, immunoglobulin G (IgG) antibodies are one of the most abundant protein forms in human serum, accounting for approximately 10–20% of plasma proteins. IgG antibodies are divided into four highly conserved (>90% primary sequence homology) subclasses. The majority of approved mAb therapeutics and those in regulatory review in the United States or Europe are IgG1, likely owing to its associated effector function activity (7). IgG2 or IgG4 is used when effector function activity is not desirable. IgG classes differ within their constant region, particularly in the hinge area and the adjacent CH2 domain, which mediates the interactions with various Fc (fragment crystallizable) gamma receptors (FcγR), including the neonatal Fc receptor (FcRn) and complement component 1q (C1q) (8).

IgG antibodies possess two identical *N*-glycosylation sites, one on each Fc region of the two heavy chains. Other proteins, like bispecific mAbs or Fc-fusion proteins, may have additional *N*-glycosylation sites. IgG are glycosylated at Asn297 on the CH2 domain of one or both heavy chains of the Fc region (**Figure 1*a***). The Fc-glycan consists of a core structure, composed of two *N*-acetylglucosamine (GlcNAc) moieties and three mannose moieties, and typically contains a fucose moiety. The two arms of the biantennary structure are defined by α1,3 and α1,6 mannose linkages that can be further extended with the addition of galactose and sialic acid (see **Figure 1*b*** for glycan structures and nomenclature). Final glycan structures typically can be divided into three types: high mannose, hybrid, and complex.

In the case of mAbs, glycans are known to affect anti-inflammatory response and effector function activity, including antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (4). This is also the case for other IgG Fc-containing therapeutics (such as bispecifics, antibody–drug conjugates, and Fc-fusion proteins). Additionally, glycan moieties such as mannose, galactose, or sialic acid can significantly impact the immunogenicity and/or pharmacokinetics of mAb and Fc-containing therapeutic proteins. It is, thus, no surprise that demonstrating control over the glycan profile is a regulatory requirement for recombinant protein therapeutics prior to use in patients (9).

N-GLYCOSYLATION INFLUENCES BIOLOGICAL ACTIVITY OF IgG AND IgG-LIKE PROTEINS

Depending on the in vivo mechanism of action of the IgG- or Fc-containing therapeutic, the Fc-glycan composition can significantly alter therapeutic activity owing to Fc-mediated interactions with components of the complement system or various immune receptors (10). As shown in **Figure 2**, IgG can engage the target antigen via the Fab (antigen-binding fragment) region. If the target is expressed on a cell surface, then, once engaged, the IgG Fc can mediate interactions with

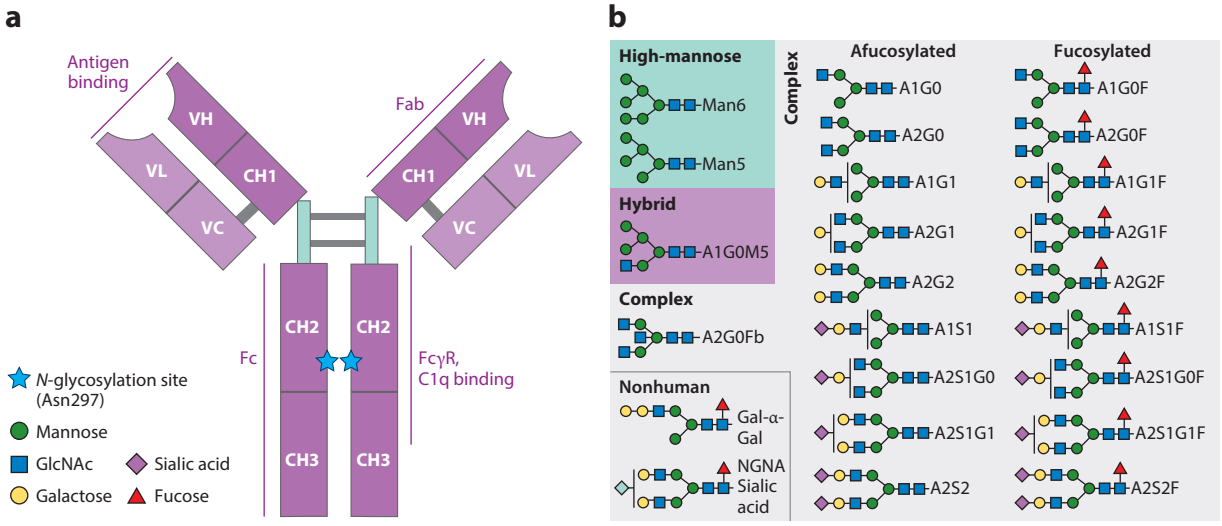


Figure 1

(*a*) Immunoglobulin G (IgG) antibody structure and locations of binding regions. The Fc *N*-glycosylation sites at asparagine 297 (Asn297) are indicated by blue stars. (*b*) Common glycan structures observed for IgG antibodies produced in mammalian cells. The three main glycan classification groups (high-mannose, hybrid, and complex) are indicated. Only one representative hybrid and bisecting glycoform are shown owing to lack of space. Two nonhuman glycan forms are shown.

FcγR expressed on effector cells, including natural killer (NK) cells, monocytes, macrophages, and neutrophils, and induce ADCC and antibody-dependent cell-mediated phagocytosis (ADCP). Alternatively, the Fc region can bind to C1q, leading to the activation of the classical complement pathway, culminating in the formation of a membrane attack complex and leading to cell lysis.

The human FcγR family consists of three structurally similar classes with a total of six family members, including FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa, and FcγRIIIb, each having a unique effector function profile (11). The FcγRs are classified as activating or inhibitory based on the type of signaling pathways they induce. The activating FcγRs include FcγRI, the only receptor that exhibits high-affinity binding to monomeric antibody. In contrast, the low-affinity receptors FcγRIIa, FcγRIIc, FcγRIIIa, and FcγRIIIb require multivalent immune complexes for their activation. FcγRIIIa exists as two allotypic variants that differ in their affinity for binding IgG. FcγRIIIa-Phe158, the most common allotype, has a lower affinity than the Val158 allotype. Cartron et al. (12) published one of the initial studies highlighting the relationship between the high-affinity Val158 allotype, FcγR-mediated effector functions, and clinical outcome, demonstrating that rituximab-treated non-Hodgkin's lymphoma patients homozygous for the Val158 allele had significantly improved objective response rates compared with non-Hodgkin's lymphoma patients who carried at least one Phe158 allele. With the exception of FcγRIIIb, intracellular signaling by the activating receptors is modulated via phosphorylation of the immunoreceptor tyrosine-based activating motifs and leads to effector functions such as ADCC and ADCP. Intracellular signaling via FcγRIIb, the only known inhibitory receptor, is modulated via phosphorylation of the immunoreceptor tyrosine-based inhibitory motif, leading to the recruitment of phosphatases that counteract the activating signals (11).

Two cell types do not coexpress activating and inhibitory receptors: (*a*) NK cells, which express only FcγRIIIa, and (*b*) B cells, which express only FcγRIIb. Although FcγRIIIb is classified as an activating receptor, it is not associated with the FcR common γ-chain. FcγRIIIb is expressed

Natural killer (NK) cell: a lymphocyte that has granules with enzymes capable of killing tumor or virus-infected cells

Monocyte: an immune cell from bone marrow that can become a macrophage

Macrophage: a white blood cell that can surround and kill bacteria and other harmful organisms

Neutrophil: immune cell, first to arrive at infection site, ingests harmful organisms and secretes enzymes to kill them

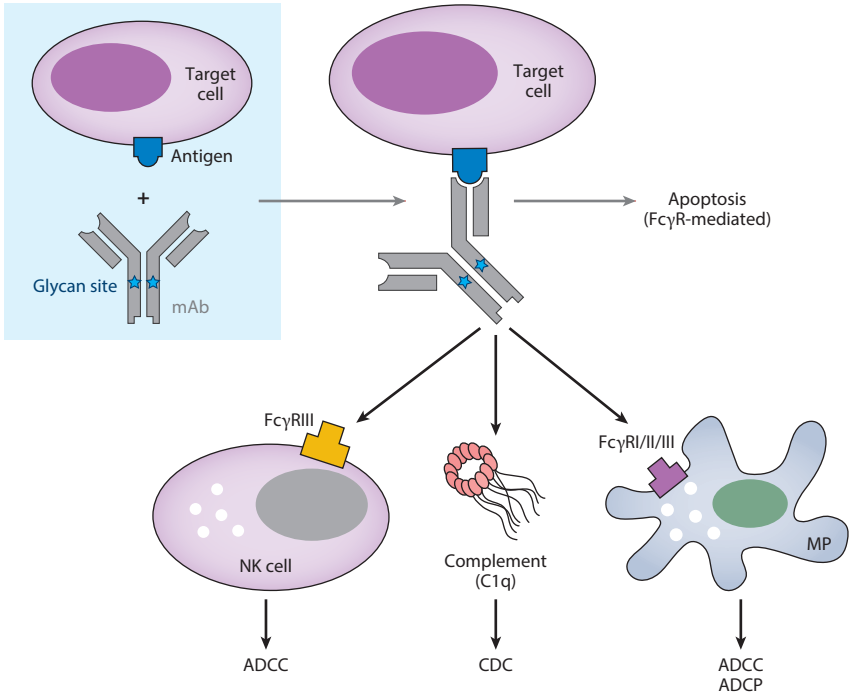


Figure 2

Potential glycan-mediated antibody mechanisms of action. Antibody binding can initiate various immune response pathways in combination with immune system actors, including ADCC, CDC, and ADCP. Abbreviations: ADCC, antibody-dependent cell cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; mAb, monoclonal antibody; MP, macrophage; NK, natural killer.

mainly on neutrophils and on a subset of basophils (13). Neutrophils also express FcγRIIa that, when crosslinked, induces phagocytosis of IgG-opsonized antigen and, in combination with the crosslinked FcγRIIIb, can also lead to neutrophil degranulation and generation of reactive oxygen species (14). The levels of FcγR at the cell surface vary by receptor and cell type. The expression levels are influenced by the activated status of the effector cell type (15).

Fc Glycosylation Modulates Effector Function Activity

Effector function activity is influenced by the IgG subclass, the FcγR (in the case of ADCC and ADCP), and Fc-glycan composition. Removal of the Fc-glycan has been shown to eliminate complement activation and CDC, as well as FγR binding, ADCC, and ADCP. The systematic assessment of the relationship between the different Fc-glycans and biological activity has been made possible by in vitro glycoengineering approaches. Boyd et al. (16) used this approach to modify Campath-1H that was produced in CHO cells (refer to sidebar titled CHO Cells: Factories for Production of *N*-Glycosylated Proteins). In these studies, a deglycosylated antibody failed to elicit CDC or ADCC without impacting antigen-binding activity. Removal of the terminal galactose moiety significantly reduced, but did not eliminate, CDC activity. In contrast, degalactosylated Campath retained full ADCC activity relative to the untreated control. Others have shown that increased terminal galactosylation of IgG1 enhances C1q binding and CDC (17). More recently, Peschke at al. (18) demonstrated that higher levels of terminal galactose improved C1q

CHO CELLS: FACTORIES FOR PRODUCTION OF *N*-GLYCOSYLATED PROTEINS

Therapeutic mAbs are primarily expressed in mammalian cells, including Chinese hamster ovary (CHO), baby hamster kidney (BHK), Sp2/0, NS0, HEK293, and PER.C6 (152). Of these cell lines, the CHO cell line continues to be the workhorse for the industry and is responsible for the manufacture of approximately 70% of all recombinant therapeutic proteins, due to their ease of genetic manipulation, scalability, capacity for adaptation to serum-free media, and familiarity to regulatory agencies (153). CHO cells contain a similar, although not identical, set of enzymes and sugars for *N*-glycosylation as those observed in humans. For example, sialic acid attachments to galactose can occur as α-2,3- or α-2,6-linkage in humans but are predominantly α-2,3-linkages in CHO cells (154). Additionally, CHO cells do not express GnTIII, resulting in the absence of bisecting *N*-acetylglucosamine (GlcNAc) glycoforms. *N*-glycans of mAbs in CHO typically include a combination of five sugar moieties: mannose, *N*-GlcNAc, galactose, sialic acid (often referred to as *N*-acetylneuraminic acid), and fucose, as can be seen in **Figure 1*a***.

binding and CDC by anti-CD20 IgG1 and IgG3; however, Fc-galactosylation alone cannot impart complement-fixing properties to IgG2 or IgG4.

Hodoniczky et al. (19) also used a variety of in vitro approaches to remodel the Fc-glycans of Herceptin and Rituxan and assessed target binding, CDC, and ADCC activity. Consistent with previous studies, degalactosylation of Rituxan significantly reduced CDC. A relationship between the levels of bisecting GlcNAc and ADCC activity was also demonstrated for a mAb modified using recombinantly expressed rat GnTIII. Rituxan containing maximal levels of bisecting GlcNAc (>80%) displayed an approximately tenfold increase in ADCC relative to the unmodified control, whereas a mAb with moderate levels (30%) of bisecting GlcNAC exhibited only a modest increase in ADCC. A similar effect was observed in Herceptin-mediated ADCC when the antibody was maximally modified with bisecting GlcNAc. Umaña et al. (20) studied the effect of bisecting GlcNAc on an antineuroblastoma IgG1 chCE7 displaying low basal levels of ADCC. In these studies, a CHO cell line with tetracycline-regulated expression of GnTIII was used to generate chCE7 samples with varying levels of bisecting GlcNAc. The results showed that chCE7 produced at optimal ranges of GnTIII expression contained the highest levels of afucosylated, bisected product and exhibited maximal ADCC activity. Although these studies pointed to a role for bisecting GlcNAc in improving ADCC, it was subsequently demonstrated that oligosaccharides that are first modified by GnTIII can no longer be modified by α1,6-fucosyltransferase (FUT8), indicating that the impact of ADCC is due to the absence of fucose rather than the addition of bisecting GlcNAc (21).

Shields et al. (22) demonstrated the relationship between core fucose in the Fc-glycan and ADCC activity, using the Lec13 cell line, deficient in the ability to add fucose owing to a mutated GDP-mannose 4,6-dehydratase (GMD) gene, to produce highly afucosylated IgG1 antibodies. These IgG exhibited increased binding to FcγRIIIa-Val158 and -Phe158, by 50- and 30-fold, respectively, as well as enhanced ADCC, relative to fucosylated antibodies expressed in wild-type CHO cells. In addition, the lack of fucose slightly improved binding to the Arg131 FcγRIIa polymorphic form and FcγRIIb but did not influence FcγRI, C1q, or FcRn binding (22). Similarly, Shinkawa et al. (23) used human peripheral blood mononuclear cells to demonstrate that the increased ADCC of an anti-CD20 antibody was associated with low fucose (9%) and not the presence of bisecting GlcNAc or galactose. The authors used lectin affinity chromatography to demonstrate that increased bisecting GlcNAc in the afucosylated Fc-glycans did not have an additive effect on ADCC, indicating that it was the lack of core fucose, rather than bisecting GlcNAc, that markedly enhanced ADCC.

GnTIII: GlcNac-transferase, transports GlcNAc from UDP-GlcNAc to *N*-glycans to make bisecting *N*-glycans; also called MGAT3 or GnT3

α1,6-Fucosyltransferase (FUT8): transports fucose from GDP-fucose to *N*-glycan

GnTI: GlcNac-transferase, transports GlcNAc from UDP-GlcNAc to a Man5 precursor *N*-glycans; also called MGAT1 or GnT1

The mechanism behind the enhanced binding observed for afucosylated antibodies and FcγRIIIa was shown in a crystallographic study that revealed a clear interaction between the afucosylated Fc-glycan and the glycan on Asn162 of FcγRIIIa (24). Removal of the glycan at Asn162 increased the affinity of the receptor for the fucosylated antibody, suggesting that the glycan on Asn162 sterically hinders interactions with the antibody Fc, and that the absence of core fucose allows greater carbohydrate–carbohydrate interactions with the Fc, increasing the overall strength of the interaction and leading to enhanced ADCC (25).

Various approaches have been used to generate antibodies with high mannose to assess the impact of mannosylation on biological activity (26, 27). Kanda et al. (26) produced glycovariants of Rituxan using a FUT8 knockout cell line, a GMD-deficient cell line, or a GnTI-deficient cell line, along with kifunensine or swainsonine, two small-molecule inhibitors, or α-mannosidase I and II. These anti-CD20 antibodies, containing high-mannose-, hybrid-, and complex-type Fc-glycans lacking core fucose, were then assessed in CDC, FcγRIIIa-binding, and ADCC assays (26). The resultant mAb that lacked core fucose, or which contained high levels of mannose (Man5, Man8, Man9), displayed significantly increased FcγRIIIa binding and peripheral blood mononuclear cell–mediated ADCC relative to Rituxan that was core fucosylated. The presence or absence of fucose had no observable impact on C1q binding or CDC. In contrast, the presence of high mannose eliminated C1q binding with significant reduction in CDC activity. Other studies using kifunensine have confirmed these results, demonstrating enhanced FcγRIIIa binding and ADCC activities with reduced binding to C1q (27, 28).

Houde et al. (29) reported the impact of glycosylation on antibody conformation using hydrogen-exchange mass spectrometry (MS). The authors postulated that increased terminal galactosylation imparts a conformational change within the CH2 domain of the Fc region, leading to increased rigidity in this area and increased affinity to FcγRIIIa. Thomann et al. (30, 31) used a combination of surface plasmon resonance (SPR) and affinity chromatography to measure FcγR binding and an NK cell–based assay to measure ADCC. They demonstrated enhanced biological activity upon enzymatic hypergalactosylation of four different mAbs produced using standard CHO manufacturing processes. These studies showed that elevated levels of terminal galactose had no impact on the ADCC activity of two different glycoengineered therapeutic antibodies, suggesting that the extent to which terminal galactose modulates ADCC depends on the background level of fucosylation (30). The authors compared the effects of galactosylation and fucosylation in the context of glycan heterogeneity and demonstrated that, although galactose can positively influence ADCC activity, the levels of afucosylated Fc-glycan remain the primary driver of this activity. In these studies, sialylation had no influence on FcγRIIIa binding or ADCC but did enhance binding to FcγRIIa. Similarly, Dashivets et al. (32) demonstrated that terminal sialic acid increases binding of antibody to FcγRIIa and FcγRIIb. Wada et al. (33) showed that core afucosylation had the most profound impact on FcγRIIIa binding and ADCC activity, with only modest improvements from terminal galactosylation. In line with previous reports, these studies demonstrated no impact of core fucosylation and a negative impact of the high-mannose glycovariants on C1q binding. In these studies, sialylation had a slight reduction in ADCC activity.

Aoyama et al. (34) generated anti-CD20 mAbs with homogeneous complex-type Fc-glycans. In line with previous reports, fully galactosylated (A2G2F) mAb showed higher CDC activities than nongalactosylated (A2G0F) mAb; however, these studies revealed functional differences associated with the isomers of A2G1F. Although the A2G1aF isomer (galactose on α1,6-Man) displayed C1q binding and CDC comparable to that of a fully galactosylated A2G2F variant, the A2G1bF isomer (galactose on α1,3-Man) displayed C1q binding and CDC activities that were no different than those of the non-galactosylated mAb. Consistent with previous reports, A2G2F showed higher FcγRIIIa binding activity than A2G0F by SPR. Similar to the results from C1q binding and CDC,

the binding activity of A2G1aF and A2G2F mAb were consistent, as were the activities of A2G1bF and A2G0F mAb. Comparable results were observed when binding to FcγRIIa, FcγRIIb, and FcγRIIIb was assessed, whereas galactosylation had no impact on FcγRI binding. Using a reporter cell–based ADCC assay, the authors demonstrated a pattern of activity (A2G2F = A2G1aF > A2G1bF = A2G0F) that was consistent with FcγRIIIa binding. The authors proposed that the interactions of the α1,6-Man arm of the Fc-glycan and a region adjacent to the CH2 domain of the IgG Fc further stabilize this interaction, leading to enhanced effector function. The glycosylation profile of mAbs has significant effects on effector function, and specific glycoforms can either contribute to or diminish the resulting immunologic reactions.

Galactose-α-1,3-galactose (α-gal): a major antigenic barrier in xenotransplantation

Non-Fc Glycosylation: Benign or Critical for Function?

Approximately 15–25% of human IgG are *N*-glycosylated within their variable region (35). Additionally, approximately 20% of IgG variable regions from the Ig sequence database have consensus *N*-linked glycosylation sites that appear to result from somatic hypermutation that occurs during antigen-specific immune responses (29). Compared with the Fc-glycan, the Fab-glycan can contain high levels of bisecting GlcNAc (for non-CHO cell production), galactose, and sialic acid, as well as reduced levels of core fucose (see **Figure 1*b*** for structures) (36). Fab glycosylation has been shown to influence antibody binding, as well as other physicochemical properties (37, 38). Fc-fusion proteins, such as the CTLA-4 Fc, often carry several *N*-glycosylation sites in the non-Fc-fusion protein portions of the molecule (39). Other Fc-fusion proteins, such as etanercept and B cell–activating factor receptor 3–Fc, also possess *O*-linked glycans on their non-Fc portions (40, 41). In the case of Fc-fusion proteins, both Fab-glycan and non-Fc-glycan sites can influence the systemic clearance of the antibody. Keck et al. (42) performed one of the initial studies linking glycosylation in non-Fc regions to serum clearance. The authors compared the *N*-glycosylation pattern of different batches of lenercept, a recombinant fusion protein consisting of the extracellular domain of human tumor necrosis factor receptor (TNFR) and an IgG Fc containing the hinge and CH2/CH3 domains (43). The glycan levels in TNFR-Fc recovered from the serum of normal human volunteers were assessed via direct measurement of GlcNAc, galactose, and sialic acid using chemical analysis or enzymatic methods. The data demonstrated that terminal GlcNAc was cleared from circulation faster, and it was hypothesized that the selective clearance was mediated by the mannose receptor (43).

N-GLYCOSYLATION AND IMMUNOGENICITY

Therapeutic mAbs produced in murine myeloid cell lines, such as Sp2/0 or NS0, contain the oligosaccharide galactose-α-1,3-galactose (α-gal) on the Fc-glycan (see sidebar titled CHO Cells: Factories for Production of *N*-Glycosylated Proteins and **Figure 1*b***) (44, 45). α-Gal is best known for its role as a major antigenic barrier in xenotransplantation (46, 47). Owing to a loss of the α-1,3-galactosyltransferase gene, humans and other primates lack the biosynthetic machinery necessary to synthesize glycoproteins with α-gal moieties. The realization that α-gal is a target for IgE-mediated hypersensitivity reactions came from efforts to understand the hypersensitivity observed by several patients with metastatic colorectal cancer who were being treated with cetuximab, an antibody specific to epidermal growth factor receptor (45). Data from structural studies with cetuximab, IgE binding/inhibition studies, and studies of alternatively glycosylated cetuximab variants demonstrated that the antibodies were specific for the α-gal residues on the cetuximab Fab heavy-chain region (48, 49).

The glycan of therapeutic mAbs produced in Sp2/0 and NS0, and to a lesser extent in CHO cells, is often modified with nonhuman sialic acid, also known as *N*-glycolylneuraminic acid

(NGNA, Neu5Gc, see **Figure 1*b***) (50, 51). Humans cannot synthesize NGNA, as the gene encoding cytidine monophosphate (CMP)-*N*-acetylneuraminic acid hydroxylase (CMAH), the enzyme responsible for converting CMP-NGNA from CMP-*N*-acetylneuraminic acid (NANA, Neu5Ac), has been irreversibly inactivated (52). However, CMAH is intact in nonhuman primates and mammalian cells used to produce glycosylated biotherapeutics. Humans have variable and sometimes very high levels of circulating antibodies directed against NGNA (53). Anti-NGNA antibodies identified in healthy human serum may result from human uptake and the incorporation of this immunogenic nonhuman dietary sialic acid (54).

Major histocompatibility complex: display peptides for antigen presentation by T cells

N-GLYCOSYLATION AND PHARMACOKINETICS

The human neonatal receptor (FcRn) is essential for the transfer of humoral immunity to the fetus (55). In the case of Fc-containing therapeutics, FcRn is important because it controls the catabolic half-life of the molecule. FcRn in the acidic endosomes binds to IgG internalized via transcytosis. The IgG is then recycled to the cell surface and released at the pH of the blood. From midgestation onward, maternal IgG antibodies are transferred to the fetus via the placenta through this highly effective, active transport mechanism. This process provides the newborn with short-term adaptive immunity until the child's own immune system is sufficiently mature. FcRn also has an important function in protecting IgG from degradation. In vascular endothelial cells, FcRn-mediated internalization and recycling of IgG are responsible for maintaining IgG levels. FcRn is a member of the major histocompatibility complex class I family and consists of an α-chain with three extracellular domains noncovalently associated with β2-microglobulin, the common subunit of class I molecules (55). The interaction of the IgG-Fc with FcRn is believed to be independent of Fc glycosylation (56).

Other receptors that are known to bind and clear proteins with specific glycans include the asialoglycoprotein receptor that binds to terminal galactose residues of *N*-glycans (57) and the mannose receptor that recognizes terminal mannose or GlcNAc (58). Goetze et al. (59) followed the distribution of Fc-glycans of one IgG1 and three IgG2 therapeutic mAbs administered in human subjects. Whereas the relative levels of IgG glycovariants with terminal galactose or GlcNAc remained constant, Man5-containing IgG2 displayed a faster elimination half-life, which was independent of the route of administration, i.e., subcutaneous or intravenous. Alessandri et al. (60) performed a similar study using an IgG1 and determined that the oligomannose species were cleared at a faster rate, whereas the clearance of fucosylated biantennary oligosaccharides remained constant over the course of the study.

Leabman et al. (61) performed a comprehensive analysis regarding the impact of glycosylation on pharmacokinetics in cynomolgus monkeys. This assessment included data from deglycosylated antibodies generated via engineering (N297A, N297G) and by production in *Escherichia coli*, an antibody with a (L234A/L235A) LALA mutation, and glycoengineered antibodies. The antibodies targeted different antigen types, including highly expressed multi-transmembrane receptors, soluble cytokines, cell-surface proteins, and ligands. The results of this comprehensive study demonstrated that antibodies with differences in glycosylation that significantly alter FcγRIIIa binding show no differences in pharmacokinetics (61). The inclusion of a broad range of antibody targets in this study increases the likelihood that these findings are broadly applicable (42).

MECHANISM OF *N*-GLYCOSYLATION AND FACTORS THAT IMPACT IT

N-glycosylation, thus, plays a critical role in recombinant protein function, safety, and serum half-life. To appropriately control the profile and levels of *N*-glycosylation during the manufacture

of therapeutic proteins, it is important to understand the mechanisms and factors that drive *N*-glycosylation. Nucleotide sugars, mainly synthesized in the cytosol, form the building blocks of *N*-glycans and are the primary substrates in glycan biosynthesis reactions. The preliminary glycan structure of $GlcNAc_2Man_9Glc_3$ is first formed on a dolichol-phosphate foundation (OS-PP-Dol) on the cytosolic side of the endoplasmic reticulum (ER) via the transfer of GlcNAc to the membrane-bound lipid dolichol phosphate. This reaction is followed by the sequential addition of GlcNAc and five mannose residues by the respective glycosyltransferases (62) (see **Figure 3**). The incomplete *N*-glycan dolichol is then translocated to the lumen of the ER by a flippase. A multiunit protein complex called the oligosaccharyltransferase (OST) then transfers the 14-sugar residue from the dolichol onto the Asn-*X*-Ser/Thr consensus *N*-glycosylation site (with *X* representing any amino acid except proline), resulting in the formation of an *N*-glycosidic bond on the nascent proteins being translated in the ER (63). The glycan subsequently undergoes a trimming process via the action of ER-resident glucosidases and mannosidases, ultimately allowing the glycoprotein to be released from the ER into the Golgi. Within the Golgi, further processing of the glycan is mediated via various glycosyltransferases and glycosidases that reside in different Golgi compartments (see **Figure 3**). Following the final maturation of the *N*-glycans in the *trans*-Golgi compartments, the glycoproteins are secreted from the cells.

Flippase: enzyme that transfers lipid-linked oligosaccharide across the ER membrane from the cytosol into the lumen

Glycans present considerable heterogeneity, owing to the presence of multiple glycosylation sites and the stochastic pairing of attached *N*-glycans with different secretory pathway enzymes and nucleotide sugars. Macroheterogeneity refers to the variation in glycan forms between unique glycosylation sites on the same protein and may be caused by factors such as enzyme site accessibility. For example, only biantennary glycans with limited sialylation can form on the *N*-glycosylation site on the Fc portion of mAbs owing to steric hindrance (64). Microheterogeneity refers to the variation of the glycan structures on the same unique *N*-glycosylation site (65). Each of the major classes of influencing factors for *N*-glycosylation are further discussed below and are illustrated in **Figure 4*a***.

Nucleotide Sugars

Protein glycosylation relies critically on the availability of activated forms of nucleotide sugars: donor substrates that act as glycosylation building blocks (66). These molecules are synthesized in the cytoplasm and are transported into the lumen of the ER and Golgi via specific antiporter membrane proteins (66) (**Figure 4*a***). Nucleotide sugar biosynthesis pathways are intimately coupled with glycolysis and the tricarboxylic acid cycle (**Figure 4*b***). Starving cells of glucose and glutamine has been used to study the impact of these limitations on nucleotide sugar biosynthetic pathways. Nyberg et al. (67) showed that glucose starvation leads to a reduction of nucleotide sugar concentrations owing to the lower abundance of nucleoside triphosphates. Changes in nucleotide sugar availability can have a significant impact on glycosylation. For example, lower uridine diphosphate (UDP)-GlcNAc concentrations resulted in reduced sialylation and antennarity of glycans because this nucleotide sugar is a precursor for CMP–sialic acid synthesis (68). In another example, Slade et al. (69) demonstrated an increase of high-mannose glycoforms when feeding with mannose as the carbon source. Higher mannose concentrations inhibit α-mannosidases in the secretory pathway, thus restricting further glycan modifications and effectively stopping the process at the high-mannose step. Because the impact of nucleotide sugar levels can have wide-ranging implications for the final glycoforms, many efforts have been made to increase nucleotide sugar levels, either rationally using metabolic pathway interventions (67) or empirically through medium manipulation. Some of these medium-supplementation strategies, including galactose, glucosamine, and ManNAc additions, are standard approaches in the industry (70). However, as Hossler et al. (71) showed, alternative supplementation options can

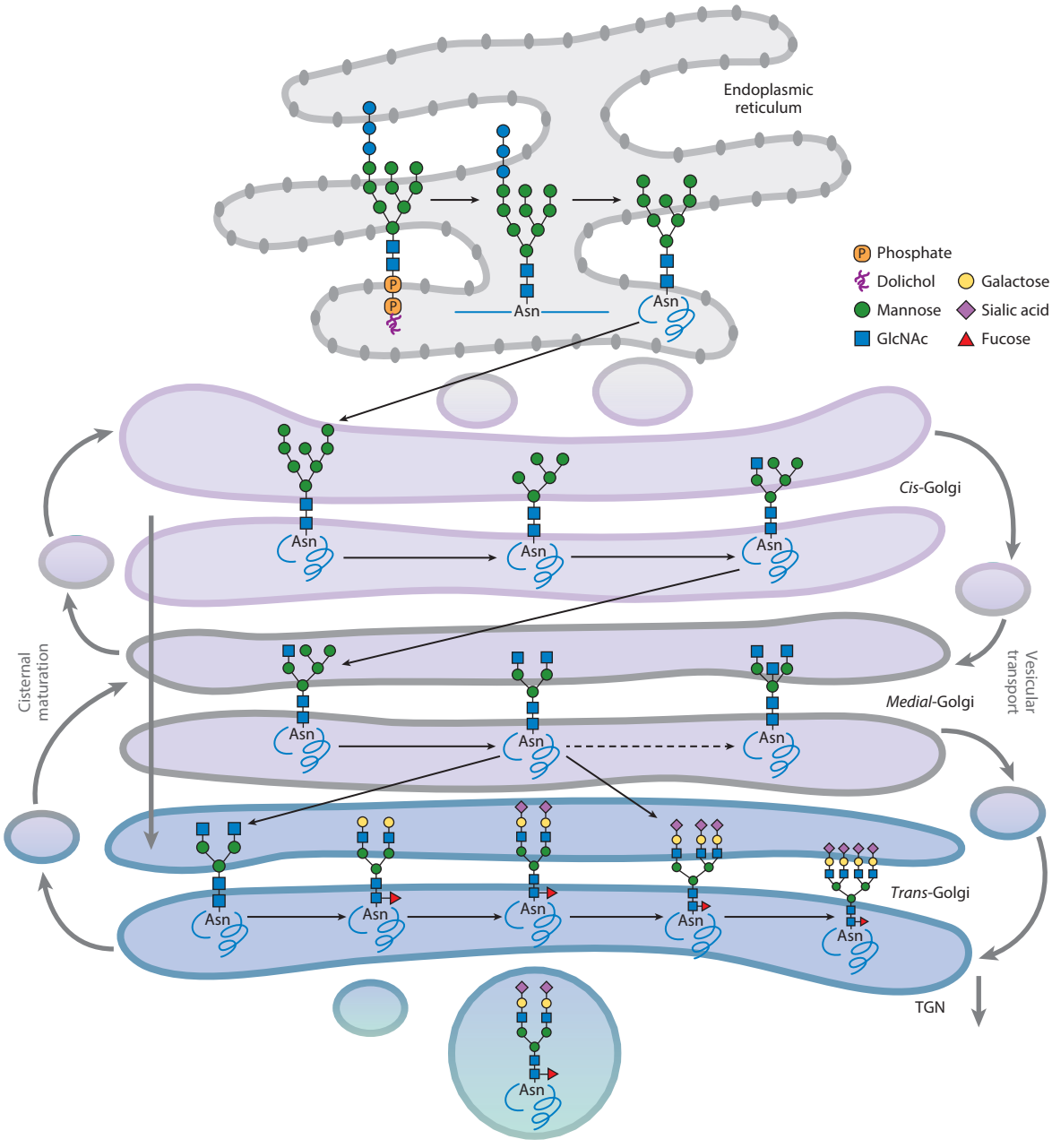


Figure 3

Overview of *N*-glycan processing of a model glycoprotein through the endoplasmic reticulum and Golgi apparatus. As the glycan moves through the secretory pathway, it is further modified by a series of glycosyltransferases to generate more complex glycan forms. On either side, an illustrated comparison of cisternal maturation (retrograde transport) and vesicular transport (anterograde transport) is shown. Please note that this figure shows only a representative set of glycans that can be generated and is not meant to be a complete representation of all possible glycoforms. Abbreviations: GlcNAc, *N*-acetylglucosamine; TGN, *trans*-Golgi network.

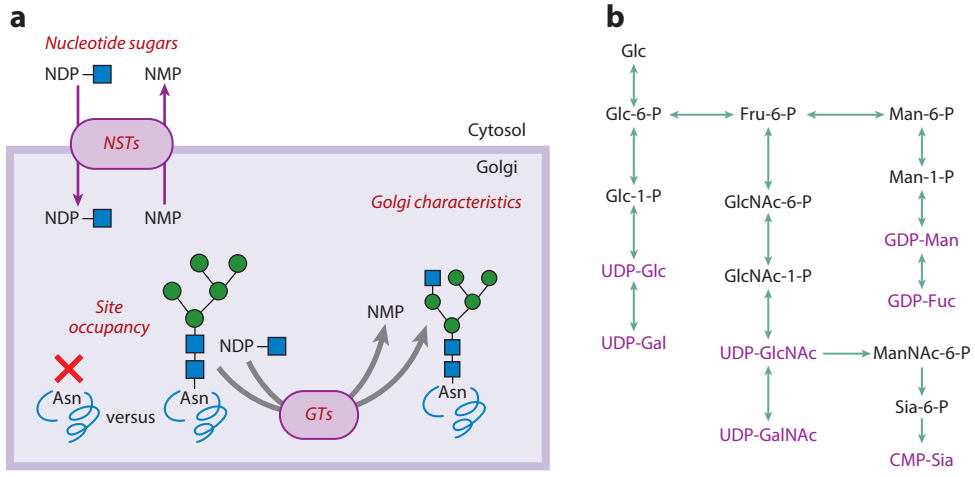


Figure 4

(*a*) Factors that impact glycosylation: nucleotide sugars, synthesized mainly in the cytosol, are transported into the Golgi through NSTs. The site occupancy is a measure of the fraction of available sites and proteins that are glycosylated. Glycans are modified by GTs and glycosidases. Finally, the Golgi environment and characteristics can have an impact on the rate and effectiveness of glycosylation machinery. (*b*) Abbreviated representation of the biosynthesis pathway of nucleotide sugars, starting from glucose. Abbreviations: GT, glycosyltransferase; NDP, nucleotide diphosphate (e.g., guanosine diphosphate); NMP, nucleotide monophosphate (e.g., guanosine monophosphate); NST, nucleotide sugar transporter.

also have a positive impact on protein glycosylation. These include the use of trehalose, raffinose, lactose, and lactulose, whose addition preferentially favors certain glycoforms. Indeed, a recent publication focused on raffinose supplementation detected a higher percentage of high-mannose species, as well as altered expression levels of glycosylation-related genes (72). For an in-depth review of nucleotide sugars, please refer to work by Naik et al. (73).

Nucleotide Sugar Transporters

Nucleotide sugar transporters (NSTs) are antiporter membrane proteins that transport nucleotide sugars into the lumen of the ER and Golgi, as can be seen in **Figure 4*a***. Owing to their specificity, these transporters are typically localized to the Golgi cisternae, where the final glycosylation reaction takes place (*cis*-, *medial*-, or *trans*-Golgi or the *trans*-Golgi network; see **Figure 3**). The distribution of transporters is often close to the location of glycan biosynthesis (74). Without rapid and efficient transport into the secretory pathway, the nucleotide sugars cannot act as substrates for glycan formation. The energy for transport is supplied by the coupled equimolar exchange of the nucleotide sugar for the corresponding nucleoside monophosphate (e.g., GDP-fucose for GMP) (75), and transport occurs only to organelles where glycosyltransferases are localized (76). The abundance and efficiency of NSTs can have dramatic effects on protein glycosylation (77).

Several methods have been developed to increase nucleotide sugar levels in the Golgi apparatus via the overexpression of relevant genes. The overexpression of the HUT1 gene (homolog of UDP-galactose transporter-related gene) in yeast increased both UDP-galactose transport and galactosylation (78). Wong et al. (77) overexpressed CMP–sialic acid transporter in CHO cells producing recombinant human interferon-γ. This overexpression resulted in a 4–16% increase in sialylation in clones overexpressing the transporter when compared with the control. There was an indication of some interplay between the NSTs owing to their related sequence. In both hamsters

and humans, the similarity between UDP-GlcNAc transporter and UDP-galactose transporter is around 50%. Overexpression of UDP-GlcNAc transporter in Lec8 CHO cells resulted in an increase in galactosylation, suggesting that the transporter took on some new functions because of an absence of UDP-galactose transporters (79). A better understanding of the interaction between transporters and the impact of overexpression on overall glycosylation could allow for improved glycoengineering approaches in the future.

GnTV: GlcNac-transferase, transports GlcNAc from UDP-GlcNAc to *N*-glycans to make tetra-antennary *N*-glycans; also called MGAT5 or GnT5

Site Occupancy

Overall, *N*-glycosylation efficiency depends on glycan site occupancy, which has been correlated to the in vivo efficacy of certain protein therapeutics (80). Glycan site occupancy was shown to be improved by two OST forms containing SST3A or SST3B subunits. Both subunits are widely expressed, with SST3A being more active but less selective and SST3B being less active and more selective (81). These two isoforms work in a sequential manner to glycosylate the full range of sites on a protein and contribute to glycan macroheterogeneity. SST3B is hypothesized to help increase site occupancy, as well as glycoprotein folding and quality control (81). Ost3 and Ost6p have been demonstrated to influence oxidative protein folding and increase site occupancy (82).

Site occupancy may also be influenced by extracellular factors. For example, in CHO cells, glucose- and glutamine-limited conditions led to decreased site occupancy (67). Additionally, lipid supplementation led to sustained increases in glycan site occupancy for interferon-γ produced from CHO cells (83). Medium supplementation of manganese and iron divalent metal ions, as well as temperature decrease and the addition of butyrate, has been shown to increase site occupancy (84). Villacrés et al. (85) showed that low glucose led to a decrease of site occupancy by 39%, whereas GDP-sugars decreased by 77% and UDP-hexoses by 90%. In the presence of zaragoic acid A, glycosylation was improved for fibroblasts deficient in Dol-P-Man synthetase-1 through an increase in the production of dolichol and the inhibition of cholesterol synthesis (86). Thus, medium composition and process conditions can impact the site occupancy of glycoproteins and overall efficiency of *N*-glycosylation, making them important parameters for the manufacturing process.

Golgi Characteristics

Golgi characteristics play an important role in influencing glycosylation and have been extensively studied within the context of protein-producing cells (87). The structure of the Golgi depends on certain Golgi proteins, such as Golgi reassembly stacking protein 55 (GRASP55), GRASP65, and golgins (88). A knockdown of GRASP proteins was shown to increase the rate of protein trafficking through the Golgi by 70%. However, in GRASP double-knockdown cells, a major decrease in complex glycans and a minor decrease in high-mannose forms accompanied the increased rate of protein export from the ER, thus illustrating the influence of GRASP proteins on glycan processing (89). The conserved oligomeric Golgi (COG) complex is a vesicle tethering complex that consists of several subunits (90) and is implicated in retrograde transport within the Golgi apparatus (91). Cells deficient in the COG complex have severely dilated Golgi cisternae (92). The major role of the COG complex is likely to ensure proper glycosylation through tight control of distribution of glycosyltransferases and other proteins in various Golgi cisternae (92). Without the proper Golgi conditions, glycan formation can be stalled or unpredictable, resulting in large-scale consequences for recombinant protein quality (93). Researchers found a 49% decrease in the volume of the Golgi apparatus of mutant Lec4 CHO cells that lack *N*-acetylglucosaminyltransferase V (GnTV), as compared with parental CHO cells (94).

Glycosyltransferases and Glycosidases

Golgi glycosyltransferases, responsible for the addition of an activated donor sugar to a glycan structure, are type-II transmembrane proteins with four domains, including a cytoplasmic tail, a transmembrane region, a stem region, and a C-terminal enzymatic domain (95). The role for the majority of these enzymes is to elongate the glycan moiety of the acceptor, whereas others aid with the initial transfer of a sugar to a lipid or polypeptide to begin glycan synthesis of the lipid-linked oligosaccharide (96). Glycosyltransferases are located in the ER and Golgi apparatus of all mammalian cells (97). Specific localization of the glycosyltransferases in the various Golgi compartments is a significant driver for the sequential order in which glycans get processed. Enzymes involved in early glycan processing are located in the *cis*- and *medial*-Golgi compartments, whereas those involved in late glycan processing are located in the *trans*-Golgi compartments (see **Figure 3**). For example, electron microscopy imaging has localized the early-acting GnTI to the *medial*-Golgi (98), whereas the late-acting glycosyltransferases β4GalT1 and ST6Gal1 were localized in the *trans*-Golgi (99). Localization of glycosyltransferases to the different Golgi compartments is influenced by various factors, including the oligomerization of different glycosyltransferases, composition of the transmembrane domain, interactions of the cytoplasmic tail with other proteins, and action of vesicle-tethering complexes (100).

Glycosyltransferases also drive the branching of glycans and, therefore, directly impact the final size of glycoforms. Two glycosyltransferases, GnTIV and GnTV, allow for the formation of tertiary and ternary branches on the glycan (3). Similarly, the addition of a bisecting GlcNAc to the first core mannose by the mechanism of GnTIII (4) inhibits the addition of fucose, thereby altering FcγRIIIa binding and ADCC (17). In contrast, glycosidases are enzymes responsible for the removal of monosaccharides from glycans (96) and aid in glycan degradation and turnover, as well as the formation of intermediate forms that act as substrates for glycan biosynthesis (74). They are imperative for the formation of higher-order complex glycans and are also employed to trim glycans within protein degradation pathways, including the calnexin/calreticulin quality-control cycle (96).

Lipid-linked oligosaccharide: the combination of sugar moieties and lipids (Glc_3-Man_9-$GlcNAc_2$-PP-dolichol) that are transferred to nascent protein in the ER

GnTIV: GlcNac-transferase, transports GlcNAc from UDP-GlcNAc to *N*-glycans to make tri-antennary *N*-glycans; also called MGAT4 or GnT4

MEASURING GLYCOSYLATION AND FACTORS THAT IMPACT GLYCOSYLATION

Given the importance of *N*-glycosylation and its associated processes, various methods have been developed to measure and characterize glycans, as well as associated biomolecules.

Analytical Methods for Glycan Characterization

For most antibodies, a single *N*-linked glycosylation site is present on the Fc region; however, for many other proteins, multiple *N*- and *O*-glycosylation sites may exist. In these cases, it may be critical to evaluate the glycan profile on a site-specific basis. Glycan analysis can be divided into three categories: (*a*) analysis of the intact glycoprotein, (*b*) analysis of glycopeptides, and (*c*) analysis of glycans released using chemical or enzymatic methods. Of these, only the first two methods preserve the site specificity of the glycans. These three methods have been reviewed widely (101–103) and are briefly summarized below and in **Figure 5*a***.

The large size of the mAb often complicates intact glycoprotein analysis via MS, predominantly owing to issues with intact glycoprotein solubility and adsorption to the stationary phases. However, alternate methods exist that require little sample preparation and can wholly characterize glycoproteins. In a protocol complementary to mass spectroscopy, Schubert et al. (104) generated a method for intact glycoprotein analysis using nuclear magnetic resonance (NMR) spectroscopy.

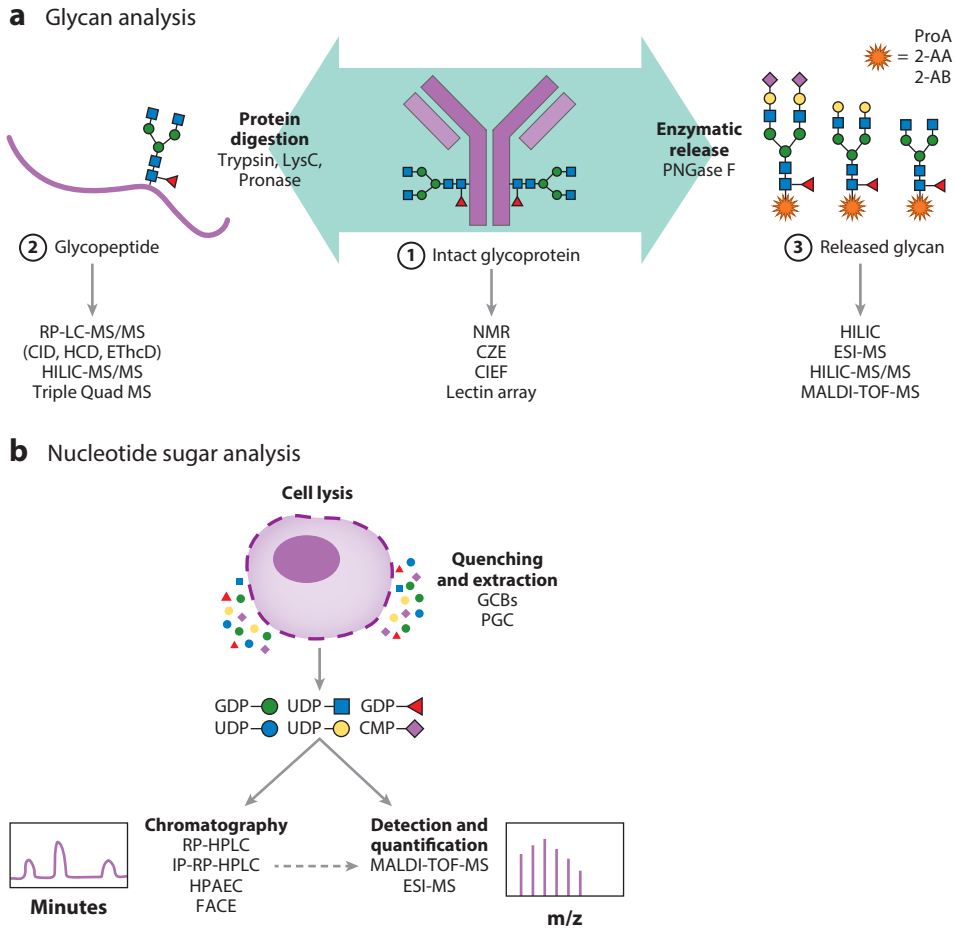


Figure 5

(*a*) Summary of analytical methods for glycan analysis: Intact glycoproteins (①) can be analyzed without further modification, or they can be digested to form glycopeptides (②). Additionally, glycans can be released from the protein with an enzyme (③) and then labeled with a fluorescent molecule and analyzed. Intact glycoprotein can also be analyzed with a lectin assay whereby the glycoprotein binds to a lectin and is then tagged with an antibody and a fluorescent dye. (*b*) Summary of analytical methods for nucleotide sugars: Nucleotide sugars first need to be quenched and extracted from the cell, which can be done with certain solid-phase columns, as well as a variety of reagents. The extracted nucleotide sugars can then be first separated and later quantified with a combination of methods or go straight to detection and quantification. Abbreviations: CID, collision-induced dissociation; CIEF, capillary isoelectric focusing; CMP, cytidine monophosphate; CZE, capillary zone electrophoresis; ESI, electrospray ionization; EThcD, electron-transfer/higher-energy collision dissociation; FACE, fluorophore-assisted carbohydrate electrophoresis; GCB, graphitized carbon black; GDP, guanosine diphosphate; HCD, high-energy collision dissociation; HILIC, hydrophilic interaction liquid chromatography; HPAEC, high-performance anion-exchange chromatography; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-associated laser desorption/ionization–time of flight; MS, mass spectrometry; NMR, nuclear magnetic resonance; PGC, porous graphitic carbon; RP-LC, reversed-phase liquid chromatography; UDP, uridine diphosphate.

NMR spectroscopy can identify the stereochemistry and linkage type of the glycan but cannot assign the glycan site. Intact glycoproteins can also be analyzed using lectin arrays, capillary zone electrophoresis, and capillary isoelectric focusing (105). Capillary zone electrophoresis separates based on size-to-charge ratios, and capillary isoelectric focusing separates based on the isoelectric point, allowing for the separation and detection of differentially glycosylated forms on the same glycoprotein. Lectins are proteins that bind to sugar moieties with high specificity and have been used for characterizing glycans from mammalian cells for decades (106). In particular, lectin binding allows for high-throughput detection of specific glycoforms on intact glycoproteins through fluorescence or SPR detection (107). However, lectin measurements may not be as quantitative as some of the other methods and are instead suitable as an initial screening tool, followed by more in-depth analysis, as appropriate.

The second category involving analysis of glycopeptides allows for site-specific glycan profiling. In this approach, the protein is cleaved with a protease like trypsin, LysC, or pronase (108) to generate shorter peptides for easier analysis. Glycopeptides have lower ionization efficiency than peptides and, therefore, often require an enrichment step (103). Enrichment can be performed with lectins, hydrophilic interaction solid-phase extraction, chromatography, or graphitized carbon solid-phase extraction (109). Tandem MS can be used to fully characterize glycopeptides using various ionization modes, including collision-induced dissociation (CID), high-energy collision dissociation (HCD), and a hybrid method combining electron-transfer dissociation with HCD termed EThcD (101, 110). CID tends to cleave glycosidic bonds but does not provide sufficient information for glycosylation sites and amino acid sequences. HCD provides diagnostic oxonium ions and partial glycopeptide information. EThcD is a two-step fragmentation strategy, obtaining multiple ion types and allowing for a more thorough glycopeptide characterization (110).

A third glycan analysis tool involves the release of the glycan followed by the attachment of a fluorescent molecule for detection and to increase MS ionization energy. This labeling is most commonly done with reductive amination, but permethylation is also often employed (102). The *N*-glycan is released from the protein using an enzyme, typically PNGase F, and then labeled with a fluorophore, such as 2-aminobenzamide, 2-aminobenzoic acid, or procainamide, via reductive amination (102). The labeled glycans are then typically separated on a hydrophilic interaction liquid chromatography column and subsequently detected with a fluorescence meter. Alternately, following separation, the glycans can be run on MS using electrospray ionization–MS (ESI-MS) or matrix-assisted laser desorption/ionization–time of flight–MS (MALDI-TOF-MS) (102). For MS analysis, labeling with procainamide can both increase the fluorescent intensity and improve ESI efficiency (111).

Enhanced Mass Spectroscopy Characterization Using Stable Isotope Labeling

The mass difference between stable isotopes can be exploited with the various MS approaches described above. The incorporation of stable isotopes allows for a distinction between labeled and unlabeled glycans, thus enabling advanced glycan characterization (112). The DuSIL (duplex stable isotope labeling) method has been optimized for detecting sialylated species, which are notorious for having lower ionization efficiency in MS analysis (113). The sialic acids are methylamidated before the reductive amination step, for the dual purposes of protecting them from degradation and of introducing a mass difference that allows for facile quantification of the number of sialic acids present on the glycan based on the resulting mass shift. Pabst et al. (114) described the use of zwitterionic hydrophilic interaction liquid chromatography, coupled with in vitro stable isotope labeling via $^{13}C_4$ succinic anhydride, to allow for a quantitative comparison of a labeled glycopeptide internal standard with an unlabeled glycopeptide sample in the same MS experiment. IDAWG (isotopic detection of aminosugars with glutamine), which uses ^{15}N-Gln, allows for a rapid

integration of the stably labeled nitrogen into the hexosamine biosynthesis pathway (115), going a step further by enabling quantitative glycan comparisons between different biological conditions via MS analysis. Stable isotope labeling coupled with MS analyses is thus an emerging approach for gaining novel understanding of *N*-glycosylation.

Analytical Methods for Nucleotide Sugar Characterization

Numerous studies have sought to analyze and quantify nucleotide sugars via analytical methods, including liquid chromatography and capillary electrophoresis (**Figure 5*b***). Before samples can be analyzed, the nucleotide sugars must be extracted. The two main methods for solid-phase extraction involve graphitized carbon, including graphitized carbon blacks and porous graphitic carbons (PGCs) (116). Braasch et al. (117) selected the ENVI-carb column after conducting a thorough screen of available analytical approaches, including quenching optimization and extractions with methanol, acetonitrile, and chloroform. Upon extraction, the nucleotide sugars were quantified using high-performance anion-exchange chromatography (HPAEC). In a separate study, all the principal nucleotide sugars, as well as the nucleotides except for ADP and CTP, were detected through HPAEC with the CarboPacP PA1 column under alkaline conditions (118). The Barnes group (119) used the ENVI-carb column and combined it with fluorophore-assisted carbohydrate electrophoresis to generate a protocol for nucleotide sugar analysis that does not require expensive analytical instrumentation. Some examples with PGC columns include work by Pabst et al. (120), who quantified nucleotide sugars using PGC in tandem with liquid chromatography–ESI-MS. Rejzek et al. (121) implemented nucleotide sugar detection using ESI followed by tandem MS. Another method to measure interactions between nucleotides and NSTs is saturation transfer difference NMR spectroscopy. Cell culture conditions can strongly impact NST expression levels. Wang et al. (122) measured NST expression at low CO_2 settings using real-time polymerase chain reaction (PCR), which led to a 78% increase in UDP-GlcNAc transporter levels and a 106% increase in UDP-galactose transporter levels, resulting in an ultimate improvement in mAb galactosylation.

Analytical Methods for Glycosyltransferase Characterization

The transcript expression of glycosyltransferases can be measured using quantitative reverse-transcription PCR, along with colorimetric and fluorescent assays (123). Colorimetric assays depend on the release of a signal upon the addition of a molecule that can react with a UDP-sugar substrate (e.g., UDP-galactose). Enzyme activity assays are another analytical tool for direct measurement of glycosyltransferase function. Shen et al. (124) were able to characterize recombinant glycosyltransferases by measuring kinetic parameters and pH values. The absorbance change of *p*-nitrophenolate was quantified to indicate the digestion of a glycosyl acceptor by an exoglycosidase. Kumagai et al. (125) were able to expand the scope of these types of assays to any substrate, including UDP-, GDP-, and CMP-sugar substrates. ADP is generated from these nucleotides and then converted to resazurin and finally to fluorescent resorufin, allowing for fluorescent detection in an assay that can be performed at the 384-well scale, suitable for high-throughput analysis. Finally, traditional molecular biology approaches such as western blotting, SDS-PAGE, and intracellular labeling can be used for glycosyltransferase detection.

Analytical Methods for Site Occupancy Characterization

Site occupancy can be quantified in several ways, including label and label-free quantification, often in combination with MS or Raman spectroscopy. One example is to use a green fluorescent

protein biomarker that remains in the ER and whose fluorescence is lost when it is glycosylated (126) in a method that has the potential for high-throughput screening. Goldman et al. (127) used micellar electrokinetic capillary electrophoresis to monitor site occupancy and performed a time-course to see how the occupancy of two glycan sites varies over time. Finally, researchers have used Raman spectroscopy to estimate glycosylated and unglycosylated antibodies in cell culture (2), which provides flexibility in allowing multiple sampling over the course of a cell culture run.

TGN38: a protein that resides in the TGN, cycles between the TGN and plasma membrane

Analytical Methods for Golgi pH and Protein Production Characterization

The pH of the cell, as well as that of the secretory pathway, can be measured by spectroscopic probes. However, because of the difficulty of reaching these inner compartments, measurements within the secretory pathway have proven challenging (128). Alternate methods for measuring Golgi pH have been developed that depend on the use of a probe molecule with a Golgi localization domain fused to a pH-sensitive dye. One example is the *E. coli* verotoxin VT1B, covalently linked with a pH-sensitive fluorescent probe, that is taken up by the Golgi via retrograde transport and retains its sensitivity after uptake (129). In another study, a chimeric protein, designed by combining the ectodomain of CD25 with domains of TGN38, was taken up through internalization to the *trans*-Golgi network (TGN). An anti-CD25 antibody linked with a pH-sensitive fluorophore allowed for the measurement of the CHO cell TGN pH of 5.95 ± 0.03 (130).

Along with Golgi pH, the rate of protein production through the secretory pathway can also impact glycosylation. At very high production rates, the protein may not spend enough time in the Golgi to be adequately glycosylated. Indeed, higher production rates of human tyrosinase, a glycoprotein with seven *N*-glycosylation sites, causes one of the glycan sites (Asn290) to be suppressed (131). A technique called pulse-chase stable isotope labeling by amino acids in cell culture (pcSILAC) allows for the measurement of newly synthesized proteins from two conditions in one experiment along with the levels of preexisting proteins (132). These types of methods can be useful for elucidating the role of protein synthesis in protein glycosylation profiles.

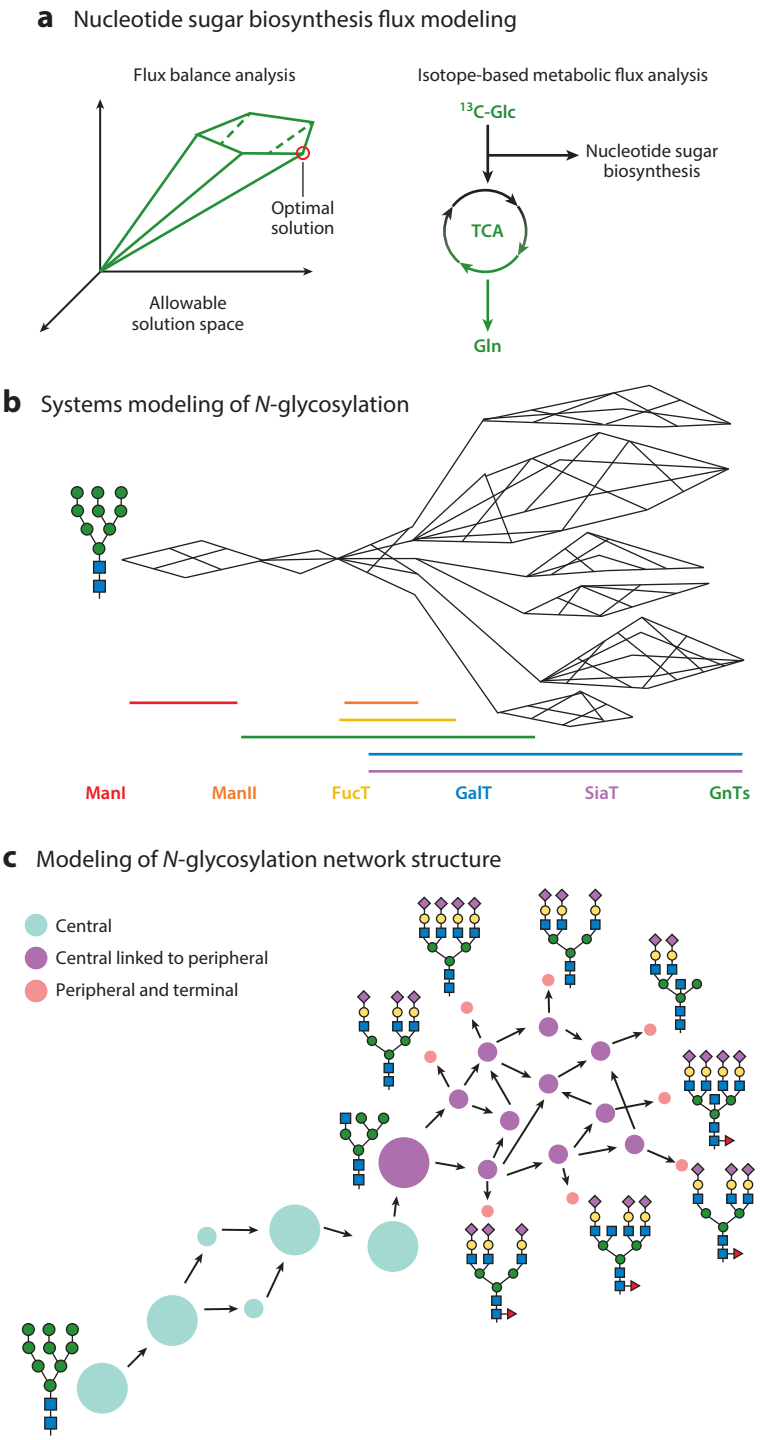
MODELING GLYCOSYLATION

The complex and non-template-driven nature of protein *N*-glycosylation, along with the variety of enzymes and substrates that may act on a specific *N*-glycan precursor, lends this process to in silico modeling approaches (133). A multitude of variables can be incorporated into the model, including the concentrations of enzymes, kinetic constants, enzyme distribution, speed of protein processing in the secretory pathway, and volume and configuration (plug-flow or well-mixed) of the Golgi (134). Additionally, metabolic flux analysis has been implemented and applied to glycosylation and nucleotide sugar biosynthesis, including both flux balance analysis and isotope-based flux analysis. These methods can quantify intracellular metabolic flux and are illustrated briefly in **Figure 6*a*** (135). Numerous models have been generated and optimized over the past two decades for CHO glycosylation, allowing researchers to better predict how different system perturbations will impact the glycan profile and to better understand the intricacies of the glycosylation process. A brief overview is provided below. Please refer to Puri & Neelamegham (133) for more detailed information about the mathematical basis behind glycosylation modeling.

In one of the first mathematical models for glycosylation, Shelikoff et al. (136) focused on determining glycosylation site occupancy and glycosylation initiation. The model used the co-translational nature of glycosylation to set it apart from other protein folding processes because glycosyltransferases are membrane bound, meaning that they can work only when the protein is translocated into the ER and Golgi. Another model constraint only allowed for nucleotide sugar

Figure 6

Summary of glycosylation modeling approaches. (*a*) Flux balance analysis uses extracellular metabolite measurements as inputs and involves optimization of a cellular objective function within an allowable solution space. Isotope-based metabolic flux analysis relies on measurement of intracellular stable isotope–labeled metabolites in conjunction with known biochemistry of metabolic reactions to calculate fluxes through metabolic pathways. (*b*) The *N*-glycosylation network, inspired by the visualization by Hossler et al. (140) using pathway maps (GlycoVis). The lines represent where each glycosyltransferase enzyme is active along the pathway. (*c*) Kim et al. (141) generated an extensive network *N*-glycosylation, of which only a fraction is illustrated here. The circle sizes indicate the influence of the glycan on the rest of the network, with larger circles representing glycans with the greatest influence. Abbreviation: TCA, tricarboxylic acid cycle.



attachment in the absence of steric hindrance on the existing glycan structure. Together, the model demonstrated the dependence of glycosylation site occupancy on protein synthesis and mRNA elongation rates. Another model by Monica et al. (137) chose to focus on a specific step in the glycosylation network: terminal sialylation. This focus allowed for the more manageable modeling of a single glycosyltransferase (sialyltransferases) and a single compartment (TGN). Next, Umaña & Bailey (UB) (134) implemented the first network-based kinetic model containing 33 glycan species, including tri- and tetra-antennary glycans. Kinetic constants used in the model were based on previously published research. Glycan distribution trends were calculated from changes in glycosyltransferase levels (134) and were illustrated, for example, for the overexpression of GnTIII.

LacNAc: *N*-acetyllactosamine, a sugar moiety that is a GlcNAc bound to a galactose, found on both glycoproteins and glycolipids

By using a network-generation algorithm based on glycan biosynthesis rules for the various glycosyltransferases, Krambeck & Betenbaugh (KB) (138) were able to expand upon the UB model by adding additional glycosyltransferase steps, including the extension of LacNAc branches and capping with sialic acid, as well as substrate concentration effects, generating a comprehensive *N*-glycosylation Golgi model. The expansion increased the number of structures possible to thousands and the number of reactions to tens of thousands. The KB model was initially developed for CHO cells and later expanded to include human *N*-glycans and associated enzymes (139). Using these models, Krambeck et al. (139) were able to fit the model to existing MS data sets for glycans by automatically annotating the spectrum and were able to calculate the predicted underlying enzyme activities responsible for the resulting glycan profile. Both the UB and KB models represented the Golgi as a series of four continuous mixing-tank reactors based on the vesicular transport mechanism, in which the compartments are stationary and cargo is transported between them through vesicles, as can be seen in **Figure 3**. Alternatively, Hossler et al. (140) modeled the Golgi as a series of four plug-flow reactors, based on the Golgi maturation mechanism, in which the cargo is stationary while the surrounding compartment matures from the *cis*-Golgi to the *trans*-Golgi, as illustrated in **Figure 3**. The simulation showed that no single enzyme played a dominant role in routing flux to a specific glycan form. To selectively generate a specific glycan, multiple enzyme levels would have to be manipulated. Additionally, the researchers generated a network visualization tool, GlycoVis, showing the interplay of various glycosylation enzymes along with their relative reaction rates, as diagrammed in **Figure 6*b*** (140).

Multiple approaches have been implemented to generate glycosylation models, including complex networks, Markov chain modeling, and discretized reaction network modeling using fuzzy parameters (DReaM-zyP) (141–143). Using a complex network approach, Kim et al. (141) generated a modular glycosylation model, which distinguished between the central and peripheral region of the glycosylation pathway, shown in **Figure 6*c***. The network helped pinpoint the 4.2% of glycan species (such as Man9 and A1G0M5, a hybrid structure with one GlcNAc, in the central or core region) that have dramatic effects on the resulting final glycan profile. Their removal has widespread effects, compared with the remaining 95.8%, which impact the generation of only 3 or fewer glycan forms. The Markov chain model, combined with flux-balance analysis, does not require external kinetic information (142). This model helped reaffirm the importance of localization of glycosyltransferases in the Golgi. For example, a Fut8 knockout prediction changed significantly if there was competition between GalT and Fut8 owing to insufficient separation within the network. Using DReaM-zyP, Kremkow & Lee (143) developed the Glyco-Mapper, a Microsoft Excel model that generates visual representations of glycan networks, showing which glycan forms will or will not be present. The model accurately predicted the glycan profiles of differently engineered CHO cell lines from previously published studies.

Building off the Golgi maturation mechanism, del Val et al. (DV) (144) expanded previous glycosylation models to include the recycling of Golgi proteins, including glycosyltransferases and

NSTs. This DV model also included the transport of nucleotide sugars from the cytosol into the ER and Golgi. By accounting for these factors, the researchers could map the location of specific glycosyltransferases in the cisternae of the Golgi apparatus, along with the corresponding enzyme concentrations. They also created a visual representation of the enzymes and crosstalk or competition between them. This model spurred the development of multiple combinations of unstructured cell culture models following the DV Golgi maturation-based model to better illustrate the intricacies and importance of the cell culture and glycosylation environments (145). Furthermore, the nucleotide sugar donor synthesis network was later incorporated, expanding upon the DV model (146), including nucleotide sugars required not only for recombinant protein production but also for host-cell protein and glycolipid biosynthesis (147). The model indicated that nucleotide sugar consumption toward host-cell protein glycosylation was significant and, therefore, a necessary factor to consider when modeling cell growth and glycosylation.

As a result of the success of these glycosylation models, the focus has recently shifted to effectively modeling scenarios that incorporate cell culture growth and productivity data to test out various process conditions. One such application has been for perfusion cell culture (148). Compared with fed-batch cultures, the three main glycan forms seen in perfusion culture (A2G0F, A2G1F, and A2G2F) did not vary more than 3% over the 20-day culture process for human recombinant antibody production with an alternating tangential flow–equipped perfusion bioreactor. Through experimental verification, it was shown that the model was able to accurately represent the impact of ammonia, manganese, and galactose supplementation on perfusion cultures. Similarly, Sou et al. (149) adapted the DV model to input the cytosolic concentration of nucleotide sugars directly into the model and applied it to a fed-batch process incorporating a temperature shift. UDP-galactose synthesis and GalT level and activity were found to be important drivers for glycosylation. The authors recommended modifying GalT expression to ensure proper glycosylation, even at lower temperatures. In a design space analysis, St. Amand et al. (150) illustrated that for some bioreactor processes, the impact of changing temperature, glucose, agitation, pH, and dissolved oxygen on glycosylation was minor. However, for the processes that were more susceptible to change, their model could predict which change allowed for the generation of the desired glycan profile. Focusing on galactosylation, a recent model combining cell metabolism, antibody production, nucleotide sugar synthesis, and glycosylation found that galactose supplementation decreased the glucose consumption rate in CHO cells (151). This optimized model yielded a process strategy that increased antibody galactosylation to 93% for all glycans with no negative process outcomes. The prediction capabilities of glycosylation models should continue to improve with the advent of a more expansive understanding of glycosylation pathways within production cells, coupled with improvements in machine learning approaches.

CONCLUSION

N-glycosylation is an important posttranslational modification that plays a critical role in the biological function of IgG and other therapeutic glycoproteins. This complex process is influenced by several contributing stochastic factors that may act intracellularly or through the external environment in which cell culture is performed; it is thus both critical and difficult to generate desirable profiles for therapeutic glycoproteins. Fortunately, many analytical approaches have been and continue to be developed that enable characterization not only of the glycans but also of the various factors that influence them. Additionally, significant developments have been made in modeling of glycosylation pathways that, coupled with the analytical methods, will continue to provide insights, aiding efforts ranging from glycoengineering to process optimization for rational glycoprotein generation during biomanufacturing in the future.

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