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Glycosylation Shapes the Efficacy and Safety of Diverse Protein, Gene and Cell Therapies

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Abstract

Over recent decades, therapeutic proteins have had widespread success in treating a myriad of diseases. Glycosylation, a near universal feature of this class of drugs, is a critical quality attribute that significantly influences the physical properties, safety profile and biological activity of therapeutic proteins. Optimizing protein glycosylation, therefore, offers an important avenue to developing more efficacious therapies. In this review, we discuss specific examples of how variations in glycan structure and glycoengineering impacts the stability, safety, and clinical efficacy of protein-based drugs that are already in the market as well as those that are still in preclinical development. We also highlight the impact of glycosylation on next generation biologics such as T cell-based cancer therapy and gene therapy.

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Author Contributions

FR, AGP, SS, JS, MW, TF, EAT and NEL all contributed to the writing of the manuscript. FR, AGP and SS created and formatted the figures. FR, EAT, TF and NEL participated in the review and editing of the manuscript

Declaration of Interests

- NEL is a co-founder and holds stock in NeuImmune, Inc. and Augment Biologics, both of which focus on glycoprotein therapeutics
- TF is a co-founder and holds stock in NeuImmune, Inc. which focuses on glycoprotein therapeutics.
- All other authors have no conflicts of interest to disclose.

Keywords

glycosylation; glycoengineering; biologic; therapeutic protein; gene therapy; cell-based therapy; monoclonal antibody

1. Glycosylation of Therapeutic Proteins

Since the commercial release of human insulin as the first therapeutic protein in the early 1980s, biologics have been the fastest-growing class of therapeutic molecules. In 2019 alone, the market share for biopharmaceuticals amounted to over 200 billion dollars in the United States(Feng et al., 2022, Moorkens et al., 2017, de Bousser et al., 2023), with over 350 new products approved for clinical use by the Food and Drug Administration by 2021(Feng et al., 2022). Therapeutic proteins make up the biggest fraction of the biologics sector, encompassing a plethora of antibodies, vaccines, immune factors, hormones, blood factors, and enzymes and are used to treat both communicable and non-communicable diseases such as cancer, diabetes, multiple sclerosis, and SARS-CoV-2, to name a few (Figure 1).

The dominance of protein-based therapeutics in the market speaks to their immense positive impact in the clinic. Compared to small molecule drugs, proteins demonstrate high target specificity, which can result in lower toxicity from fewer off-target effects and improved pharmacological potency. However, these biopharmaceuticals are not without issues, such as intrinsic limitations in their physicochemical and pharmacological characteristics. Thus, a focal point of biologic development has been to generate more efficacious formulations of therapeutic proteins through protein and cellular engineering.

Many protein-based drugs are engineered glycoproteins that are recombinantly expressed in animal cell-lines, and almost all such biopharmaceuticals undergo post-translational modification (PTM). Perhaps the most important class of PTM for many biologics is glycosylation, a process that occurs on most eukaryotic secreted and membrane proteins. Glycosylation involves the covalent addition of carbohydrates (glycans) to a protein through two major linkages: (a) the amide nitrogen atom on an asparagine (Asn) residue (N-linked glycosylation), and (b) the hydroxyl oxygen on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues (O-linked glycosylation). These carbohydrate groups can be a single monosaccharide or chains of branched or linear oligosaccharides(Reily et al., 2019, Varki et al., 2022) (Figure 1). Furthermore, a glycoprotein can have many different “glycoforms”, with variations pertaining to either glycosylation site occupancy (macroheterogeneity) or differences in glycan structure (microheterogeneity). Not only does glycosylation increase protein structural diversity, glycan heterogeneity is also a crucial contributor in determining biophysical, and pharmacological properties of glycoproteins. Glycoengineering—the manipulation of glycan composition—has therefore been an invaluable tool in generating products that demonstrate optimal therapeutic efficacies(Sola et al., 2007, Sola and Griebenow, 2009, Ma et al., 2020, Sinclair and Elliott, 2005, Chen et al., 2022, Dammen-Brower et al., 2022). This process can pertain to the addition or removal of glycosylation sites on a protein, or the alteration of its native glycosylation profile. Because of the impact

of glycans on protein structure, function, and dynamics, we view glycoengineering as an essential protein engineering method that complements and amplifies changes introduced by mutagenesis. In this review, we describe how glycosylation significantly impacts key characteristics of protein-based drugs such as their stability, transport and uptake, half-life, therapeutic efficacy, and immunogenicity. We also discuss how glycoengineering can be applied to improve newer classes of biologics such as T cell- and oligonucleotide-based therapies.

1.1 Stability

Proteins are innately prone to degradation due to physical and chemical processes like denaturation, proteolysis, aggregation, oxidation and hydrolysis. Overcoming the inherent instability of glycoproteins is key to therapeutic protein development. Preservation of glycoprotein conformation ensures that they remain intact and functionally active during storage and after they have been administered to patients. Degradation of therapeutic proteins can result in reduced or complete loss of efficacy and compromised safety.

The presence of large, hydrophilic groups such as glycans on a protein can improve their stability by preventing aggregation, contributing to increased thermal and chemical stability, and making them more resistant to enzymatic degradation(Lis and Sharon, 1993, Mitra et al., 2006, Shental-Bechor and Levy, 2008, Sola and Griebenow, 2009, Sola et al., 2007, Zheng et al., 2011, Zhou and Qiu, 2019, Wang et al., 1996, Lee et al., 2015). Glycosylation can lead to an increase in internal electrostatic interactions, and strengthen hydrogen bonds and hydrophobic interactions within the protein, making it more resistant to denaturation(Sola and Griebenow, 2009, Sola and Griebenow, 2006, Sola et al., 2007, Lee et al., 2015). Glycans around the peptide backbone also make the protein less accessible to proteases through steric hindrance, thereby making them less susceptible to proteolysis(Nishiyama et al., 2000, Sola and Griebenow, 2009). One mechanism through which glycosylation improves solubility is to increase the solvent-accessible surface area of the glycoprotein facilitated by the presence of the glycans(Tams et al., 1999, Paul et al., 2021, Sola and Griebenow, 2006). This can prevent protein aggregation, which often leads to an increased likelihood of adverse immune reactions and accelerated clearance from the bloodstream(Lundahl et al., 2021, Pham and Meng, 2020, Wang, 2005). Resistance against aggregation is therefore important in preserving both the safety and efficacy of the molecule.

Most biologic drugs are typically more stable and less prone to aggregation, and denaturation than their non-glycosylated counterparts (Supplementary Table 1). Biologics such as interferon-β (IFN-β)(Runkel et al., 1998, Karpusas et al., 1998, Farrell et al., 2012), alpha-1 antitrypsin (AAT)(Jeppsson et al., 1975, Travis et al., 1985, Kwon and Yu, 1997), granulocyte stimulating factor (GCSF)(Oh-eda et al., 1990), erythropoietin (EPO)(Narhi et al., 1991) become more susceptible to aggregation and thermal degradation upon removal of glycan moieties, to name a few examples. Protection against protease digestion can also be imparted just by the presence of glycosylation, such as in the case of interferon-γ(Sareneva et al., 1995), interferon-α(Ceaglio et al., 2008, Ceaglio et al., 2010) and GCSF(Carter et al., 2004). Furthermore, the abundance and type of attached carbohydrates can also be tweaked to improve physical characteristics of the protein. Site specific addition of glucosyl moieties

on human insulin improved physical stability in solution(Baudys et al., 1995, Uchio et al., 1999). Indeed, a glycoengineered variant of insulin that is O-mannosylated at Thr27 (B-chain) has a comparable activity to the naturally occurring protein while being more resistant to enzymatic degradation and oligomerization(Guan et al., 2018).

1.2 Half-life

The therapeutic efficacy of a biopharmaceutical is largely impacted by how long it remains functional in circulation. Glomerular filtration by the kidneys eliminates small proteins and peptides, and depends mostly on size and charge, with molecules under ~30 kDa typically being eliminated through this route(Tryggvason and Wartiovaara, 2005, Maack et al., 1979, Mahmood and Green, 2005, Dammen-Brower et al., 2022). Since the glomerular filter is negatively charged, anionic peptides are typically repelled by the charge similarity and therefore less likely to be eliminated(Rennke et al., 1975, Bocci, 1989, Tryggvason and Wartiovaara, 2005, Mahmood and Green, 2005). Apart from renal filtration, protein clearance is also mediated by receptors that recognize and bind specific types of glycans. Two avenues of serum clearance are through the mannose receptor (MR) (Stahl, 1992, Ashwell and Harford, 1982) and the asialoglycoprotein receptor (ASGPR) (Stockert, 1995, Ashwell and Harford, 1982, Ashwell and Morell, 1974) (Figure 2A). Both are C-type lectin receptors that recognize and bind specific carbohydrates decorating glycoproteins, thereby facilitating their elimination from the blood. Mannose receptors are found primarily in liver Kupffer and endothelial cells(Hubbard and Stukenbrok, 1979, Schlesinger et al., 1978, Linehan et al., 1999, Takahashi et al., 1998) and immune cells, such as macrophages(Shepherd et al., 1982, Stahl et al., 1978, Stahl and Gordon, 1982, Wileman et al., 1986, Largent et al., 1984, Martinez-Pomares, 2012) and immature dendritic cells(Sallusto and Lanzavecchia, 1994, Martinez-Pomares, 2012). They bind glycans bearing a terminal mannose, fucose, or N-acetylglucosamine (GlcNac)(Stahl, 1990, Taylor and Drickamer, 1993). ASGPRs, on the other hand—while also abundantly found in liver cells—recognize terminal β-linked galactose or N-acetylgalactosamine (GalNac) that have been desialylated; hence the prolonged serum longevity of highly sialylated glycoproteins(Stockert, 1995, Ashwell and Harford, 1982, Morell et al., 1968, Morell et al., 1971, Ashwell and Morell, 1974, Van Den Hamer et al., 1970, Park et al., 2003). Additionally, ASGPRs can bind and clear proteins bearing a terminal Sia2,6GalNac and Sia2,6Gal(Park et al., 2005), suggesting that in addition to sialic acid capping, engineering the terminal linkage to be α-2,3 instead of α-2,6 could prolong the half-life of a therapeutic protein(Andre et al., 2004, Andre et al., 1997, Unverzagt et al., 2002, Tian et al., 2019).

Because a protein's circulatory half-life can be significantly impacted by glycosylation, evading and bypassing the different serum clearance mechanisms through glycoengineering is important aspect in biopharmaceutical design (Supplementary Table 2). Adding or modifying sugar groups to increase the size, hydrodynamic radius and net negative charge of the protein can decrease the rate of removal via kidney filtration. Incomplete glycosylation or glycan removal has resulted in recombinant human proteins with much shorter *in vivo* half-lives compared to their fully glycosylated counterparts—such as in the case of AAT(Travis et al., 1985, Ross et al., 2012, Yu and Gan, 1978, Weber et al., 1985), EPO(Wasley et al., 1991, Yamaguchi et al., 1991, Fukuda et al., 1989) and Granulocyte-

Macrophage Colony Stimulating Factor (GM-CSF)(Okamoto et al., 1991). Conversely, simply adding more attached carbohydrates to proteins—such as EPO(Su et al., 2010, Elliott et al., 2004), follicle-stimulating hormone (FSH)(Perlman et al., 2003, Weenen et al., 2004) and interferon-α(Ceaglio et al., 2008)—can prolong circulation and activity *in vivo*. Glycoengineering to increase in levels of sialylation or sialic acid capping of terminal galactose or GalNac is also utilized to inhibit ASGPR binding and limit hepatic clearance. This approach has been applied to improve serum longevity for therapeutic proteins such as EPO(Egrie et al., 2003, Egrie and Browne, 2001), human growth hormone(Flintegaard et al., 2010), α-galactosidase(Sohn et al., 2013a), iduronate sulfatase(Muenzer et al., 2007, Muenzer et al., 2006), and β-glucuronidase(Cadaoas et al., 2020).

1.3 Transport and Uptake

In addition to eliminating molecules from circulation, glycan-binding cell-surface receptors also participate in the cellular targeting and tissue distribution of therapeutic glycoproteins, particularly those used for enzyme replacement therapy (ERT). Lysosomal storage diseases (LSDs) are inherited metabolic disorders with a deficiency of lysosomal enzymes and an accumulation of unwanted metabolites, ultimately resulting in clinical dysfunction in peripheral organs and the central nervous system(Futerman and van Meer, 2004, Parenti et al., 2013, Bonam et al., 2019). Many LSDs are treated by ERT, whereby patients receive intravenous supplementation of the deficient enzyme—which is itself a glycoprotein. Trafficking and uptake of these lysosomal enzymes are mediated largely by the mannose-6-phosphate dependent pathway, which involves recognition and binding by mannose-6-phosphate receptors (MPRs)(Sly, 1985, Parenti et al., 2013, Varki and Kornfeld, 1980, Achord et al., 1978, Stahl et al., 1978) (Figure 2B). Like ASGPRs and MRs, MPRs are lectins that recognize and bind specific glycosylation features on glycoproteins, particularly those that bear mannose-6-phosphate residues(Kaplan et al., 1977, Seo and Oh, 2022). It is therefore no surprise that glycan manipulation of ERT enzymes for better lysosomal targeting has improved their cellular uptake and therapeutic efficacy.

Gaucher's Disease is one of the very first LSDs for which ERT was developed(Barton et al., 1991). This disorder is typified by a deficiency in β-glucocerebrosidase(Brady et al., 1966, Brady et al., 1965), a lysosomal hydrolase internalized by macrophages through an MR-dependent pathway(Sato and Beutler, 1993, Shaaltiel et al., 2007). Intravenous supplementation of the β-glucocerebrosidase is the most prevalent treatment modality among individuals affected with Gaucher's disease. Enriching for exposed terminal mannose residues on this enzyme leads to better engagement of macrophages through MRs and increased uptake by affected cells(Doebber et al., 1982, Furbish et al., 1981, Friedman et al., 1999). Thus, the three commercially available versions of β-glucocerebrosidase (Imiglucerase(Grabowski et al., 1995), Velaglucerase alfa(Zimran et al., 2007) and Taliglucerase alfa(Shaaltiel et al., 2007)) were developed to contain high mannose structures(Tekoah et al., 2013).

Additional ERTs developed for the management of other LSDs include α-galactosidase for Fabry Disease(Desnick, 2001, Schiffmann et al., 2000, Schiffmann et al., 2001), α-glucosidase for Pompe Disease(Kishnani et al., 2006, Rossi et al., 2007, Van den Hout

et al., 2004, Chen et al., 2009), α-L-Iduronidase for Mucopolysaccharidosis (MPS) Type I(Kakkis et al., 2001, Clarke et al., 2009, Parini and Deodato, 2020, Wraith et al., 2004, Dornelles et al., 2017), iduronate sulfatase for MPS Type II(Muenzer et al., 2002, Garcia et al., 2007, Muenzer et al., 2007, Muenzer et al., 2006, Sohn et al., 2013b, Wraith, 2008), GalNac-6-Sulfatase for MPS Type IVA(Hendriksz et al., 2014, Hendriksz et al., 2018), GalNac-4-Sulfatase for MPS Type IV(Harmatz et al., 2006, Harmatz et al., 2004), and β-glucuronidase for MPS Type VII(Fox et al., 2015, Wang et al., 2020, Harmatz et al., 2018); all of these rely on MPR signaling to facilitate lysosomal targeting and delivery (Seo and Oh, 2022, Oh, 2015). Similar to β-glucocerebrosidase, these therapeutic protein products have been glycoengineered to improve cellular uptake and biodistribution by enriching for glycoforms bearing mannose-6-phosphate residues (Oh, 2015, Seo and Oh, 2022, Lee et al., 2003, Sakuraba et al., 2006, Zhu et al., 2004, Zhu et al., 2009, Park et al., 2018, Lachmann, 2011, Tiels et al., 2012, Kakkis et al., 1994, Muenzer et al., 2007, Togawa et al., 2014, Parini and Deodato, 2020, Tomatsu et al., 2007) (Supplementary Table 3).

1.4 Immunogenicity

Even more important than biological potency, product safety is paramount in biologics. Adverse immunological responses to therapeutic proteins undermine both the safety of the subject and the molecule's therapeutic efficacy. In addition to the development of unwanted acute allergic or inflammatory reactions against a recombinant protein, unwanted immune engagement can also lead to a partial or complete loss of pharmacological activity due to binding by neutralizing antibodies(Porter, 2001, Wadhwa et al., 2015, Wang et al., 2008) or accelerated serum clearance(Ehrenpreis, 2017, Lundahl et al., 2021, Filipe et al., 2014). The development of anti-drug antibodies has been documented among patients treated with recombinant EPO(Mayeux and Casadevall, 2003, Casadevall et al., 2002), interferon-α(Bonetti et al., 1994, Douglas et al., 1993, Fossa et al., 1992), interferon-β(Zang et al., 2000, Konrad et al., 1987, Larocca et al., 1989), insulin(Di Mario et al., 1986, Fineberg et al., 1983), thrombopoietin(Li et al., 2001), and factor VIII(Pratt and Thompson, 2009)—to name a few examples. Stimulation of the immune response against therapeutic proteins is driven by several factors, including aggregate formation(Pham and Meng, 2020, Lundahl et al., 2021, Wang, 2005)—which in turn, can be controlled through glycan manipulation (see Section 1.1). Numerous glycosylated therapeutic proteins, such as interferon-β(Runkel et al., 1998, Kivisakk et al., 2000) and EPO(Elliott et al., 1996), are less immunogenic compared to their deglycosylated counterparts due to increased stability and a decreased propensity for aggregation. In some cases, increasing protein sialylation could also be applied to minimize the antigenicity of the drug. Desialylated EPO for example, has demonstrated higher immunoreactivity(Wide et al., 2003), and decreased antigenicity was observed with increased sialylation for asparaginase(Fernandes and Gregoriadis, 2001) (Supplementary Table 4).

The presence of non-human glycan structures in recombinant biotherapeutics can also trigger adverse immune responses. Although host expression systems in bacteria, yeast and plants have been utilized in the past, mammalian systems such as Chinese Hamster Ovary (CHO) cells are predominantly used to manufacture recombinant glycoproteins with the aim of producing glycoforms that most closely resemble their human-derived counterparts.

Despite this, inherent genetic differences between the glycosylation machineries of nonhuman mammals, such as murine myeloma cell lines (i.e. NS0 and Sp2/0), relative to humans can lead to the addition of glycan moieties that are normally absent in humans, and thus trigger adverse immunological reactions. The most commonly reported immunogenic glycan epitopes are the α-Gal epitope (Galα1,3-Gal) and N-glycolylneuraminic acid (Neu5GC)(Butler and Spearman, 2014, Goh and Ng, 2018) (Figure 2C).

Antibodies against α-Gal are naturally abundant in human serum (approximately 1% of circulating immunoglobulins), due to continuous antigenic stimulation from normal gut flora(Galili et al., 1988). This can lead to hypersensitivity against proteins terminating in α-Gal residues. Unfortunately, many monoclonal antibodies generated from mouse-derived cell lines contain α-Gal epitopes(Sheeley et al., 1997, Yoo et al., 2002, Macher and Galili, 2008), which undermines their clinical safety. Such was the case with cetuximab, a monoclonal antibody for treating colorectal and head neck cancers. A subset of patients treated with cetuximab experienced anaphylaxis due to the pre-existing population of α-Gal IgE antibodies in their bloodstream(O'Neil et al., 2007, Chung et al., 2008). Roughly 30% of the 21 glycoforms present in cetuximab were capped by α-Gal residues(Qian et al., 2007). Neu5GC is a modified sialic acid that is also potentially immunogenic glycan structure common to many nonhuman mammalian cell lines. It can be assimilated from exogenous sources into newly synthesized glycans and presented on human cells, but as with α-Gal epitopes, humans produce circulating antibodies against Neu5GC(Altman and Gagneux, 2019, Padler-Karavani et al., 2008, Tangvoranuntakul et al., 2003, Nguyen et al., 2005). This co-existence of anti-Neu5GC antibodies and epitope incorporation correlates well with chronic inflammation-mediated diseases(Okerblom and Varki, 2017, Varki, 2017, Dhar et al., 2019). Adverse reactions have been observed among patients treated with rabbit-derived anti-thymocyte globulin, likely due to the immune response triggered by Neu5GC(Salama et al., 2017, Amon et al., 2017, Yehuda and Padler-Karavani, 2020). A comparative study of murine myeloma-derived cetuximab, bearing terminal Neu5GC residues, and CHO-derived panitumumab, containing negligible Neu5GC residues, also revealed that when exposed to human serum containing high levels of anti-Neu5GC antibodies, immune complex formation was only observed against cetuximab(Ghaderi et al., 2010); this suggests that patients who have higher levels of anti-Neu5GC antibodies could be prone to adverse reactions when treated with cetuximab (Supplementary Table 4).

Given the importance of glycosylation to modulating the glycoprotein immunogenicity, tweaking its composition can help elevate the immune response when necessary—as in the case of vaccines. Vaccines come in a variety of formulations that can consist of the whole pathogen itself, or protein-, polysaccharide-, and nucleic acid-based molecules(Cid and Bolivar, 2021); however, for protein-based subunit vaccines, glycosylation can vary substantially depending on the heterologous expression host and may not reflect the natural glycan pattern found on the native pathogen. Many viruses, such as SARS, influenza, and HIV, have surface proteins that are extensively coated by host-derived glycans. In addition to being essential to binding and entry into the host cells, these attached sugars form a glycan shield, masking the viral epitopes that can be neutralized by circulating antibodies and allowing them to evade the host immune system(Wanzeck et al., 2011, Fenouillet et al., 1994, Watanabe et al., 2020, Tate et al., 2014). In some cases, the presence

of the glycan structures is integral to the immunogenic epitope and are necessary for engagement by neutralizing antibodies(Behrens et al., 2016). Modifying glycosylation on subunit viral vaccine candidates, such has hemagglutinin (influenza), S protein (SARS) and ENV protein (HIV), can significantly alter immune response and improve clinical efficacy. By altering glycans on hemagglutinin (HA) to trim heterogenous complex-type glycans down to only N-linked GlcNacs, better binding and neutralization was obtained, along with broader cross-strain activity in mice (Chen et al., 2014, Wang et al., 2009). Likewise, an S protein vaccine against SARS-CoV-2 that was enzymatically modified to be mono-GlcNac-decorated induced a stronger immune response compared to normally glycosylated S protein, and protected vaccinated animals against wild-type virus and other variants of concern(Huang et al., 2022). Furthermore, an mRNA vaccine candidate with specific glycosites removed in the S2 domain of the S protein resulted in higher antibody neutralization and CD8+ T cell activity against variants of concern relative to the wild-type SARS-CoV-2 mRNA vaccine(Wu et al., 2022). One caveat, however, of mRNA vaccines is that while genetic engineering of glycan occupancy can be achieved, optimizing glycan microheterogeneity at specific sites is not possible on this platform, due to the fact that the protein glycosylation machinery is completely dependent on the host cell. The stronger and broader immune engagement observed in vaccine candidates after glycan shield removal is likely due to the exposure of conserved previously hidden epitopes on the protein surface. Alternatively, the addition/removal of glycan structures can also lead to the creation of neoepitopes not found in the native pathogen, which can subsequently be targeted by the immune response. Using this approach, immune response to HIV vaccine candidates can be redirected. By knocking in a novel glycosite on an HIV Env trimer vaccine candidate, they masked a previously immunodominant epitope and instead created new epitopes on the protein that could be recognized by neutralizing antibodies by knocking out multiple N-linked glycosites(Ringe et al., 2019). It is important to note, however, that full removal of glycans could decrease vaccine efficacy for sites where the glycans impact protein conformation(Chen et al., 2014, Huang et al., 2022, Wu et al., 2022). Moreover, eliminating glycans entirely runs the risk of creating neoepitopes that are not recognized by the immune system upon infection by the native virus, as seen with deglycosylated HIV env (Zhou et al., 2017). Nevertheless, these examples demonstrate that by leveraging informed glycan design, protein-based subunit vaccines could provide stronger and longer lasting protection, and also be effective against a broader spectrum of strains and variants, reducing the frequency of needed updates to formulations in response to new mutants.

2 Monoclonal Antibodies

Monoclonal antibodies (mAb), such as recombinant immunoglobulins of the IgG1 subtype, are monospecific in terms of the epitope recognized. Therapeutic mAbs account for 80% of total antibody sales in the United States(IAVI/Wellcome, 2020). Furthermore, the global therapeutic mAb market is expected to generate more than $300 billion globally per year (Lu et al., 2020). Therefore, understanding their critical quality attributes, specifically glycosylation, ensures their safety and similarity as effective therapies.

IgG antibodies contain two heavy chains and two light chains to form three major domains: two identical Fab domains for antigen binding and an Fc domain (dimeric base of the

antibody)(Schroeder and Cavacini, 2010). Asparagine(N) 297-linked glycosylation occurs in the Fc region and in the form of biantennary complex structures(Mimura et al., 2018, Varki et al., 2022) (Figure 3). Glycosylation at the Fc region of IgG proteins can greatly impact antibody structure and effector functions (Jefferis, 2009a, Jefferis, 2012).

The constant region of antibodies contains binding sites for immune effector molecules such as the complement system or Fc receptors (Vidarsson et al., 2014). These receptors help recruit immune mediators, generally via Fc receptor binding. Antibody Fc receptors mediate the cell killing effects of mAbs by complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), or antibody-mediated phagocytosis by monocytes/macrophages(Weiner et al., 2010, Ludwig et al., 2003, Jefferis, 2009b). In ADCC, this is accomplished by engaging immune complexes with FcγRIIIa on natural killer (NK) cells, or by directly inducing tumor cell apoptosis through the suppression of pro-survival ligands or inhibition of signal receptor dimerization. CDC is activated by the binding of complement component C1q to the mAb Fc region to initiate the complement cascade (Raju, 2008, Dekkers et al., 2017, Zhou et al., 2008b, Pereira et al., 2018). These mechanisms are particularly important for cancer immunotherapy; indeed, Fc-mediated effector cell recruitment and functions, such as ADCC, are crucial to tumor-targeting antibodies. For instance, rituximab or trastuzumab activity was abolished in genetically modified mice that lacked FcγR expression or had defective FcγR signaling. In contrast, FcγRIIb knockout mice showed enhanced efficacy(Clynes et al., 2000, de Haij et al., 2010). Although glycosylation at the N297 region accounts for only 2–3% of antibody mass, IgG-Fc glycosylation and structure are critical for Fc effector functions, such as ADCC and CDC (Chan and Carter, 2010, Jefferis, 2009b). Aglycosylated Fc-IgGs reduce binding affinity to FcγRI and eliminate binding to FcγRII and FcγRIII receptors, and C1q-mediated processes such as phagocytosis, ADCC, and CDC are abated or severely impaired in aglycosylated IgG. (Sazinsky et al., 2008, Raju et al., 2000, Nose and Wigzell, 1983, Pound et al., 1993, Sarmay et al., 1992, Tao and Morrison, 1989, Woof and Burton, 2004).

2.1 Fc Glycosylation and mAb Effector Function: Fucosylation

Core fucosylation of Fc N-glycans on mAbs occurs when α-1,6-linked fucose is attached to the innermost GlcNAc moiety (Garcia-Garcia et al., 2021). The removal of the core fucose increases Fc affinity for FcγRIIIa in all IgG subclasses, thereby inclusively augmenting ADCC activity and improving therapeutic efficacy(Shields et al., 2002, Shinkawa et al., 2003). Due to the impact of Fc fucosylation on FcRγIIIa binding, many monoclonal antibodies and antibody-drug conjugates being developed for the clinic are being engineered or “glyco-optimized” to decrease fucose(Golay et al., 2022, Tong et al., 2021, Pereira et al., 2018), with a few already commercially available or in clinical trials, such as obinutuzumab (anti-CD20)(Tobinai et al., 2017), mogamulizumab (anti-CCR4)(Watson and Marx, 2019), belantamab mafodotin (anti-BCMA)(Lassiter et al., 2021), benralizumab(anti-IL5Rα)(Pelaia et al., 2018), imgatuzimab (anti-EGFR)(Temam et al., 2017) and tomuzotuximab (anti-EGFR)(Fiedler et al., 2018) (Supplementary Table 5). Furthermore, an increase or decrease in ADCC activity produced by other glycan features always occurs in the context of core fucosylation(Li et al., 2017, Shinkawa et al., 2003).

2.2 Fc Glycosylation and mAb Effector Function: Sialylation

Whether or not sialic acids are well-suited in ADCC- and CDC-related therapies, sialylated IgGs have recently garnered substantial interest as immunosuppressants for autoimmune and inflammatory diseases, as demonstrated by intravenous immunoglobulin (IVIG) therapy (Nimmerjahn and Ravetch, 2008, Kaneko et al., 2006). IVIG therapy involves administering concentrated IgG derived from pooled plasma to patients (Seite et al., 2008). As a highly effective biologic in treating several autoimmune diseases, including idiopathic thrombocytopenic purpura (ITP), chronic inflammatory demyelinating polyneuropathy, and myasthenia gravis, IVIG consumption has increased approximately 400-fold since 1980, and approximately 100 tons are consumed per year(Orange et al., 2006, 2019). Sialylated IgGs initiate anti-inflammatory responses through murine C-type lectin-like receptor-specific intracellular adhesion molecule-grabbing non-integrin R1 (SIGN-RI) (DCSIGN in humans), expressed by macrophages and dendritic cells. As a result, FcγRIIb is upregulated, increasing Treg cell populations and suppressing inflammatory responses (Anthony and Ravetch, 2010, Anthony et al., 2008, Kaneko et al., 2006, Sondermann et al., 2013). Although IgG sialylation is not the main determinant of anti-inflammatory effect of IVIG therapy, IgG sialylation does enhance the efficacy(Schwab and Nimmerjahn, 2013). Increased endogenous IgG sialylation improved treatment of Kawasaki disease during IVIG therapy(Ogata et al., 2013), and sialylated human IgG reduced the severity of rheumatoid arthritis in mouse models, an effect that was not observed using desialylated human IgG(Kaneko et al., 2006). Similarly, a 10-fold increase in anti-inflammatory activity was seen with tetra-Fc sialylation compared to asialylated IVIG across several animal models(Washburn et al., 2015).

2.3 Fc Glycosylation and mAb Effector Function: Galactosylation

The effector functions of galactosylation, particularly ADCC, should be addressed on a case-by-case basis. In some cases, the terminal galactose residue content had no effect on ADCC activity. For example, the degalactosylation of rituximab and other recombinant mAbs with variable galactose contents confirmed that ADCC activity was unaffected(Hodoniczky et al., 2005). To complicate matters, increased galactosylation can promote ADCC activity or inhibit ADCC activity(Houde et al., 2010, Nimmerjahn et al., 2007, Kumpel et al., 1994, Pereira et al., 2018, Zhang et al., 2020b, Aoyama et al., 2019, Thomann et al., 2016). Interestingly, site-specific galactose attachment on the N-glycan structure in afucosylated palivizumab influences FcγRIIIA binding and ADCC activity, as shown with enzymatic transglycosylation using chemically defined N-glycans(Hatfield et al., 2022). Although terminal galactose may only play a minor role in ADCC activity, it is critical for CDC activity(Hodoniczky et al., 2005). Galactosylated rituximab had higher CDC than degalactosylated glycoforms due to its higher affinity to C1q receptors; when Campath-1H was deglycosylated, CDC activity was reduced by 50%(Peschke et al., 2017, Hodoniczky et al., 2005, Boyd et al., 1995). Based on these findings, additional research on the effects of galactosylation on ADCC activity and its subclasses is required, and different glycoforms should be considered whenever developing new drugs.

2.4 Fc Glycosylation and mAb Effector Function: Bisecting GlcNac

Human serum contains ~10% IgGs by protein mass, and many of their N-glycans contain bisecting GlcNAc structures(van de Bovenkamp et al., 2016, Gudelj et al., 2018). These features can only be produced in human or murine cells (e.g., S20); CHO cells are unable to produce N-glycans with bisecting GlcNAc because of a lack of active N-acetylglucosaminyltransferase-III (GnT-III) required for its synthesis(Campbell and Stanley, 1984, Sallustio and Stanley, 1989, Umana et al., 1999), although the enzyme can be genetically activated(Karottki et al., 2020, Shamie et al., 2021). Overexpression of GnT-III was applied to increase bisecting GlcNAc attachment to N-glycans in therapeutic mAbs, thereby improving FcR-binding(Davies et al., 2001). Specifically, ADCC activities were 10–30-fold higher when mAbs containing bisecting GlcNAc N-glycans bound to FcRIIIa receptors (Davies et al., 2001, Shinkawa et al., 2003). For example, adding bisecting GlcNAc without removing core fucosylation improved ADCC by approximately 10-fold, indicating that N-glycans with bisecting GlcNAc structures can enhance ADCC activity. Furthermore, trastuzumab-bearing N-glycans modified with bisecting GlcNAc increased ADCC 10-fold, comparable to that observed for similarly modified rituximab(Hodoniczky et al., 2005). However, these finding have been contradicted. Since incorporation of bisecting GlcNAc is not a suitable substrate for 1,6-fucosyltransferase, N-glycans containing such structures are always associated with loss of core fucosylation. As a result, removing the core fucose rather than bisecting GlcNAc may still have the greatest impact on the therapeutic antibody ADCC activity(Shinkawa et al., 2003, Schachter, 2000).

2.4 Fc Glycosylation and mAb Effector Function: Mannosylation

The prevalence of high-mannose N-glycans on recombinant mAbs can vary substantially (more than 1–20% in both CHO and murine cells), but endogenous human IgG contains only trace amounts (<0.1%) of these glycovariants(Flynn et al., 2010, Goetze et al., 2011). ADCC activity is enhanced in mAbs with high-mannose glycoforms(Liu, 2015, Yu et al., 2012, Shi and Goudar, 2014, Brady et al., 2015, Liu et al., 2017). However, similar to the other glycoforms mentioned previously, changes in ADCC are more directly associated with the loss of core fucosylation(Zhou et al., 2008a, Brady et al., 2015). Furthermore, mAbs with high mannose structures can have a negative impact on CDC activity by lowering the binding affinity with C1q(Walsh, 2018). Other studies have reported similar results for high-mannose mAbs that reduce CDC activity by lowering binding to C1q. Thus, Fc mannosylated mAbs have a positive effect on ADCC but a negative effect on CDC activity(Yu et al., 2012, Zhou et al., 2008a, Hiatt et al., 2014).

3. Future Perspectives: Next Generation Biologics

Glycoengineering of therapeutics has been applied to a variety of therapeutic glycoproteins(Walsh, 2018, Majewska et al., 2020). Beyond protein-based drugs, however, next-generation biologics, such as cell therapy- and nucleic acid-based therapeutics, offer opportunities for treating cancer, infectious diseases, immune disorders, and inherited genetic diseases(Kulkarni et al., 2021, Weber et al., 2020). Here we describe how glycosylation can impact cell therapy, gene therapy, and drug delivery platforms, including specific examples of glycoengineering to improve biological activity and clinical efficacy.

3.1 T-Cell Therapy

T-cell based immunotherapies, in particular chimeric antigen receptor (CAR) T-cells, are gaining traction especially for cancers of the blood and bone marrow(June et al., 2018, Feins et al., 2019). For these, T-cells are collected and transfected to express surface receptors that bind to and eliminate tumor cells. T-cell therapies rely on interactions between tumor cell ligands or antigen presenting cells (APCs) and co-stimulatory receptors on T-cells, such as CD28, inducible costimulatory (ICOS), and 4–1BB, which promote T-cell proliferation and cytotoxicity (Chen and Flies, 2013) (Figure 4A). Receptors on immune cells—including T-cell receptors (TCRs)—are glycosylated, and specific glycosylation patterns are pivotal in immune function, including communication between immune cells and the modulation of their anti-tumor activity(Sun et al., 2021, Mereiter et al., 2019). For example, inhibiting N-glycosylation of CD28 increased interaction with its CD80 ligand, thereby enhancing its co-stimulatory signaling activity(Ma et al., 2004). Sialidase treatment of T-cells and APCs also enhanced CD28-mediated activation of T-cells and reactivation of exhausted T-cells, possibly by eliminating sialic acid-containing glycans that compete for CD28-CD80 interactions(Edgar et al., 2021). Removing specific glycosites in ICOS led to intracellular sequestration of the receptor(Kamei et al., 2010) and altered its ligand binding affinity(Kamei et al., 2010, Rujas et al., 2020). Galectin-9 also binds 4–1BB, a co-stimulatory signaling receptor on T-cells, thus controlling T-cell function by facilitating 4–1BB surface expression(Madireddi et al., 2014). Deglycosylation of 4–1BB results in reduced galectin-9 binding(Madireddi et al., 2014). Furthermore, the mutation of N-glycosylation sites reduces membrane expression of 4–1BB and decreases polyubiquitination, thereby inducing multimerization of 4–1BB, which may hamper 4–1BB receptor signaling(Sun et al., 2022).

Co-inhibitory signaling pathways mediated by receptors on T-cells (e.g., PD-1, CTLA-4, and TIM-3) induce exhaustion and apoptosis and inhibit cytotoxic function(Chen and Flies, 2013) (Figure 4A). The PD-1/PD-L1 axis has been a target of cancer immunotherapy, and its inhibitory function depends on PD-1 glycosylation. PD-1 depends on core fucosylation, as the inhibition of FUT8 attenuated PD-1 cell surface expression and promoted PD-1 degradation, resulting in augmented T-cell activity and anti-tumor responses(Okada et al., 2017, Zhang et al., 2020a). Among the PD-1 N-glycosylation sites, N116 mediates the interaction between galectin-9 and PD-1, which induces TIM-3/PD-1 dimerization and attenuates galectin-9/TIM-3-mediated cell death(Yang et al., 2021). Furthermore, PD1 N-glycosylation maintains its expression and its interaction with PD-L1 (Sun et al., 2020). PD-1 N-glycosylation varies when produced by different host systems, leading to different the binding affinities of camrelizumab, an anti-PD-1 monoclonal antibody(Liu et al., 2020). Another co-inhibitory receptor expressed on T-cells, CTLA-4, interacts with CD80/86 proteins to transmit the inhibitory signal. N-glycosylation (N78/110) contributes to CTLA-4 dimerization and T-cell activity(Darlington et al.). CTLA-4 surface expression was decreased in Mgat5-negative T-cells, and increased upon hexosamine treatment, implying N-glycan branching enhances the CTLA-4 surface retention and suppresses its endocytosis(Grigorian et al., 2007, Lau et al., 2007). Furthermore, defective N-glycan branching reduced CTLA-4 surface expression and promoted multiple sclerosis(Mkhikian

et al., 2011). Finally, TIM-3 N-glycosylation is critical its interaction with galectin-9, which inhibits T-cell effector function(Zhu et al., 2005a).

Because of the importance of glycosylation on T-cell development, function and activation, manipulation of glycan structures on key ligands or even the cell itself can also be applied to improve immunotherapeutic outcomes of T-cell therapeutics. The mutation of PD-1 N-glycosylation at site N74 decreased PD-1 surface expression in CAR T-cells, thereby enhancing their cytotoxicity and cytokine secretion(Shi et al., 2019). Also, Deactivation of Mgat5—which catalyzes the addition of branched, β1,6-GlcNac N-glycans on T cells—led to a reduction of β1,6-GlcNac N-glycan branching on the surface of CAR T-cells, and enhanced their expansion rate and antitumor activity compared to the wild-type population (de Bousser et al., 2023). Exofucosylation can increase surface sialyl-Lewis X, a glycan that enhances E-selectin binding, thereby improving CAR T-cell targeting efficiency(Mondal et al., 2019). Furthermore, N-glycosylation on the CAR hinge domain derived from CD28 contributes to CAR surface expression and CAR-T cytotoxicity(Hirobe et al., 2022). Drugs, including small molecules, sugar analogues, and mAb, can also be utilized to target glycosylation on various proteins to support immunotherapy(Zheng et al., 2022). BMS1166 inhibits PD-L1 glycosylation and blocks its endomembrane transport, resulting in T-cell activation(Chen et al., 2020). Another small molecule, N-linked glycosylation inhibitor-1, targets oligosaccharyltransferase, thus inhibiting N-glycosylation of epidermal growth factor receptor, cyclooxygenase-2, B7-H4 and other proteins to impede tumor cell proliferation(Lopez-Sambrooks et al., 2016, Rinis et al., 2018, Song et al., 2020). Similarly, inhibition of N-glycosylation with 2-deoxy-D-glucose (2DG) enhances T-cell cytotoxicity and promotes memory T-cell differentiation (Sasawatari et al., 2020). 2DG hindered the N-glycosylation of target proteins, including MICA/B and PD-L1, and enhanced the anti-tumor activity combined with other treatments (Andresen et al., 2012, Shao et al., 2018, Kim et al., 2020). 2-fluoro-L-fucose has also inhibited fucosylation of PD-1 and B7-H3, resulting in T-cell proliferation and activation(Okada et al., 2017, Huang et al., 2021). Sialic acid mimetics can block sialylation to suppress tumor growth and increase the proportion of NK cells, CD8+ T-cells, and CD4+ T-cells with reduced regulatory T-cells(Bull et al., 2018). Enhanced T-cell activity is achieved by mAbs with superior binding affinity to the N-glycosylation at site N58 of PD-1(Liu et al., 2020, Wang et al., 2019b, Liu et al., 2019, Lu et al., 2022). Fusion proteins also show promise, such as a sialidase-conjugated mAb that targets human epidermal growth factor receptor 2 and enhances the NK cell antitumor activity by desialylating HER2-positive tumor cells(Xiao et al., 2016). Understanding and ultimately manipulating the glycoproteome can therefore be invaluable to immunotherapies.

3.2 Viral Drug Delivery

Successful delivery of therapeutic nucleic acids, such as plasmid DNA, mRNA, antisense oligonucleotide (ASO), and small interfering RNA (siRNA) remains challenging since their negative charges inhibit transfer across the plasma and nuclear membranes(Molle et al., 2022). However, delivery platforms such as viral vectors, including adeno-associated viruses (AAV), adenoviruses, and lentiviruses overcome this challenge and are used in gene therapy(Bulcha et al., 2021, Sharon and Kamen, 2018). However, the target specificity, vector yield and transduction efficiency of viral vectors, such as AAV, is determined by

capsid serotypes and capsid protein PTMs, such as ubiquitination, SUMOylation, and O-glycosylation (Wang et al., 2019a, Mary et al., 2019a).

Glycans have been detected on capsid proteins in several AAV serotypes, including AAV2, 3, 5, 7, 8, 9, rh10 (Mary et al., 2019a). N-glycosylation of viral proteins can affect infectivity and the immune response by assisting in protein folding and trafficking and modulating the interaction between virus and host immune system(Vigerust and Shepherd, 2007). Inhibition of N-glycosylation increased AAV2 transduction efficiency and decreased vector yield(Mary et al., 2019a, Mary et al., 2019b), and the site-specific mutation of glycosylation sites revealed that modulation of glycosylation increased the hepatic and ocular gene transfer of AAV2(Mary et al., 2019a, Mary et al., 2019b). Proteomic analysis of AAV5-producing HEK293 showed an upregulation of MAN2A2, an alpha-mannosidase which trims high-mannose structures resulting in complex N-glycans in Golgi, suggesting that the glycosylation pathway can be targeted to improve AAV production(Strasser et al., 2021). Furthermore, bioconjugation of GalNAc on capsid proteins can increase AAV2 transduction efficiency and reduce neutralizing antibody production against AAV2 and AAV8 (Mevel et al., 2019).

Glycans and glycan-binding proteins (lectins) are also important to virus and host interactions, including viral entry and tissue tropism(Raman et al., 2016) (Figure 4B). Cell-surface glycans, such as heparan sulfate proteoglycans (HSPG), sialic acids, and terminal galactose, aid in attachment and infection of AAV serotypes(Stroh and Stehle, 2014, Meyer and Chapman, 2022). Membrane-associated HSPG are receptors for AAV2 and AAV3, so disrupting HSPG synthesis genes and/or treatment with heparin inhibits attachment and infection (Summerford and Samulski, 1998, Handa et al., 2000, Rabinowitz et al., 2002). Terminal sialylation is also critical for AAV infectivity; heparan sulfate and sialic acids are necessary for binding and transduction of AAV6 (Halbert et al., 2001, Wu et al., 2006, Ng et al., 2010), while AAV4 and AAV5 use O-linked and N-linked sialic acids as a primary receptor, respectively(Chen et al., 2005, Walters et al., 2001, Kaludov et al., 2001, Walters et al., 2002). Inhibiting sialylation also decreases binding and transduction efficiency of AAV1 (Wu et al., 2006, Chen et al., 2005). For AAV9, however, enzymatic digestion of sialic acids increased the surface binding and transduction, suggesting that N-linked galactose facilitates its binding and transduction (Shen et al., 2011, Bell et al., 2011). Understanding the glycan binding specificity of different AAV serotypes can help improve gene therapy vectors (Mietzsch et al., 2014), and engineering capsid protein affinity to glycans is being attempted (Madigan and Asokan, 2016).

3.3 Non-Viral Drug Delivery

Nucleic acids and other therapeutic molecules can also be administered as naked molecules or with non-viral delivery platforms. Many cell types recognize and bind specific glycan structures, such as terminal mannose, fucose, galactose, GalNac and GlcNac; conjugation of carbohydrate moieties to oligonucleotide-based drugs can improve their stability, cell specificity, and delivery (Bakowski and Vogel, 2022) (Figure 4C). This can impact cancer therapy, where lectin receptors are often overexpressed in tumors versus healthy tissue(Berthe et al., 2003, Ishiwata et al., 1997, Pavelic et al., 2003, Laube, 2009, Hebert,

2006), and glycoengineering can be exploited to reduce drug toxicity in healthy cells and improve clinical efficacy.

Galactosylated polyethylene glycol and mannose 6-phosphate polyethylene glycol can be covalently linked to siRNA to successfully inhibit gene expression in hepatocytes *in vitro* (Zhu and Mahato, 2010), and conjugation of siRNA to GalNAc (a ligand for ASGP receptors), facilitated targeted delivery and robust gene silencing both *in vitro* and *in vivo* (Nair et al., 2014). Different designs of GalNAc conjugation have been tested to optimize synthesis and targeting efficiency(Matsuda et al., 2015, Rajeev et al., 2015). Furthermore, conjugation of multivalent GalNAc to antisense oligonucleotides and siRNAs using GalNAc phosphoramidite monomer has extended the structural flexibility of the number of GalNAc units for effective silencing(Yamamoto et al., 2016, Sharma et al., 2018).

Glycoengineering of delivery platforms can also improve uptake or alter biodistribution of nanoparticles (NP) and extracellular vesicles (EV) by leveraging glycan receptors(Bakowski and Vogel, 2022, Bost et al., 2021) (Figure 4C). NPs coated with fucose can transfer liposomes into pancreatic cancer cells(Yoshida et al., 2012), and galactosylated liposomes and lipid nanoparticles (LNP) can deliver therapeutic molecules such as azidothymidine, doxorubicin, paclitaxel, and siRNA (Garg and Jain, 2006, Wang et al., 2010, Jain et al., 2015, Wang et al., 2016, Yang et al., 2018). Mannosylated NPs demonstrated improved delivery of both DNA and RNA into dendritic cells(Kim et al., 2006, Markov et al., 2015, Goswami et al., 2019), and mannose-based NPs containing dasatinib showed efficient uptake into macrophages(Rushworth et al., 2020). Coating liposomes with mannose-6-phosphate also increased cellular uptake of a cytotoxic molecule C6Cer, and selectively induced apoptosis in cancer cells(Minnelli et al., 2018). The surface of EVs are naturally enriched with glycoproteins, and glycosidase treatment impacts EV binding affinity, showing that surface glycans are important in target cell uptake(Williams et al., 2019). Indeed, the inhibition of EV surface N- and O-glycosylation enhanced uptake, and O-glycan removal significantly increased the EV accumulation into lung tissues *in vivo* (Nishida-Aoki et al., 2020). Similarly, removal of sialic acid on EV surfaces shifted their biodistribution from the liver to the lungs (Royo et al., 2019). As with nanoparticles, adding glycans to EVs can target cells with specific lectin receptors. For example, compared to unconjugated EVs, mannose-conjugated carriers containing an immune stimulant MPLA, demonstrated higher cellular uptake and elevated cytokine secretion in dendritic cells, which primarily express mannose receptors (Choi et al., 2019). These findings underscore the immense potential impact of glycosylation on improving the efficacy and safety profiles of new and current platforms for drug delivery.

3. Conclusion and Outlook

There is no denying that optimizing glycosylation in the drug discovery and development workflow can immensely improve protein-based, oligonucleotide-based and cell-based therapeutic products. The last two decades have seen significant advancements with regard to the creation of glycoengineering platforms that involve genetic modification of plant(Grabowski et al., 2014), yeast(Jacobs et al., 2009, Beck et al., 2010, Arico et al., 2013), human(Hart et al., 2017, Meuris et al., 2014), and hamster(Shitara, 2009, Pereira

et al., 2018) producer cell lines to generate recombinant products with homogenous and tailored glycan structures for improved safety and efficacy. By combining these technologies with systems biology approaches that aim to better understand the cell's highly complex glycosylation machinery(Spahn et al., 2017, Spahn et al., 2016, Liang et al., 2020, Krambeck et al., 2017), we may be better able to predict and design optimal glycoprofiles and build the capacity to produce glycoengineered biologics more reliably and cost-effectively at scale.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Glycosylation is a common feature of a wide variety of protein-based drugs and profoundly impacts their stability, safety, and therapeutic efficacy
- Manipulating glycan heterogeneity is a powerful tool that is utilized to develop products with optimal physical and biological properties
- Glycoengineering can also be used to improve the efficacy of next-generation biologics such as T cell immunotherapy and oligonucleotide-based therapeutics

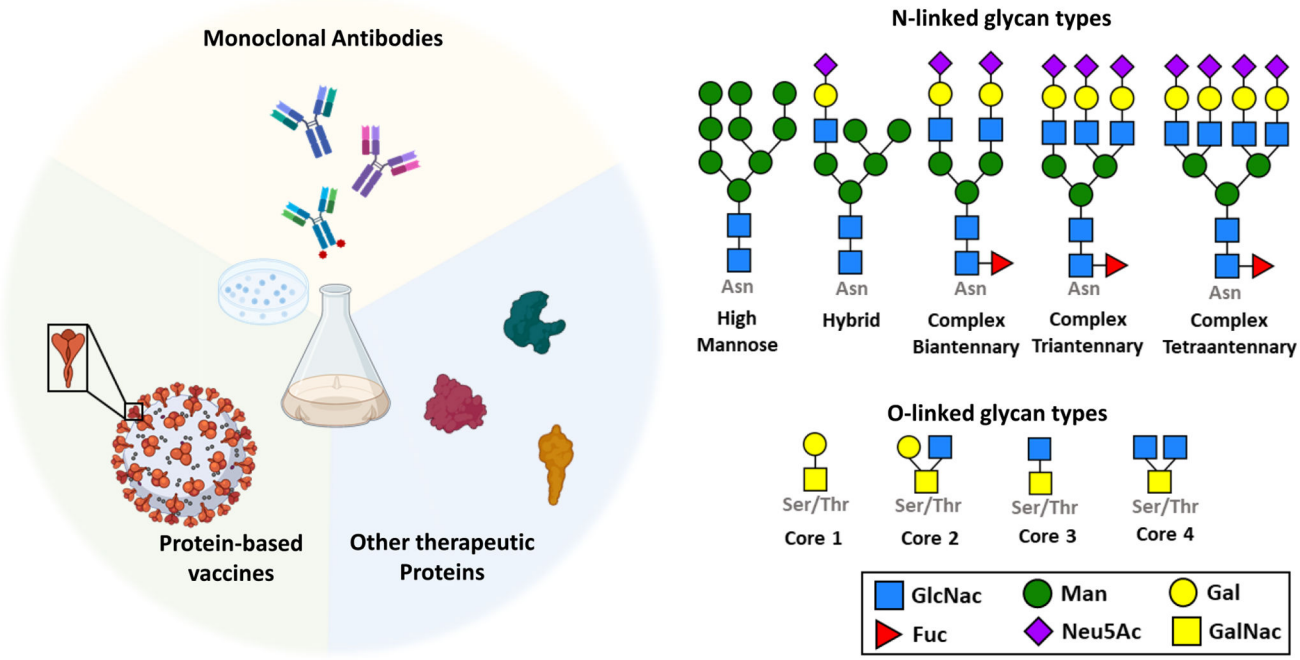

Figure 1. Most protein-based drugs undergo N-linked or O-linked glycosylation.

Most therapeutic proteins such as subunit vaccines, monoclonal antibodies, hormones, enzymes and immune factors undergo N-linked or O-linked glycosylation. N-linked glycans consist of carbohydrate molecules that are attached to the nitrogen atom on Asparagine (Asn) residues in the protein, while O-linked glycans consist of carbohydrates linked to the oxygen atom on Serine (Ser) or Threonine (Thr) residues in the protein.

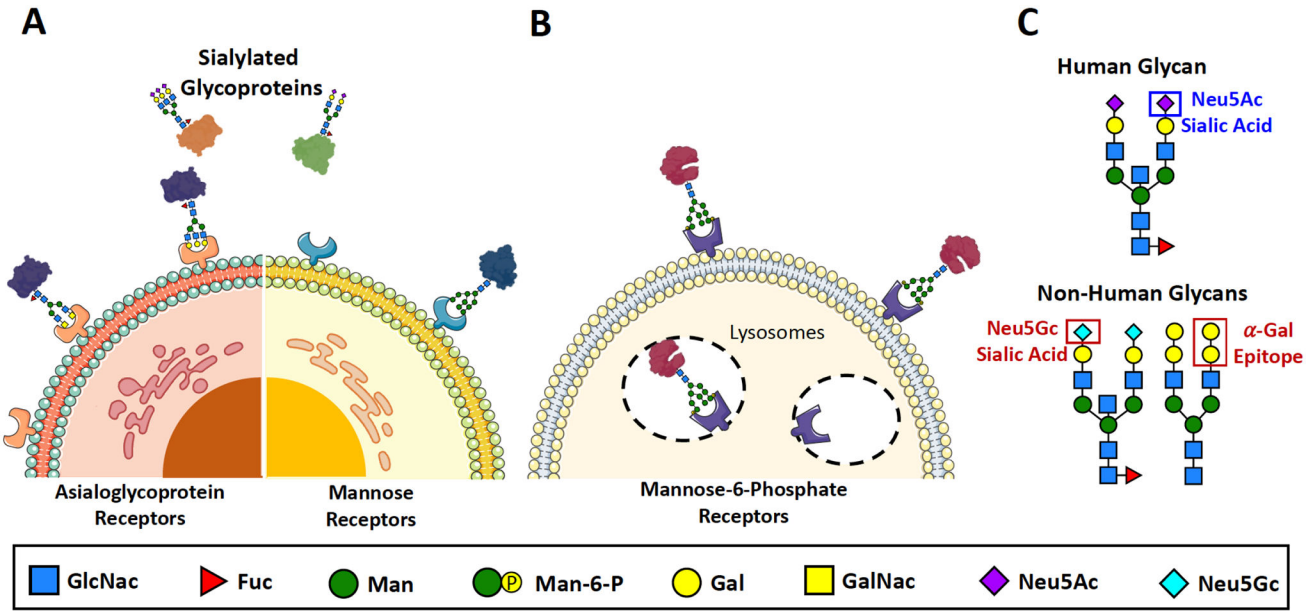

Figure 2. Protein glycosylation is a critical quality attribute that has a significant impact on the stability, efficacy, and safety of the drug

(A) Sialylation of therapeutic proteins prolongs circulatory half-life by helping them evade capture by lectin receptors that facilitate clearance from the bloodstream. The presence of the terminal sialic acid masks the sugars that would otherwise be bound by asialoglycoprotein receptors (terminal Gal/GalNac) and mannose receptors (terminal Man/Fuc/GlcNac). **(B)** The presence of Mannose-6-Phosphate residues on therapeutic proteins facilitate better targeting and uptake by cells—such as macrophages—that express Mannose-6-Phosphate receptors. **(C)** Some glycan structures are associated with adverse immune responses among human subjects due to their structural dissimilarity to human-derived glycans. Such immunogenic glycans include the α-Gal Epitope and the Neu5Gc residue—both of which are derived from non-human, mammalian protein expression systems.

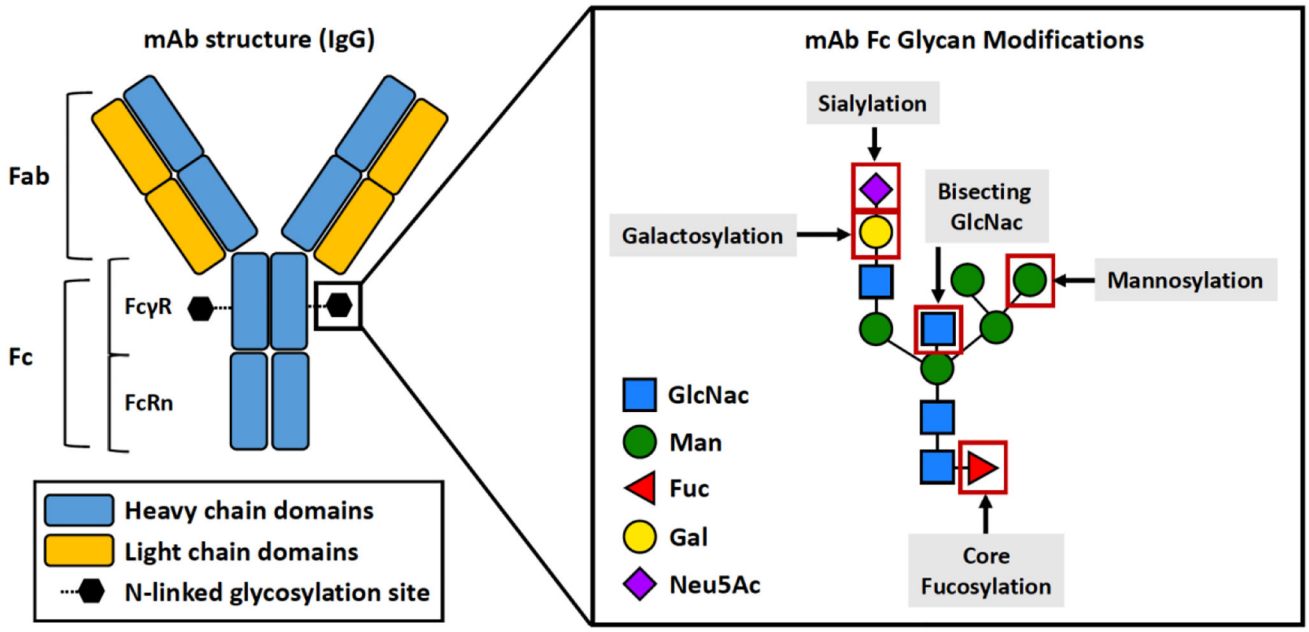

Figure 3. Fc Glycosylation impacts monoclonal antibody effector function.

All IgG antibodies are N-glycosylated at Asn-297 on their Fc region. The absence or presence of core fucose, bisecting GlcNac, terminal mannose/galactose/sialic acid residues on the Fc Asn-297 glycosite are crucial to regulating antibody function.

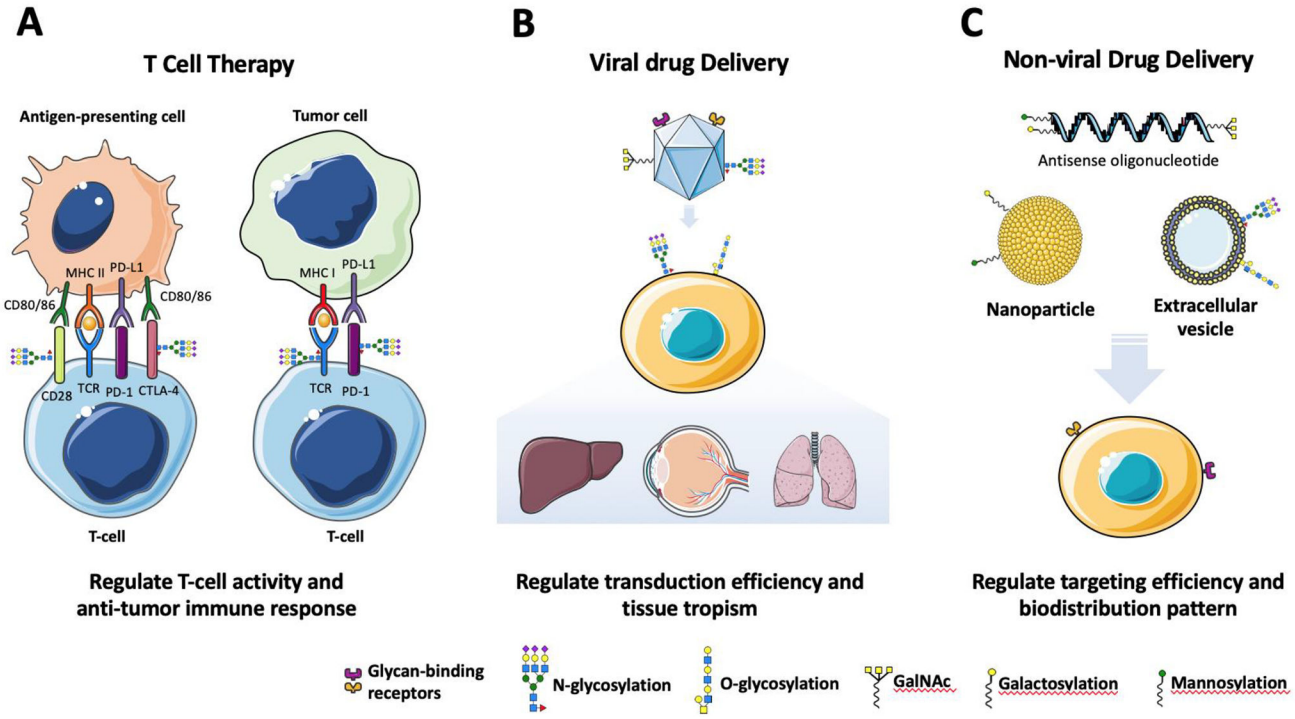

Figure 4. Glycosylation impacts therapeutic efficacy of biologics beyond protein therapeutics

(A) Anticancer T-cell therapies rely on interactions between the T-cell and the tumor cell or antigen presenting cell. Some of these important interactions include CD28⇔CD80/CD86 which positively regulates T-cell activation and promote downstream anti-tumor activity, while PD1⇔PDL1 and CTLA-4⇔CD80/CD86 negatively regulate T-cell activation and reduce anti-tumor activity. N-linked glycan heterogeneity on these interacting proteins significantly impacts their binding affinity, expression level and cellular localization and therefore play a role in ensuring the therapeutic efficacy of such cell-based treatment modalities. **(B)** The presence/absence and identity of N-linked glycans on AAV capsid proteins can affect host cell infectivity, vector yield and immune response. Furthermore, the primary receptors of all AAV serotypes are O-linked and N-linked glycans such as terminal sialic acid and terminal galactose residues found on the host cell surface. N- and O-linked glycosylation, therefore, are key factors that modulate AAV transduction efficiency and tissue tropism. **(C)** Because different cell types usually express a distinct array of glycan receptors, conjugation of glycan structures to naked oligonucleotide-based drugs and delivery platforms such as nanoparticles and extracellular vesicles can improve targeting efficiency as well as modulate biodistribution *in vivo*.