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Glycotherapy: New Advances Inspire a Reemergence of Glycans in Medicine

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Abstract

The beginning of the 20th century marked the dawn of modern medicine with glycan-based therapies at the forefront. However, glycans quickly became overshadowed as DNA- and protein-focused treatments became readily accessible. The recent development of new tools and techniques to study and produce structurally defined carbohydrates has spurred renewed interest in the therapeutic applications of glycans. This review focuses on advances within the past decade that are bringing glycan-based treatments back to the forefront of medicine and the technologies that are driving these efforts. These include the use of glycans themselves as therapeutic molecules as well as engineering protein and cell surface glycans to suit clinical applications. Glycan therapeutics offer a rich and promising frontier for developments in the academic, biopharmaceutical, and medical fields.

Glycans are a universal and essential component to life as we know it. They can be found as large structural polysaccharides, secreted mucus components, or protein and lipid conjugates, ranging in size from a single monosaccharide to polysaccharides thousands of units long (Ju et al., 2011; Hanisch, 2001; Wennekes et al., 2009; Apweiler et al., 1999; Somerville, 2006). Sugars coat the cells of every organism and are estimated to be the most abundant class of organic molecules on Earth (Mohanty et al., 2000; Weinbaum et al., 2007). However, while the structures of the monosaccharides were first elucidated by Fischer in the mid-1880s (Fischer and Bergmann, 1909), it took nearly a century before scientists began to appreciate the complex roles that these molecules played in biology (Bertozzi and Kiessling, 2001; Rademacher et al., 1988; Varki, 1993). This lag in understanding glycan structure and function is in part due to the complexity inherent to the regulation and assembly of these biomolecules. Glycans are not directly encoded by the genome and thus their biosynthesis and make-up is dictated by metabolism, signal transduction, and cellular status (Dennis et al., 2009; Parker and Kohler, 2010; Yarema and Bertozzi, 2001). Additionally, they can be connected by an array of linkage regiochemistries

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and stereochemistries, leading to large structural diversity that can then be further elaborated by functional group modifications (Cummings, 2009; Gabius et al., 2004; Muthana et al., 2012).

It is now well recognized that glycans play an essential role in a myriad of biological events including cellular adhesion and migration, organism development, disease progression, and the modulation of immunological responses (Haltiwanger and Lowe, 2004; van Kooyk and Rabinovich, 2008; Ohtsubo and Marth, 2006; Spiro, 2002). Although much effort has been spent on the study of glycans and how they affect disease, clinicians and medicinal chemists rarely consider glycans as biological targets or drugs (Ernst and Magnani, 2009). This unfamiliarity is beginning to change as improved methods for carbohydrate synthesis (Boltje et al., 2009; Lepenies et al., 2010; Zhu and Schmidt, 2009), sequencing (Alley et al., 2013; Zaia, 2008), and biological analysis (Laughlin and Bertozzi, 2009; Liang et al., 2008) of glycans become more sophisticated and widely available. This review focuses on a redefined approach to engineer glycan components for biomedical purposes that has emerged from the assimilation of carbohydrate chemistry, chemical biology, and glycobiology. Built on decades of carbohydrate research and tool development, scientists are creating improved or novel glycan products to control human health and disease. The realm of glycoengineering remains a young and exciting yet largely unexplored area in the creation of new therapeutics and medical treatments.

The History of Glycan Structures in Medicine

Much like protein and DNA biomolecules, glycans have had a very rich history in medicinal use. However, with the discovery of the genetic code and the ensuing DNA technologies, glycans and lipids became less appreciated as the other two main molecular constituents of life. Nevertheless, this brief omission has not reduced their importance or potential for therapeutic relevance (Marth, 2008). This is especially apparent with the rise in obesity and type II diabetes in which the role of lipids and glycans are essential to understanding and treating this burgeoning epidemic (Smyth and Heron, 2006). This section of the review will focus on the emergence of glycans themselves as administered therapies in the clinic, which provided some of the first major breakthroughs in modern medicine (Figure 1).

In 1900, Karl Landsteiner reported on the discovery of three blood types, A, B, and O, which governed compatibility in blood donor matching and allowed for the first successful blood transfusion to be performed by 1907 (Landsteiner, 1900). This discovery would garner him the Nobel Prize in Medicine in 1930 but the structures of the ABO constituents were not revealed until 50 years later. Studies to identify the chemical identities of the various blood types did not become fruitful until the 1950s, when both a source for copious active compound was found (ovarian cysts) and plant lectins that agglutinated blood group specific cells were discovered (Watkins, 2001). Work by Kabat, Morgan, and Watkins demonstrated that the main component of the H antigen was the monosaccharide fucose upon which N-acetylgalactosamine (GalNAc) or galactose (Gal) were added to form the A and B antigens, respectively (Kabat and Leskowitz, 1955; Morgan and Watkins, 1953; Watkins and Morgan, 1955). The full structures were then elucidated in the 1960,’s with the

clever use of selective alkylation chemistry coupled with enzymatic and acid/base hydrolysis to determine the monosaccharide linkages and components (Watkins, 2001).

The discovery and use of the polysaccharide heparin for treatment of thrombosis in humans has made a huge and lasting impact on the medical community. Heparin was first discovered in 1916 (McLean, 1916) and through advances in isolation from animal sources, it was used clinically by the 1930s (Lever et al., 2012). By the mid-20th century, most industrial heparin was isolated from porcine mucosa, which remains the main source for the 100 tons of heparin used per year. Investigations into heparin's mode of action led to the discovery of antithrombin III, which was found to be necessary for heparin's inhibition of the clotting cascade initiators, thrombin and factor Xa (Brinkhous et al., 1939; Lindahl et al., 1979). The structure of the basic disaccharide unit of heparin was not elucidated until much later and found to consist of sulfated glucosamine and iduronic acid, identifying heparin as a glycosaminoglycan (GAG; Lindahl et al., 1980). Interestingly, endogenous human heparin is found exclusively in a subset of mast cells where it appears to control the constituents of its granules used for immunological protection (Humphries et al., 1999). These discoveries, along with its clinical success, have made heparin a billion-dollar industry and rich source for further investigations discussed in later sections.

In 1917, Dochez and Avery reported that a "soluble-specific substance" shed from Pneumococcus could react with type-specific antisera from patients infected with the pathogen (Dochez and Avery, 1917). Five years later, Avery teamed up with Heidelberger, an early leader in the field of antibodies, to report that this substance was a type-specific polysaccharide-based soluble material (Heidelberger and Avery, 1923). Although this was initially met with much skepticism (Van Epps, 2005), by 1930, Francis and Tillett identified that this capsular polysaccharide could be used as a main component for vaccine development against *Pneumococcus* (Francis and Tillett, 1930; Heidelberger et al., 1950; MacLeod et al., 1945). Therapeutic products based on this polysaccharide have historically had a variety of clinical uses and are used in the vaccine Pneumovax (PPV23), which contains 23 purified capsular polysaccharides from *Streptococcus pneumonia* (Barocchi et al., 2007). While few subsequent polysaccharides from other pathogens were able to alone provide adequate antibody responses for vaccination, these discoveries proved that carbohydrates could make successful vaccines and gave the impetus to explore their further use, a main topic in this review.

Aminoglycosides are a class of amine containing small molecule glycans synthesized by the *Streptomyces* and *Micromonospora* genus of Gram-positive bacteria. The first aminoglycoside, streptomycin, was discovered in 1943 and found expedient clinic use as the first antibiotic for the successful treatment of tuberculosis (Schatz et al., 1944). Other members of this widely used class of antibiotics include gentamicin, kanamycin, and neomycin. Most function as protein synthesis inhibitors though the exact mechanism of all the aminoglycosides is not fully understood (Wang et al., 2012). Unfortunately, the rapid onset of bacterial resistance to aminoglycosides has led to a steady decline in their clinical use, but the increase in multidrug resistant strains has renewed interest to block resistance or engineer new target compounds (Becker and Cooper, 2013).

While the concept behind the imaging modality, positron emission tomography (PET), was first developed in the 1950s, it was the synthesis and use of 2-fluorodeoxy-D-glucose (FDG) 20 years later that brought this technology to the forefront of clinical oncology (Kelloff et al., 2005; Reivich et al., 1979). ^{18}FDG is taken up more quickly by cells with high metabolic demand than other cell types, and thus paved the way for detection of tumors and imaging of the brain (Ametamey et al., 2008). It would take several decades before alternative methods for the direct imaging of glycans and other biomolecules would appear, which has been covered extensively and not discussed further in this review (Laughlin and Bertozzi, 2009; Boons, 2010; Cipolla et al., 2011; Chang and Bertozzi, 2012).

Advances in Glycobiology Enable Gycoengineering of Novel Therapeutic Agents

Despite these early successes in bringing carbohydrates to the clinic, the remainder of the 20th century was fraught with difficulties in the field of carbohydrate research. Synthetic endeavors failed to produce a facile method to produce large quantities of glycans for studies while the analytical methods to separate heterogeneous natural polysaccharides were still in their infancy. Several key findings in the latter half of the 20th century pertaining to specific glycan structure activities led to the development of new techniques and the founding of glycobiology as its own field of research (Bertozzi and Kiessling, 2001; Rademacher et al., 1988). It wasn't long before scientists began to implement this information to engineer carbohydrates with distinct properties for use in the clinic.

Engineered Glycans and Glycan Mimics as Therapeutic Agents

Glycan Small Molecule Drugs

The majority of small molecule glycan-based drugs thus far have been carbohydrate binding protein (lectin) or glycosidase inhibitors (Figure 2; Asano, 2003; Brown et al., 2007; Ernst and Magnani, 2009). The most successful drugs to come from engineered sugar moieties have been the antiviral compounds zanamivir (Relenza) (von Itzstein et al., 1993) and oseltamivir (Tamiflu; Asano, 2003). The life cycle of the influenza virus involves the binding of viral hemagglutinin (HA) to sialic acid-containing glycans on host cell surfaces. These same glycans then serve as substrates for viral neuraminidase (NA) to ensure virus release and maturation (Shriver et al., 2009). Zanamivir and oseltamivir function by binding with nanomolar affinity to NA and halting viral budding as well as viral entry into cells (Matrosovich et al., 2004). Acquired resistance to oseltamivir has provided the impetus to design new drugs that target the influenza virus. In this regard, the Withers group has recently designed a difluorinated covalent NA inhibitor (Kim et al., 2013).

Other successful glycan pharmaceuticals include the type II diabetes mellitus drugs miglitol (Glyset) and acarbose (Precose, Glucobay), which inhibit glucosidases and amylases in the gut for the control of blood glucose levels. Miglustat (Zavesca, N-butyl-deoxynojirimycin) is a drug developed by Actelion and used primarily to treat the lysosomal storage disease type 1 Gaucher disease. Miglustat is an imino sugar first synthesized by Butters and Dwek who found that N-alkylated analogs of the natural product deoxynojirimycin were inhibitors of the glucosyl-transferase involved in glucosylceramide biosynthesis (Abian et al., 2011;

Platt et al., 1997). Treatment with miglustat was shown to alleviate the built up of sphingolipid seen in Gaucher disease. Many factors that manage bacterial adhesion on host cells are lectins and thus much work has been directed toward creating glycan based molecules to inhibit this interaction (Ernst and Magnani, 2009; Imberty et al., 2008). The most successful have been targeted to binding the PA-IL and PA-IIL virulence factors in *Pseudomonas*. *aeruginosa* and FimH of *Escherichia*. *coli*, both of which are opportunistic pathogens with increasing antibiotic resistance.

Glycosaminoglycans (GAGs) are a class of highly charged linear polysaccharides found secreted and connected to the cell surface that play a large role in physiology and development (Bishop et al., 2007; Sasisekharan et al., 2006). The structural elucidation of the most famous clinical GAG, heparin, in 1982 (Thunberg et al., 1982) enabled the synthesis of the fully active heparin pentasaccharide 1 year later (Linhardt, 2003). This advance led to the development of the first synthetically defined small molecule heparin, fondaparinux (ARIXTRA') which has been on the market since 2002 (Petitou and van Boeckel, 2004). Due to its small size and longer half-life, fondaparinux has enhanced potency and reduced risk of heparin-induced thrombocytopenia (Maccarana and Lindahl, 1993). The chemoenzymatic synthesis of a pentasaccharide heparin has also recently been reported (Xu et al., 2011). The importance of generating a defined heparin therapeutic was emphasized in 2008 when an oversulfated chondroitin sulfate contaminant led to an international health crisis (Liu et al., 2009). A similar approach has been used to synthesize a 3-O-sulfated heparin octasaccharide, which binds Herpes simplex virus type 1 (HSV-1) and blocks its entry into the cell (Copeland et al., 2008).

The discovery of selectins in the late 1980s was a major advance in the field of glycobiology that has inspired several decades of therapeutic development. The selectins control the attraction of leukocytes to sites of inflammation through the binding of the sialyl Lewis x (sLe^x) and sialyl Lewis a (sLe^a) carbohydrate structures (Bevilacqua et al., 1991; Lasky, 1995). Beyond leukocyte homing to sites of inflammation (Imhof and AurrandLions, 2004), sLe^x also plays a significant role in tumor migration (Schultz et al., 2012) and has recently been shown to be a necessary ligand for human sperm binding to the zona pellucida during fertilization (Pang et al., 2011). Despite early excitement in designing small molecule glycan structures such as Cylexin (CY-1503) to inhibit the selectins, many clinical trials have been stopped (Kerr et al., 2000). Nevertheless, fucosylated mimics of the Lewis structures are providing promising leads in current clinical trials for the treatment of asthma (Bimosiamose, TBC-1269; Kogan et al., 1998) and sickle cell crisis (GMI-1070; Chang et al., 2010). Recently, Ernst and coworkers used an nuclear magnetic resonance-guided fragment screen to design ligands with a sLe^x scaffold attached to a second site ligand (Egger et al., 2013). This approach afforded E-selectin inhibitors with low nM affinities, although these antagonists have yet to be tested in vivo.

The dynamic intracellular glycosylation of proteins with N-acetylglucosamine termed O-GlcNAcylation has also gained the attention of the medicinal community due to its altered regulation in cancer, diabetes, obesity, and Alzheimer disease (Bond and Hanover, 2013; Slawson et al., 2010). The O-GlcNAcylation modification is controlled by two enzymes, glycosyltransferase uridine diphosphate-N-acetyl-D-glucosamine:polypeptidyl transferase

(OGT) and O-GlcNAcase (OGA), which respectively install and remove O-GlcNAc from Ser and Thr residues. Although small molecule regulation of these enzymes is still in its infancy, the laboratory of David Vocadlo has developed a selective, low nM inhibitor of OGA, thiamet-G (Yuzwa et al., 2008). Treatment of rodents with thiamet-G was found to increase O-GlcNAcylation levels in the brain, which decreased tau aggregation and neuronal cell loss in a mouse model of Alzheimer's disease (Yuzwa et al., 2012).

New methods employing carbohydrate scaffolds are being utilized to screen the glycan chemical space for more potent inhibitors of lectins involved in disease states (Diot et al., 2011; Ferreira et al., 2010; Weïwer et al., 2009). In addition to the compounds reported above, many natural products are glycosylated, which influences their efficacy and specificity. These products constitute a rich field that is beyond the scope of this review (La Ferla et al., 2011; Ostash et al., 2010).

Polyvalent Glycan Structures and Inhibitors

A major constraint with small molecule, monovalent glycan structures as targets is that their lectin binding interactions are often too weak to be employed as suitable drug candidates. In this regard, the carbohydrate and polymer chemistry communities have successfully developed multivalent glycoconjugates which take advantage of the known "cluster glycoside effect" to enhance lectin avidity (Becer, 2012; Deniaud et al., 2011; Lundquist and Toone, 2002). These approaches have resulted in three major types of glycosylated macromolecular structures: glyconanoparticles, glycopolymers, and glycodendrimers (Becer, 2012; Chabre and Roy, 2008; Marradi et al., 2013). While many of these materials are still in their infancy in terms of biological application, they represent the next wave of glycan-based technologies to fill the therapeutic pipeline.

The most clinically promising scaffold employed thus far has arguably been the glycodendrimer (Chabre and Roy, 2008; Mintzer et al., 2012). Dendrimers are single molecular weight, globular structures which contain a central core, branching layers, and numerous functionalized end groups (Newkome and Shreiner, 2008, 2010). Though glycodendrimers have been synthesized for decades (Yuan, 1978), one of the first therapeutically relevant successes was the design of STARFISH; an oligovalent dendron-like compound bearing Gb_3 trisaccharide analogs (P^k) attached to a glucose pentavalent core (Figure 3A). The Gb_3 glycan is a known ligand for the multimeric Shiga-like toxins I and II, found in pathogenic *E. coli*. The multivalent display of P^k in STARFISH demonstrated an in vitro inhibitory activity against Shiga-like toxin that was 1–10-million-fold higher than any other known univalent ligands (Kitov et al., 2000). Similar approaches have been used to also bind and neutralize cholera toxin and heat-labile enterotoxin. Dendrimers bearing the sialic acid containing pentasaccharide, GM1, were generated that could increase in vitro binding to the toxins and inhibit their cellular toxicity (Pukin et al., 2007; Sisu et al., 2009).

Dendrimer structures have also been used effectively to block or image mammalian carbohydrate binding proteins. Chaikof and coworkers demonstrated that a high valency poly(ethylene oxide) (PEO) star dendrimer bearing highly sulfated lactose ligands could bind with high affinity to the selectins and reduce inflammation when administered in a mouse model (Figure 3B; Rele et al., 2005). The Davis research group has also created

multimeric selectin ligands by conjugating sLe^x to glyconanoparticles consisting of cross-linked amine-functionalized iron oxide (Figure 3H). Because iron oxide is an approved MRI contrast agent, the ability of the particles to target selectins enabled the successful in vivo imaging of cerebral inflammation in animal models of multiple sclerosis and stroke (van Kasteren et al., 2009).

Due to its role in the dissemination of many pathogens, dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN) has been a popular target for many dendrimer constructs. DC-SIGN is a C-type lectin present on both macrophages and dendritic cells that is exploited by pathogens such as HIV, Ebola, and *Mycobacterium tuberculosis* to spread and evade the immune system (van Kooyk and Geijtenbeek, 2003). In 2008, Wong and coworkers developed an oligomannose dendron that inhibited binding of the mannosylated HIV envelope protein, gp120, to anti-HIV antibodies and a recombinant dimeric DC-SIGN with IC_{50} values in the nanomolar range (Wang et al., 2008b). Since then, other groups have shown they could inhibit HIV trans-infection by blocking HIV with nanoparticles bearing mannose dendrimers (Martínez-Avila et al., 2009). Recent evidence has demonstrated that a high mannose dendrimer mimic can successfully inhibit DC-SIGN-mediated HIV infection in cellular and human uterine cervix explant models (Figure 3C; Berzi et al., 2012; Sattin et al., 2010).

The Davis research group has developed methodology in which glycodendrimers are attached to multiple sites on a protein to produce a highly valent “glycodendriprotein” (Rendle et al., 2004). This technology was employed in the conjugation of mannose dendrimers to Qβ self-assembling protein icosahedrons. The resulting “glycodendronanoparticles” are the most highly valent glycodendrimeric constructs yet seen with diameters of up to 32nm corresponding to 1,620 glycan units (Figure 3G). Using these supervalent constructs, they were able to inhibit the Ebola infection of dendritic cells by blocking viral binding to DC-SIGN (Ribeiro-Viana et al., 2012). In an attempt to increase delivery of vaccines to dendritic cells (DCs), the van Kooyk lab has generated poly(amido amine) (PAMAM) dendrimers conjugated to the tetrasaccahride Le^b, a known DC-SIGN ligand (García-Vallejo et al., 2013). When these dendrimers were attached to the OVA peptide as an antigen, they produced stronger DC activation and subsequent T cell stimulation than previous Lewis glycan conjugates to the OVA protein alone (Singh et al., 2009).

Sialic acid binding immunoglobulin-like lectins (Siglecs) are a class of cell surface receptors that are differentially expressed in hematopoietic cells and have been regarded as promising targets for drug delivery and cancer therapies (Jandus et al., 2011; O’Reilly and Paulson, 2009). The Paulson group has developed diversified sialoside libraries to identify new carbohydrate-based ligands for the Siglecs (Blixt et al., 2008; Rillahan et al., 2012). They have utilized these specific ligands for therapies to direct toxic virus-like particles and lipid nanoparticle carriers to particular leukocytes and lymphomas based on their Siglec expression (Figure 3I; Rhee et al., 2012; Chen et al., 2010, 2012b; Nycholat et al., 2012). These approaches provide new targets and support ongoing efforts in targeted gene delivery and targeted anticancer therapies.

The Kiessling research group has made important contributions to the glycopolymer field showing that the architecture of multivalent ligands is a key aspect governing activity. In effect, a designed scaffold can be required in some instances to enhance avidity and specificity (Gestwicki et al., 2002; Kiessling and Grim, 2013). Working on this notion, Bundle and coworkers improved their previous STARFISH inhibitor by creating glycopolymers composed of a monomer containing the P^k glycan linked to cyclic pyruvate ketal (CP) called (S)-PolyBAIT (Figure 3D; Kitov et al., 2008). CP is a known binder of serum amyloid P component (SAP), an endogenous protein that is able to target bound ligands for clearance and plays an important role in Shiga-like toxin clearance. An intravenous injection of this polymer protected 100% of mice that were subcutaneously injected with a lethal dose of Shiga-like toxin I. Interestingly, mice that were given a copolymer in which P^k and CP were randomly distributed developed severe signs of Shigatoxemia. These results highlight the importance of ligand placement on a polymeric scaffold in recruiting the necessary protein components for toxin clearance and drug efficacy.

Further demonstrating the importance of ligand placement in a glycan construct, the Davis group was able to generate a synthetically defined mimic of P-selectin-glycoprotein-ligand-1 (PSGL-1). Using a mixture of the copper-catalyzed azide alkyne cycloaddition (CuAAC) and their glycomethanethiosulphonates (glyco-MTS) ligation technology (Davis et al., 1998), they were able to site-specifically introduce sLe^x and a sulfated tyrosine into the bacterial enzyme SSβG. Because these two constituents are required for specific binding to P-selectin, the galactosidase (*lacZ*-type) activity of this glycoengineered SSβG could be used to stain for P-selectin in chronically inflamed rat cortex (van Kasteren et al., 2007).

Whereas homogeneous natural GAGs are nearly impossible to obtain from natural sources, the Hsieh-Wilson group has pioneered the use of ROMP to synthesize GAG glycopolymer mimics. Previous studies have suggested that the sulfation patterns of chondroitin sulfate (CS) were important for controlling healing in the nervous system but were limited by the ability to precisely control chondroitin sulfation patterns (Busch and Silver, 2007; Lin et al., 2011). Hsieh-Wilson and coworkers showed that synthesized ROMP-CS mimics were able to inhibit neurite outgrowth (Rawat et al., 2008), and in follow-up studies, they pinpointed this activity to sulfation of the 4- and 6-position of the galactosamine within CS (Figure 3E; Brown et al., 2012). Using this synthetic glycan enabled this group to create an antibody specific to the CS-E epitope that could promote axon regeneration in a mouse model of glial scarring in the optic nerve.

The glycopolymer field has recently been propelled by the use of newly developed controlled radical polymerization techniques that allow for the assembly of highly defined polymer structures (Vaázquez-Dorbatt et al., 2012). While many of these materials have yet to be tested biologically, Haddleton and coworkers recently reported a method using single-electron transfer living radical polymerization (SET-LRP) to create a linear copolymer where for the first time, the position of the sugars could be controlled (Figure 3F; Zhang et al., 2013). They constructed multi-block glycopolymers with acrylate monomers consisting of mannose, glucose, and fucose residues and demonstrated that only certain clustered mannose monomer structures could bind to DC-SIGN.

Carbohydrate-Based Vaccines

Carbohydrate-based vaccines have been at the forefront of efforts to bring carbohydrate chemistry into clinically relevant platforms (Boltje et al., 2009; Seeberger and Werz, 2007; Tarp and Clausen, 2008). Many currently utilized vaccines are comprised of glycans including vaccinations against *Neisseria meningitides* (Menactra), *Streptococcus pneumonia* (Prevnar), *Haemophilus influenzae* type b (Hib; Hiberix, Comvax), and *Salmonella typhi* (TYPHIM Vi; Morelli et al., 2011; Seeberger and Werz, 2007). However, these constructs are derived from natural sources and utilize nonspecific conjugation methods to carrier proteins. Herein, we will focus on recent developments in using synthetic glycoengineered vaccines to create more defined therapeutics. Advances in the fields of protein conjugation have also expanded the techniques available to generate carbohydrate vaccines selectively attached to protein carriers (Gamblin et al., 2009; Grayson et al., 2011). For those interested, several reviews are available that cover more in-depth progress of carbohydrate-based vaccines against bacteria (Pozsgay, 2008), HIV (Wang, 2006), and cancer (Cipolla et al., 2008; Ouerfelli et al., 2005).

The most successful case to utilize a synthetically defined vaccine has been the production of the Cuban Hib vaccine; the first clinically approved, fully synthetic carbohydrate vaccine based on the structure of the capsular polysaccharide antigen from Hib (Figure 4B; Verez-Bencomo et al., 2004). The pentasaccharide conjugated to tetanus toxin (TT) is produced under GMP conditions in scales large enough to be incorporated into Cuba's current routine vaccination system. A similar approach was taken by the Seeberger research group to generate an antimalarial vaccine by automated synthesis of the glycosylphosphatidylinositol (GPI) from the causative agent of malaria, *Plasmodium falciparum* (Figure 4A; Hewitt et al., 2002; Schofield et al., 2002). Recipient mice were protected against many of the damaging symptoms normally found during parasite infection including malarial acidosis, pulmonary edema, cerebral syndrome, and death. The same group has also successfully made a vaccine against the *Bacillus*. anthracis spore tetrasaccharide, which was used to raise antibodies against anthrax for detection and imaging purposes (Tamborrini et al., 2006).

Mulard and coworkers have reported on a synthetic pentadecasaccharide consisting of three repeating units of the O-specific polysaccharide (O-SP) domain of *Shigella flexneri* 2a (SF2a) LPS (Figure 4C). This glycan coupled to tetanus toxoid (TT) elicited a better serum anti-LPS 2a antibody response in mice than shorter synthetic O-SP sequences (Beálot et al., 2004; Phalipon et al., 2006). The immunized mice successfully produced glycoconjugate-induced anti-LPS antibodies which afforded them protection against SF2a infection. Preclinical data suggest these results are also applicable to human treatments (Phalipon et al., 2009). While these previous endeavors were based on archetypal conjugation techniques with monovalent epitopes, newer methods are employing multivalent epitope structures. A polymer bearing the β-mannan trisaccharide epitope from the *Candida albicans* cell wall conjugated to chicken serum albumin was shown to have more robust IgG production in mice than the trisaccharide-TT conjugate vaccine alone (Lipinski et al., 2011). In a follow-up study, Bundle and co-workers were able to use these antibodies to identify a minimal disaccharide epitope vaccine that could protect rabbits from fungal burden when challenged with live *C. albicans* (Figure 4D; Bundle et al., 2012). The Pier and Nifantiev labs have also

reported a series of synthetic oligoglucosamine vaccines that recognize β-(1→6)-poly-*N*-acetyl-d-glucosamine (PNAG), a common constituent in the capsular polysaccharide of many pathogenic bacteria. Antibodies from rabbits immunized with this construct were able to protect mice from *S. aureus* and *E. coli* infections (Gening et al., 2010).

One of the major discoveries to entice the glycobiology field in the past decade was the isolation of broadly neutralizing antibodies for HIV that bind the high mannose, Man_9 glycan of the HIV envelope protein, gp120 (McCoy and Weiss, 2013; Pejchal et al., 2011; Walker et al., 2009). The most highly studied variant is the 2G12 clone which neutralizes by slowing the entry of the virus into cells and can inhibit replication after passive transfer into patients (Platt et al., 2012). Other isolated clones such as PG9 can bind to the glycans from both gp120 and gp140 envelope proteins (Julien et al., 2013). Early attempts to elicit an immune response against the high mannose epitope of gp120 in a BSA conjugate were performed in rabbits, but the resulting antibodies were unable to bind the native gp120 N-glycans or neutralize the virus (Astronomo et al., 2008; Joyce et al., 2008).

These results spurred the Davis group to create a vaccine that used synthetic analogs in place of mannose to create a more immunogenic, non-self epitope (Figure 4E). Conjugation of this oligomannose mimic onto the virus-like particle Qβ bound with high affinity to 2G12 and elicited a greater antibody response than vaccines constructed with only mannose (Doores et al., 2010). Immunization in rabbits produced antibodies that were able to bind well to Man_8 and Man_9 conjugated to cowpea mosaic virus. Unfortunately, the antibodies did not bind native gp120 or reduce virus infection implying that the protein carrier is also crucial to eliciting a successful neutralizing antibody. The Danishefsky group has recently reported on the synthesis of a glycopeptide containing the epitope for the PG9 antibody (Aussedat et al., 2013). The glycopeptide contained the two closely spaced *N*-glycans at Asn^{160} and Asn^{156} of gp120 and could bind with high affinity to the PG9 antibody, whereas the glycans alone were not good ligands. Experiments are currently underway to evaluate their immunogenicity and HIV protection in animal models.

Alterations in the glycosylation of transformed cancer cells have been observed for decades (Fukuda, 1996; Kloppel et al., 1977; Sell, 1990). This characteristic change in glycosylation especially pertains to an increase in branched and highly sialylated structures that is often accompanied by an increase in mucinous proteins such as MUC1 and MUC16 (Hollingsworth and Swanson, 2004; Kufe, 2009). Unique recurring glycan epitopes are referred to as TACAs (tumor-associated carbohydrate antigens) and have been a predominant target for cancer vaccinations (Figure 4F; Pashov et al., 2011). Samuel Danishefsky was an early pioneer in the use of synthetically defined carbohydrate vaccines, creating the first synthetic Globo H vaccine for clinical use (Gilewski et al., 2001; Slovin et al., 1999). His research group has followed these advances with a vaccine that contains five prostate and breast cancer-associated carbohydrate antigens, Globo-H, GM2, STn, TF, and Tn conjugated to the carrier protein KLH (Figure 4F; Zhu et al., 2009). After initial studies demonstrating a successful IgG/IgM response in mice, this construct is currently in clinical trials. Wong and coworkers have also reported on an optimized vaccine against the hexasaccharide Globo-H. This approach improved upon previous reports by testing different carrier proteins and adjuvants to improve the immunogenicity and safety profile (Huang et

al., 2013). Globo-H conjugated to diphtheria toxoid and coadministrated with an α-galactosylceramide analog adjuvant induced a robust IgG response against Globo-H including other related structures specifically found on breast cancer and cancer stem cells. The Boons research group has reported a three-component vaccine composed of a TLR2 agonist, a promiscuous peptide T-helper epitope, and a tumor-associated glycopeptide. The all-in-one construct was able to elicit high titers of IgG antibodies in mice that could recognize tumor-associated carbohydrates on cancer cells (Ingale et al., 2007).

MUC1 was adopted early on as a cancer vaccine target due to its expression in a wide variety of cancers (Cheever et al., 2009). However, most of these initial approaches relied on traditional vaccination means with unglycosylated epitopes (Gilewski et al., 2000). The Boons group applied their three component approach to create a MUC1 vaccine consisting of a GalNAc-glycosylated MUC1 peptide, the T-helper epitope and the Pam_3CysSK_4 TLR agonist (Lakshminarayanan et al., 2012). This was the first example of a MUC vaccine that could elicit both humoral and cellular immunity leading to high titer production of IgG against the epitope in mice. The Payne research group has found comparable success in a similar three component vaccine approach that can assemble into nanoparticles for adjuvant purposes (Wilkinson et al., 2012). Embracing a multivalent synthetic approach, Dumy and coworkers have created self-adjuvanting glyco-lipopeptides bearing their regioselectively addressable functionalized templates (RAFT) construct (Renaudet and Dumy, 2003). These include a RAFT made of four α-GalNAc molecules (Tn antigen), a $CD8^+$ T cell epitope and a universal $CD4^+$ T-helper epitope attached to the Pam adjuvant (Bettahi et al., 2009). This construct produced robust IgG/IgM in mice that recognized tumor cell lines and elicited a reduction of tumor size in mice inoculated with syngeneic murine MO5 carcinoma cells. Another self-adjuvating approach involves the use of self-assembling fibrils from the Q11 peptide domain, which when attached to glycosylated MUC1 peptides, can induce an adjuvant-free response (Huang et al., 2012b).

Protein Glycoengineering to Enhance Efficacy and Therapeutic Value

Most therapeutic proteins are produced as a mixture of glycoforms each with its own biological efficacy and properties that must be fine-tuned for the therapeutic application (Arnold et al., 2007; Hossler et al., 2009; Sethuraman and Stadheim, 2006). For example, the physical properties of glycosylation can protect proteins from proteolysis or increase stability and solubility (Sinclair and Elliott, 2005; Solá and Griebenow, 2009). Additionally, each protein glycoform can have a distinct biological response with differences in pharmacodynamics and effector functions (Ferrara et al., 2011; Jefferis, 2012; Raju, 2008). Yet, isolating homogeneously glycosylated products and defining their individual in vitro and in vivo functions remains a daunting task. Even in the simplified case of human IgG antibodies, which have one N-glycosylation site on each of two heavy chains, up to 500 glycoforms may exist (Jefferis, 2009). Using current methodology, protein therapeutics must be constantly monitored and even blockbuster drugs show changes in glycosylation status over time (Rathore and Winkle, 2009; Schiestl et al., 2011). Two main tactics have emerged to produce homogeneously glycosylated products and address these complications. One embraces a chemically defined approach using synthetic methodology to generate glycosidic linkages on synthetic or recombinant proteins. Another pursuit uses engineered enzymatic

pathways in vitro or in the expression host itself to afford pure protein glycoforms (Bennett and Wong, 2007; Rich and Withers, 2009; Wang and Lomino, 2012; Yuan et al., 2010).

In the late 1970s and early 1980s, it became apparent that the conserved N-glycan on human IgG was important for not only its secretion, but also for determining the effector functions of the antibody through antibody-dependent cellular cytotoxicity (ADCC) and complement activation (Figure 5; Hickman and Kornfeld, 1978; Nose and Wigzell, 1983; Tao and Morrison, 1989). It was further shown that this conserved N-glycan at Asn297 of the Fc moiety was essential for binding to Fc receptors (Nimmerjahn and Ravetch, 2005), mannose-binding lectin (Malhotra et al., 1995), and the collectin-related molecule C1q (Raju, 2008). These discoveries led to the creation of engineered Chinese hamster ovary (CHO) and human embryonic kidney (HEK) expression hosts that produced specific glycosylation structures to direct effector functions toward a particular therapeutic application (Crispin et al., 2009; North et al., 2010). This work began with a CHO line that was engineered to express β-1,4-*N*-acetylglucosaminyltransferase III (GnTIII), which adds a bisecting GlcNAc onto the N-glycan core (Davies et al., 2001; Umanñа et al., 1999). Antibodies produced from these cells showed a substantial increase in ADCC and provided the first report of a cell line glycoengineered to produce antibodies with enhanced efficacy for killing cancer cells. It was later found that the bisecting GlcNAc also blocked core fucosylation and that the defucosylated Fc was the predominant feature for increased ADCC. This has led to continued efforts to produce nonfucosylated antibodies with enhanced Fc receptor binding (Satoh et al., 2006; Shields et al., 2002; Shinkawa et al., 2003; Zhou et al., 2008). Glycart Technology, now owned by Roche, has focused on the production of nonfucosylated antibodies for enhanced biotherapeutics. Seattle Genetics has also developed the fucose inhibitor 2-fluorofucose, which reduces fucosylation in vitro and in vivo for production of antibodies with increased ADCC (Okeley et al., 2013).

In addition to proinflammatory properties, the glycosylation status of IgG has also been to shown to exhibit anti-inflammatory effects. Intravenous gamma globulin (IVIg) therapy in which a heterogeneous population of IgG is injected in the patient has been implemented for decades to treat inflammatory diseases (Dwyer, 1992). The Ravetch research group was the first to demonstrate that this property was due to the sialylation of the Fc N-glycans. The antennary sialic acid reduced binding affinity to the activating Fcγ receptor-III and increased expression of the inhibitory Fcg receptor-IIB (FcγRIIB) (Kaneko et al., 2006). They further demonstrated that α2,6-linked sialic acid conferred this property which allowed the construction of an antibody containing increased α2,6-sialic acid for enhanced anti-inflammatory properties (Anthony et al., 2008). The galactosylation of N-glycans on IgG1 has also been shown to promote cooperative signaling of FcγRIIB with dectin-1, resulting in an inhibitory pathway that blocks the complement cascade and proinflammatory effector functions (Karsten et al., 2012). For those interested in further reading, the potential for controlling IgG-based therapeutics through glycoengineering has recently been reviewed (Jefferis, 2009).

Beyond antibodies, the extent and composition of glycans can also affect the efficacy of many other therapeutic proteins. Recombinant EPO (Epogen) has been widely used for the treatment of anemia, but its expression in mammalian hosts must be closely monitored as the

extent of sialylation on its N-glycans can heavily affect its pharmacokinetics (Fukuda et al., 1989). In fact, glycosylation affects the in vivo stability so much that removal of EPO's glycans decreases its plasma half-life in rodents from 5-6 hr to less than 2 min (Erbayraktar et al., 2003). Using this observation for inspiration, Amgen was able to design a hyperglycosylated form of EPO, darbepoetin alfa (Aranesp), which possesses two additional N-glycosylation sites and a 3-fold prolonged half-life in humans (Elliott et al., 2003). Darbepoetin alfa is currently the top choice for EPO treatment as the prolonged half-life increases potency and reduces the number of necessary injections. The sialic acid content and extent of N-glycan branching on follicle-stimulating hormone (FSH) also determine the protein's in vitro activity as well as its in vivo permanence in circulation (Creus et al., 2001). Introducing two additional N-glycosylation sites into FSH increased its size and charge to reduce renal clearance and enhance bioactivity (Perlman et al., 2003). This same approach was applied to bispecific single-chain diabodies, where adding extra N-glycosylation sites also improved pharmacokinetic profiles (Stork et al., 2008).

A new approach aiming to harness the beneficial effects of sialylation has been the incorporation of polysialic acid (PSA) onto therapeutic proteins produced in engineered HEK cells (Chen et al., 2012a). A polysialylated anti-HER2 single-chain variable fragment (scFv) showed an almost 30-fold increase in blood half-life but, unfortunately, PSA attachment also decreased receptor-mediated endocytosis. A sialylation approach has also been utilized to engineer insulin with a terminal 2,6-SialylLacNAc dendron. This glycodendrimer construct displayed an increased half-life and in vivo potency in mice while adding a LacNAc-only dendrimer did not have this effect (Sato et al., 2004). Sialylated glycans have also shown to prolong the serum half-life of quantum dots in addition to providing excellent water solubility and long-term stability (Ohyanagi et al., 2011), whereas quantum dots with nonsialylated glycans can lead to hepatotoxicity (Kikkeri et al., 2009).

The previous methods discussed include the production of recombinant proteins from mammalian cell hosts. However, these systems are still very susceptible to glycosylation changes through alterations in growth environment and are very costly (Datar et al., 1993; Hossler et al., 2009). Thus, a considerable amount of effort has been spent on engineering simpler expression hosts to produce human-like, single glycoform proteins. The mammalian cellular machinery employs two predominant pathways to glycosylate proteins—N-glycosylation and O-glycosylation. N-glycosylation of eukaryotic proteins begins with a common oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, that is transferred to the Asn residue of nascent polypeptides in the endoplasmic reticulum. This is followed by a set of glycosidases and glycosyltransferases that construct tailored N-glycan structures specific to the organism and cellular status. Mammalian mucin-type O-glycosylation, identified by a core GalNAc linkage to Ser/Thr, is more complex in its regulation and is not present in many lower eukaryotes (Hang and Bertozzi, 2005; Hanisch, 2001).

Gerngross and colleagues at GlycoFi engineered a *Pichia pastoris* yeast host that could produce a terminally sialylated EPO that had full potency in mice. In comparison, the unengineered host produced mostly high mannose N-glycans, which had minimal in vivo activity (Hamilton et al., 2006). This technology was also used to produce sialylated or nonsialylated humanlike N-linked glycoforms of recombinant human lactoferrin. Only the

sialylated structure was able to retain its ability to protect immune cells from methotrexate-induced death (Choi et al., 2008). GlycoFi has also used these glycoengineered yeast to produce single antibody glycoforms which piqued the interest of Merck to recently acquire the company (Hamilton et al., 2006; Li et al., 2006).

Callewaert and colleagues have provided an alternative method to produce glycoengineered yeast using patented *P. pastoris* strains and vectors which allow an individual company or researcher to produce their own glycoengineered yeast line. This technology was shown to produce relatively homogeneous glycoforms of murine IL-10, GM-CSF and IL-22 with a glycan structure matched to the particular *Pichia* strain used in its production (Jacobs et al., 2009). Efforts have also begun to introduce the mucin-type O-glycosylation machinery into yeast expression hosts. These glycoengineered yeast were capable of generating a fully active O-glycosylated human podoplanin (Amano et al., 2008). Furthermore, plants have now been engineered that can produce fully N- and O-glycosylated EPO containing sialylated biantennary structures (Castilho et al., 2012). The DeLisa research group has recently reported on an *E. coli* host containing the yeast *N*-acetylglucosaminyl- and mannosyltransferases and the bacterial oligosaccharyltransferase from *Campylobacter jejuni*. The engineered *E. coli* could successfully generate N-glycosylated proteins, such as IgG-Fc and hGH, bearing the eukaryotic pentasaccharide core (Valderrama-Rincon et al., 2012).

Instead of engineering the expression host to produce homogeneous glycans, another approach is to enzymatically alter the glycans postexpression (Figure 6). A classic example of this enzymatic tailoring approach is the 1997 paper by Witte and Wong in which a single glycoform of ribonuclease B was generated through treatment with glycosidases and glcosyltransferases (Witte et al., 1997). This type of engineering is currently utilized for treatment of the lysosomal storage disorder, Gaucher disease. To alleviate this enzymatic deficiency disorder, a glucocerebrosidase enzyme (imiglucerase, Cerezyme) is used that is in vitro deglycosylated, using α-neuraminidase, β-galactosidase, and β-N-acetylglucosaminidase, to expose terminal mannose residues, which improves its targeting and internalization (Pastores, 2010). The Lai-Xi Wang group has had great success in using glycosynthases, which are glycosidases with engineered glycosyltransferase activity (Lairson et al., 2008). Implementing activated donors as substrates, whole glycans can be transferred to create homogeneously glycosylated proteins (Huang et al., 2009). Complicated by low yields and the discrete substrate specificities of the endo-β-*N*-acetylglucosaminidases, Endo-A and Endo-M (Fujita and Yamamoto, 2006; Wang et al., 1997), Wang and coworkers have optimized a method using an Endo-S mutant to create fully sialylated antibodies with increased anti-inflammatory activities, nonfucosylated antibody glycoforms for enhanced FcγIIIa receptor-binding, and azide-tagged glycosylated antibodies for further modification of the natural N-glycan structure (Huang et al., 2012a). They have also utilized this methodology to remodel the N-glycans produced from a glycoengineered *E. coli* expression host (Schwarz et al., 2010). The Tolbert research group has successfully combined enzymatic glycan remodeling with native chemical ligation (NCL) to introduce an RGD integrin binding motif into a homogeneously glycosylated Fc construct for targeting cancer cells expressing the $\alpha_v\beta_3$ integrin (Xiao et al., 2009).

Approaches utilizing genetic and enzymatic engineering have arguably provided material that is closest to clinical implementation and therefore has been a heavy focus in this section. While synthetic chemistry has successfully provided homogeneously glycosylated peptides and proteins for investigating glycan function (Figure 6; Buskas et al., 2006; Pratt and Bertozzi, 2005), the synthetic methodology remains a technically challenging hurdle toward producing commercially relevant quantities. Nevertheless, we would like to present a few key highlights in the field, although we also direct the reader to more thorough reviews (Gamblin et al., 2009; Yuan et al., 2010).

The Kajihara research group has synthesized a fully synthetic EPO derived from piecing together solid phase peptide synthesis fragments by NCL and glycosylation at two native sites by cysteine alkylation. The synthetic EPO showed cellular activity comparable to that of the recombinant, naturally glycosylated protein (Hirano et al., 2009). They have also recently reported on a synthetic IFN-β construct glycosylated at the natural Asn80 with a complex sialylated or asialo N-glycan (Sakamoto et al., 2012). Surprisingly, the IFN-β with a sialylated N-glycan was a more potent antitumor agent compared to asialo or commercial IFN-βin a mouse tumor model, perhaps leading to the production of a better therapeutic. The Danishefsky lab has made progress toward the total synthesis of FSH by also combining solid phase peptide synthesis and NCL (Nagorny et al., 2009, 2012). However, the biological efficacy of the fully glycosylated product has yet to be tested. This group's recent advances in NCL combined with metal-free dethiylation to produce fully functional human proteins will hopefully be used to expedite the future chemical synthesis of fully glycosylated proteins (Li et al., 2012).

To bridge the high protein production of recombinant systems with the structural homogeneity of chemical glycan synthesis, many groups have employed unnatural linkages to introduce synthetic carbohydrates onto peptide or protein backbones (van Kasteren et al., 2007; Lee et al., 2009; Watt et al., 2003). Most of these approaches rely on bioorthogonal and chemoselective reactions that undergo specific conjugations without affecting native biological functional groups (Dirksen and Dawson, 2008; Sletten and Bertozzi, 2009). Only a few examples exist that include site-specific chemical glycosylation of full-length recombinant proteins. The Davis group has focused on site-specific conjugation with synthetic glycans through disulfide formation or thiyl radical addition to alkene-containing nonnatural amino acids (Chalker et al., 2011). The Bertozzi and Wong groups have utilized oxime formation to introduce chemoenzymatically derived aminooxy glycans onto specific protein sites (Hudak et al., 2011; Liu et al., 2003). Of recent note, the group of P. G. Wang introduced a method to glycosylate proteins with maltose- and dextrose-based polysaccharides as an alternative to conjugation with polyethyleneglycol (PEG), which is not as readily biodegradable (Styslinger et al., 2012). Clinical applications for this burgeoning field are on the near horizon as the techniques for protein conjugation as well as chemical and enzymatic glycan synthesis become more advanced and readily scalable.

Cell Surface Glycoengineering Offers Distinct Modes of Control and Targeting

While efforts in glycan engineering and remodeling have focused on glycans on proteins or as small molecules, many glycanprotein interactions in biology occur at cellular interfaces. Thus, there is a need for tools that can monitor or regulate the density of specific glycan structures in the context of cellular surfaces to better understand and control the biological response. In 1992, Reutter and colleagues took a step in this direction when they demonstrated that administering the monosaccharide derivative, N-propanoyl-D-mannosamine, to rats led to the subsequent incorporation of N-propanoyl-sialic acids into their cellular membranes and serum glycoproteins (Kayser et al., 1992). This notion of cell surface glycan remodeling was further established by the Bertozzi group, which developed metabolic oligosaccharide engineering (MOE) (Boyce and Bertozzi, 2011; Luchansky et al., 2003). This methodology has expanded the chemical space of the glycome to introduce ketone, azide, thiol, and alkyne functional groups into the glycans of cells and living animals (Figure 7; Laughlin and Bertozzi, 2009).

Beyond its use for imaging and analyzing glycans (Agard and Bertozzi, 2009), MOE can also be used for targeting specific glycans in therapeutic endeavors. Immunization of rabbits with a ketone-containing sialic acid bound to the carrier KLH produced significant titers of antibodies that were specific for this unnatural sialic acid moiety (Lemieux and Bertozzi, 2001). The antibodies were capable of binding to cancer cells that were fed the unnatural biosynthetic precursor and could successfully promote their eradication through complement-mediated lysis. This approach to target cancer has also been shown with N-propionyl-mannosamine which was incorporated into leukemic cell surface polysialic acids as N-propionylpolysialic acid. Cell death could then be induced by treatment with a specific antibody against α2-8-N-propionylated polysialic acid (Liu et al., 2000). Further efforts in this direction are being pursued by the Guo research group to increase the immunogenicity of specific TACAs for cancer immunotherapy (Wang et al., 2007, 2008a).

Using the unique reactivities of the non-natural sugars (Figure 7), a bioorthogonal reaction strategy has also been employed to target the pathogen *Helicobacter pylori* (Kaewsapsak et al., 2013). By feeding the bacteria an azide containing N-acetylglucosamine (GlcNAz), which is incorporated more efficiently into bacterial than mammalian cell surfaces, the azide on the *H. pylori* could be labeled with a 2,4-dinitrophenyl (DNP) phosphine by the Staudinger ligation (Saxon and Bertozzi, 2000). The *H. pylori* could then be eliminated by binding to naturally found anti-DNP antibodies. Aside from antibody targeting, the reactive functional groups of non-natural sugars can also target drug delivery vehicles as has been demonstrated in the reaction of hydrazide-coated nanoparticles with cell surface glycan ketones (Iwasaki et al., 2007). Because many pathogens and tumor cells display glycan structures not normally found on healthy human tissue, one can imagine a slew of modalities to target these epitopes via MOE.

MOE can also be used to engineer cell- and stem cell-based therapies for regeneration and disease studies (Du and Yarema, 2010). In fact, many stem cell markers are glycans such as the stage specific embryonic antigens SSEA-1, −3, and −4 or heavily glycosylated mucins

such as CD34 (Muramatsu and Muramatsu, 2004). The field of cell therapy is quickly expanding to include an array of therapeutic applications utilizing donor cells ranging from stem cell progenitors to terminally differentiated tissues (Stephan and Irvine, 2011). As a testament to their increasing therapeutic value, there are currently over 500 companies based on cell-based therapies and products (Kirouac and Zandstra, 2008). Bioengineering methods such as MOE can play a large role to increase their safety and efficacy.

In the late 1990s, N-propanoyl sialic acid incorporated into the glycoproteins of cells from neonatal rat brains was shown to stimulate the proliferation of astrocytes and microglia but not of oligodendrocyte progenitors (Schmidt et al., 1998). The same treatment was also shown to stimulate neurite outgrowth in cerebellar neurons (Büttner et al., 2002). The Yarema research group has utilized a thiol-containing mannosamine derivative to install thiols into surface displayed sialic acids. When stem cells bearing these cell surface thiols were grown on a gold surface, the strong gold-thiol bonds that formed induced the stem cells to adopt a neuron-like morphology and accumulate β-catenin, a marker in neuronal differentiation (Sampathkumar et al., 2006). Bertozzi and Gartner have also used the reaction with azide bearing sugars to install DNA on cell surfaces (Gartner and Bertozzi, 2009). By installing complementary DNA strands on different cell types, they were able to assemble defined microtissues that can be used for studying disease progression or organ development.

Other approaches in cell surface engineering have used biochemical techniques to introduce specific glycan structures onto cell surfaces to control their behavior and localization. In a key example, the Sackstein group identified that hematopoietic stem cells (HSCs) expressed a CD44 glycoform (HCELL) that contained sLe^x, which enabled HSCs to home to the bone marrow via binding to endotheial E-selectin (Dimitroff et al., 2001). The McEver group further demonstrated that human cord blood can home significantly better to the bone marrow upon treatment with exogenous α1-3 fucosyltransferase VI which increased cell surface sLe^x and thus selectin binding (Xia et al., 2004). The Sackstein group followed up on this study by treating mesenchymal stem cells (MSCs) with α1-3 fucosyltransferase VI, which introduced the sLe^x CD44 glycoform, HCELL, onto the MSCs (Figure 8A; Sackstein, 2012; Sackstein et al., 2008). These HCELL-engineered human MSCs demonstrated efficient homing to bone marrow; a process that was previously a limiting factor in MSC-based treatments for bone and cartilage diseases. In a similar approach, the Karp research group has decorated cell surfaces with biotin, which can be subsequently functionalized with a biotinylated sialyl $Lewis^x$ through a streptavidin bridge (Figure 8B; Sarkar et al., 2008, 2010). The glycoengineered cells showed enhanced binding to selectins and initiated rolling on activated endothelium. Mesenchymal stem cells engineered in this manner were able to home to inflamed tissue in a mouse (Sarkar et al., 2011).

In addition to introducing specific carbohydrate structures onto the cell surface, the removal of glycan epitopes has also garnered interest in therapeutic applications. A paramount example is the continuing endeavor to create universal blood through the removal of the group A and B blood antigens. Early pursuits in the 1980s identified an α-galactosaminidase from the coffee bean that could convert group B RBCs to the O blood type (Goldstein et al., 1982). While the converted blood proved safe and effective in human trials, a low pH

optimum and the required grams of enzyme per blood unit hampered advances to the clinic (Kruskall et al., 2000). A collaboration between the laboratories of Henrissat and Clausen led to the identification of two new bacterial glycosidase gene families that remove group A (GalNAc) and B (Gal) antigens from the underlying H antigen core on human red blood cells (RBCs; Liu et al., 2007). Treatment of group A RBCs with an α-N-acetylgalactosaminidase from *E. meningosepticum* and group B RBCs with an α-galactosaminidase from *B. fragilis* produced an O antigen blood type while maintaining RBC viability and activity.

While impressive, these previous techniques cannot introduce structurally defined glycans or control their density on cell surfaces. Yarema and Bertozzi previously showed that MOE could be used to glycoengineer cell surfaces by reaction with synthetic aminooxy glycans (Yarema et al., 1998). This approach, however, only added glycans onto randomly distributed cell surface glycoproteins and potentially obscured natural glycan epitopes. In an effort to emulate natural multivalent glycoproteins, the Bertozzi group developed a platform to engineer a cell's glycocalyx with synthetic glycans by generating glycopolymers end-functionalized with phospholipids that could passively insert into cell membranes (Rabuka et al., 2008). This technique enables the introduction of chemically defined glycan structures onto live cell surfaces, which is demanding to achieve through conventional biological methods alone. This platform was recently used to elucidate the roles of specific sialosides in mediating Siglec-based immunoevasion. A panel of sialylated glycopolymers was designed that could engage Siglecs on natural killer (NK) cells and control NK-mediated cytotoxicity against cancer cells (Figure 8C; J.E.H, C.R.B., and S.M. Canham, in press). This aided in defining a mechanism of cancer immunoevasion in which cancer hypersialylation can protect against NK cell recognition and activation. The most efficacious of these polymers could also mask xenogeneic porcine and allogeneic bone marrow cells from NK cell killing for applications in cell-based transplantation.

Conclusions

Eighty years ago, the structure of the DNA double helix was solved and ushered in a new era of biology. The ability to sequence and alter the genetic code is reaching its technological pinnacle and changing the way we approach treating disease. This is now also true for the glycocode. Much like genomic technology has enabled protein engineering (Brannigan and Wilkinson, 2002), the same can now be said for glycoengineering. Through advances in physical and biological chemistry, the glycobiology field now has the tools necessary to decipher the sequence and the structure of cell- and protein-associated glycans. Furthermore, cellular biologists and chemists have established novel ways in which we can alter and exploit glycan structure function. As this field has matured, it has become obvious that the biomedical community can no longer ignore the details of glycosylation. Glycans are ubiquitous and the ability to understand and control their functions are going to be vital to pioneering future biological and therapeutic breakthroughs.

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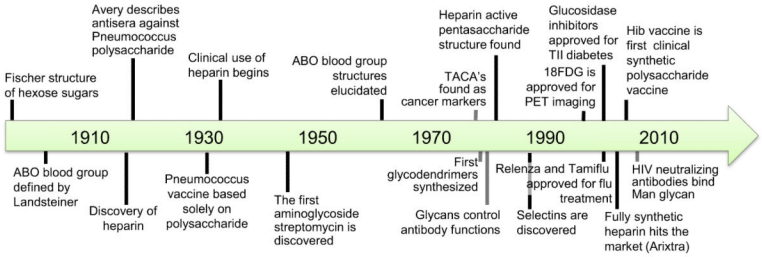

Figure 1. Timeline of Glycans in Medicine

The first half of the 20th century was marked with major breakthroughs in glycan-based treatments. However, further progress was dampened by a lack of structural understanding that was not available until the past 60 years. A large number of discoveries in the 1980s elucidated the molecular and mechanistic details of glycan-mediated biological events and provided the impetus to expand the use of glycans in therapeutic endeavors.

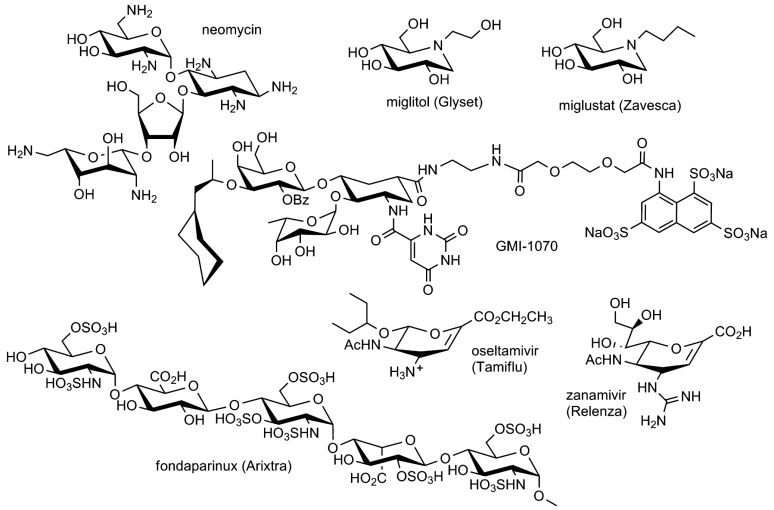

Figure 2. Structures of Glycan-Based Small Molecule Drugs Currently on the Market or in the Pipeline

All compounds are natural products or based on a carbohydrate scaffold. Their uses range from the prevention of bacterial (neomycin) and viral infection (oseltamivir, zanamivir) to the treatment of glycan-based diseases (miglitol, diabetes; miglustat, Gaucher), sickle cell crisis (GMI-1070), and as an anticoagulant (fondaparinux).

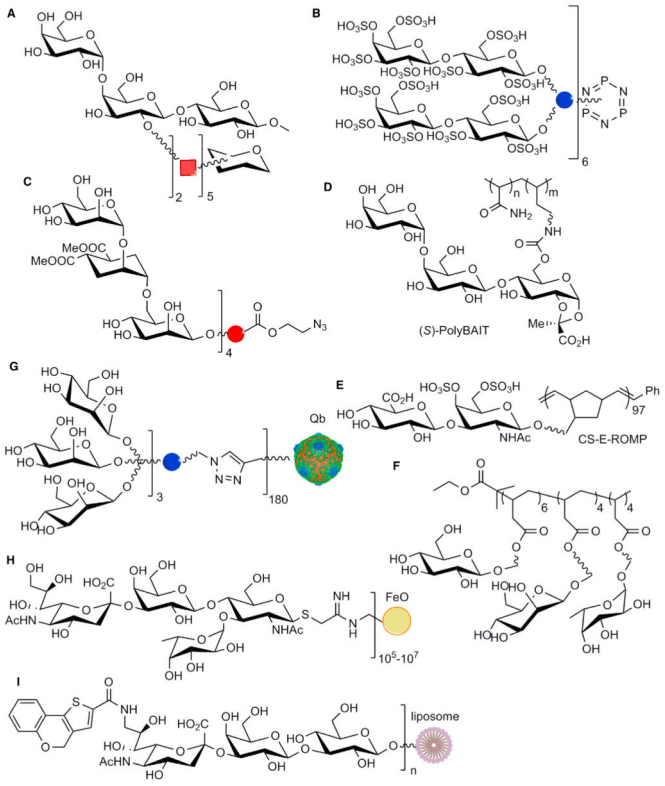

Figure 3. Polyvalent Glycan Structures Designed for Increased Avidity to Target Lectins

(A) STARFISH, a Shiga-like toxin inhibitor.

(B) Sulfated lactose PEO star dendrimer to inhibit selectins.

(C) High mannose dendrimers, which inhibit DC-SIGN mediated HIV infection.

(D) PolyBAIT, which inhibits Shiga-like toxin and increases its clearance.

(E) CS-E glycopolymer mimic for controlling neuronal healing.

(F) SET-LRP glycopolymers with controlled assembly for DC-SIGN binding.

(G) Qβ glycodendronan particle that inhibits DC-SIGN-dependent Ebola infection.

(H) Iron oxide sLe^x nanoparticels for selectin imaging.

(I) Lipid nanoparticles for delivery to Sialoadhesin cells.

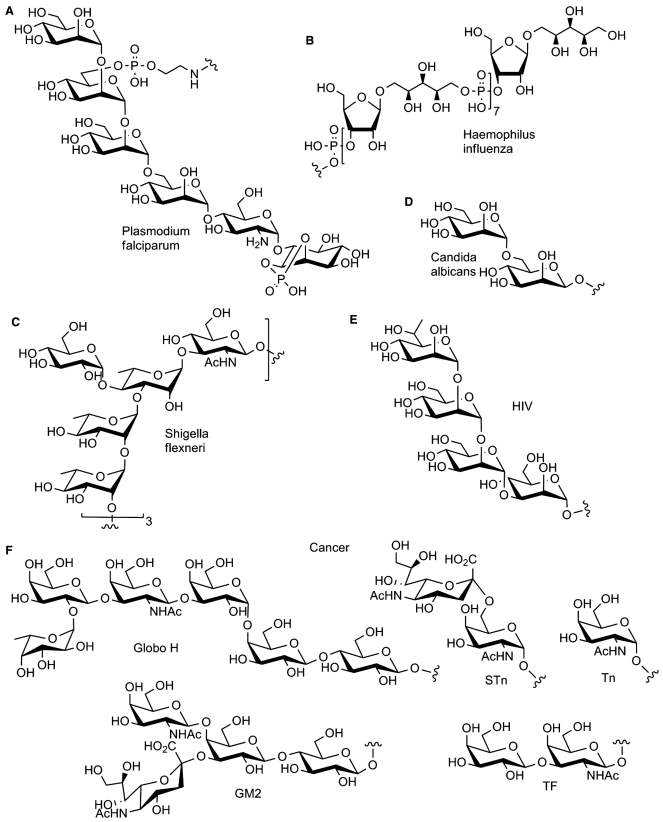

Figure 4. Synthetic Carbohydrate-Based Vaccines

Representative vaccines against microbial pathogen- (A–D), HIV- (E), and cancer-associated (F) glycan epitopes.

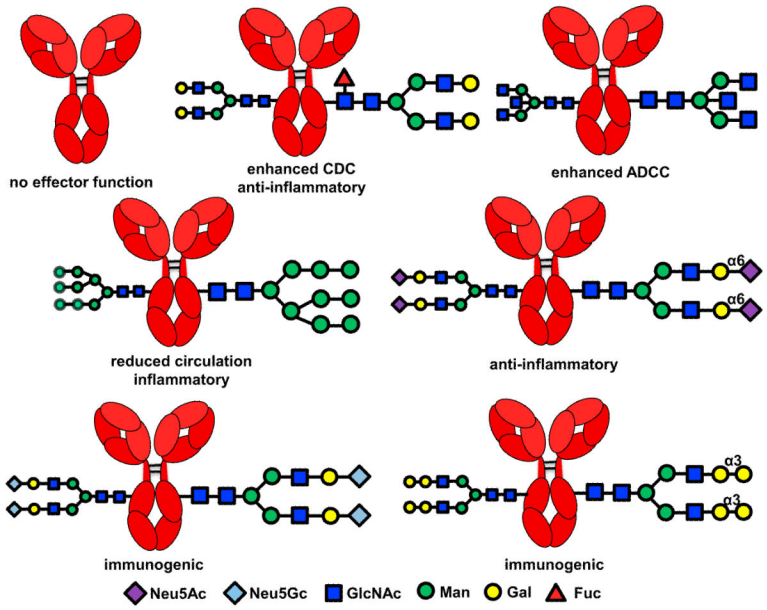

Figure 5. Effect of Glycan Structure on IgG Antibody Effector and Immune Function

Without glycosylation, IgG does not bind Fc receptors or activate complement, whereas the addition of different sialic acids can elicit anti-inflammatory or immunogenic effects.

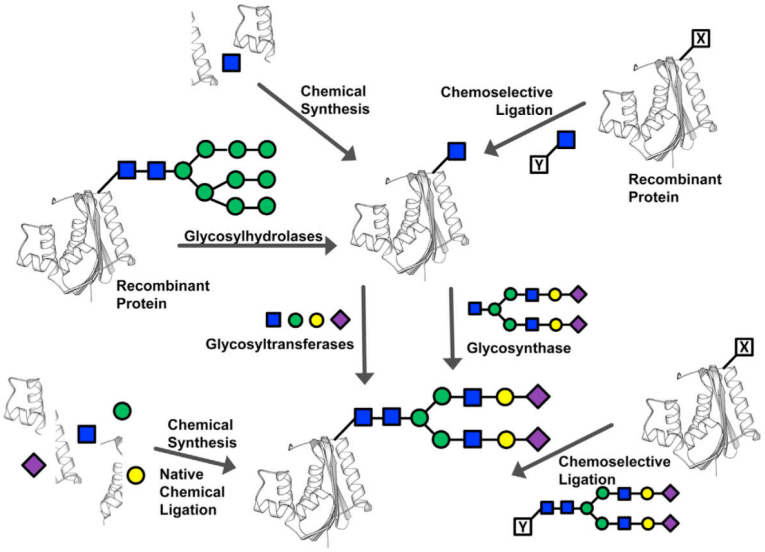

Figure 6. Synthetic and Enzymatic Methods for the Production of Homogeneous Glycoproteins

A glycoprotein with an initial monosaccharide can be generated by total synthesis, enzymatic removal of a heterogeneously glycosylated precursor, or attachment by a chemoselective reaction. This intermediate can then be further elaborated by enzymatic methods. Additionally, the full-length, fully glycosylated protein can be synthesized de novo or the complete glycan installed by a chemoselective reaction. X and Y represent functional groups that undergo chemoselective reactions.

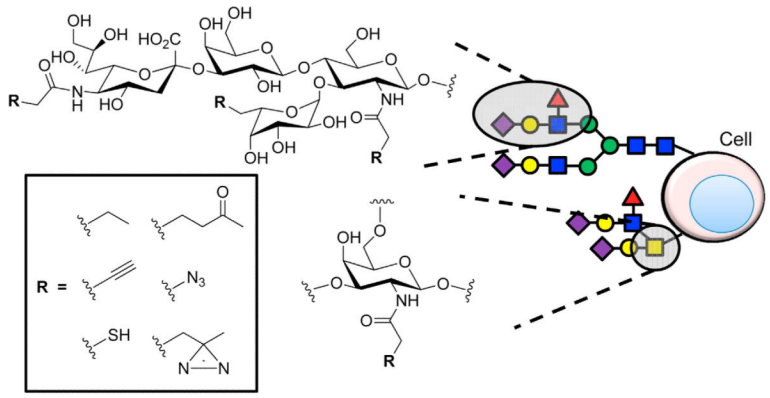

Figure 7. Modified Monosaccharides that Can Be Incorporated via Metabolic Oligosaccharide Engineering Display a Range of Functional Groups on the Cell Surface

Four modified monosaccharides have been incorporated into vertebrate glycans (Neu5Ac, GalNAc, GlcNAc, and fucose), which contain handles for chemoselective reactions or photoaffinity tags.

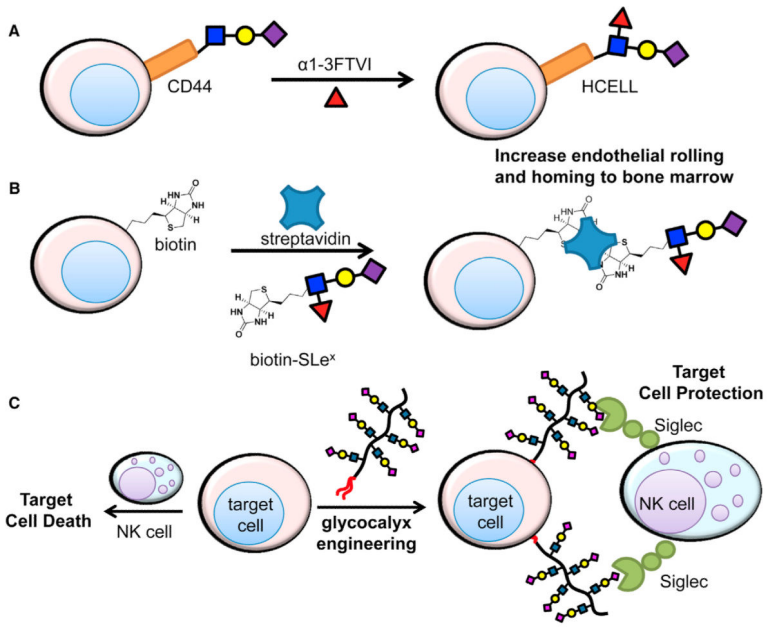

Figure 8. Cell Surface Glycoengineering

Glycosylation can be modified by exogenous enzymes (A) or materials (B and C) to alter glycosylation status for various functions. Cells with increased surface sLe^x bind to the selectins, which increase rolling along the endothelium or increase the efficiency for bone marrow homing (A and B). Additionally, glycocalyx engineering with cell surface-integrating sialic acid polymers can be used to protect cells from NK cell-mediated cytotoxicity (C).