Understanding the Chemistry and Biology of Glycosylation with Glycan Synthesis

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Abstract

Glycoscience research has been significantly impeded by the complex compositions of the glycans present in biological molecules and the lack of convenient tools suitable for studying the glycosylation process and its function. Polysaccharides and glycoconjugates are not encoded directly by genes; instead, their biosynthesis relies on the differential expression of carbohydrate enzymes, resulting in heterogeneous mixtures of glycoforms, each with a distinct physiological activity. Access to well-defined structures is required for functional study, and this has been provided by chemical and enzymatic synthesis and by the engineering of glycosylation pathways. This review covers general methods for preparing glycans commonly found in mammalian systems and applying them to the synthesis of therapeutically significant glycoconjugates (glycosaminoglycans, glycoproteins, glycolipids, glycosylphosphatidylinositol-anchored proteins) and the development of carbohydrate-based vaccines.

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INTRODUCTION

Carbohydrates, together with nucleic acids, proteins, and lipids, are the primary constituents of cells. Among these four major classes of biomolecules, carbohydrates are the least understood with regard to their biological function, mainly due to the lack of effective tools and methods for their study. Nevertheless, during the past 25 years, impressive progress has been achieved in our understanding of the biology of glycosylation (1). In addition to participating in metabolic events and other intracellular processes, oligosaccharides are often conjugated to lipids and proteins displayed on the cell surface and are involved in various intercellular communications and carbohydrate-mediated recognition processes. Because the biosynthesis of glycans depends on the expression of genome-encoded enzymes, the composition of cell-surface carbohydrates is specific to the cell type and alters as the cell undergoes developmental, physiological, and pathological changes. Not surprisingly, determining the biological functions of glycans is a complex task, and it requires the development of effective methods of glycan analysis and synthesis. Thus, glycobiology, initially formed at the interface of chemistry and biochemistry, has expanded to encompass areas of molecular and cellular biology, as well as physiology, and is expected to continue its integration into a variety of biomedical disciplines (2).

Heparin: a highly sulfated derivative of heparan sulfate; a commonly used anticoagulant

Antigen: recognized by the immune system as a foreign structure, thus activating the cascade of events that leads to antigen-specific antibody production

Glycosaminoglycans (GAGs): linear polysaccharides consisting of repeating units containing UA-HexN and Gal-GlcNAc

In addition to making fundamental contributions, research in glycoscience also has implications for biotechnology and medicine. Various carbohydrate scaffolds are found in a number of prescription drugs, which include nucleosides and macrolides, enzyme inhibitors (e.g., zanamivir), oligo- and polysaccharides (e.g., acarbose, heparan sulfate, heparin, and fondaparinux), vaccines, and therapeutic glycoproteins (e.g., bevacizumab, erythropoietin, and darbepoetin alfa) (3, 4). Although carbohydrate antigens and glycosaminoglycans (GAGs) isolated from natural sources have been used in the clinic, their heterogeneity could be a liability, as illustrated by the case of heparin sulfate contaminated with oversulfated chondroitin sulfates, which led to nearly 100 deaths (5). Fortunately, current chemical and enzymatic methods allow the generation of structurally defined oligosaccharides, glycoproteins, and other glycoconjugates. The approved synthetic carbohydrate-based vaccine against *Haemophilus influenzae* type b infection is the first successful example of this trend (6).

Despite impressive progress in developing methods of oligosaccharide synthesis, which has been reviewed recently (7–11), the field is still expanding, responding to the ever-growing demands of glycoscience. A major bottleneck in glycoscience research is the lack of homogeneous forms of glycans and the shortage of reliable and scalable methods of glycan synthesis. This review covers general aspects of the chemoenzymatic synthesis of glycans commonly found in mammalian systems and their application to the synthesis of GAGs, glycoproteins, glycolipids, glycosylphosphatidylinositol (GPI)-anchored proteins, and carbohydrate-based vaccines. The biosynthesis of different classes of oligosaccharides is briefly discussed to provide background information on the structural diversity of glycans and the enzymatic pathways required for target synthesis. Along the way, readers will be referred to more specialized reviews, and the most prominent contributions to the field will be discussed.

METHODS OF GLYCAN SYNTHESIS

Chemical Synthesis

Carbohydrate chemistry is an old discipline, which traces its origin to the pioneering work of Emil Fisher (11a) on the interconversion of monosaccharides and the determination of glucose (Glc) structure and its isomers. For some time, the field was mainly driven by the challenge of synthesis, rather than by practicality. Difficulties associated with stereoselective glycosidic bond formation and protecting group (PG) manipulations have hindered access to these biomolecules and limited our knowledge of glycosylation in biology. Nonetheless, when the need for carbohydrate probes emerged, synthetic chemists were equipped with the basic set of PGs, leaving groups (LGs), and promoters, which has been substantially expanded over the past 25 years. In this review, we outline the basic principles behind oligosaccharide synthesis. More detailed discussion can be found in a number of reviews covering methods of glycosidic bond formation (12, 13) and strategies for glycan assembly (8, 14, 15).

One of the pivotal challenges of oligosaccharide synthesis is selective glycosidic bond formation, which can proceed with either *trans* or *cis* regioselectivity between the substituents at C-1 and C-2 (**Figure 1**). The stereoselectivity of *O*-glycosylation depends on multiple factors; however, the structural features of the glycosyl donor and PGs have the most prominent roles in the reaction's outcome. Apart from discriminating the faces of the anomeric center by steric constraints (e.g., $R^2 = OCHPh_2$, **Figure 1**) (16), PGs can electronically participate in the stabilization of the glycosyl cation intermediates. In addition, cyclic PGs at remote positions can control stereoselectivity by imposing conformational constraints. Although there is no universal method for synthesizing diversely substituted saccharides, certain procedures designed for specific carbohydrate scaffolds can deliver a high degree of stereocontrol. The most effective strategies are summarized in **Figure 1**.

The classic concept of neighboring group participation usually refers to the 2-*O*-acyl group and analogs that can participate in stabilizing the oxocarbenium ion via an acetoxonium-type intermediate, leading to *trans* selectivity (**Figure 1*a***). Synthesis of the *cis* glycosides can be accomplished in the presence of nonassisting groups and usually results in a mixture of isomers; however, the concept of neighboring group participation has been now expanded to the construction of 1,2-*cis* glucoses and 1,2-*cis* galactoses (**Figure 1*a***) (17). The chiral auxiliary at C-2, the (1*S*)-(phenylthiomethyl)benzyl PG, stabilizes the oxocarbenium ion via the *trans*-decalin sulfonium intermediate and effectively prevents the *trans* face attack by the glycosyl acceptor.

The new and highly efficient solution to β-mannosidic linkages relies on the remote stereocontrol provided by the 4,6-*O*-benzylidene acetal PG (**Figure 1*b***) (18). In a similar manner,

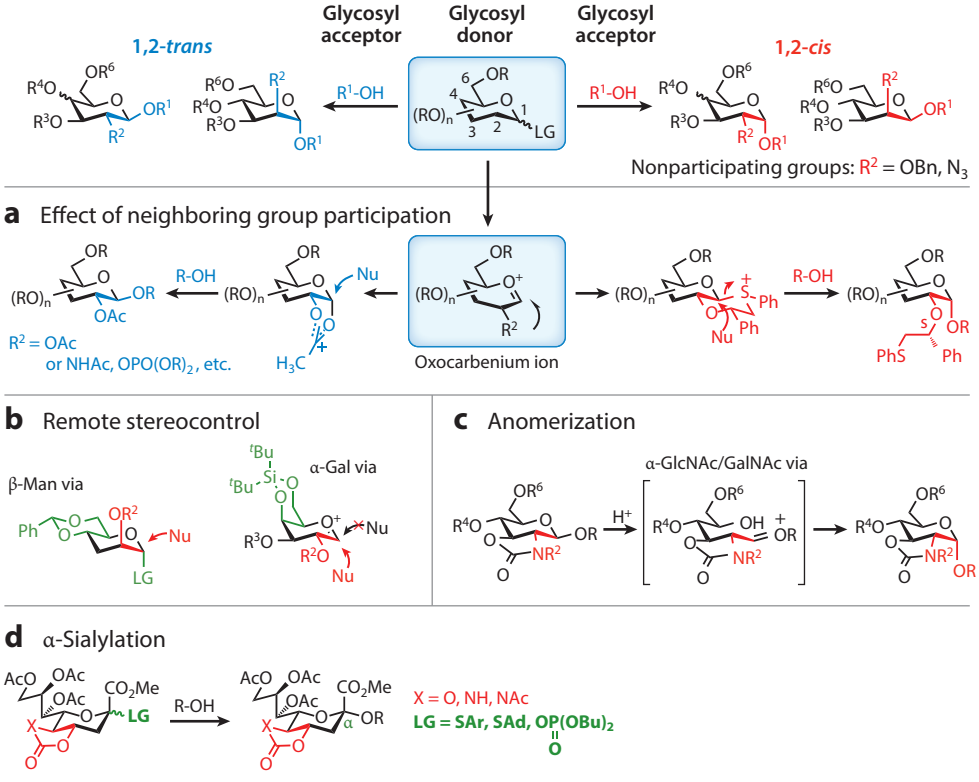


Figure 1

Chemical synthesis of oligosaccharides depends on reliable glycosylation methods, which can selectively provide 1,2-*cis* or 1,2-*trans* linkages. Structural features and protecting groups of the glycosyl donor are used to control the stereoselectivity of chemical glycosylation. Abbreviations: Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose.

the 4,6-*O*-silyl acetal group offers excellent *cis* selectivity in the α-Gal (galactose) and α-GalNAc (*N*-acetylgalactosamine) series (19).

Locking the C-2 and C-3 substituents in the oxazolidinone ring gives good *cis* selectivity for the aminoglycosyl derivatives as the result of acid-catalyzed anomerization (**Figure 1*c***) (20–22). Among all glycosylations, α-sialylation is regarded as the most difficult transformation, due to the poor control of stereoselectivity at the tertiary anomeric center and the absence of neighboring participating groups. A solution to this challenge has come in the form of cyclic carbonate and carbamate protection (18) used together with the α-phosphate LG (**Figure 1*d***) (23), which ensures high α-selectivity. This method has been successfully used to prepare sialoside arrays and to synthesize α-(2,9)oligosialic acids and *Neisseria meningitidis* capsular oligosaccharides for vaccine development (24, 25).

Another factor that controls stereoselectivity in glycosylation is the nature of the glycosyl acceptor, which in most cases cannot be easily changed, therefore further optimization can be accomplished by tuning the reactivity of the LG, the choice of the promoter, the order of addition, and the reaction temperature, pressure, and concentration. The effects of exogenous nucleophilic additives and media on glycosylation reactions have been extensively studied and recently reviewed (26).

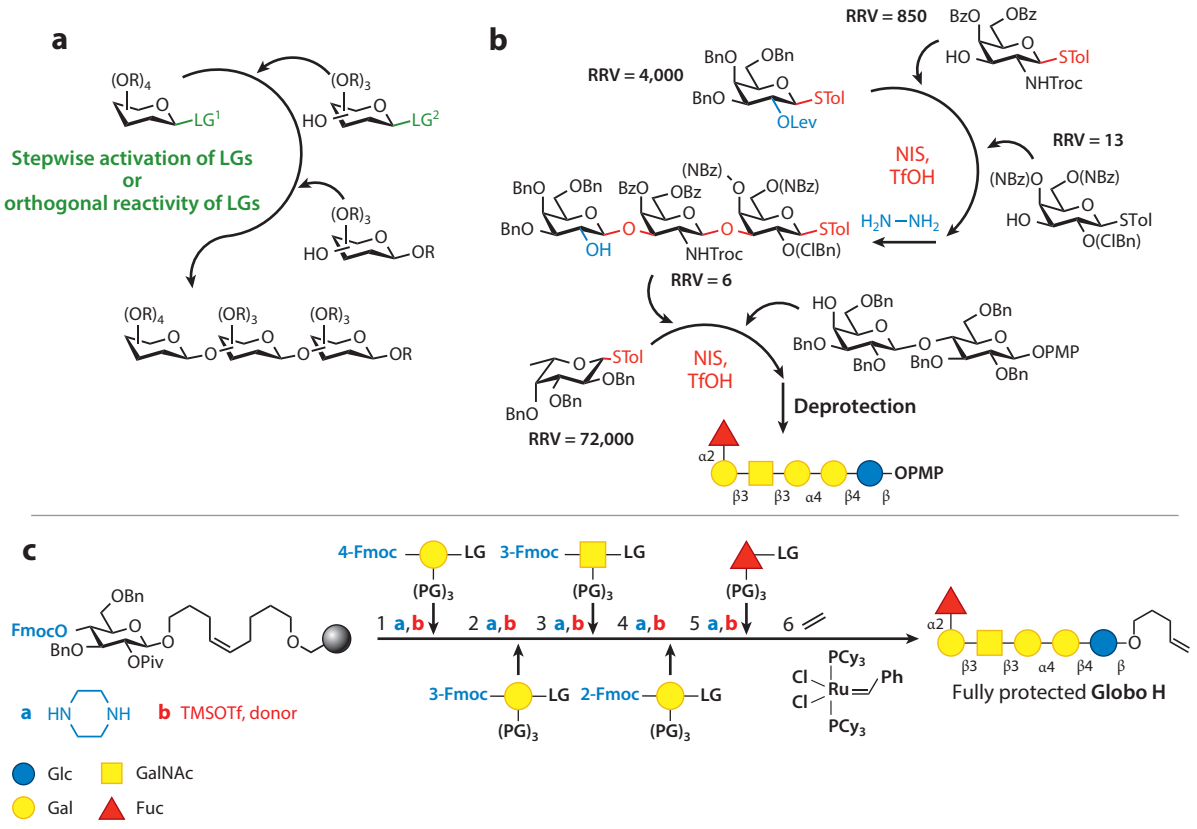


Figure 2

Strategies used for oligosaccharide assembly: (*a*) chemoselective glycosylation with different LGs and promoters for stepwise and orthogonal one-pot procedures, (*b*) reactivity-based, programmable one-pot synthesis, and (*c*) solid-phase automated synthesis of Globo H. Abbreviations: Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; LG, leaving group; RRV, relative reactivity values.

In addition to addressing the problem of stereoselective *O*-glycosylation, notable effort has been directed toward developing a simple, yet efficient, method of glycan assembly. One of the major advances in this area has been the introduction of a one-pot procedure (**Figure 2*a*,*b***). The original one-pot procedure reported by Raghavan & Kahne (27) took advantage of the different reactivities of LGs; later variations of one-pot procedures are based on the PG-controlled reactivity of glycosyl donors. Building on the observation that glycosyl donors with electron-donating PGs undergo faster hydrolysis than glycosyl donors equipped with electron-withdrawing groups, Fraser-Reid and colleagues (28) formulated the concept of the armed–disarmed glycosyl donors for carbohydrate synthesis, which was further expanded to address the stereoelectronic and torsional effects of PGs (29, 30). The first quantitative assessment of the PG-based reactivity of glycosyl donors was performed by the Ley group (31).

The concept of one-pot glycosylation has further led to the development of the iterative one-pot reaction (32) and the orthogonal one-pot procedure (15, 33). Other notable examples include one-pot protocols for synthesizing suitably protected monosaccharides (34, 35).

One-pot: refers to the multistep reaction performed in a single setup, thus eliminating the need to isolate intermediates and shortening the experimental time frame

Programmable and Automated Synthesis of Oligosaccharides

The complexity of the chemical synthesis of glycans, especially in PG and LG manipulations, represents a major impediment in the field. Although the one-pot strategy has proven to be quite efficient for synthesizing complex oligosaccharides, its utility is restricted to those with expertise in carbohydrate chemistry. Anticipating the transformation of highly specialized oligosaccharide synthesis into a routine and automated process, a programmable strategy has been introduced by the Wong group (**Figure 2*b***) (36). A software program called OptiMer has been designed to aid chemists in selecting building blocks for the one-pot assembly of oligosaccharides. The selection and assessment of the PGs that affect the anomeric center reactivity of thioglycosides were determined and expressed as relative reactivity values. The database contains more than 400 building blocks and has been used to prepare various oligosaccharides (from tri- to hexa-), including colon cancer antigen Lewis Y (Le^y), sialyl Lewis X (sLe^x), fucosyl GM_1, heparan pentasaccharide, and the embryonic stem cell surface carbohydrates Lc_4 and $IV^2Fuc\text{-}Lc_4$ (8). As an example, the Globo H antigen was assembled in an efficient manner using the one-pot procedure (**Figure 2*b***) (37), a considerable improvement over the original multistep synthesis.

Automated oligosaccharide synthesis on solid supports also holds great promise; the most advanced prototype machine, developed by the Seeberger group (14, 38), utilizes the concept of a peptide synthesizer. The logic behind automated solid-phase synthesis can be illustrated with the preparation of Globo H (**Figure 2*c***) (39). After the initial attachment of the acceptor to the solid support through an orthogonally cleavable linker, the repetition of deprotection–coupling–capping cycles is performed until the desired oligosaccharide is assembled. Some of the notable targets synthesized by this technology include Le^y and Le^x antigens; *N*-linked glycan cores; short glycopeptides; heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS) oligosaccharides; α-mannosides; and GPI glycolipids (14). The main drawbacks of the method are the need for selective deprotection after each coupling step and the high cost of the monomeric building blocks, which are used in excess to achieve high-yield conversions. These restrictions could be partially lifted with solution-phase methods using flow reactors (40), fluorous-tag-assisted techniques (41, 42), or reagent-free donor activation, as in the case of the electrochemical synthesis of oligoglucosamines (43). Moving forward, for the programmable and solid-phase methods to be routinely used for the synthesis of oligosaccharides, all required building blocks and equipment would have to become readily available to the community.

Yamada & Nishimura (44) applied solid-phase methods to biocatalytic transformations and developed a prototype of an automated glycosynthesizer, coined the artificial Golgi, with enzymes immobilized on solid supports (4, 8). Others have followed this direction with the development of digital microfluidic devices (45, 46).

Chemoenzymatic Synthesis

Although chemical methods are still commonly practiced in academic laboratories because they can quickly provide new structures for exploratory research, they cannot compete with the efficiency of the enzymatic methods used for industrial-scale synthesis in which there is no PG manipulation and the reaction proceeds in aqueous solution at ambient temperature. The repertoire of enzymes available for glycan synthesis and oligosaccharide modifications includes glycosyltransferases (GTs), glycosidases, phosphorylases, sulfotransferases, and others. This section outlines the general methods of enzymatic synthesis; in-depth discussion can be found in a number of reviews (8, 47, 48).

In contrast to the chemical synthesis of oligosaccharides, which relies heavily on PGs, nature's solution to stereospecific glycosyl bond formation is rooted in the choice of the LG and the

GT. The majority of mammalian GTs are of the Leloir type; GTs of this type utilize nine basic nucleotide-activated building blocks (donors) for the stepwise synthesis of complex oligosaccharides. Despite the high fidelity and efficiency of glycosylations with mammalian GTs, microbial GTs may be more suitable for in vitro synthesis because (*a*) the substrate flexibility of bacterial enzymes allows analog synthesis, (*b*) they have better solubility profiles, and (*c*) the ease of expression in *Escherichia coli* or other species enables preparative synthesis.

Early attempts to use GTs for the synthesis of oligosaccharides required expensive nucleotide donors and suffered from feedback inhibition caused by nucleoside–phosphate by-products. These challenges were resolved with the simple concept of nucleotide–phosphate recycling, reported by Whitesides and colleagues (49), as demonstrated by the large-scale preparation of *N*-acetyllactosamine (LacNAc) with a multienzyme system containing the in situ generation of uridine 5′-diphosphogalactose via uridine 5′-diphosphoglucose, from uridine 5′-triphosphate and glucose 6-phosphate. Since then, multienzymatic protocols for the regeneration of other nucleotide donors have been developed (50) and applied to the synthesis of sLe^x (51), sialyl-T antigen (52), disialyllacto-*N*-tetraose (53), heparin oligosaccharides (54), and hyaluronic acid (55). The power of the efficient recycling systems is best illustrated by the preparation of Globo H and SSEA4 antigens for use in cancer vaccine development (**Figure 3*a***) (56). Multigram synthesis was enabled by the sugar kinase-mediated generation of sugar-1-phosphate, which was subsequently converted to a sugar nucleotide. The fidelity of GTs ensures high yields, albeit with complexity in the reaction mixture.

In addition to its high efficiency, GT-mediated synthesis proceeds with excellent regio- and stereoselectivities, thus making GTs irreplaceable for the glycosylation of complex scaffolds, particularly the terminal sialylation of glycans (57, 58). Despite its potential, the availability of GTs poses the main obstacle to the widespread application of GT-mediated synthesis. Expanding the list of synthetically relevant GTs to cover all biologically significant glycosylic linkages and increasing the number of commercially available enzymes would undoubtedly benefit the field (47).

Another class of enzymes applied to glycan synthesis is glycosyl hydrolases (GHs), which are commonly used in the industrial processing of polysaccharides. Due to the reversibility of hydrolysis, certain conditions can be used to skew the equilibrium toward the glycosylation product. More than 2,500 GHs, targeting nearly every glycosidic bond, are known. Many GHs are commercially available or can easily be expressed in *E. coli*, making these catalysts synthetically attractive. Comprising more than 130 different families with diverse structures, the GHs feature a fairly conserved active site. Hydrolysis with GHs occur with high specificity and produce products with retention (**Figure 4*a*,*c***) or inversion at the anomeric carbon. Structural variations within the binding site control the position of glycosidic bond cleavage; *endo*-acting enzymes have a continuous binding groove, which is blocked in the *exo*-glycosidases (GHs). GHs possess a well-defined site for donor recognition, but show a high degree of acceptor promiscuity. The challenges of synthesis with GHs include poor regioselectivity, the problem of self-condensation, and low yields due to competing hydrolysis. Thus, synthesis with native GHs under thermodynamic conditions generally gives low yields, but it could be synthetically viable under kinetic conditions, for example, with the use of activated glycosyl donors (fluorides, oxazolines), organic cosolvents, or excess of the glycosyl donor.

A major breakthrough in the area of enzymatic glycan synthesis was the introduction of *exo*-glycosyl synthase (GS) by Withers and colleagues (60). The mutation of the nucleophilic residue at the active site of GH abolishes its hydrolytic function and generates GS, which is suitable exclusively for synthesis (**Figure 4*b***). In the past, many GHs used for synthetic purposes were of the *exo*-type; however, in the last decade *endo*-GH and *endo*-GS have become invaluable in the synthesis of homogeneous glycoproteins (**Figure 4*d***).

Leloir glycosyltransferases: these use nucleotide-phosphate-activated monosaccharides as glycosyl donors; non-Leloir glycosyltransferases recognize phosphate or polyprenyl phosphate donors, or starch-derived oligosaccharides

SSEA: stage-specific embryonic antigen

GHs: glycosidases, glycoside hydrolases or glycosyl hydrolases

***Exo*-glycosidases (GHs):** these break the glycosidic bond of terminal sugar residue (or disaccharide), whereas *endo*-glycosidases hydrolyze the internal bond, thus cleaving more than one sugar

GS: glycosyl synthase

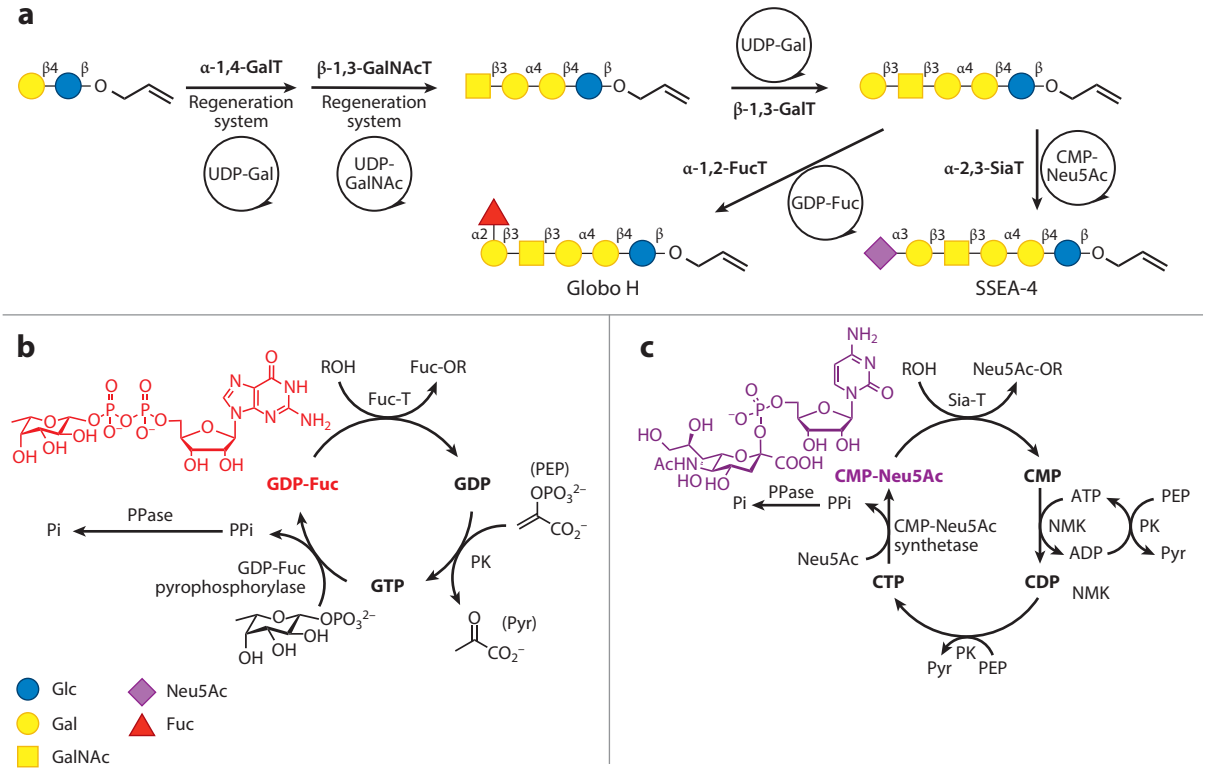


Figure 3

(*a*) Enzymatic multigram synthesis of Globo H and SSEA4 antigens with one-pot regeneration of sugar nucleotides. Examples of sugar nucleotide regeneration systems starting from a free sugar or sugar-1-phosphate to the sugar nucleotide for GT reaction: regeneration of (*b*) GDP-Fuc and (*c*) CMP-Neu5Ac. Abbreviations: CMP-Neu5Ac, cytidine-5′-monophospho-*N*-acetylneuraminic acid; GDP-Fuc, guanosine 5′-diphosphofucose; GT, glycosyltransferase; NMK, nucleoside monophosphate kinase; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PPase, pyrophosphatase; Pyr, pyruvate; SSEA, stage-specific embryonic antigen; UDP-Gal, uridine 5′-diphosphogalactose; UDP-Glc, uridine diphosphoglucose.

Transglycosylation with transglycosidases or phosphorylases is another practical method for glycosidic bond formation. Phosphorylases are highly specific toward donor substrates, yet they demonstrate relaxed acceptor specificity. As they are cheap and robust, phosphorylases have been adopted to the industrial synthesis of certain simple di- and trisaccharides (61, 62).

SYNTHESIS OF GLYCAN TARGETS

Having reviewed the basic principles of chemical and enzymatic methods of oligosaccharide synthesis, in this section we discuss the scope and limitations of the methods available for synthesizing naturally occurring glycoconjugates that are immediately related to human physiology.

Glycosaminoglycans

The GAGs are essential polysaccharides that mediate numerous processes in multicellular organisms and certain microbes that have adopted GAGs as a part of their host mimicry. GAGs are

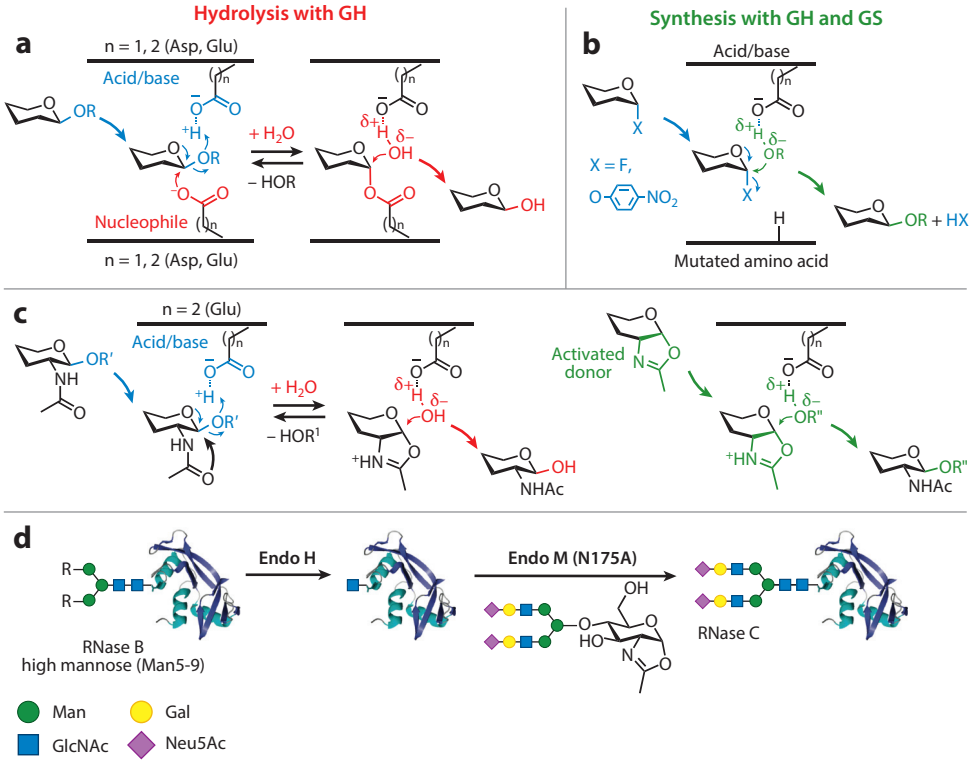


Figure 4

Synthetically relevant mechanisms of the oligosaccharide transformations with GHs and GSs. (*a*) The catalytic site of retaining GHs contains two amino acid residues, with one acting as an acid or base (Glu or Asp) and the other as a nucleophile (Glu or Asp, sometimes Tyr). (*b*) Removal of the nucleophilic site in artificial GSs prevents hydrolysis of the product. (*c*) Substrate-assisted retaining GHs can be used for hydrolysis or synthesis with activated oxazoline donors. (*d*) Application of *endo*-GHs and GSs for the glycan remodeling of RNase (59). Abbreviations: Asp, aspartic acid; Endo, endoglycosidase; Gal, galactose; GH, glycosyl hydrolase; GlcNAc, *N*-acetylglucosamine; Glu, glutamic acid; GS, glycosyl synthase; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; RNase, ribonuclease; Tyr, tyrosine.

widely distributed in the extracellular matrix and on the cell surface; they bind to a variety of proteins and participate in regulating blood coagulation, cell proliferation, differentiation, adhesion processes, and cell–pathogen interactions. Composed of repeating disaccharide units (GlcA-HexN or Gal-GlcNAc), GAG chains are subject to further modifications that increase their structural diversity and substantially complicate the synthesis of these macromolecules (**Figure 5*a***). Compared with other polysaccharides, heparin and heparan sulfate (HS) are regarded as the most challenging synthetic targets, as they display all possible modifications: *O*-sulfation, *N*-deacetylation and *N*-sulfation, and C_5-epimerization of D-glucuronic acid (GlcA) to L-iduronic acid (IdoA) (63, 64). During the biosynthesis of HS, installation of *N*-sulfated glucosamine (GlcNS) residues in heparosan is crucial for starting a cascade of the sequential modifications catalyzed by C_5-epimerase (65), 2-*O*-sulfotransferase (2-OST), 6-OST, and 3-OST (66). Incomplete conversions of the sulfation reaction and reversibility of the epimerization step result in structural heterogeneity in GAGs. At the end, modifications are dispersed throughout the polysaccharide chain, creating highly sulfated domains (so-called NS domains) separated from unmodified domains (or NAc

Heparosan: $[GlcA\beta1,4\text{-}GlcNAc\alpha1,4]_n$ is the unmodified backbone of heparin

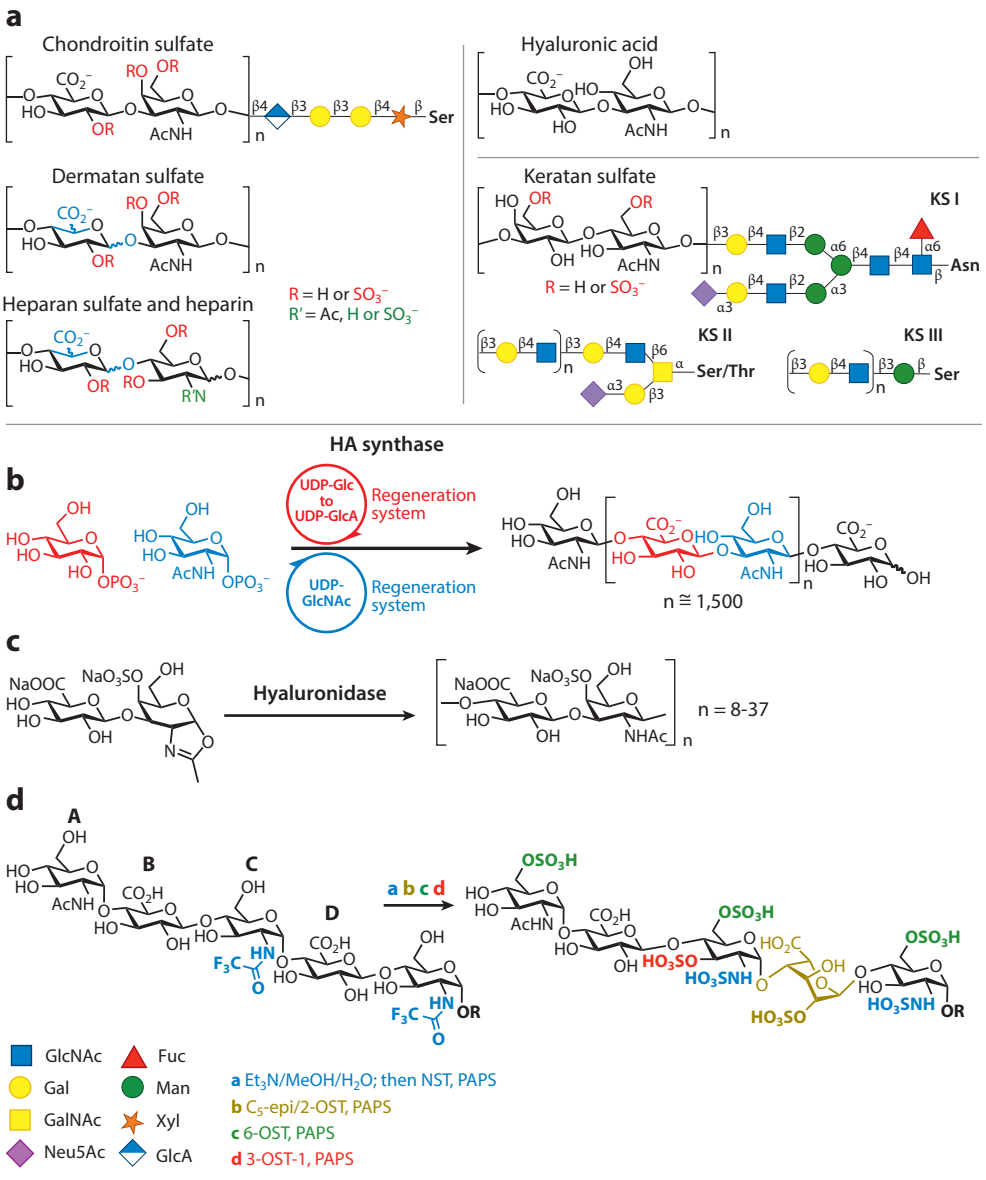


Figure 5

(*a*) Examples of naturally occurring polysaccharides: HA and HS (GlcNAc-GlcA repeating units), CS and DS (GalNAc-GlcA), and KS (GlcNAc-Gal). Except for HA, other polysaccharides contain domains with different modification patterns: *O*-sulfations (present in CS, DS, and HS), C_5 epimerization (DS, HS), and *N*-sulfations (HS). In vivo sulfation of GAGs is the result of the synergistic action of STs and sulfatases. Polysaccharide chains of CS, DS, and HS are bound to a protein core (Ser) through a conserved glycan sequence. KS is linked through conserved oligosaccharide sequences to either Asp (KS I), Thr (KSII), or Ser (KS II, KS III). (*b*) Synthesis of HA with regeneration of nucleotide–phosphate donors. (*c*) Polymerization of oxazoline disaccharides with hyaluronidase. (*d*) Enzymatic synthesis of homogeneous heparin oligosaccharides. Abbreviations: Asn, asparagine; CS, chondroitin sulfate; DS, dermatan sulfate; Fuc, fucose; GAG, glycosaminoglycan; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcA, D-glucuronic acid; GlcNAc, *N*-acetylglucosamine; HA, hyaluronic acid; HS, heparan sulfate; KS, keratan sulfate; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; Ser, serine; ST, sulfotransferase; Thr, threonine; UDP-Glc, uridine 5′-diphosphoglucose; Xyl, xylose.

domains) by transitional mixed NAc and NS sections. The biological activity of HS resides in the densely functionalized NS domains and can be attributed to charge-based, nonspecific interactions, and modification-specific and domain-specific interactions (67). Remarkably, the sulfation pattern of HS is an important factor in the regulation of embryonic stem cell (ESC) fate. Although nonsulfated HS is characteristic of pluripotent stem cells and their self-renewal ability, highly sulfated HS is associated with ESC differentiation. Several studies have shown the possibility of neural cell generation from mice ESCs in the presence of GAG probes (68–70). However, the precise sulfated epitopes, the mechanisms involved in ESC differentiation, and the production of specific cell lineages are still largely unknown and call for systematic study involving structurally defined GAGs and human ESCs, as the required oligosaccharide epitopes may vary among species.

Methods of GAGs synthesis can be divided into two categories: (*a*) cost-efficient methods of GAG production for the therapeutic applications and (*b*) modular methods, which can provide access to all possible structural variations of GAGs for the structure–activity relationship (SAR) study (71). Because biological functions are predominantly defined by the sulfation pattern of a side chain and not by the protein core, the task of synthesis is usually reduced to the production of polysaccharide chains with desired modifications. The majority of commercial methods are based on extraction of GAGs from animal tissues; however, this approach gives heterogeneous samples with broad molecular mass distributions, and the samples may be contaminated with harmful entities (such as prions or pathogens). These liabilities could be alleviated by preparing GAGs in bacteria, as is the case for the microbial production of HA (72).

The established methods for the synthesis of structurally defined GAGs include GT-mediated polymerization with uridine 5′-diphosphate sugar donors with a nucleotide regeneration system (**Figure 5*b***) (55), hyaluronidase-mediated polymerization of the oxazoline donors (**Figure 5*c***) (73), and stepwise synthesis with GTs. The last approach is particularly useful for preparing densely functionalized oligosaccharides, as illustrated by the synthesis of ultralow molecular weight heparins (**Figure 5*d***) (74).

The key features of the process include an optimized order of sulfo-group installations and the selection of hepta- and larger oligosaccharide substrates that are more susceptible to sulfotransferases. Another important issue is epimerization of GlcA to IdoA (**Figure 5*d***, residue D, step b), which is accomplished by meeting the following two criteria: (*a*) for the C_5-epimerase-mediated transformation of GlcA to IdoA (residue D) to take place, the adjacent sugar at the nonreducing end has to be GlcNS (residue C); and (*b*) in order to ensure the irreversibility of the epimerization step and lock the IdoA structure, the sugar (residue A) that is three residues upstream of GlcA has to be GlcNAc (and not GlcNS, GlcN, or absent) (75). Further reaction optimization using mammalian enzymes and scale-up, with the incorporation of the 3′-phosphoadenosine-5′-phosphosulfate regeneration cycle into the enzymatic reaction (52, 54), may lead to improved commercialized processes. Chemical methods can provide hexa- and smaller oligosaccharides (76).

A cost-efficient approach toward developing structurally defined GAGs employs alterations and modifications of polymers produced in vivo. The trimming of natural GAGs and subsequent transglycosylation with hyaluronidase provide sulfated domains for synthesizing chimeric GAGs (77). Transglycosylation with *endo*-β-xylosidase permits transfer of the entire GAG chain (78). Although transfer onto the serine residue of a selected peptide remains challenging, alkyne-modified GAGs obtained by this method have been used to synthesize neoproteins and other glycoconjugates. Multivalent artificial scaffolds (dendrimers, polymers, and glycan arrays) decorated with short oligosaccharides have been used as probes to study GAG function. Recent reports have shown that brush-like synthetic polymers carrying the disaccharide epitopes of CS-E, HS, or heparin could mimic the biological activities of natural proteoglycans (68, 69, 79).

Glycoproteins

Glycoform: a homogeneous form of a glycoprotein

Microheterogeneity: a diversity of glycans associated with a single glycosylation site

Macroheterogeneity (occupancy): refers to nonregular glycan expression at possible glycosylation sites

Glycosylation is one of the most common post- and cotranslational modifications of proteins, which affects protein structure, function, stability, trafficking, and receptor recognition. Static modifications of the membrane-bound and secreted proteins by glycosylation create elaborated oligosaccharide structures (*N*- and *O*-glycans), whereas dynamic modification regulates cytosolic and nuclear proteins by adding and removing *O*-GlcNAc. More than half of all human proteins are estimated to be glycosylated by the competitive actions of approximately 200 genome encoded GTs (not including other carbohydrate-modifying enzymes), resulting in the formation of multiple glycoforms with distinct pharmacological profiles (80). As a result, controlling the glycosylation state of recombinant proteins becomes extremely important for developing and manufacturing glycoprotein pharmaceuticals (81, 82). From a different perspective, access to glycoproteins with a well-defined, tailored glycosylation state is needed to advance our knowledge of the glycosylation effect on protein folding and pathological conditions associated with aberrant glycosylation. Although defective glycosylation pathways can be directly linked to congenital disorders (83), the relation between abnormal protein glycosylation and glycoproteostasis and associated pathologies is far more complex and requires systematic investigation (84). In this section, we highlight methods of homogeneous glycoprotein synthesis and major challenges related to the issues of *N*- and *O*-glycosylation. More focused discussions on homogeneous glycoprotein synthesis can be found in a number of reviews (47, 85, 86). Unlike the situation with GAGs, where the task of synthesis can be reduced to preparing oligosaccharides with specific sulfation patterns, the synthesis of glycoproteins has to address not only the issue of glycan microheterogeneity but also the efficiency of glycan coupling to the carrier molecule and the installation of oligosaccharides at a specific amino acid residue within the polypeptide backbone, that is, macroheterogeneity. Therefore, defining the correct target molecule becomes a significant challenge, and this has inspired the evolution of glycoproteomic techniques and instrumentation.

The diversity of glycan structures is easy to understand by following their biosynthetic transformations (80, 87). The synthesis of *N*-glycans commences in the endoplasmic reticulum (ER), where a lipid-linked oligosaccharide, $Glc_3Man_9GlcNAc_2$, is transferred en bloc to the acceptor Asn-X-(Ser/Thr) (X ≠ Pro) peptide sequons by oligosaccharyltransferase (OST). After removal of the two terminal glucose residues, the $GlcMan_9GlcNAc_2$-associated polypeptide undergoes chaperone-guided protein folding, a series of transformations that is part of the protein quality control system (80, 88). The correctly folded proteins are then transferred to the Golgi apparatus for further structural modifications. Universal to all cells, the ER machinery produces the stem region of the *N*-glycan, which is trimmed down to the conserved $Man_3GlcNAc_2$ sequence in the *cis*-Golgi, also the location of initiation of *O*-glycan biosynthesis. The final microheterogeneity of *N*- and *O*-linked glycans is a result of the biosynthetic pathways that take place in the rest of the Golgi compartments. The expression and localization of the competing GTs, which have overlapping acceptor preferences, and the availability of glycoprotein substrates and activated sugar donors directly affect the final composition and heterogeneity of oligosaccharide sequences. Although the theoretical number of possible structural variations is high, the LacNAc (hybrid and complex glycans) and mannose (Man) (oligomannose-type glycans) residues are the most commonly observed units in the eukaryotic oligosaccharide sequences. Termination of glycan biosynthesis often occurs by sialylation or fucosylation. In addition to asparagine and serine (or threonine) glycosylations, other less common protein modifications include C-mannosylation, among others (89).

As one may expect, the manufacturing of many biopharmaceuticals, including glycoproteins, is carried out in mammalian systems, mainly Chinese hamster ovary (CHO) cell lines, which employ

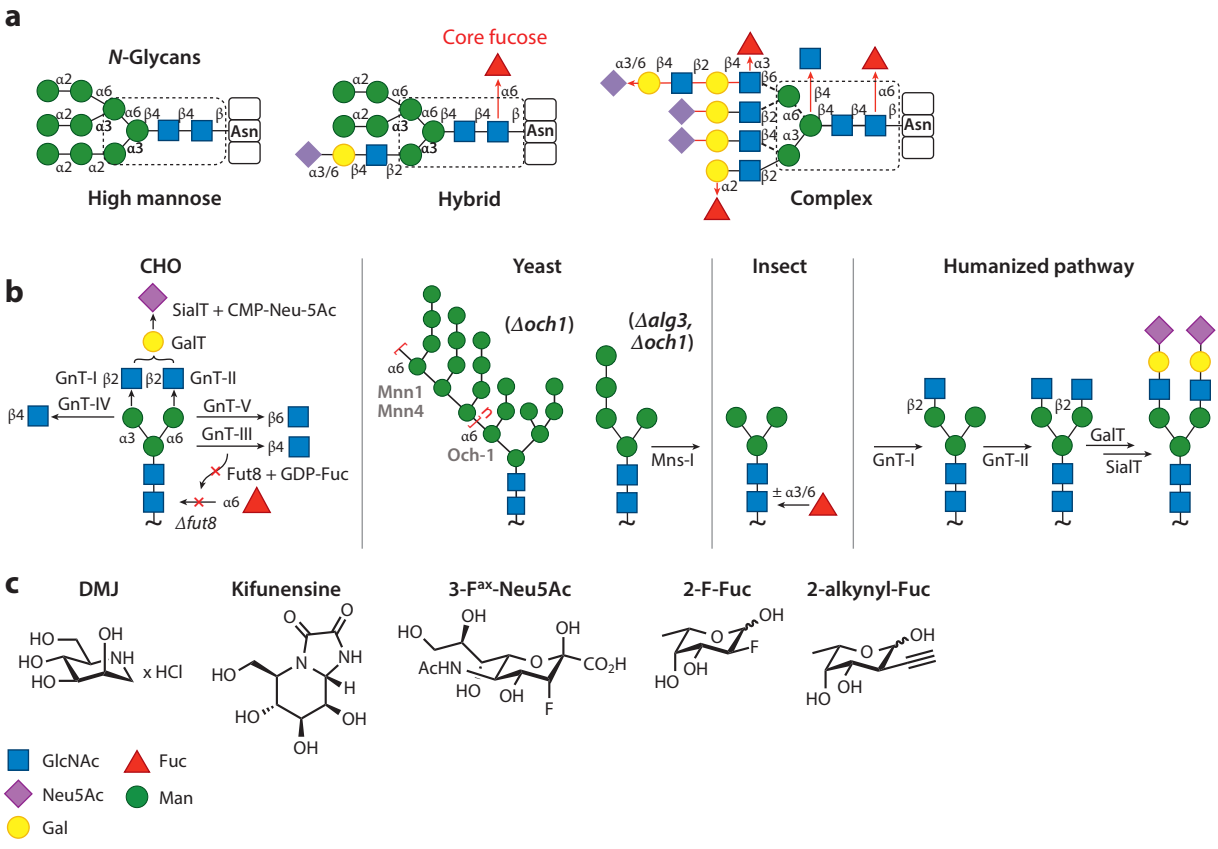


Figure 6

Examples of the most abundant mammalian oligosaccharide sequences found in glycoproteins. (*a*) *N*-linked glycans consist of the conserved core ($Man_3GlcNAc_2$) and elongated branches. Elongating units of the complex-type *N*-glycans can contain the sequences of LacNAc, LacdiNAc, and neoLacNAc and are usually terminated by an α-linked sialic acid or fucose. (*b*) Examples of *N*-glycosylation pathway engineering for recombinant protein production. (*c*) Examples of inhibitors of GTs for production of recombinant proteins enriched with a specific glycoform. Abbreviations: CHO, Chinese hamster ovary; DMJ, deoxymannojirimycin; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GT, glycosyltransferase; LacNAc, *N*-acetyllactosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid.

human-like glycosylation pathways (90). The control over the *N*- and *O*-glycosylation states of proteins in mammalian cells is performed by using a combination of standard techniques: gene mutation, knockout, and pathway inhibition (**Figure 6*b***) (47, 81). In a recent study, a comprehensive knockout screen of glycosyltransferase genes with zinc-finger nucleases was performed to identify key glycogenes and to design a matrix for genetic manipulation of protein *N*-glycosylation in CHO cells, including introducing human-like α2,6-sialylation (91). New precision genome editing methods, such as the CRISPR/Cas system, are expected to become effective tools for producing the desired glycoforms of recombinant proteins.

Major progress has been made in producing proteins with controlled sialylation of glycan structures, predominantly with *N*-acetylneuraminic acid (Neu5Ac) and not *N*-glycolylneuraminic acid (Neu5Gc), which is immunogenic in humans (92), and production of afucosylated recombinant glycoproteins via concurrent deletion of *fut8* and inhibition of the GDP–fucose substrate

generation pathway (**Figure 6*b***). Inhibition of GTs represents a well-established approach for preparing glycoproteins with altered glycoforms. Some of the classical examples include the use of imminocyclitols, deoxymannojirimycin, and kifunensine as α−mannosidase I inhibitors for the in vivo synthesis of high-mannose-type glycoproteins (**Figure 6*c***) (7). More recent cases include the use of 3-F^{ax}-Neu5Ac and 2-F-Fucose (Fuc) inhibitors of sialyl- and fucosyltransferases for remodeling cell-surface glycans (93). In another study, 2-F-Fuc and 2-alkynyl-Fuc inhibitors were used to produce fucose-free monoclonal antibodies in CHO cells (94).

Erythropoietin (EPO): a glycoprotein that stimulates production of erythrocytes and is widely used as a treatment for anemia

Glycoengineering in yeast (95) and bacterial cultures (96) can improve cost-efficiency of biologics synthesis and prevent potential cross-contamination with mammalian-borne infections (**Figure 6*b***). The most successful adaptation of humanized pathways was achieved for *N*-glycosylation (specifically, for complex biantennary structures) in *Pichia pastoris* strains (97). Unfortunately, creating the humanized *O*-glycosylation pathways in yeast is still problematic. Although the glycoengineering of cell lines may provide a high degree of glycan homogeneity, current processes employed in glycoprotein manufacturing are optimized mostly to minimize safety risks and to eliminate immunogenic sequences.

Optimizing therapeutic effects and identifying the most potent glycoforms can be accomplished by using chemoenzymatic methods, which can deliver glycoproteins in a pure, homogeneous form. For instance, the commercial sample of rituximab, produced in CHO cells, contains more than 50 glycan variations at Asn297 of the fragment crystallizable (Fc) region. Although our knowledge of how different glycoforms affect immunoglobulin G (IgG) function remains limited, some SAR studies have been reported (98). Specifically, removal of the core fucose enhances the anticancer potency of rituximab via improving binding to the FcγIIIa receptor (FcγRIIIa) (99–101), thus enhancing the antibody-dependent cellular cytotoxicity (ADCC). The Wong group (102) has recently demonstrated that the α2,6-linked sialic acid of the biantennary glycan can also improve antibody interaction with FcγRIIIa and FcγRIIa and results in the enhancement of ADCC and vicinal effects. In addition, the anti-inflammatory activity of IgG, the desired property of therapeutics targeting autoimmune disorders, is the result of IgG interactions with FcγRIIb and correlates with α2,6-sialylation (103, 104). These studies have led to the identification and preparation of homogeneous antibodies with a common glycan at the Fc region to maximize anticancer, anti-infective, and anti-inflammatory activities (102).

In the case of human erythropoietin (EPO), the link between serum stability and the number of terminal sialic acids in EPO has led to the development of a novel erythropoiesis-stimulating protein, darbepoetin alfa. The improvement in the half-life and dosing regimen for darbepoetin alfa was achieved by increasing the number of sialic acids (from 14 to 22) via the introduction of two additional *N*-glycosylation sites (105). Conversely, asialo EPO has been shown to possess potent neuroprotective activity (106). These examples illustrate the value of a well-defined composition for biologics and highlight the necessity for efficient and reliable methods for homogeneous glycoprotein synthesis.

Synthesis of *N*-Glycans and *N*-Glycoproteins

The synthesis of homogeneous glycoproteins encompasses the tasks of oligosaccharide synthesis and ligation of the desired glycan structure to the protein core. Alternatively, oligosaccharides can be directly linked to polypeptide sequences, which can then be assembled by native chemical ligation. Methods of peptide ligation are independent of the glycan's nature and can be applied equally to the synthesis of both *N*- and *O*-linked glycoproteins.

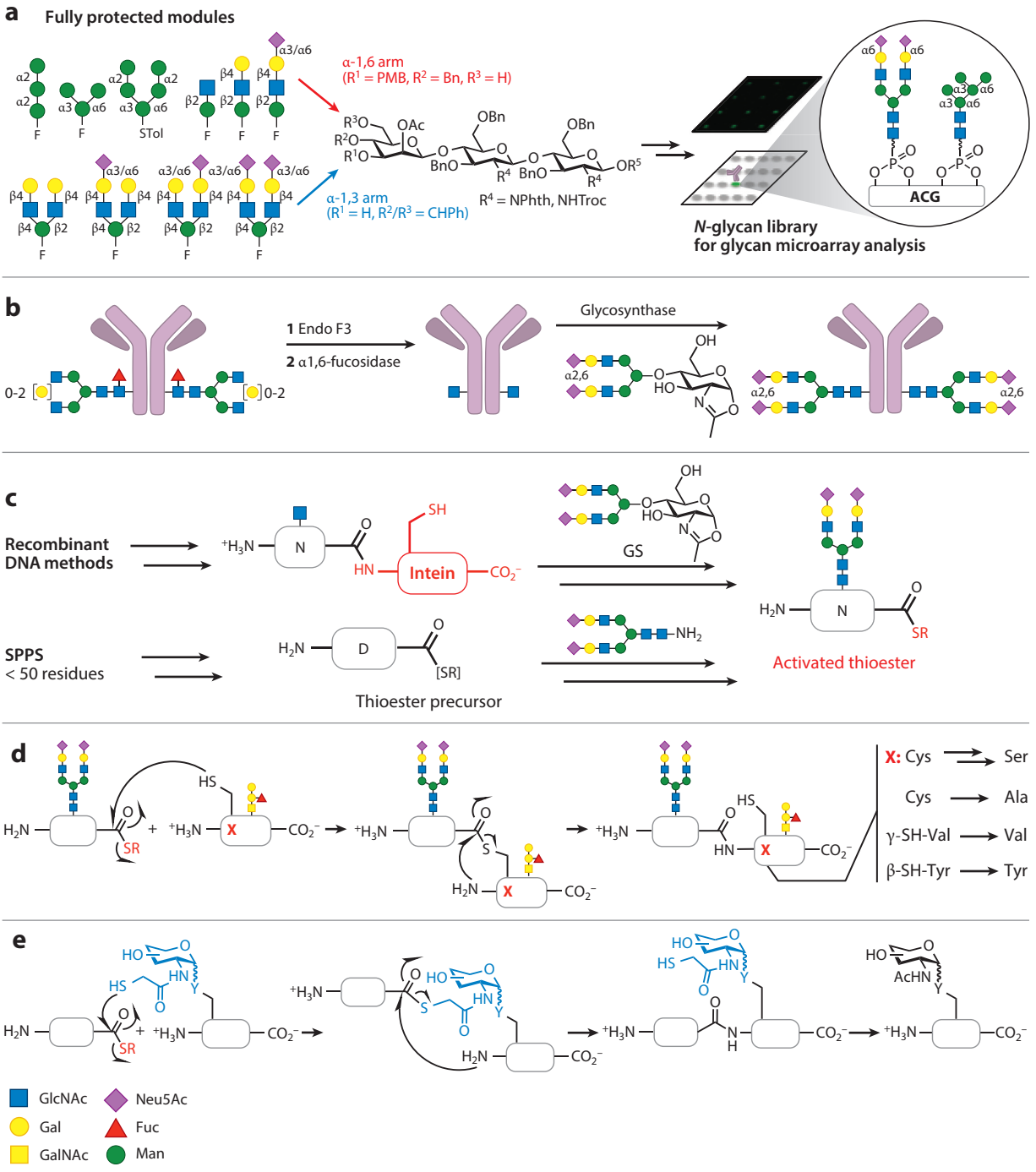
Neither purely chemical nor enzymatic methods can deliver the substantial amounts of material required for developing new and improved therapeutic glycoproteins. However, chemical

synthesis can be considerably simplified by incorporating enzymatic steps, for example, late-stage introduction of the challenging linkages, such as α2,3- or α2,6-sialylation of the LacNAc branches (57, 58), and core fucosylation (107).

Access to diverse sequences of *N*-glycans (estimated to exceed 20,000 structures), particularly asymmetrically branched multiantennary structures, is essential not only for glycoprotein synthesis but also for developing new analytical and diagnostic tools. Making complex *N*-glycans available, especially the structures that are isomeric and nondistinguishable by mass spectrometry, is instrumental for developing new methods of glycan sequencing. In addition, diverse, well-defined *N*-glycan samples are needed to prepare comprehensive glycan arrays. To achieve these goals, Boons and colleagues (108) reported a general strategy for chemoenzymatic synthesis of asymmetrically branched, complex *N*-glycans, taking advantage of the orthogonal protection of the branched trimannosyl core and selective modifications of PG-differentiated branches with GTs. In a separate study, a similar strategy was applied to the preparation of a library of biantennary *N*-glycans (109). However, the modular strategy developed by the Wong group (110) could provide general access to the diverse multiantennary complex and hybrid-type *N*-glycans (**Figure 7*a***). It utilizes a library of branching units with reducing end mannose, which are synthesized using the chemoenzymatic method, and then chemically transformed into glycosyl fluoride donors for the reaction with the selectively protected $Man_3GlcNAc_2$ core. The high efficiency of the α3- and α6-Man–Man coupling reactions permitted the preparative synthesis of various *N*-glycans, which were further used to prepare a mixed glycan microarray to enable the detection of heteroligand binding of broadly neutralizing antibodies isolated from HIV-positive patients. This new glycan array method is expected to facilitate the design and development of carbohydrate-based vaccines against HIV-1.

Among other examples is a report describing the synthesis of variously substituted high-mannose *N*-glycans using the top-down chemoenzymatic approach. The synthetic high-mannose structures were modified with glucose, galactose, and GlcNAc monosaccharides. These artificial structures were then used as substrates for orthogonal oligosaccharide trimming with GHs, thus generating a library of high-mannose type *N*-glycans (111).

Remodeling of oligosaccharide sequences of recombinant proteins can be readily accomplished in two steps: first, by trimming heterogeneous glycans of a protein to a homogeneous glycoform containing a core glycan or single GlcNAc residue with glycosidases, and, second, by subsequently modifying the homogeneous glycoform with GTs (112) or *endo*-glycosidases (ENGases) (**Figure 7*b***) (113). Synthesis with ENGases and mutated synthases, coupled with the use of activated oxazoline donors, has become particularly effective for preparing homogeneous glycoproteins and glycopeptides. Advanced by the Wang group (85, 86), this approach has been applied to the synthesis of several notable targets, including the CD52, HIV-I V3 and C34 glycopeptides, and the IgG1 Fc. The last example illustrates the specificity of Endo S, which permits manipulations of the complex fucosylated glycans on IgG targets (114). Despite the generality of the approach, ENGase-mediated synthesis requires the use of 50- to 100-fold excess of the oxazoline donor to achieve high conversions, which makes it expensive and can lead to nonspecific modification of other nucleophilic residues of proteins with oxazoline substrate. Therefore, alternative LGs and enzymatic methods are being investigated, specifically direct protein glycosylation catalyzed by OST (115, 116). Although the use of mammalian OST for in vitro synthesis is difficult due to the complexity and instability of the transmembrane complex, some progress has been achieved using bacterial analogs of OST, a single-subunit PglB from *Campylobacter jejuni* (96). In a proof-of-concept study, PglB was applied for in vivo (*E. coli*) and in vitro synthesis of several glycoprotein targets (117). The challenges that need to be resolved for this method to become practical include


a
Fully protected modules
α-1,6 arm
(R1 = PMB, R2 = Bn, R3 = H)
α-1,3 arm
(R1 = H, R2/R3 = CHPh)
R4 = NPhth, NHTroc
ACG
N-glycan library
for glycan microarray analysis
b
1 Endo F3
2 α1,6-fucosidase
Glycosynthase
0-2
0-2
c
Recombinant
DNA methods
Intein
SPPS
< 50 residues
Thioester precursor
GS
Activated thioester
d
X: Cys → Ser
Cys → Ala
γ-SH-Val → Val
β-SH-Tyr → Tyr
e
GlcNAc
Gal
GalNAc
Neu5Ac
Fuc
Man

improving the efficiency of synthesis and substrate specificity for human sequences and glycolipid substrates.

The methods discussed above are restricted to native glycoprotein preparations; however, considerable efforts are also being directed toward preparing neoglycoproteins. One notable example is direct glucose (Glc) transfer onto the NXS/T consensus sequence by *N*-glycosyltransferase and subsequent modification of Glc with complex and oligomannose glycans by ENGases (118). Other examples can be subsumed under the tag-and-modify approach (119) in which an oligosaccharide is coupled to activated cysteine, lysine, or unnatural amino acid tags. These methods allow introduction of the desired functionality into the target proteins; although, the problems associated with the immunogenicity of the non-native linkages are usually overlooked. Using artificial glycoconjugates as probes to study multivalent carbohydrate interactions at cell surfaces, however, can provide valuable information (68, 69, 120, 121). Glycocalyx engineering is an emerging technology that employs carbohydrate epitopes covalently attached to the membrane-anchoring unit to study and regulate a specific biological response (3).

Glycocalyx: an extracellular, negatively charged layer of glycans and glycoconjugates that participates in intercellular processes

A solution to the issue of the macroheterogeneity of glycoproteins with multiple glycosylation sites is de novo synthesis via ligation of peptide sequences (122, 123). The majority of ligation procedures require generation of an activated thioester intermediate, which can be obtained in a stabilized precursor form as an intein-fusion protein through coexpression or as a thioester by solid-phase peptide synthesis (**Figure 7*c***). Glycan installation is carried out at this stage using either enzymatic or chemical methods. The next step involves activation of the thioester intermediate and coupling to the cysteine-terminated glycosylated peptide block (**Figure 7*d***). The native chemical ligation (NCL) method has advanced through the development of new orthogonal PGs for thiols, masked thioesters, and unnatural thiolated amino acids, which after the ligation step can be converted into the native structures. This allows extra flexibility during the design of the synthetic route, as cysteine residues are rare and may be missing at the desired ligation junctions. In many cases, a desulfurization reaction is performed allowing the Cys → Ala transformation; however, the Cys → Ser transformation has also been reported (124). Unfortunately, these steps require the selective protection of any additional cysteine residues that may be present in the protein. In this regard sugar-assisted ligation provides a convenient way of assembling peptide fragments, particularly with occupied *O*-glycosylation sites (**Figure 7*e***) (125, 126). Modification of the acetamido moiety of GalNAc or GlcNAc with a sulfhydryl group enables transthioesterification with the peptide thioester, which then triggers the S →N acyl transfer to form a peptide bond. During the next step, the thiol auxiliary or the entire monosaccharide unit can be selectively removed. The most attractive advantage of sugar-assisted ligation is its broad sequence tolerance at ligation sites (127, 128).

Perhaps the most impressive example of chemical glycoprotein synthesis is a total synthesis of a homogeneous EPO realized by the Danishefsky group (129). Years of research devoted to this notable target resulted in a number of groundbreaking synthetic methods (130), which may

Figure 7

(*a*) Modular synthesis was used to prepare a library of *N*-glycans and fabrication of mixed glycan arrays on aluminium oxide–coated glass slides for profiling antibodies targeting the HIV-1 virus (110). (*b*) In vitro glycan remodeling and synthesis of homogeneous rituximab (102). (*c*) Generation of activated thioester for peptide ligation. Ligation strategies used for glycoprotein and glycopeptide synthesis rely on thiol-ester-mediated coupling and include (*d*) native chemical ligation, expressed-protein ligation, Staudinger ligation (not pictured), and (*e*) sugar-assisted ligation. Abbreviations: Ala, alanine; Cys, cysteine; Endo, endoglycosidase; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; Ser, serine; SPPS, solid-phase peptide synthesis; Tyr, tyrosine; Val, valine.

eventually provide a means to study the effect of a specific glycan on the structure and function of a glycoprotein with multiple glycosylation sites. Other useful techniques include thiol auxiliary-based cysteine-free ligation and protease-assisted ligation (47, 123).

Glycosylated Proteins for Structural Studies

Because the early steps of *N*-glycan biosynthesis are directly implicated in protein folding and quality control during the calnexin–calreticulin cycle in the ER, the *N*-glycosylation-induced effect on protein structure has become the subject of several biophysical studies. Early reports demonstrated the effects of carbohydrate composition on local peptide conformations (131) and modulation of the disulfide bond formation (132). Although the effect of *N*-glycosylation varies from one protein to another, and has to be considered together with the peptide core, some general rules of glycan effects on protein folding and stability can be formulated for simple glycoprotein models.

The cell adhesion and signaling molecule CD2, which is expressed on T lymphocytes and natural killer cells, has a functional 105 amino acid extracellular adhesion domain (hCD2ad), which bears a single glycosylation site at Asn65 in the human homolog, and has no disulfide bonds or proline residues to interfere with the effect of glycosylation on its folding, making hCD2ad a perfect study model. Results of folding experiments have shown that glycosylation of hCD2ad both increased the folding rate (kinetic contribution) and stabilized the folded state (thermodynamic contribution). Comparisons of glycoform samples have suggested that the first GlcNAc residue attached to asparagine is the most important component for structure stabilization. Approximately 3 kcal/mol of thermodynamic stabilization has been attributed to the Man-GlcNAc-GlcNAc trisaccharide, in which the first GlcNAc (**Figure 8**, part *i*) contributes two-thirds of the energy, the second and the third sugars (**Figure 8**, parts *ii* and *iii*) of the core trisaccharide provide one-third, and the outer sugars have negligible effects on structure stabilization. The results suggest that the core trisaccharide is sufficient for intrinsic acceleration of folding and stabilization in hCD2ad, and the presence of this trisaccharide as the common and conserved core in all *N*-linked glycans may not be coincidental (133). Further study of the system has revealed that introducing an aromatic phenylalanine residue at the ($i - 2$) position to GlcNAc-Asn (*i*) provided an additional 0.8–1 kcal/mol stabilization (134, 135). A structural study of IgG1 Fc glycoforms has revealed that the transition from hybrid to complex-type glycans increases the stabilization of the Fc structure (136). In a report by the Imperiali group (137), analysis of the kinetic and thermodynamic contributions of *N*-glycosylation (GlcNAc-GlcNAc) at different sites in the bacterial immunity protein Im7 revealed the effect of glycan on the local conformational preferences of glycosylated sequences. The most prominent stabilizing effect of *N*-glycosylation has been observed at the compact turn motifs between segments of ordered secondary structures, thus providing an explanation of the preferential expression of *N*-glycan at the transition zones between different types of secondary structures.

Protein *O*-glycosylation has also been found to affect protein structure, function, and stability. Since the discovery of intracellular protein *O*-glycosylation with β-GlcNAc by Torres & Hart (138), a great deal of effort has been directed toward understanding this biological process (139) and its interplay with the posttranslational phosphorylation of proteins (140). An early study of the *O*-GlcNAc modification of RNA polymerase II demonstrated the importance of glycosylation to protein folding, as observed by nuclear magnetic resonance imaging (141). In a more recent report, *O*-GlcNAc modification at Ser75 of EZH2 methyltransferase was shown to have an essential role in its stability and enzymatic activity. EZH2 methyltransferase facilitates trimethylation of the K27 site of histone H3, resulting in the inhibition of tumor suppression (142).

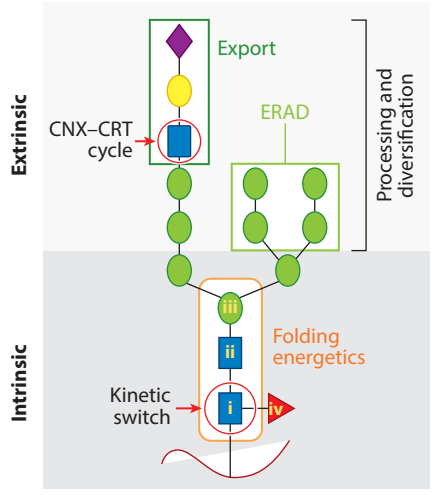


Figure 8

GlcNAc (*i*) speeds up folding by 0.8 kcal/mol and provides stabilization of the folded structure by 2 kcal/mol; an additional 1.1 kcal/mol comes from GlcNAc-Man (*ii*,*iii*). Fucose (*iv*) has no effect on protein stabilization. Abbreviations: CNX–CRT, calnexin–calreticulin; ERAD, endoplasmic reticulum–associated protein degradation; GlcNAc, *N*-acetylglucosamine; Man, mannose.

Synthesis of *O*-Glycans and *O*-Glycoproteins

In general terms, *O*-glycosylation is defined by the addition of oligosaccharide structures (GalNAc, galactose, GlcNAc, mannose, fucose, glucose, or xylose) to the hydroxyl group of amino acids (e.g., serine, threonine, tyrosine, hydroxylysine) (143). More commonly, however, *O*-glycosylation refers to the mucin-type *O*-linked α-GalNAc oligosaccharides. Mucins are densely *O*-glycosylated proteins (up to 70% by mass), which are expressed by mucosal epithelial cells and create a protective mucin barrier in the ocular epithelium and the respiratory, gastrointestinal, urinary, and reproductive tracts. Glycosylated domains (approximately 600–1,200 amino acids) in mucins are rich in serine, threonine, and proline. These long glycosylated domains are separated by shorter nonglycosylated domains (20–70 amino acids). Most mucins are negatively charged due to the presence of terminal sialic acids and sulfated sugars. These bottlebrush-like, firm structures can either extend from the glycocalyx or be secreted as oligomers into the extracellular matrix. The 16 human mucins are divided depending on their biophysical properties: secreted gel-forming, secreted nongel-forming, and cell surface mucins (144). In the healthy state, mucins fulfill an important function by acting as a protective barrier for cells against mechanical damage and infections; they also act as decoys for bacterial adhesins and initiate intracellular signaling pathways in response to pathogen invasion (145). Additionally, *O*-glycosylated proteins have been shown to play prominent parts in embryonic development, organogenesis, and tissue homeostasis (146). Compared with *N*-glycosylation, our knowledge of mucin *O*-glycosylation is surprisingly limited. The lack of defined amino acid consensus sequences combined with the great diversity of *O*-glycan structures makes mucin analysis and the study of sequencing and SAR extremely difficult, although these issues provide plenty of opportunities for research. Nevertheless, biochemical studies of the enzymes involved in mucin biosynthesis can help to elucidate the apparent complexity and heterogeneity of this class of compounds.

The first step in mucin biosynthesis, the installation of the GalNAc sites, is completed by the successive action of several polypeptide *N*-α-acetylgalactosaminyltransferases (ppGalNAcTs).

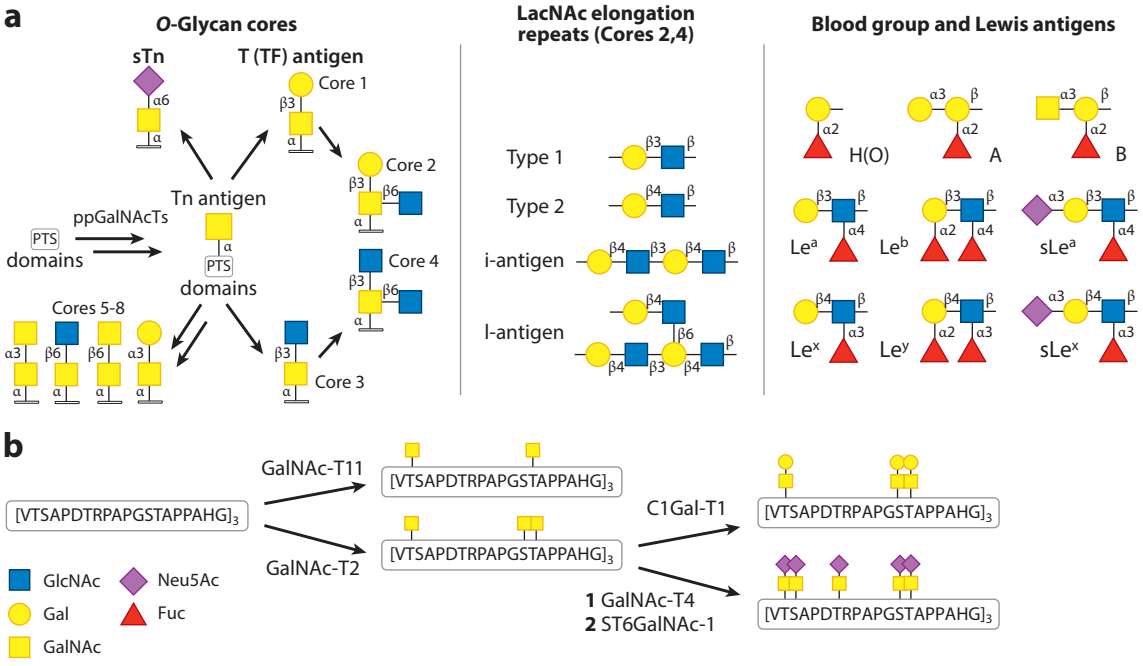


Figure 9

(*a*) Examples of *O*-glycan structures. Conserved cores 1–8 can be elongated or terminated by fucosylation and sialylation. Termination of *O*-glycans with Tn and T (TF) antigens is characteristic of cancerous cells. In the healthy state, *O*-glycans are typically terminated with blood group antigens or, less commonly, with Le and sLe antigens. (*b*) Synthesis of Tn and sialyl Tn glycoforms of the MUC1 peptide. Abbreviations: Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; LacNAc, *N*-acetyllactosamine; Le, Lewis; Neu5Ac, *N*-acetylneuraminic acid; sLe, sialyl Lewis; TF, Thomsen–Friedenreich.

Biochemical characterization of ppGalNAcT isoforms and systematic study of their substrate specificities have revealed a complex balance between redundancy and hierarchy within this family of enzymes (147). For instance, ppGalNAcT isoforms 1, 2, and 5 prefer non- and monoglycosylated peptide substrates, and they participate in the initial step of peptide glycosylation. A further increase in glycosylation sites is realized by the middle stage transferases (e.g., ppGalNAcT-3 and -4) that prefer peptides with two GalNAc sites. Yet even a higher glycosylation density is achieved by the action of the late stage ppGalNAcT-10, which prefers substrates with three and four glycosylation sites (148). A particular feature of ppGalNAcTs, which is responsible for their selectivity, is the presence of poorly conserved R-type lectin domains that bind to the preexisting GalNAc sugars and guide the catalytic domain toward distal glycosylation sites. In-depth discussions of the structural features, activities, and differential expression of ppGalNAcTs in healthy and disease states can be found in recent reviews (143, 147).

After its installation, the GalNAc sugar (the Tn-antigen) is further modified by downstream GTs: T-synthase (core 1), C3GnT, C2GnT1, and C2GnT2 (**Figure 9*a***) (149). The generated *O*-glycan cores can be further extended with LacNAc repeats (for cores 2 and 4) or terminated with the blood group antigens and sialic acids. Dysregulation of *O*-glycosylation pathways is related to a number of disorders, mainly cancers, which are usually characterized by overexpression of truncated (Tn, sTn, and T antigens) and overfucosylated structures (Lewis antigens).

Due to the complexity and heterogeneity of the glycan constituents of mucin-type proteins, no attempts at preparing naturally occurring mucins have been reported. However, synthetic

nonglycosylated MUC1 peptides and their truncated glycoforms have been extensively studied as tumor-associated carbohydrate antigens for the development of anticancer vaccines (150). In vitro enzymatic synthesis based on the direct glycosylation of a synthetic MUC1 peptide has been also reported (**Figure 9*b***) (151).

In the case of a typical glycoprotein (nonmucin type), *O*-glycosylation sites occur fairly rarely and display relatively short oligosaccharides. Synthesis of such *O*-glycans and glycoproteins can be accomplished by using the chemoenzymatic methods and ligation techniques discussed in the previous sections. If *O*-glycosylation is not essential for the activity of the glycoprotein, it can be substituted with glycoPEGylation (152) or polysialyl modifications (153), which are used to improve the pharmacokinetic properties of glycoproteins.

Glycolipids

Glycolipids comprise another class of glycosylated natural products that have been implicated in the intracellular signaling network and are crucial for the sustainable functioning of multicellular organisms (154, 155). Although disruptions of glycolipid biosynthetic pathways result in only a handful of disorders because many lead to in utero lethality, defects in the *exo*-GHs responsible for glycolipid catabolism are associated with a large number of lysosomal storage diseases (156). In addition, many glycolipids are known tumor-associated antigens (e.g., GM2, GD2, Globo H, SSEA4). Because Globo H, SSEA3, and SSEA4 are expressed in at least 16 different types of cancers but not on normal cells, these antigens are especially promising targets for cancer vaccine development (157) (see the sidebar, Carbohydrate-Based Therapeutic Vaccines Against Cancer).

The structural composition of glycolipids, such as conjugation of a highly polar sugar to the lipid carrier, defines their unique biophysical properties, localization at the membrane surface, and aggregation into glycosynapses and lipid rafts, together with other signaling molecules (e.g., GPI-anchored proteins, transmembrane proteins, receptors, and ion channels). Apart from cholesteryl-glucoside, phosphatidylglucoside, and seminolipid, most mammalian glycolipids use ceramide as a lipid carrier and, thus, are termed glycosphingolipids (GSLs) (**Figure 10*a***). The heterogeneity of GSLs arises both from the glycosyl substituent and the ceramide unit, specifically the fatty acyl group on the sphingosine base, which can be of variable length and saturation and can be modified with hydroxyl substituents. Glycosylation of the ceramide with galactose or glucose gives the two

Glycosphingolipids (GSLs): glycosylated lipids in which the lipid part consists of sphingosine modified at the amino group with fatty acyl (C16–C36)

CARBOHYDRATE-BASED THERAPEUTIC VACCINES AGAINST CANCER

The ability of the immune system to discriminate foreign antigens from self-expressed epitopes is exploited in the development of carbohydrate-based vaccines (170, 171). In addition to classical preventive vaccines against human pathogens, therapeutic vaccines are emerging that target cancer cells with altered glycosylation states (172). The most advanced vaccine candidate, GH-KLH-QS21, has been further improved by the new generation GH-DT vaccines with α-galactosylceramide-type (C34) adjuvant (**Figure 10*c***); this has been shown to induce antibody class switch and gave the highest titers of anti–Globo H IgG compared with the GH-KLH-QS21 combination (173, 174). The induced antibodies showed specificity against Globo H, SSEA3, and SSEA4, which are specific to breast cancer and its stem cells, as well as 15 other types of cancer cells. Another study has highlighted the appeal of non-self antigens, that is, a vaccine candidate based on Globo H modified with $6N_3$-Fuc induced a robust IgG immune response, much greater than that of the parent unmodified GH-DT conjugate (175). This progress was made possible by the synthesis of Globo H using programmable one-pot (37) and enzymatic methods (56). An historical perspective on the development of the Globo H vaccine can be found in a recent review (176).

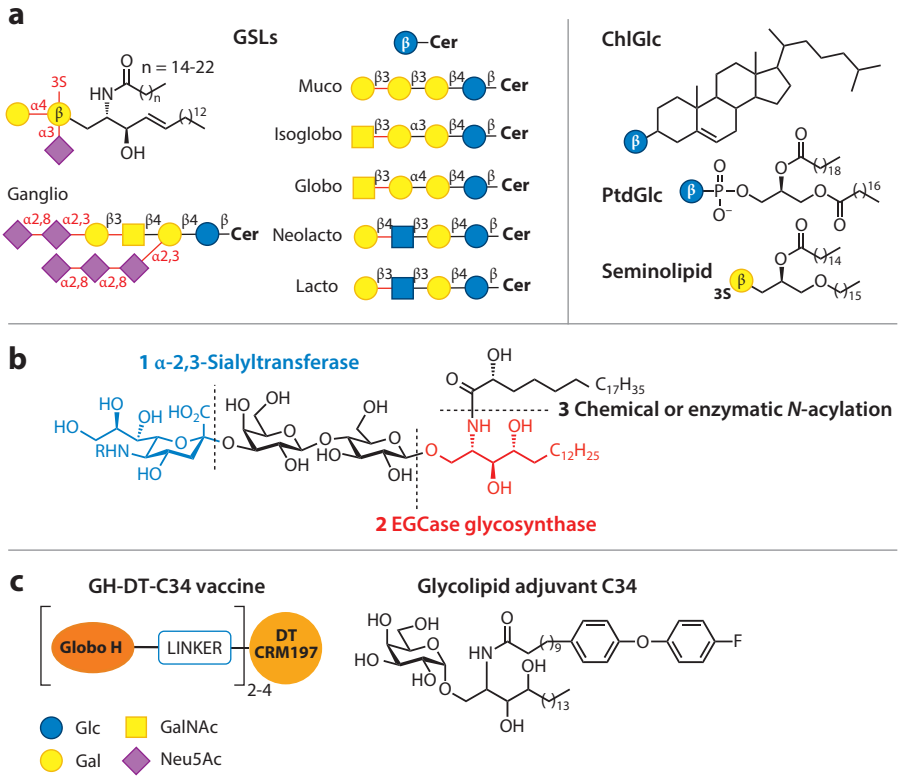


Figure 10

(*a*) Examples of lipid structures and GSL series. (*b*) Steps in the chemoenzymatic assembly of starfish ganglioside LLG-3. (*c*) Composition of GH-DT-C34 vaccine candidate. Abbreviations: ChlGlc, cholesterylglucoside; DT CRM 197, diphtheria toxin cross-reacting material 197; EGCase, *endo*-glycoceramidase; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GSL, glycosphingolipid; Neu5Ac, *N*-acetylneuraminic acid; PtdGlc, phosphatidylglucoside.

simplest GSL structures: β-Gal-Cer and β-Glc-Cer. Further modifications of β-Glc-Cer with GTs give rise to several classes of tissue-specific GSLs. Structurally, the most challenging, highly sialylated GSLs—gangliosides—are populated in the nerve cells and represent one of the most intriguing areas for research (158).

Oligosaccharide sequences found in complex GSLs are well established and can be accessed using the chemoenzymatic methods discussed earlier. The Wong group has reported a preparative enzymatic synthesis of Globo series oligosaccharides (**Figure 3*a***) (56), which can be adapted for preparing other classes of complex GSLs. The main difficulty with glycolipid synthesis, however, is associated with the attachment of a water-soluble glycan to the hydrophobic lipid chain. The Withers group engineered several *endo*-glycoceramidase mutants with broad lipid specificity (159). The utility of the *endo*-glycoceramidase method was later demonstrated with the synthesis of starfish ganglioside LLG-3 (**Figure 10*b***) (160). The reported chemoenzymatic synthesis provides a general approach for assembling GSLs. Another prevalent area of research focuses on bacterial glycolipids, which are structurally more complex than their human counterparts. In this regard, the Wong group reported a preparative enzymatic synthesis of bacterial lipid II and analogs in which structurally complex targets were assembled in only two steps from GlcNAc (161).

Glycosylphosphatidylinositol-Anchored Proteins

Many functionally diverse proteins, including hydrolytic enzymes, adhesion molecules, and receptors, can be expressed as GPI proteins. Compared with transmembrane proteins, GPI-anchored proteins maintain greater mobility due to the nature of lipid anchors. The lipid tail that secures a GPI protein at the membrane's surface does not completely extend through the cell membrane, thus permitting a GPI protein to migrate from cell to cell; however, cleavage of the phosphate linkage with phospholipases can release the protein in a soluble form. Apart from being anchoring devices, GPIs have been shown to affect the conformation of the attached protein and the organization of lipid rafts (162). However, the biological significance of GPI anchoring, as well as the correlation between GPI structural variations and their functions, are not fully understood. Access to homogeneous forms of GPIs remains difficult, thus impeding research in that direction. Several chemical strategies for synthesizing the glycolipid have been developed; however, conjugation of the GPI anchor to a full-size protein remains problematic. As a result, there have been no practical methods established for the synthesis of native and homogeneous GPI-anchored proteins. Nevertheless, the synthesis of GPI analogs for the study of their function has been reported and reviewed (162, 163).

The conserved core of most eukaryotic GPIs consists of a phosphoethanolamine linkage, Man_3-GlcN saccharide, inositol, and phospholipid. In humans, additional modifications to the conserved core result in approximately 20 distinct isoforms and may include glycosylation and phosphoethanolamine attachment to the carbohydrate unit, acylation of inositol, and structural variations within the lipid tail (**Figure 11*a***). An even higher level of structural complexity is found among parasitic GPIs, many of which can act as virulence factors or toxins and, therefore, are important targets for the development of antiparasitic vaccines (164).

So far, only chemical methods have been reported for synthesis of structurally defined glycolipid parts of GPIs. Currently, research aims to develop flexible synthetic strategies that will allow access to a wide variety of structures for the SAR study. Synthetic GPI anchors can be coupled to the desired peptide by NCL; however, this requires introducing a cysteine residue at the phosphoethanolamine group (**Figure 11*b***) (165, 166). Alternatively, GPI anchors suitable for NCL can be generated by yeast expression of prion proteins bearing a TEV protease cleavage site (167). Another useful procedure for synthesizing GPI-anchored protein analogs uses ligation mediated

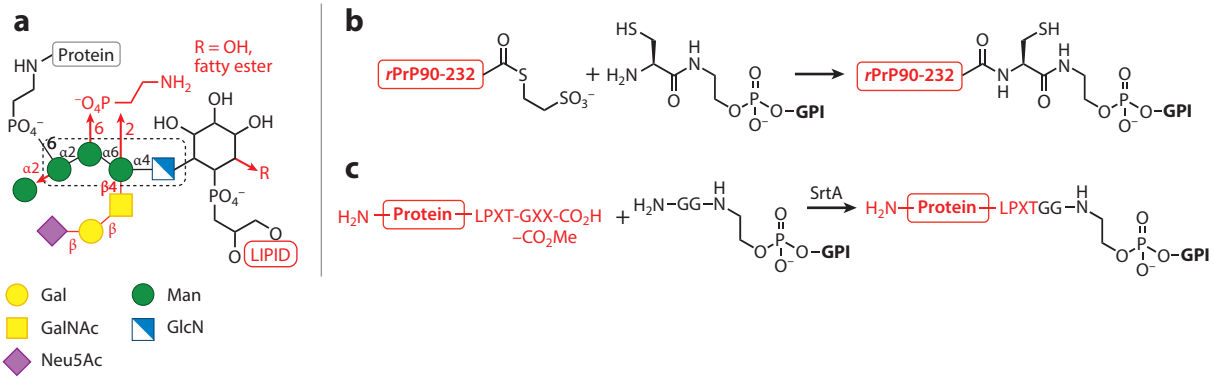


Figure 11

(*a*) General structure of GPIs and possible variations (in *red*). Ligation methods used for GPI analog synthesis include native chemical ligation, (*b*) expressed-protein ligation, and (*c*) SrtA-mediated ligation. Abbreviations: Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcN, glucosamine; GPI, glycosylphosphatidylinositol; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; SrtA, sortase A.

by sortase A, which relies on the presence of a nonnative LPXTG sorting signal (**Figure 11*c***) (168, 169).

CONCLUSIONS

The many methods developed during the past 25 years have substantially enriched the repertoire of accessible, physiologically relevant oligosaccharides and led to the introduction of new technologies, such as cell-surface engineering (3) and glycan microarrays (177), that have proved useful for addressing the questions posed by fundamental and applied research. Many groups that entered the field with carbohydrate chemistry expertise now incorporate the subject of disease biology into their research, thus ensuring the long and prosperous growth of glycoscience.

SUMMARY POINTS

1. The progress of glycoscience directly depends on the availability of structurally defined, homogeneous samples of oligosaccharides and glycoconjugates.
2. In the area of chemical synthesis, the goal is to develop simplified and automated methods for preparing designer carbohydrates.
3. Priorities for the enzymatic synthesis of glycans and glycoconjugates include expanding the repertoire of GTs and GSs, as well as engineering high-yielding, cost-efficient enzymatic systems.
4. The most powerful methods of oligosaccharide synthesis are based on combined chemoenzymatic approaches, which enable fast generation of libraries of glycans for the SAR study.
5. Modern methods of glycan synthesis can provide access to many mammalian-type oligosaccharides and some glycoconjugates, particularly glycolipids and homogeneous glycoproteins.

FUTURE ISSUES

1. Research in glycoscience revolves mostly around the mammalian glycome; however, deciphering the glycomes of microbes is no less important and should lead to a better understanding of microbiota and discovery of new antibacterial treatments and vaccination regimens.
2. With carbohydrate-based vaccines pushing forward the standards of existing cancer treatments, we expect to see more studies that focus on developing structurally defined glycotherapeutics.
3. The identification of disease-associated biomarkers, as well as imaging and monitoring of disease-affected glycosylation pathways, will continue to be dominating research areas.
4. Systematic SAR studies of glycoproteins should provide an enhanced understanding of the physiology of lysosomal storage diseases and disorders associated with aberrant glycosylation. Perhaps, progress in glycobiology will eventually deliver a solution to human aging, which, among other symptoms, is characterized by an altered glycosylation state.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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