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Modifications of Glycans: Biological Significance and Therapeutic Opportunities

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Abstract

Carbohydrates play a central role in a wide range of biological processes. As with nucleic acids and proteins, modifications of specific sites within the glycan chain can modulate a carbohydrate's overall biological function. For example, acylation, methylation, sulfation, epimerization, and phosphorylation can occur at various positions within a carbohydrate to modulate bioactivity. Therefore, there is significant interest in identifying discrete carbohydrate modifications and understanding their biological effects. Additionally, enzymes that catalyze those modifications and proteins that bind modified glycans provide numerous targets for therapeutic intervention. This review will focus on modifications of glycans that occur after the oligomer/polymer has been assembled, generally referred to as postglycosylational modifications.

Nucleic acids, proteins, and carbohydrates are the three major biopolymers that mediate biological processes in living organisms. It is well appreciated that functions of nucleic acids and proteins are frequently modulated by chemical modifications of the main polymer. For example, phosphorylation of serine, threonine, or tyrosine residues on proteins can lead to dramatic changes in protein function, and methylation of specific sites within DNA can lead to silencing of gene expression. Carbohydrates are also involved in many biological processes and play a key role in numerous diseases. Like other biopolymers, biological functions of carbohydrates can be modulated by modifying specific sites within an oligosaccharide/polysaccharide chain. Modifications can involve a variety of functional groups but most often entail derivatization of hydroxyls or amino groups, such as acylation, sulfation, methylation, and phosphorylation (for representative examples, see Figure 1).(1–4) As a result, significant efforts are underway to identify carbohydrate modifications and link them with specific biological functions. Insights into the biosynthesis and functions of modified glycans should translate into new therapies for infectious, inflammatory, malignant, and degenerative diseases (Figure 2).

Carbohydrates and their modifications are extremely difficult to study. Carbohydrates, or glycans (free carbohydrates or carbohydrate fragments of glycoproteins and glycolipids), are composed of monosaccharides linked together to form oligosaccharides or polysaccharides. Variations in linkage stereochemistry, linkage regiochemistry, and branching generate natural glycans of enormous structural diversity. Further adding to this diversity, modifications at various sites yield additional structures that can change dynamically. The biosynthesis of glycans is not template-driven like translation of polypeptides, and it is regulated by many factors including availability of nucleotide donors and expression of enzymes. Thus, protein molecules with the same polypeptide sequence can have distinct glycans appended to them, resulting in an array of glycoforms.(5) As a result, predicting or

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controlling glycan expression can be difficult. Moreover, methods to detect, characterize, and sequence glycans can be slow and arduous. Therefore, the full repertoires of glycans and glycan modifications present in organisms (their glycomes) are unknown.

Our understanding of the roles of carbohydrate modifications, and glycobiology in general, has been primarily driven by studies on the proteins that bind to carbohydrates and the enzymes involved in the biosynthesis and catabolism of carbohydrates. It has been estimated that over 400 proteins in the human glycome are involved in the biosynthesis, catabolism, and binding of carbohydrates.(6) Although the exact number of proteins that contribute to glycan modifications is not known, a significant number are involved in postglycosylational modifications(2) of carbohydrates or recognition of modified glycans. Moreover, many proteins that interact with unmodified glycans can be inhibited when the target substrate or ligand is modified.

Below, we provide an overview of the current understanding of post-glycosylational modifications (sulfation, acylation, phosphorylation, methylation, and epimerization) and highlight recent progress in field. In addition, we discuss current challenges and barriers that impede progress in the field. Finally, we describe opportunities for development of new basic research tools and new therapeutics related to post-glycosylational modifications.

SULFATION

Sulfated glycans are a major class of enzymatically modified glycans with important biological functions. All classes of glycans can be sulfated. Sulfated glycosaminoglycans (GAGs) are found on cell surfaces and within the extracellular matrix, where they mediate binding interactions and provide structural support. Biological activity depends on the pattern of sulfation, sometimes referred to as the “sulfation code.”(7) Sulfation similarly tunes the biological activity and physical properties of glycoproteins. Finally, sulfoglycolipids are essential components of testis and the myelin sheath surrounding neurons.(8)

Sulfated Glycosaminoglycans (GAGs)

Glycosaminoglycans are unbranched polysaccharides made up of repeating disaccharide units. There are five major classes of GAGs: hyaluronan, chondroitin, dermatan, keratan, and heparin/heparan sulfate.(9) Of these, hyaluronan is not sulfated. Chondroitin, dermatan, and keratan contain sulfate groups on hydroxyls, but *N*-linked sulfation does not occur for these GAGs. Heparin and heparan sulfate can contain both *O*- and *N*-linked sulfates. In general, heparins are more sulfated with higher content of iduronic acid (IdoA) than heparan sulfates. Figure 3 shows representative types of sulfated GAGs. Since varying degrees of sulfation are possible and different domains within a polysaccharide can have different sulfation patterns, a very large number of distinct structures are possible.

Sulfated GAGs are products of a highly orchestrated process within the Golgi apparatus. For example, the syntheses of heparin and heparan sulfate involve coordinated removal of some *N*-acetyl groups, addition of *N*-sulfates, epimerization, and addition of *O*-sulfates. Thus far, 30 genes encoding sulfotransferases that modify GAGs have been identified (summarized in Figure 3). These sulfotransferases are localized to the Golgi apparatus, and they use 3′-phosphate-5′-phosphosulfate (PAPS) as the donor sulfate group. Each enzyme modifies a specific position on the GAG backbone, but there is considerable overlap in the enzymatic function of these sulfotransferases.(10) The C3 of GlcNAc in heparan sulfate, for example, can be sulfated by enzymes encoded by 6 genes. The seeming redundancy presumably allows sulfotransferases to be expressed in a cell-specific manner and change during development or in response to stimuli.

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Although biosynthesis and modification of GAGs is not template driven, it appears to be tightly regulated through complex mechanisms. For example, recent sequencing of the glycan portion of bikunin found a single, defined sequence of chondroitin out of the billions of distinct GAGs possible.(11) Understanding how this biosynthetic machinery produces sulfated GAGs with remarkable fidelity will require substantial research into the multiple levels of cross-talk between sulfotransferases. In some cases, enzymatic activity of one enzyme is coupled to another since activity of sulfotransferases depends on production of precursor carbohydrates.(12)

After biosynthesis, *endo*-acting sulfatases known as sulfs can remove 6-*O*-sulfates from heparan sulfates.(13) Two heparan sulfate endosulfatases are known in humans, Sulf-1 and Sulf-2. They function extracellularly, and can dynamically alter the sulfation patterns by removing 6-*O*-sulfate. The spatiotemporal pattern of 6-*O*-sulfate is determined by expression of the heparan sulfate 6-*O*-sulfotransferases within the Golgi apparatus and the extracellular activity of the sulfatases Sulf-1 and Sulf-2.

Detection and characterization—Characterization of sulfated GAGs is essential for understanding relationships between structure and function, but it remains challenging. Some monoclonal antibodies are available for the detection of certain epitopes found in GAGs, and these have been used to evaluate sulfation changes in tissues,(14, 15) but it's difficult to obtain specific structural information with these reagents or to determine the precise location of sulfates along the GAG backbone. Detailed information on the positions of sulfates requires sequencing of sulfated GAGs. Recently, the first sulfated GAG was sequenced via mass spectrometry.(11)

Biological and medical significance—Sulfated GAGs are expressed on proteoglycans, which are key cell-surface receptors and components of the extracellular matrix. The ability of sulfation to tune the function of these proteoglycans is a recurring theme. Sulfation patterns can alter properties of the extracellular matrix, influence cell-cell interactions, and modify ligand binding to receptors. For example, the sulfation pattern of heparan sulfate determines its affinity for growth factors, which influences their retention and diffusion within the extracellular matrix.(16)

Genetic disorders of carbohydrate sulfotransferases illustrate that sulfated GAGs are especially important for development of connective tissue. Spondylepiphyseal dysplasia (Omani Type), for instance, is caused by a missense mutation in carbohydrate sulfotransferase 3 (*CHST3*), the gene encoding chondroitin 6-*O*-sulfotransferase-1 (C6ST-1).(17) This mutation causes skeletal abnormalities (club feet, congential joint dislocation) as well as deafness and a congenital heart defect (ventricular septal defect).(18) Mutation of *CHST14*, which encodes dermatan-4-sulfotransferase 1 (D4ST1), causes another syndrome of skeletal abnormalities, the musculocontractural type of Ehlers-Danlos syndrome.(19) Loss of *CHST6*, which encodes corneal GlcNAc-6-sulfotransferase, leads to macular corneal dystrophy, in which painful opacities develop within the cornea. Affected individuals often require a corneal transplant, but abnormalities in other organs are subtle or absent.

As another example, the glial scar, which forms after spinal cord injury, illustrates how specific sulfation patterns influence biological function. In response to nerve damage, astrocytes upregulate expression of chondroitin sulfate proteoglycans, likely due to increases in TNF-α. Chondroitin sulfate within the glial scar repels growing axons as they attempt to cross the injury site. Removal of sulfates using chondroitinase ABC stimulates axon regeneration in a rat model of CNS injury.(20) *In vitro* models have confirmed that chondroitin sulfate is chemorepulsive for neurons and the potency varies with the sulfation

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pattern (e.g., chondroitin-4-sulfate versus chondroitin-6-sulfate). For example, chondroitin-4-sulfate (CS-A) was found to be a significantly more potent chemorepellent than chondroitin-6-sulfate (CS-C),(21) and chondroitin-4,6-bisulfate (CS-E) was more chemorepulsive than chondroitin-4- sulfate (CS-A).(22) Downregulation of chondroitin-4,6-bisulfate (CS-E) was shown to improve nerve attachment and extension in vitro.(22)

Additionally, highly sulfated domains of heparan sulfate create “S-domains” that are critical for binding many protein ligands, such as chemokines and growth factors (see Figure 4). 6-*O*-sulfation of glucosamine modulates signaling pathways by enhancing and stabilizing heparan sulfate’s interactions with ligands such as Wnts, GDNF, and FGFs. Sulfs, which dynamically regulate sulfation patterns through 6-*O*-desulfation, direct embryogenesis(23) and contribute to carcinogenesis(24) and inflammation.(25)

Heparan sulfates also facilitate uptake of metabolites and ligands via endocytosis. Cell-surface heparan sulfate proteoglycans are believed to function as receptors for lipoproteins. Studies of conditional mouse knockouts of uronyl 2-*O*-sulfotransferase (*Hs2st*) suggest that hepatic receptors for chylomicrons (circulating lipid transport particles) require heparan-2-sulfate to clear plasma proteins.(26) Knockout mice lacking heparan-2-sulfate within hepatocytes have elevated plasma triglycerides due to delayed clearance of chylomicrons. Knockout of glucosaminyl 6-*O*-sulfotransferase-1 (*Hs6st1*) had no effect on plasma triglycerides, which again shows the dependence of biological function on pattern of sulfation.

Due to the wide-spread distribution of sulfated GAGs throughout the body, they are likely to have additional as of yet undescribed functions. Epigenetic studies have found that many cancers silence *HS3ST2*, but the functional consequences of the resulting changes in heparan sulfate proteoglycans are not clear.(27) Also, genome wide-association studies have linked sulfated GAGs to unexpected diseases. For example, response of schizophrenic patients to antipsychotics has been linked to genetic variants (known as single nucleotide polymorphisms or SNPs) in the carbohydrate sulfotranserase 8 (*CHST8*).(28)

Recent advances in chemical biology are providing systematic approaches for understanding the biological significance of sulfated GAGs. Chemical and chemo-enzymatic synthesis of sulfated GAGs is providing materials for detailed analysis of relationships between structure and function, which had been unfeasible using only glycans purified from natural sources. Advances in microfluidics, for example, enable enzymatic synthesis of complex sulfated GAGs analogous to their natural production in the Golgi apparatus.(29) Printing these sulfated glycans on a microarray(30) in a multivalent manner to mimic native conformations(31) should accelerate progress in deciphering the “sulfation code” and linking biological function with specific structural motifs.

Towards therapeutic applications—Knockout studies have shown that sulfated GAGs are valuable therapeutic targets for many diseases, as discussed above. To translate these discoveries into new therapies, technologies are needed to modulate sulfation of GAGs in living cells and animals. Highly specific inhibitors of carbohydrate sulfotransferases would offer a new class of potential therapies and provide a toolbox for elucidating the function of sulfated glycans. Inhibitors that change the pattern of sulfation could be treatments for inflammation, viral infection, and cancer.(32) For example, a small molecule screen identified some kinase inhibitors with activity for sulfotransferases (IC_{50} = 20–40 μM).(33) Peptide inhibitors provide an alternative strategy with comparable potency.(34) Overall, however, there are relatively few reported small molecule inhibitors, and enhancements to potency are needed.

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In addition to sulfotransferases, mounting evidence indicates that sulfatases may be valuable targets for anti-cancer therapeutics. Since these are extracellular esterases, they c=ould be targeted with inhibitors that are not cell-permeable. Inhibitors for sulfatases would complement inhibitors of carbohydrate sulfotransferases, but there are very few examples of sulfatase inhibitors. Sulfamates are one class of molecules that have been examined as substrate analogue inhibitors of Sulf-1 and Sulf-2 (IC_{50} of ≈ 100 μM).(35) A heparin mimetic was also found to inhibit heparan sulfatases with micromolar potency.(36) Better structural and mechanistic understanding of these carbohydrate sulfotransferases and sulfatases should facilitate rational design of inhibitors with improved potency and specificity.

Other Sulfated Glycoproteins

Sulfation is not restricted to GAGs. Other types of glycans found *N*- and *O*-linked to proteins can also be sulfated. Sialyl Lewis X, which is a terminal structure essential for many cell-cell interactions, can contain a sulfate group on the C6 position of GlcNAc. Also, analysis of mucins produced by patients with cystic fibrosis identified sulfation on the GlcNAc-6-sulfate, Gal-3-sulfate, and Gal-6-sulfate.(37, 38) Additionally, sulfated GalNAc and Gal have been found on the cancer-associated mucin MUC1 from breast cancer cells lines.(39)

Biosynthesis of these sulfated glycoproteins occurs within the Golgi apparatus using the same or similar enzymatic machinery that produces sulfated GAGs. Two GlcNAc-6-*O*-sulfotransferases (GlcNAc6ST-1(40) = encoded by *CHST2*, and GlcNAc6ST-2(41) = encoded by *LSST*) are known to produce 6-sulfo sialyl Lewis X.

Detection and characterization—Like sulfation of GAGs, identification and characterization of sulfated *O*-linked and *N*-linked glycans can be slow and difficult. The primary tools now available for assaying sulfated glycans are monoclonal antibodies and mass-spectrometry. Several monoclonal antibodies specific for sulfated carbohydrates have been developed.(42–44) Monoclonal antibodies can be used for high-throughput analysis of tissue samples, but care must be taken when interpreting the results due to subtle differences in specificity for structurally related glycans. Moreover, monoclonal antibodies are only available for a tiny fraction of the sulfated glycans present in the human glycome.

Mass-spectrometry, in particular MALDI-TOF, provides detailed structural information about sulfated glycans. For example, it was used to characterize differences in sulfation of mucins expressed by healthy controls and patients with cystic fibrosis.(38) Although useful in linking changes in sulfation with specific diseases, current methods for characterizing sulfation are limited in their ability to track sulfation changes within living organisms. Also, quantifying changes in sulfation requires a high level of technical expertise that is not widely accessible. Additionally, there is a need for *in vivo* imaging of dynamic changes in sulfation, which would significantly improve our understanding of how sulfation contributes to normal physiologic functions and disease pathogenesis.

Biological and medical significance—Sulfated glycoproteins have important functions in inflammation. The preferred ligands for L-selectin (a receptor that lymphocytes use to bind endothelial cells) are sulfated glycoproteins containing 6-*O*-sulfated GlcNAc, such as 6-sulfated sialyl Lewis X on GlyCAM-1(45, 46) and MAdCAM-1.(47) Because of its importance to L-selectin binding, sulfation is a key regulator of tethering and rolling of lymphocytes. Mice deficient in GlcNAc6ST-1 and GlcNAc6ST-2 had impaired homing of lymphocytes to the lymph nodes and diminished contact hypersensitivity.(48) Similarly, an antibody specific for 6-sulfo sialyl Lewis X and its defucosylated structure bound to *N*- and

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O-glycans on endothelial cells of lymphoid tissue, and it inhibited contact hypersensitivity and lymphocyte homing to the spleen in mice.(43)

Sulfation of MAdCAM-1 has a prominent role in the pathogenesis of ulcerative colitis (UC),(47) an autoimmune mediated inflammation of the colon that increases the likelihood of colon cancer and can require surgical resection of the colon. Interestingly, flares of ulcerative colitis are associated with increased sulfation of MAdCAM-1. During the remission phase of UC, endothelial cells within the high endothelial venules (HEVs) express comparable levels of the Lselectin receptor MAdCAM-1 relative to the active phase; however, sulfation of MAdCAM-1 is largely absent when UC is quiescent. It appears that active UC occurs when HEVs express GlcNAc6ST-1 that sulfates MAdCAM-1, which triggers inflammation by recruiting lymphocytes to the colonic lamia propria.

Additionally, abnormally sulfated mucins appear to be central to respiratory infections of patients with cystic fibrosis. Since the 1970s, it has been known that cystic fibrosis patients produce highly anionic mucins containing abundant sialic acid and sulfates.(49) These modifications are believed to contribute to excessive production of viscous mucous, increased inflammation, and colonization with *Pseudomonas aeruginosa*, which contribute to respiratory failure. Although mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel have been identified as the genetic cause of cystic fibrosis, it is unclear how exactly this mutation produces the symptoms of cystic fibrosis. It is possible that a defective chloride channel might lead to inadequate acidification of the Golgi lumen,(50) thereby causing abnormal sialylation and sulfation. Alternatively, chronic inflammation associated with CF could induce expression of TNF-α that upregulates carbohydrate sulfotransferases.(51) A major challenge for the field is determining how a defective chloride channel alters mucin sulfation and whether abnormally sulfated mucins contribute to the pathogenesis of CF.

Towards therapeutic applications—Thus far, sulfated glycoproteins have been linked to diseases in critical need of new therapies, such as cystic fibrosis and ulcerative colitis. A challenge for developing new therapies for these diseases is identifying the specific structures of disease-related sulfated glycoproteins along with the carbohydrate sulfotransferases required for their production. There is an opportunity to clarify therapeutic targets and develop strategies to interfere with their production or function. As with sulfated GAGs, inhibitors of carbohydrate sulfotransferases would provide important tools for studying sulfated glycoproteins that could be translated into new therapies, but relative few are known. Additionally, sulfated glycomimetics that block L-selectin on lymphocytes are promising new therapies for inflammation.(52)

Sulfoglycolipids

The two major mammalian sulfoglycolipids are sulfatide (Figure 1e) and seminolipid containing a 3-*O*-sulfo-galactose linked to a lipid (ceramide and diacylglycerol, respectively). Sulfatides are biosynthesized in the Golgi apparatus of oligodendrocytes, and they constitute 4–6% of glycolipids that form the myelin sheath surrounding neurons.(53) Seminolipids are the major glycolipid of spermatogenic cells.

Sulfatides are necessary for proper function of the nervous system.(54) They appear to facilitate transmission of axon potentials by organizing the paranodal junctions.(54) Knockout mice lacking sulfatides show deficiencies in learning and memory.(55) They also have irregular neuronal activity in the hippocampus and cortex.(56) In a postmortem study of humans, brain tissue of Alzheimer's disease (AD) patients had substantially lower sulfatide content relative to controls.(22) A shotgun lipidomics study, moreover, has found lower sulfatide levels in both cerebral gray and white matter of individuals with very mild

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cognitive impairment relative to controls.(57) Although investigations continue into the underlying biological basis for the link between sulfatides and dementia, sulfatides could contribute to the pathogenesis of AD through effects on clearance of amyoloid-β (Aβ) peptide by apolipoprotein E (ApoE).(58) Seminolipids are known to be essential for spermatogenesis,(59) but their precise functions remain unclear.

O-ACYLATION OF GLYCANS

O-Acylation is a common, biologically important post-glycosylational modification of many glycans. *O*-Acylation of glycans may include transfer of a relatively small group like an acetyl group or a more complex structure, such as ferulate, to a sugar hydroxyl (see Figure 1a and 1f). Acylated glycans are found on cell surfaces, capsular polysaccharides of certain bacteria, and on glycoconjugates such as glycoproteins, glycolipids, proteoglycans, and glycosylphosphatidylinositol (GPI) anchors. Acylation and de-acylation of glycans are catalyzed by acyltransferases and esterases, respectively. Changes in the acylation levels of glycans can significantly affect their specific molecular recognition events, such as binding and degradation, as well as physical properties, such as solubility and hydrophobicity. More importantly, acylation of glycans can play a role in human immunology, disease pathogenesis, and cancer progression.

O-Acetylated Glycans

O-Acetylation, the most common form of acylation, has been reported in various species from bacteria to higher animals including humans. *O*-Acetylation generally occurs within the Golgi apparatus, where an *O*-acetyltransferase adds an acetyl moiety from acetyl CoA to a specific hydroxyl group of the glycan.(60, 61) Once on the cell surface, acetylesterases can remove acetyl groups to remodel the glycans. The regulation of *O*-acetyltransferases and acetylesterases is not well understood.

O-Acetylation of sialic acids is one of the most commonly reported modifications. Sialic acids are negatively charged monosaccharides with a nine-carbon backbone that are typically found as the terminal residues of glycoconjugates (see Figure 1b). They are among the most structurally diverse sugars, and are involved in many biological and pathological processes.(60) Although *O*-acetylation is the most common modification of sialic acids, numerous other modifications of sialic acid are known, including sulfation, phosphorylation, methylation, and lactylation.(60) *O*-Acetylation of the C4, C7, C8, and C9 positions of sialic acid has been reported.(60, 62) Acetylation of sialic acids has been shown to be involved in many biological and pathological processes such as cancer and viral infections.(60, 61, 63)

Sialate-*O*-acetyltransferase and sialate-*O*-acetylesterase catalyze *O*-acetylation and de-*O*-acetylation of sialic acids.(62, 64) At least two genes (*NeuO and NeuD*) are known to be responsible for the *O*-acetylation of sialic acids. Acetyl groups are believed to be first incorporated into the C7 position of sialic acid; however, they can subsequently migrate to the C9 position.(65–67) Migration of the acetyl group to the 9-position may occur spontaneously under physiological conditions,(68) or under the influence of enzymatic isomerization.(69) Alternatively, direct acetylation of the C9 position may occur.(65–67) Acetylation at the C4 position is less common and has been found in some animals including guinea pigs, bovine, horses, and mice.(70–73)

O-Acetylation of polysaccharides produced by bacteria and other microorganisms is also known. In one example, *O*-acetylation occurs on alginates, which are polysaccharides produced by brown seaweeds and certain bacteria. Alginates contain 1,4-linked β-D-mannuronic acid and α-L-guluronic acid. In alginates from bacteria, such as mucoid *P. aeruginosa*, one or both of the O2 and O3 positions of the mannuronate residues are

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acetylated. The presence of *O*-acetyl groups modulates virulence in patients with cystic fibrosis (CF) since these acetylated glycans inhibit opsonic killing and prevent activation of the alternative complement pathway.(74) As detailed in a recent review,(75) the bacterial alginate biosynthesis pathway involves polymerization of GDP-mannuronic acid to form a linear polymer, which is subsequently modified by *O*-acetylation of some of the D-mannuronic acid residues along with epimerization of non-acetylated D-mannuronic acid residues to L-guluronic acid. It has been demonstrated that *algF, algJ, and algI* genes encode the biosynthetic machinery of acetylation.(76) Mutation of these genes in *P. aeruginosa* blocked *O*-acetylation of alginate.(76)

Detection and characterization—Identification and characterization of *O*-acetylation in biological samples, such as tissues, can be challenging. Techniques such as NMR spectroscopy can be used to determine the structure of glycans and to precisely define the position of the *O*-acetyl groups, but this method typically require substantial amounts of a homogeneous glycan, which is often difficult to obtain from a complex sample. Therefore, such a method is not wellsuited to high-throughput evaluation of many biological samples, such as large numbers of cancer biopsy samples.

Another commonly used method for the structure determination of *O*-acetylated glycans is mass spectrometry (MS). This technique can be applied to complex biological samples, but it is difficult to define the exact position, glycan sequence, and stereochemistry of glycosidic linkages associated with the acetylated residue. In addition, chemical methods used to prepare glycans for MS analysis, such as basic or acidic treatment, may result in the loss or migration of the acetyl group from one position to another. Therefore, in order to improve characterization of these glycans, MS is often coupled with other detection methods including enzymatic degradation, radiolabeling, binding assays, and various chemical and spectroscopic methods.(67, 77–81)

Biological and medical significance—*O*-Acetylation of glycans plays an important role in human immunology, disease pathogenesis, and cancer progression. Cancer is one particular area where *O*-acetylation displays alterations and contributes to the disease. For example, more than 50% of sialic acids present in human colonic mucins are *O*-acetylated, (80) and a reduction in the level of *O*-acetylated sialic acids is a major alteration related to progression of colorectal cancer.(82) In addition, *O*-acetylated sialic acids are over expressed in the tumor-associated antigens sialyl Lewis X, GD3 and GM3.(79, 83–85) *O*-Acetylation of sialic acids mediates the survival of peripheral blood mononuclear cells (PBMC) of acute lymphoblastic leukaemia (ALL) patient.(86) A recent study demonstrated a correlation in the level of acetylated sialic acid in the cytosolic and lysosomal fractions from lymphoblasts of ALL patients with sialate-*O*-acetylesterase activity,(87) which suggests that sialate-*O*-acetyltransferase and sialate-*O*-acetylesterase activities are responsible for the enhanced level of 9-*O*-acetylated sialic acid in ALL lymphoblasts.

O-Acetylated glycans also play a critical role in bacterial and viral infections. Overproduction of alginate is believed to produce a thick biofilm that protects bacteria from the host defense mechanism and antibiotic treatment, which is a major problem for treating patients with CF.(88–90) Finally, *O*-acetylation can play an important role in viral adhesion to host cells. For example, whereas 9-*O*-acetylation of sialic acids is required for the binding of influenza C viruses, it prevents attachment of both influenza A and B viruses.(91)

O-Acetylated glycans are also involved in other biological processes. For example, *O*-acetylation of sialic acids is thought to regulate inhibitory signaling in B lymphocytes.(92) Additionally, a defect in sialate-*O*-acetylesterase activity is linked to other human diseases including rheumatoid arthritis, type 1 diabetes, and some autoimmune disorders.(93, 94)

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Towards therapeutic applications—Enzymes that catalyze the transfer and hydrolysis of *O*-acetyl groups are potential therapeutic targets for cancer and infectious diseases, but this area has been largely understudied. For example, mutated *P. aeruginosa* incapable of *O*-acetylating alginate had disrupted bacterial cell walls due to accumulation of alginate polymers in the periplasm.(95) A small molecule inhibitor specific for bacterial acetyltransferase, therefore, might help treat patients with CF. While CoA has been reported to be a general inhibitor for *O*-acetyltransferases,(67) there are no published selective inhibitors of sialate-*O*-acetyltransferases and sialate-*O*-acetylesterases.

Other Types of *O*-Acylation

Lactylation is a less common modification, but lactyl groups have been reported in the glycolipids of *M. smegmatis*(96) and in higher animals including humans.(97) The presence of a 9-*O*-lactyl-N-acetylneuraminc acid has been reported in human gastric aspirates.(97) Additionally, 4-*O*-acetyl-9-*O*-lactyl-N-acetylneuraminic acid was identified from horse submandibular gland.(98) However, the biological functions of this type of modification remain unknown.

In addition to the simple *O*-acylation of carbohydrates with acetic acid, acylation with longer chain fatty acid are found in bacteria and plants.(99–102) For example, lipopolysaccharide (LPS) is a major component of the outer membrane in Gram-negative bacteria and is often referred to as an endotoxin. LPS protects bacteria against antibiotic treatment and stress, and it plays a role in the ability of bacteria to cause diseases. It is divided into three regions: lipid A, core polysaccharide, and *O*-antigen repeats.(103) The LPS structure can vary in different bacteria and often determines the virulence of the bacteria and how it interacts with receptors of the innate immune system.(104) The *O*-antigen is the most variable region and can bear repeats of three to five sugars, which can undergo further modifications. The majority of enzymes and genes coding for the biosynthesis and transport of LPS have been identified, and they are targets for the development of new antibiotics and vaccines.(105–107)

Plant glycans can also be acylated with ferulate, which can undergo radical coupling reactions that causes cross-linking between cell wall polysaccharides of some plants such as maize bran.(108) The cross-linking of polysaccharides by dehydordiferulates may contribute to the plant’s defenses against insects and diseases.(108)

PHOSPHORYLATION

Phosphorylation of carbohydrates is also an important feature in glycobiology. Phosphorylated glycans play key roles in protein transport, bacterial pathogenesis, and human diseases. (109–111) Carbohydrates can be phosphorylated with simple phosphate groups or with more complex phosphodiester groups. Phosphorylation of carbohydrates occurs pre- or co-glycosylationally. Post-biosynthesis phosphorylation of carbohydrates is rare; nevertheless, a few examples of phosphorylated carbohydrates are known.

Mannose-6-phosphate (M6P) is one of the most studied phosphorylated glycans. Biosynthesis of M6P proceeds via a two-step enzymatic process. Initially, GlcNAc-1-phosphate is transferred by UDP-GlcNAc:lysosomal enzyme GlcNAc-phosophotransferase to the terminal mannose residues of selected high mannose glycans, followed by removal of GlcNAc residues by *N*-acetylglucosamidase to give M6P (Figure 1d), which allows the transport of proteins to the lysosome by interacting with the M6P receptor.(4, 112)

In Gram-positive bacteria, phosphorylated polymers known as teichoic acids are attached to the peptidoglycan layer via a phosphodiester linkage.(113) Teichoic acids contribute to

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bacterial resistance to human lysozyme,(114) and play a role in biofilm formation.(115) The enzymes and genes coding for the biosynthesis of teichoic acids from *Staphylococcus aureus* have been identified.(116, 117) Understanding the biosynthetic pathways of teichoic acids may lead to the development of inhibitors that can be useful for treating infections caused by gram-positive bacteria.

A variety of other phosphorylated glycans have been found in nature including cyclicphosphate- containing capsular polysaccharides isolated from *Vibrio cholera* O139,(118) phosphorylcholine-glyconjugates in nematodes,(119) and lipophosphoglycan in *Leishmania* parasites.(120) *Leishmania* is a sandfly-transmitted parasite that is responsible for the Leishmaniasis disease. The parasite expresses lipophosphoglycan which enable the parasite to survive defense mechanisms of the host.(120)

Detection and characterization

Mass spectrometry and NMR are also commonly used for the characterization of phosphorylated glycans.(121, 122) NMR spectroscopy can provide structural information and precisely define the phosphorylated site(s) within the glycan. However, this method requires large amount of sample, which is often hard to obtain from complex samples. Alternatively, with recent advances in MS characterization of these glycans can be achieved with much less sample. However, accurate assignment of the positions of the phosphate groups within the glycan still challenging. The MS analysis can be further complicated by the instability of the phosphorylated glycans.

Biological significance and therapeutic applications

O-Phosphorylation of glycans plays key roles in protein transport, bacterial pathogenesis, and human diseases. For example, mutations in the genes encoding subunits of GlcNAc-1-phosphotransferase enzyme in humans result in lysosomal disorders,(123–125) and mannose phosphorylation is important for controlling the secretion and extracellular levels of leukemia inhibitory factor (LIF).(126) Using mouse embryonic stem cells, it was demonstrated that phosphorylated mannose of LIF stimulated cell differentiation. A similar mechanism may apply to other cytokines and proteins bearing M6P to see if they can be regulated. Defects in the phosphorylation modifications of *O*-mannosyl glycans may be responsible for some diseases including Fukuyama congenital muscular dystrophy and muscle-eye-brain disease.(121) Additionally, mutation of the dystroglycan gene *Dag1* impairs dystroglycan function by inhibiting the post-translational modification.(109) However, the biological functions of phosphorylated glycans remain poorly defined. With better understanding of the mechanism and functions of these glycan, opportunities for therapeutic applications will be possible.

METHYLATION

O-Methylated carbohydrates have been found in various organisms and are most common in bacteria. Neutral *O*-methylated *N*-glycans have been isolated from gastropods.(3, 127) *O*-Methylation of these oligosaccharides occurs at the C3 position of terminal mannose residues (Figure 1g). An 8-*O*-methylated sialic acid has been reported in the starfish *Asterias forbesi*(128), and as glycoconjugate-bound sialic acids in *Asterias rubens*.(129) Additionally, *O*-methylated glycans have been found in some nematodes including *Toxocara canis* (*T. canis*) which bear *O*-methylated oligosaccharides similar to the mammalian blood-group antigen H.(130) They contain one or two methyl groups on the O2-position of the terminal fucose and the O4-position of galactose.(130) In general, *O*-methylation occurs on mature glycans by *O*-methyltransferases that utilize *S*-adenosylmethionine (SAM) as the methyl donor. Detection and characterization of *O*-

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methylated glycans are achieved using various chemical and spectroscopic methods similar to those previously described including NMR and mass spectrometry.(131)

Biological significance and therapeutic applications

O-Methylated glycans can play a role in human infections and diseases. Although it is commonly found in dogs, the *T. canis* parasite is known to infect humans, especially young children. (132) The parasite larvae can damage tissues it enters, and give rise to visceral larva migrans (VLM) or ocular larva migrans (OLM).(132) Synthetic glycoconjugates of these *O*-methylated glycans induce parasite-specific antibodies in approaches to generate a vaccine.(133) The ability of parasite glycans to modulate host immune response may be an important first step toward designing effective molecular or glycoconjugate-based vaccines that can offer lifelong protection against the parasite.

In addition, mycobacteria are known to cause serious human diseases such as tuberculosis. Some of the mycobacteria express *O*-methylated polysaccharides (PMPS) on their surfaces as part of their GPL, which is important for their survival. It has been suggestion that PMPS play a role in the regulation of fatty acid metabolism by forming complexes with fatty acyl chains and acyl-CoAs; however, additional studies are needed to confirm this hypothesis. (134, 135) Understanding the biosynthetic pathways and biological functions of these molecules may lead to potential new drugs and vaccine for diseases caused by mycobacteria including tuberculosis.

EPIMERIZATION

While most modifications result in the decoration of oxygen or nitrogen atoms linked to monosaccharide residues, epimerization alters the stereochemistry at one of the carbon atoms producing a new monomer residue. For example, D-glucuronyl C5-epimerase converts Dglucuronic acid residues within an oligosaccharide/polysaccharide chain to L-iduronic acid residues by epimerizing the C5 position. This can lead to major effects on glycan structure and function.

One area where epimerization plays a key role is in the biosynthesis of certain glycosaminoglycans, including heparin, heparan sulfate, and dermatan sulfate.(136) Conversion of glucuronic acids to iduronic acid residues is an important step in the formation of these saccharides, and this process affects the biological activity of the GAG chains. For example, an increase in iduronic acid content increases the anti-proliferative activity of dermatan sulfate in human lung fibroblasts, and iduronic acid- rich heparin and heparan sulfates displayed anti-proliferative effects on mesothelioma cells.(137, 138) Alterations in epimerase levels have also been associated with disease. A recent study has shown a significant reduction of D-glucuronyl C5-epimerase expression in human breast cancer.(139) Since this enzyme is normally expressed in most human tissues, detection of this change may be useful as a diagnostic and/or prognostic marker in cancer.

In bacteria, epimerization of the C5 position of mannuronic acid to produce guluronic acid was observed in the biosynthesis of alginates.(75) Epimerization of D-mannuronic acid to L-guluronic acid occurs in the periplasm by mannuronan C5-epimerase, and the gene (*AlgG*) responsible for this conversion has been identified. The mannuronan C5-epimerase could be targeted to disrupt the biosynthesis of alginate in bacteria, which play a role in protecting the bacteria against host defenses and antibiotic treatment.

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OTHER MODIFICATIONS

Certain microorganisms are reported to produce enzymes that can carry out postglycosylational modifications on host glycans. For example, certain bacteria and viruses express glycosidases that can cleave off sugars from host glycans to allow for their colonization and pathogenesis.(140, 141) For example, the influenza virus contains a neuraminidase that cleaves sialic acid residues from host's cell surface glycans. This enzyme is crucial for the spread of the virus, and inhibition of this enzyme forms the basis of several FDA approved treatments. Additionally, certain bacteria found in the gut, such as *Bacteroides thetaiotaomicron*, express glycosidases that remove hexose sugars from host cell surface glycans.(142) They also express genes that encode various sialic acid-specific 9-O-acetylesterase, a mucin-desulfating sulfatase, and a chondroitin lyase.(142) These examples illustrate how microbes can modify host glycans and support targeting these enzymes for the development of new therapeutics.

PERSPECTIVE AND FUTURE DIRECTIONS

As key modulators of glycan function, post-glycosylational modifications play a critical role in many normal and disease processes. At present, however, there are still many challenges and barriers that impede progress towards understanding and fully exploiting this area of biology. As a result, this is a rapidly evolving field with numerous opportunities for major contributions.

First, new technologies and new information are needed to define the key players: the modified glycans themselves, their modifying enzymes, and the proteins that bind to modified glycans. One major need is a high-throughput, high-content technology for rapidly identifying and characterizing glycan modifications in complex biological samples, such as biopsy samples. Most techniques currently used to study glycans and glycan modifications are slow, require large amount of pure glycan, are unsuitable for high-throughput studies, and/or provide only cursory information about structure. Furthermore, the lability of many modifications hinders their characterization, particularly by mass spectrometry. In addition to the glycans, many of the enzymes that modify glycans have yet to be identified, sequenced, and characterized. Moreover, many of the proteins that bind to modified glycans are unknown, although glycan 1array technology is at least partially addressing this issue. (30, 143, 144) Identification of the key players is crucial for a more complete understanding of the roles of glycan modifications.

Second, new tools and techniques are needed to modulate and control functions of the key players. Gene knock-out experiments and siRNA inhibition are powerful tools for studying protein function, but these techniques are not well-suited for controlling glycan expression. While these techniques can be applied to the associated proteins, knowledge of the target gene(s) is an important prerequisite. Antibodies and small molecule inhibitors can also be used to modulate and control biomolecule function. For example, they are commonly used as agonists or antagonists to define the basic functions of proteins and validate them as therapeutic targets. At present, however, there are very few small molecule inhibitors of proteins that produce or interact with modified glycans, and most possess only modest activity. In addition, few antibodies specific for modified glycans or related proteins are available. Therefore, development of potent and selective small molecule inhibitors and/or antibodies could have a significant impact on the field.

Finally, these new tools, information, and technologies will provide better insight into many fundamental questions about glycan modifications. How do genetic and environmental factors contribute to the repertoire of glycan modifications *in vivo*? How are they regulated? What other biological processes and diseases do they affect? With the many advances over

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the last few years and rapid growth of new information in the coming years, the field is poised for significant expansion.

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Keywords with Definitions

Carbohydrate	polyhydroxy aldehydes or ketones
Glycan	oligosaccharide, polysaccharide, or carbohydrate portion of glycoconjugate
Post-glycosylational modification	structural change to a mature glycan that occurs after the formation of linkages between adjacent monosaccharide units
Sulfation	addition of a sulfate group
Phosphorylation	addition of a phosphate group
Acylation	addition of an acyl group
Methylation	addition of a methyl group
Epimerization	forming an epimer by changing one asymmetric center

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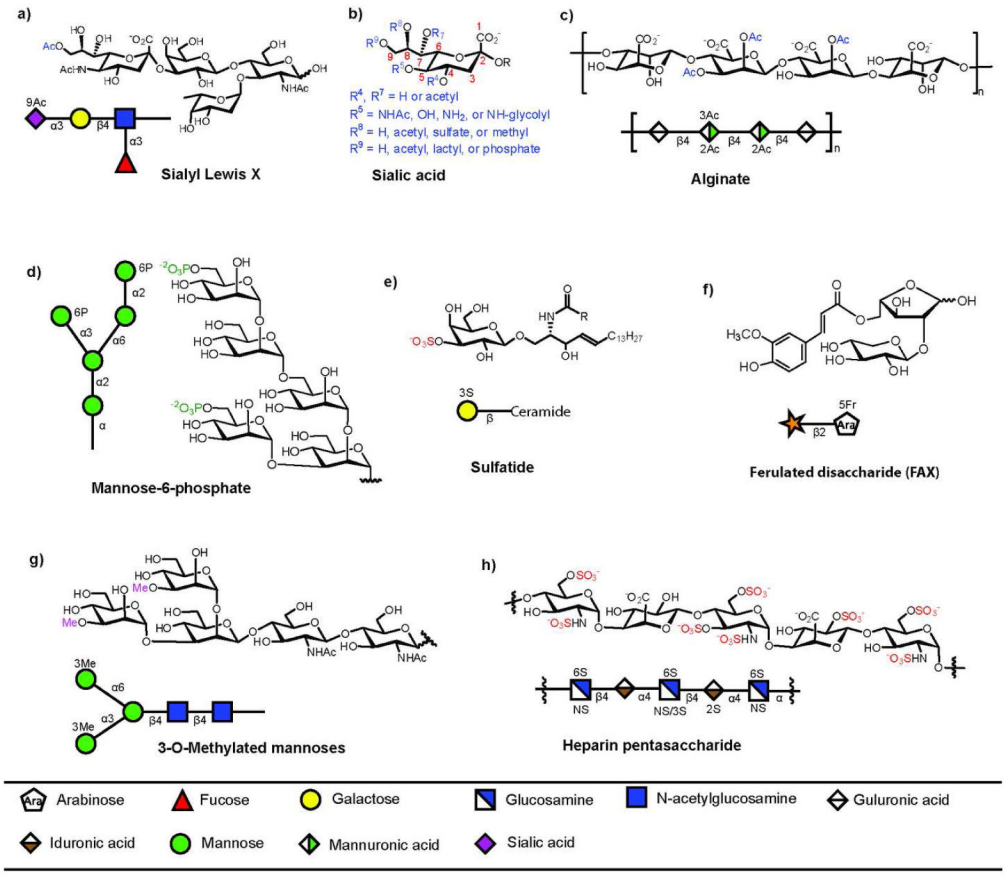

Figure 1.

Representative examples of common carbohydrate modifications in nature. Symbols for each monosaccharide component are identified in the legend. Glycosidic linkages are identified by α or β with a number that identifies the carbon atom of the acceptor sugar. *O*-Acetylation is indicated by Ac, *O*-phosphorylation is indicated by P, *O*-methylation is indicated by Me, *N*-sulfation is indicated by NS, *O*-sulfation is indicated by S,O-ferulyl is indicated by Fr, and the numbers indicate the carbon atom where the modification occur on the monosaccharide. (**a**) Structure of 9-O-acetylated sialyl Lewis X. (**b**) Structure diversity of sialic acids. (**c**) Alginate structure from *P. aeruginosa*. (**d**) Mannose-6-phosphate (Man 5). (**e**) Structure of sulfatide. (**f**) β1,2–D-Xylopyranosyl-5-O-*trans*-ferulyl-L-arabinofuranose (FAX). (**g**) *O*-Methylated glycan from gastropods. (**h**) Structure of heparin pentasaccharide.

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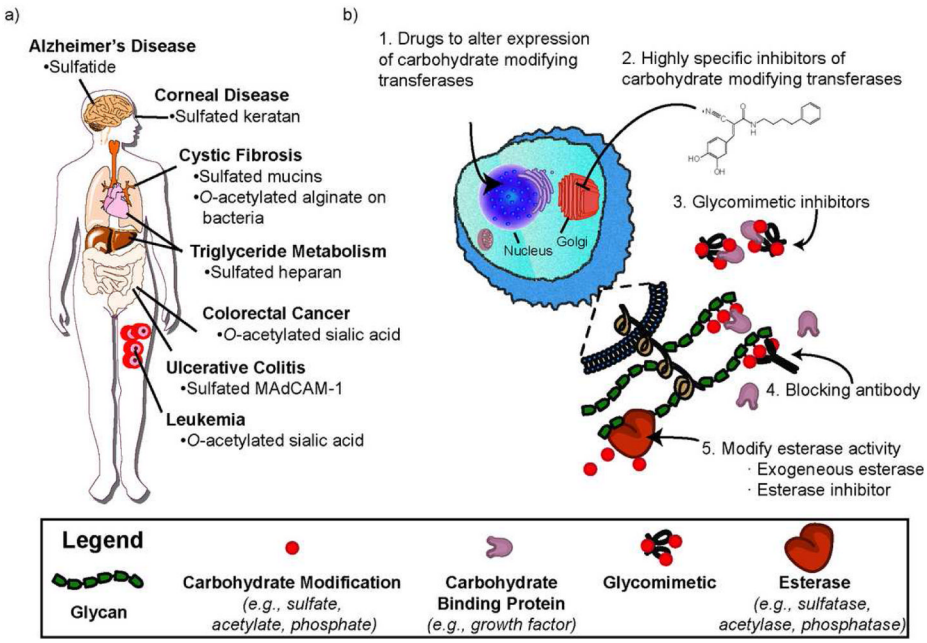

Figure 2.

Medical significance of modified glycans. (**a**) Modified glycans mediate biological functions across various organ systems, and they have been linked to malignant, degenerative, infectious, and inflammatory diseases. (**b**) Strategies that intervene in these functions could lead to new classes of therapies. Production of modified glycans can be downregulated by targeting their biosynthetic machinery, either by blocking expression or activity of carbohydrate modifying enzymes. Antibodies and glycomimetics can disrupt the ligand-receptor interactions of modified glycans. Finally, therapeutic strategies can target the extracellular esterases that remodel patterns of glycan modifications.

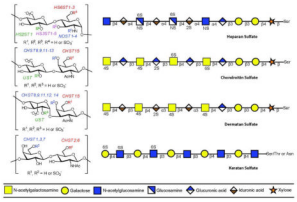

Figure 3.
Biosynthesis and structures of sulfated GAGs. A family of carbohydrate sulfotransferases catalyzes the sulfation of GAGs at specific positions within the repeating disaccharide repeat units.

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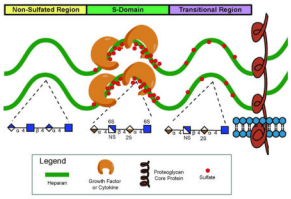

Figure 4.

Sulfation tunes biological activity of GAGs. Sulfates are not uniformly positioned along the GAG backbone. Cells modulate the linkage and density of sulfates added to the GAG core as one way of regulating a sulfated GAG's biological function. Ligands that bind to sulfated GAGs recognize specific patterns of sulfation, sometimes referred to as the "sulfation code". In this example, the sulfation pattern of heparan sulfate determines the binding affinity for cytokines and growth factors. Some regions, known as S-domains, are heavily sulfated and have high affinity for ligands. Other regions contain fewer or no sulfates.

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