Carbohydrate biomarkers for future disease detection and treatment

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Carbohydrates are considered as one of the most important classes of biomarkers for cell types, disease states, protein functions, and developmental states. Carbohydrate “binders” that can specifically recognize a carbohydrate biomarker can be used for developing novel types of site specific delivery methods and imaging agents. In this review, we present selected examples of important carbohydrate biomarkers and how they can be targeted for the development of therapeutic and diagnostic agents. Examples are arranged based on disease categories including (1) infectious diseases, (2) cancer, (3) inflammation and immune responses, (4) signal transduction, (5) stem cell transformation, (6) embryo development, and (7) cardiovascular diseases, though some issues cross therapeutic boundaries.

carbohydrates, biomarkers, imaging agents, boronolectins

1 Introduction

Carbohydrates, traditionally considered as bioenergy suppliers and structural components, have been found to have a wide variety of biological and physiological functions. Therefore, together with proteins and peptides, nucleic acids and oligonucleotides, and lipids, carbohydrates are considered one of the most important classes of biomacromolecules [1–4]. In addition, the relationship among these four classes of biomolecules is often intertwined. For example, protein glycosylation is very important to its conformation, transportation, function, and fate [5–16]; glycosylated lipids (glycolipids) are essential biomolecules [3, 17–22]; and nucleic acid glycosylation, whether synthetic [23] or natural [24–31], has recently been recognized as important in affecting its distribution and function. Because of all these properties, it is not surprising that carbohydrates are biomarkers for cell types, disease states, protein functions, and developmental states [2, 32–40]. Recent years have seen a rapid increase in knowledge related to all these areas mentioned. Such advancements can largely be attributed to the development in new techniques, such as NMR and mass spectrometry, and molecular biology, and the ready availability of genomic information. As a result, recent advancements in glycobiology and glycomics have also opened new doors for the development of new therapeutics and imaging agents through carbohydrate recognition. Therefore, compounds that can specifically recognize a particular carbohydrate have very important applications [41–52]. They can be used as sensors for detection, diagnosis, and prognosis, as “blockers/inhibitors” for therapeutics development if the target carbohydrate is involved in pathogenesis, and as vectors for the targeted delivery of imaging and therapeutic agents. Critical to all these potential applications are two issues: (1) the identification of carbohydrate biomarkers and (2) the design and synthesis of “binders” that can specifically recognize the target biomarker with high affinity and specificity. In this review, we
present selected examples of important carbohydrate biomarkers and how they can be targeted for the development of therapeutic and diagnostic agents. It should be noted that there are many books on glycobiology and there are many review articles on how one can target carbohydrates for various applications [45, 51, 53–55] and it would be impossible to cover the entire topic in detail. Therefore, this article does not strive to be comprehensive. Instead, it is meant to be “tutorial,” which we hope would help to generate more interests in carbohydrate recognition as a way for the development of new therapeutics and diagnostics. Carbohydrate recognition research impacts essentially all areas in which carbohydrates play a role. Examples include (1) infectious diseases, (2) cancer, (3) inflammation and immune responses, (4) signal transduction, (5) stem cell transformation, (6) embryo development, and (7) cardiovascular diseases. In the following sections, we divide the discussions based on disease categories though some issues cross therapeutic boundaries. In the last section, we present a few simple examples of how to target carbohydrate biomarkers for diagnostic and therapeutic applications.

2 Cancer

Among all pathologically relevant glycosylation changes, cancer is probably the most extensively studied. Even in normal cells, surface carbohydrate structures are known to be characteristic markers for different types of cells [56–61]. Transformations of normal to cancerous cells are often associated with the alteration of cell surface carbohydrates and the expression or over-expression of certain carbohydrates has been closely correlated with cancer [56–61]. Therefore, many carbohydrates are considered cancer associated antigens (CAA). Among all carbohydrate-based CAA, Globo H and the Tn antigen are probably the most common. It is known that the Tn antigen is found on the cell surface of over 90% of solid tumor [62]. The formation of the Tn antigen is because of deficiency of an enzyme named β-1,3-galactosyltransferase, which results in the incomplete conversion of the Tn antigen to the T antigen. Other important cancer-related carbohydrates include the sialylated carbohydrates. For example, sialyl Lewis X (sLeα) has been shown to mediate lung colonization of B16 melanoma cells [63] and yet excessive sLeα expression is shown to lead to rejection by natural killer cells [64]; serum sLeα and cytotkeratin 19 fragment are said to be predictive factors for recurrence in patients with stage I non-small cell lung cancer [65]; and sLeα and sialyl Lewis a (sLeβ) have been shown to mediate adhesion of urothelial cancer cells to activated endothelium [66]. Changes in sLeα and sLeβ levels in cancer have been attributed to both “neosynthesis” and “incomplete synthesis” of pathways involving sulfation or sialylation [67] and variations of enzyme levels can be directly correlated with certain changes in glycosylation [68]. With all these aberrant glycan expressions, “binders” that can recognize these carbohydrates will be very useful research tools, diagnostic agents, and possibly therapeutic agents [51].

In addition to cell surface carbohydrate biomarkers, post/co-translational protein glycosylation often carries signatures of malignant transformations [69–93]. In terms of the biological significance of protein glycosylation, usually it is not a question of whether there is glycosylation; rather it is the glycosylation pattern that marks different pathological states including malignancy. For example, the glycosylation patterns of prostate specific antigen (PSA) from cancer cells in culture [94] and prostate cancer patients’ tissue and sera [69, 93, 95] are different from that of normal prostate; human pancreatic RNase 1, a glycoprotein secreted mostly by pancreatic cells, has completely different oligosaccharide chains when produced from pancreatic tumor cells [39, 71, 96, 97]; pregnancy-related human chorionic gonadotropin (hCG) can be biomarkers for cancer. Down syndrome, and pregnancy failure depending on their glycosylation patterns [98, 99]; and specific glycosylation patterns of haptoglobin (Hp) and alpha-fetoprotein (AFP) have a much higher degree of correlation with cancer than the total Hp/ AFP levels [100, 101]. In such cases, “binders” that can recognize glycosylation variations can be very useful diagnostic tools.

3 Infectious diseases

There are several ways in which carbohydrates are involved in the pathogenicity of infectious agents. In the case of viral infections, the human influenza virus is an excellent example. Flu viral infection involves sialic acid for binding to hemagglutinin and infection. After infection, the budding of mature viruses from infected cells involves the cleavage of sialic acid by neuraminidase in order for the virus to detach [102, 103]. Flu drug such as tamiflu functions by inhibiting neuraminidase and thus inhibit viral replication [104, 105].

In the case of human immunodeficiency virus (HIV), a critical protein, gp120, is glycosylated with polymannose [106, 107]. Infection of cells by HIV-1 requires the fusion of the viral membrane with cellular membrane [108]. This fusion is mediated by gp120 and gp41 along with cell surface receptors (CD4 and chemokine receptor) on the target cells [109]. Conceivably, agents that interact with gp120 may interfere with viral entry into target cells [110]. Binding to glycans on the viral envelope may also force the virus to delete a portion of its glycan shield, making the virus more susceptible to attack [111]. Along this line, several lectins have been studied for their anti-HIV activities. For example, cyanovirin-N (CV-N) is a 11kDa protein with 101 amino acids and has affinity for high-mannose glycans especially α-(1,2)-linked mannose oligomers [112, 113]. CV-N inactivates T-lymphocyte-tropic, laboratory strains of HIV type 1 and HIV type 2, as well as T-tropic, M-tropic and
dual tropic primary clinical isolates of HIV-1, presumably through inhibition of viral entry by blocking part of gp120. In CEM-SS cells, CV-N has an EC50 of 0.1 nM. As a control, treatment of uninfected CEM-SS cells in the presence of high concentrations (9000 nM) of CV-N did attenuate the lethal effect of the virus. Other lectins have also shown similar effect, presumably by binding to the polymannose portion of gp120. These include SVN (scytovirim), which has affinity for α(1,2)-α(1,6)-mannose trisaccharide units and can inhibit HIV infection in T-tropic laboratory strain HIV-1 in CEM-SS cells with an EC50 of 0.3 nM [114], and actinohivin, which has affinity for mannose-type glycans [115] and can inhibit T-cell and macrophage infection by HIV-1 in cell culture (IC50 of 60 to 700 nM). All such results indicate that the viral envelope glycans play an important role in their pathogenicity.

In bacterial pathogenicity, carbohydrates also play very important roles. One prominent example is lipopolysaccharides (LPS), also known as endotoxin, from the outer membrane of cell wall of Gram-negative bacteria. One such example is Pasteurella multocida, an encapsulated, Gram-negative coccobacillus that causes a wide range of animal diseases including avian fowl cholera [116]. When infections by Gram-negative bacteria occur, LPS stimulate the immune system in an attempt to clear the bacteria and the infection that may result. The lipid A component of LPS is primarily responsible for this inflammatory response. As the infection proceeds, the presence of a large amount of LPS can result in an overproduction of inflammatory mediators that result in damage to tissues, septic shock, organ failure, and death [117]. LPS has also been shown to play an important role in the pathogenesis of P. multocida, in which modification of LPS structure negatively affects the viability of P. multocida in vivo [118]. In addition to functioning as toxins, carbohydrates can also be the target for bacterial recognition. For example, many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids [119]; Pseudomonas aeruginosa and Pseudomonas cepacia isolated from cystic fibrosis patients bind specifically to gangliotetraosylceramide (asialo GM1) and gangliotriaosylceramide (asialo GM2) [120]; the pili of P. aeruginosa strains PAK and PAO bind specifically to the carbohydrate sequence beta GalNAc(1-4)beta Gal found in glycosphingolipids asialo-GM1 and asialo-GM2 [121]; adherence of P. aeruginosa and Candida albicans to glycosphingolipid (Asialo-GM1) receptors is achieved by a conserved receptor-binding domain present on their adhesions [122]; lacto- and ganglio-series glycolipids are adhesion receptors for Neisseria gonorrhoeae [123]; and there are many other examples [124]. In addition to all these, serotyping of Gram-negative bacteria is primarily based on their LPS structures [125]. Because of the many ways that carbohydrates can affect bacteria (and fungi) infections, one can envision situations where artificial “binders” of the target carbohydrates can be used for detection and treatment of bacterial and fungal infections.

4 Inflammation and immune responses

Carbohydrates are involved in mediating inflammatory processes in many ways. The most widely known example is probably sLeα-mediated white blood cell adhesion to infection/damaged sites through interactions with L-selectin. Recent studies unveiled that 6-O GlcNAc sulfate modification of the sLeα tetrasaccharide is of importance in L-selectin activity in animal and in leukocyte invasion into different human tissues [126]. Furthermore, the de novo induction of endothelial sLeα or its sulfated form through interactions with L-selectin is a common event in many organs, including thyroid gland, heart, skin and colon, thus, suggesting a crucial role for these glycans in the early-stage induction of tissue inflammation [127].

The most prominent examples of carbohydrate-mediated immune responses originate from the ABO blood group antigens, which are entirely carbohydrate-based [128]. Figure 1 shows the structures of these antigens. The difference between the A and B blood types is due to their terminal galactosamine N-acetylation on red blood cells, while type
O antigen lacks the terminal sugar. Therefore, persons of one blood type, such as A, would have natural antibodies against the other type (such as B) by targeting the difference in the terminal sugar structures. However, since O-type antigen does not have the terminal sugar and only has the common sugar structure, no antibody against the common core is produced by individuals of any blood type, which is the reason that O-type is “universal” [129].

Another type of carbohydrate-mediated immune response is the natural immunity in human against animal tissues. For example, pig cell surface has an antigen including the α-Gal-Gal moiety [130], which is absent on human cells. Therefore, humans have natural antibodies against α-Gal-Gal [131]. The acute immune response against α-Gal-Gal is a major hurdle in organ transplant using pig organs. A few years ago, genetically engineered pigs were produced [132, 133], which lack the α-Gal-Gal moiety and thus allow for organ transplant using pig organs to move one step closer to reality. In addition, targeted delivery of the α-Gal-Gal moiety has also been used to elicit immune response at a specific location or cell type [134].

5 Stem cell differentiation and embryo development

Fundamentally, stem cell differentiation and embryo development are the same. The process of transforming pluripotent stem cells into those of specialized functions is marked by different stages, which have characteristic stage-specific biomarkers. Such biomarkers are often carbohydrate-based [135–137] and can be targeted in “binder” development for various applications [138]. For example, Lewis X (Le⁺) has been identified as a stage-specific embryonic antigen, which can be used for identifying and isolating specific cell types from heterogeneous populations [139, 140]. Along this line, it was reported that sorting SVZ cells on the basis of Le⁺ was a good strategy to enrich a restricted but highly proliferative neutral stem cell population [141]. Other examples of carbohydrate changes at various developmental stages include (1) enriched fucose incorporation into macromolecules was found on cell surface at the 8- to 16-cell stage in pre-implantation mouse embryos [142]; (2) sialic acid was found on the 12th day of incubation of metanephros while before that time only N-acetyl-D-glucosamine and alpha-D-mannose were found to exist ubiquitously [143]; (3) in chick embryo development, glycopeptides were found to be mainly N-linked on the 8-day and both O- and N-linked on the 16th day with increased sialylated small glycopeptide contents [144]; and (4) glycosylation of two identical polypeptide chains was found to be organ specific by analysis of chicken serum transferrin and ovotransferrin glycans [145]. There are also many other examples of these biomarkers, such as SSEA-1, -3, and -4 and tumor rejection antigen (TRA)-1-60 and -1-81 [137, 139, 146]. All such results indicate that carbohydrate biomarkers play very important roles in the stem cell differentiation and embryo development. In stem cell research, one critical element is the ability to purify cells of the same differentiation stage and lineage. “Binders” that can recognize stage-specific biomarkers will be very useful for the purpose of separation and identifications. Along this line, aptamers can be very useful since their selection does not require prior knowledge of the nature and structure of the biomarkers in questions. There have been successful examples of selecting aptamers for cell-surface biomarkers using whole cells for the selection [138, 147–151]. For example, liver cancer-specific aptamers were developed by using whole live cells [152]. This study demonstrates that cell-based aptamer selection can specifically recognize cells from multiple cell lines, even for two cell lines with minor differences [152, 153]. In another study, a series of aptamers were selected for leukemia cells. These aptamers have dissociation constants (Kd) in the nano to pico-molar range. The selected aptamers could specifically recognize leukemia cells when mixed with normal human bone marrow aspirates. These aptamers were also used to identify cancer cells closely related to the target cell line in real clinical specimens [154]. Therefore, cell-based selection is a very promising method of developing specific molecular probes for cell-surface biomarker recognition. Similar approaches can be applied to stem cells for the selection of aptamers capable of recognizing stage-specific carbohydrate biomarkers. The recent development of boronic acid-modified DNA-based aptamers [155] allows for the selection of high affinity “binders” for glycoproteins with the ability to differentiate variations in glycosylation patterns and should tremendously enhance the chance of finding high affinity aptamers for such cell-surface carbohydrate biomarkers because of the intrinsic affinity of the boronic acid moiety for carbohydrates [51, 55]. Recently, the Schultz lab has developed a way of engineering boronic acid-modified protein [155, 156]. This will also be very useful in selecting artificial lectins for carbohydrate recognition.

6 Signal transduction

In the signal transduction area, the most prominent example is probably with glycosphingolipids (GSLs), which are involved in a whole host of activities. GSLs have the general structure of two hydrophobic tails (ceramide, consisting of SpH and fatty acid) and one carbohydrate chain, which are oriented perpendicularly. GSLs are held in the membrane by ceramide such that the carbohydrate chain is accessible to various ligands (antibodies, lectins, and complementary carbohydrates) (Figure 2) [157]. GSLs are an integral part of cellular membrane, which functions as antigens, receptors for microbial toxins, and mediators of cell adhesion and
modulators of signal transduction. Many functions were attributed to the unique property of GSLs to form clusters. Glycosynapse refers to glycosphingolipids-enriched microdomain, and glycosynaptic domains, which control GSL-dependent or -modulated cell adhesion, growth, and motility, are formed by the interaction of GSL clusters and functional membrane proteins. Modulation of glycosynapse functions can lead to new strategies in cancer therapy, and elucidation of the molecular mechanism of interaction among components in glycosynaptic domain might shed lights on new possible approaches to disrupt or promote such interactions [119, 121, 122, 157–161].

Protein glycosylation is known to be involved in regulating signal transduction as well. For example, the dynamic glycosylation of serine or threonine residues on nuclear and cytosolic proteins by O-linked β-N-acetylgalactosamine (O-GlcNAc) has been implicated in regulating protein-protein interaction(s) and/or protein function [162]; the Notch signaling pathway could be regulated by alterations of O-fucose structures [68]; O-fucose modification of Cripto is essential for Nodal-dependent signaling [68]; glycosylation of human CRLR at Asn123 is required for ligand binding and signaling [163]; elevated nucleocytoplasmic glycosylation by O-GlcNAc results in insulin resistance associated with defects in Akt activation [164]; alternative O-glycosylation/O-phosphorylation of serine-16 regulates the activities of murine estrogen receptor beta [165]; NFκB activation is associated with its O-GlcNAcylation state under hyperglycemic conditions [166]; glucose deprivation stimulates O-GlcNAc modification of proteins through up-regulation of O-linked N-acetylgalactosaminyltransferase [167, 168]; a mitotic GlcNAcylation/phosphorylation signaling complex alters the posttranslational state of the cytoskeletal protein vimentin [169]; O-glycosylation of Sp1 by insulin seems to enhance its transcriptional activity and results in activation of CaM gene transcription [170]; and glycosylation of guanylyl cyclase C affects its conformation and functional ability [134]. There are many other reports of similar nature.

In 1993, a uniquely glycosylated base, β-D-glucopyranosylxymethyluracil (also called base J), was identified in the nuclear DNA of *Trypanosoma brucei* [171]. This is a rare, if not the only, case where DNA glycosylation is known to play a very important physiological role in signal transduction and gene silencing [24, 25, 27, 172–175]. Specifically, base J is said to be the first hypermodified base found in eukaryotic DNA in the telomeric repeats and to be present in all kinetoplastid flagellates analyzed and some unicellular flagellates closely related to trypanosomatids [176]. In one study, Sabatini and coworkers proposed a model in which chromatin remodeling by J Binding Protein (JBP2) regulates the initial sites of J synthesis within bloodstream form trypanosome DNA, with further propagation and maintenance of J by JBP1. Synthesis of J within telomeric DNA of *Trypanosoma brucei* correlates with the bloodstream-form-specific epigenetic silencing of telomeric variant surface glycoprotein genes involved in antigenic variation [177, 178]. All such results suggest that binding and modulation of glycans of various proteins, lipids, and even DNA may allow for regulation of signal transductions.

### 7 Cardiovascular diseases

In the cardiovascular area, the case of glycosylated hemoglobin (technically it should be glycated since the attachment of a sugar moiety is through chemical reactions) is most widely recognized. Specifically, glycated hemoglobin (GlcHb) level is a strong indicator of cardiovascular disease (CVD) risk in diabetic patients [179–184], who die of CVD at rates 2–4 times higher than those without diabetes [181]. In vivo, glycation, normally defined as a nonenzymatic reaction of glucose with amino groups in protein to form the Amadori product [185] (Scheme 1), is generally considered the first step in the Maillard reaction [186, 187]. Later stages of Maillard reaction lead to the formation of sugar-derived protein adducts and advanced glycation end-products (AGEs), which play an important role in the pathogenesis of chronic diseases [182]. The term GlcHb generally refers to the full spectrum of glycated hemoglobins, including those containing glycated valine (such as HbA1c) and/or lysine residues. In a normoglycemic person, GlcHb accounts for ~24% of total hemoglobin at the end of the erythrocyte lifespan (~120 days), including 4% HbA1c [182, 188]. However, elevated glucose concentration in diabetic patients significantly increases this glycation reaction. For example, some diabetic patients have about 2–4-fold increases in HbA1c [189]. Although certain important questions regarding the prevention of CVD in diabetic patients remain unresolved, epidemiologic analyses suggested that a 1% increase in glycated hemoglobin elevates the risk for CVD by approximately 18% [181].

Another example of glycosylation’s effect on cardiovascular diseases is with fibrinogen, which circulates in the blood as the precursor of fibrin, the structural component of blood clot. It plays a key role in platelet aggregation, the final step of the coagulation cascade, and is a major deter-
minant of plasma viscosity and erythrocyte aggregation [190]. Fibrinogen is a glycoprotein with a molecular weight of 340 kDa [191] and contains approximately 3% carbohydrate consisting of NeuAc, Gal, Man, and GlcNAc [192]. From amino acid sequence studies, it has been determined that carbohydrate is linked to Asn52 on the γ chain and Asn364 on the Bβ chain [193, 194]. The carbohydrate moieties play important roles in fibrinogen functions [195, 196] and are involved in the clotting process, although some contradictory results have been reported [197–199]: deglycosylated fibrinogen could accelerate polymerization and increase lateral aggregation of fibrin fiber [200]; the desialylated oligosaccharide chains of fibrinogen could potentially mediate plasma clearance via the hepatic galactose/galactosamine binding lectin [201]; altered amounts of carbohydrate in fibrinogen are known to be directly related to certain types of dysfibrinogenemia [202]; extra negative charges (sialic acids) on fibrinogen can impair fibrin polymerization [203, 204], presumably due to the repulsive forces of the charges; and steric hindrance presented by additional glycan structures can also impair fibrin polymerization [203]. Fibrinogen-related abnormality is also implicated in other diseases such as hepatoma [202], pancreatic [33, 205] and other cancers [33, 206–213], tumor metastasis [208, 214–216], human hemopoietic cell proliferation [217], and embryogenesis and reproduction [218]. Some of these pathological changes are directly related to abnormal glycosylations [203, 204, 219–228]. In addition, there are many other situations where glycosylation affects the health of the cardiovascular system. For example, certain congenital disorder of glycosylation has been linked to intracranial hemorrhage [229].

The above examples are far from a complete list and are at best some highlights of examples where glycosylation variations play an important role in determining the biological outcome. However, even from this incomplete list, it can be seen that “binders” that can recognize a carbohydrate biomarker with high specificity and affinity would have great application potentials as sensors for concentration analysis, diagnostics, and possibly therapeutic agents. Below is a section describing the general concept of artificial “binder” design. It should be noted that there are several extensive reviews and book chapters on this subject. The following section is far from comprehensive. It can only be treated as some basic conceptual description. Readers are referred to published reviews [45, 51, 54, 55, 230, 231] and book chapters [232, 233] for more details.

8 Carbohydrate recognition

As discussed above, “binders” that can recognize certain carbohydrates with high affinity and specificity should be very useful research tools and potential diagnostic and therapeutic agents. However, achieving high affinity and high specificity carbohydrate recognition is not a trivial issue. In molecular recognition, antibody is often the gold standard. However, in the case of carbohydrates, raising antibodies is often a difficult task. More often than not, low affinity IgM antibodies are obtained. Recently, Boons [234] and colleagues have developed a remarkable synthetic approach to carbohydrate-protein conjugates, which elicits strong immune responses with very high titer. Naturally occurring protein “binders” include lectins [235, 236]. A few hundreds of lectins have been identified, about 60 of which are commercially readily available. Lectins all have certain specificity based on the overall topology and sugar compositions. For example, there are lectins that recognize polymannose structures and others that can recognize galactose connected to different structures. However, essentially all lectins have cross reactivity issues [237, 238].

Recent years have seen a great deal of interest in developing artificial lectins for various applications. These artificial lectins can generally be divided into two types: boronic acid-based and non-boronic acid based. The reason that boronic acid plays such an important role is that it can form tight complexes with diol-containing compounds. Sometimes single hydroxyl group interactions are sufficient in reinforcing highly specific macromolecular interactions. All these aspects have been discussed in detail in a recent re-
view and will not be duplicated here [51]. One thing that needs to be emphasized is the misperception that boronic acids only interact with linear diols and diols on five-membered ring. There are ample literature precedents demonstrating that boronic acid interactions with single hydroxyl groups or other nucleophiles/Lewis bases can also play a very important role in designing/selecting carbohydrate “binders” [51]. Since all boronic acid-containing carbohydrate “binders” function in a similar fashion as lectins, we have termed them as boronolectins [55]. Within the boronolectin category, there are small molecule- and macro-molecule-based boronolectins including boronic acid-based carbohydrate sensors [51, 55, 239], nucleic acid-based boronolectins (NABL) [240], peptide boronolectins (PBL) [241–244], and protein boronolectins (PrBL) [155, 156]. Each of these categories is discussed briefly below.

8.1 Non-boronic acid based lectin mimics

In this approach, the design is mostly based on hydrophobic and hydrogen bond interactions for recognition. Though early efforts were mostly on recognition in organic solvent, recently there has been remarkable progress in making non-covalent carbohydrate binders which showed reasonably high affinity in water, especially those by the Davis lab [245–249]. One example comes from the successful design of such a binder (1, Figure 3) for all-equatorial disaccharides, such as D-cellobiose, with good affinity and selectivity in aqueous solution [248]. Receptor 1 has two building blocks: (1) a-meta-terphenyl structure providing the “roof” and “floor” for hydrophobic interactions with carbohydrates and defining the length of the binding cavity; (2) isophthalamide units serving as pillars, with the potential to form hydrogen bonds, promote solubility, as well as prevent the cavity from collapsing. The binding constants between reporter 1 and selecting carbohydrates were determined to be 5–910 M⁻¹ with good selectivity.

Recently, Boons and Davis reported another analog 2 (Figure 4) using the same design concept, with a biphenyl “roof” and “floor” as well as isophthalamide “pillars” [249]. In a previous study, receptor 2 showed weak bind for the β-glucosyl unit (K_a = 9 M⁻¹ for β-glucose (α/β = 40/60) and 5 M⁻¹ for D-glucose (α/β = 72/28); K_a = 27 M⁻¹ for methyl-β-glucoside and 7 M⁻¹ for α-anomer) [247]. In an expanded study, 2 was recently reported as a strong and selective receptor for β-GlcNAc. The apparent binding constant between GlcNac β-OMe (Figure 4) and receptor 2 was determined to be 630 M⁻¹, which competes well with one lectin (WGA, K_a = 730 M⁻¹). Furthermore, receptor 2 has higher selectivity for GlcNac β-OMe than for the α-anomer and other N-acetylaminosugars.

8.2 Small molecule boronolectins (SBL)

Among all the carbohydrate sensors, boronic acid emerges as the most commonly used functional group for recognition, due to its strong interactions with diols [51–55, 231, 239, 250–263], aminoalcohols [264–266], α-aminoacids [267], α-hydroxyl acids [268–271], alcohols [55, 233, 272–287] as well as cyanide [288, 289] and fluoride [290–294]. The intrinsic ability for boronic acids to interact with nucleophiles is described in Scheme 2. The boron atom has only 6 valence electrons in its trigonal neutral form, which makes boronic acid a Lewis acid and capable of strong interactions with Lewis bases/nucleophiles. As a result, the boronic acid (3, Scheme 2) group is able to react with a protic solvent and convert to its anionic tetrahedral form (4). Both 3 and 4 are able to form tight and reversible complexes with 1,2- and 1,3-substituted Lewis base donors such as hydroxyl, amino, and carboxylate groups. Several factors such as

![Figure 3](structure_of_all-equatorial_disaccharide_receptor_1.png)

Figure 3 Structure of all-equatorial disaccharide receptor 1.

![Figure 4](structures_of_reporter_2_and_glcnaclist.pdf)

Figure 4 Structures of reporter 2 and GlcNAcβ-OMe.
O-C-C-O dihedral angle, $pK_a$ values of the diol, buffer, ionic strength, as well as solvent all affect the complexation [55, 295, 263, 296].

Small molecule boronolectins (SBLs) have drawn a great deal of attentions in carbohydrate biomarker recognition and targeting [51]. Several recent reviews and research papers comprehensively summarize the use of boronic acids in sensor designs for carbohydrates [51, 54, 55, 231, 239] fluoride [290–294], cyanides [288, 289], as well as in-depth discussions of factors [55, 263, 296] that should be considered along the line of designing such sensors. Besides, there have also been quite a few recent reviews [51, 53] and research papers [44, 156, 297–312] on boronic acids that change fluorescent properties upon binding to a nucleophilic analyte or pH changes. Readers are referred to the above-referenced papers for details. Below, several representative examples are discussed to highlight applications.

One such example comes from the Wang lab, which developed an anthracene-based diboronic acid compound for sialyl Lewis X (sLeX). The anthracene-based boronic acid was first developed by the Shinkai group [253], whose fluorescence can be quenched by nitrogen lone pair electrons and recovered if lone pair electrons are masked through protonation after binding with carbohydrates [140]. By taking advantage of this, Wang and co-workers successfully designed and synthesized a series of anthracene-based diboronic acid compounds with different linkers, rigidity, and spatial orientation for recognition of sLex on cell surface [254, 313]. Among all the designed compounds, sensor 7 (Figure 5) stands out as an excellent receptor, which was able to label sLeX-expressing cells at low concentrations (0.5 μM) without cross-reactivity to Ley-expressing cells. This represents the first example of a small organic molecule used to fluorescently label cells based on the cell-surface carbohydrate structures. Further development along this line could lead to a number of small molecule boronolectins for labeling, drug delivery, and selective imaging applications.

In another example, the Hall lab in 2006 reported an ortho-hydroxymethyl phenylboronic acid (8, Scheme 3), which competes well with the well-established dialkylamino (Wulff-type) analogs with better binding affinity and solubility [272, 306]. The most significant finding of compound 8 was the weak but encouraging binding with model glycopyranosides. In aqueous media at physiological pH (7.4), the apparent binding constant between 8 and methyl α-D-glucopyranoside was determined to be 22 M⁻¹, which was slightly lower than that for glucose ($K_a = 36$ M⁻¹). This system has been used by the Hindsgaul lab for the detection of the terminal glycosylation of a glycoprotein with the aid of colored ortho-hydroxymethyl phenylboronic acid conjugates [314].

Another area developed recently in carbohydrate sensor design is nucleic acid-based boronolectins (NABL). The Wang lab is working on incorporating boronic acid-modified thymidine into DNA for aptamer selection work for glycoproteins. A boronic acid-labeled thymidine triphosphate (BTTP 9, Figure 6) was successfully synthesized [46]. It has been demonstrated that DNA polymerase can recognize BTTP as a substrate and the boronic acid-labeled DNA as a template, which are critical issues for aptamer selection work. One challenging task of carbohydrate recognition is the differentiation of glycosylation patterns of a glycoprotein. By taking advantage of the general aptamer selection method developed about 18 years by the labs of Szostak [315], Joyce [316], and Gold [317], as well as the intrinsic affinity of boronic acids for carbohydrates, it is reasonable to believe that incorporation of a boronic acid into the DNA aptamer would allow for the selection process to gravitate toward the glycosylation site and therefore allow for differentiation of glycosylation patterns.

Figure 5  Structures of boronic acids sensor 7.

Figure 6  Binding between ortho-hydroxymethyl phenylboronic acid 8 and glycoconjugates.
Anslyn and co-workers invented a method of using aptamers to fine-tune the selectivity of boronic acid-based synthetic small molecule receptors [240]. Specifically, organic receptor 10 (Figure 7) was immobilized on glyoxal agarose beads through reductive amination to form immobilized receptor 11, which was in complexation with tartrate as the target for the selection. Control compound 12, incapable of binding tartrate, was used for counter selection. The progress of the selection was monitored by incorporating a radiolabel into the RNA pool. By this approach, the author successfully selected an aptamer with good selectivity for the complex between bis-boronic acid receptor 11 and tartrate (>14 for tartrate, \( K_d = 2.1 \times 10^{-4} \text{M} \) and \( K_d < 3 \times 10^{-3} \text{M} \) for citrate in 20% MeOH). One explanation for the selectivity is that aptamer might form a pocket more precisely to accommodate the receptor-tartrate complex, while excluding citrate via steric interactions or charge repulsion. This work can lead to the applications for improving the specificity of synthetic receptors and the development of biosensors for small organic analytes.

### 8.4 PBL

In addition to nucleic acid-based boronolectins (NABL), there have been efforts in making peptide boronolectins (PBL) for the same purpose [241–244]. For examples, the Anslyn lab group developed a chemosensor array of PBL for saccharides, saccharide derivatives, and even sucralose in a real world beverage sample with good water solubility and high sensitivity at physiological pH [241]. The Duggan lab prepared solid-supported PBL derived from 4-borono-L-phenylalanine and studied their affinity for alizarin [242]. The Hall lab developed a general solid-phase approach to the synthesis and isolation of functionalized boronic acids, which should be very useful in combinatorial library synthesis of boronic acid-based carbohydrate sensors [243]. The Lavigne lab reported their PBL sensors for glycomics recognition with the potential for cancer diagnosis [244]. Along a similar line, Hall and coworkers have also established a prototypic bead-supported split-pool library of triamine-derived triboronic acid receptors [318].

### 8.5 PrBL

Recently, the Schultz lab successfully demonstrated the feasibility of adding the boronate functionality to the genetic code of \( E. coli \) in high yield and efficiency [155, 156]. Specifically, \( p \)-boronophenylalanine (13, Figure 8) was incorporated into proteins. The intrinsic affinity of the boronic acid group allows for the selection of high affinity PrBL. In addition, this method has the potential to be used for purification of native protein sequences in a one-step scarless affinity procedure.

### 8.6 Carbohydrate labeling in living systems

Recently, the Bertozzi lab has developed labeling approaches to probing the functions of glycans in living system and application of these tools to studies of glycobiology such as the identification of novel glycan-based tumor biomarkers [319–334].

The general principle relies on the availability of a toolkit of “azido sugars” for metabolically labeling different classes of glycans. These azido sugars can be recognized by their respective processing/incorporation enzymes and can be used for tagging via two bio-orthogonal reactions: Staudinger ligation (Figure 9) and [2+3] cycloaddition (click reaction) (Figure 10) [335–337]. Along this line, a
In using the Huisgen [3+2] dipolar cycloaddition with alkynes, the Bertozzi lab developed strained alkynes (14 and 15, Figure 10) for copper-free cycloaddition [320, 322, 324, 326, 328]. The Boons lab subsequently also reported their own strained alkynes for copper-free cycloaddition with azido compounds (16, Figure 10) [338]. The availability of such alkynes for copper free cycloaddition is especially important in live cell imaging because of the toxicity issues of copper in living systems. With this technique, the dynamics of glycan trafficking and a population of sialoglycoconjugates with unexpectedly rapid internalization kinetics were studied. In another example, 14 was used for the noninvasive imaging of glycans in live developing zebrafish [327]. In this experiment, zebrafish embryos were first treated with an unnatural sugar to metabolically label their cell-surface glycans with azides and then visualized by using an in vivo Cu-free click reaction with fluorophore-conjugated 14. The Bertozzi group also performed a spatiotemporal analysis of glycan expression and trafficking and identified patterns by using a multicolor detection strategy [324].

In conclusion, carbohydrates serve very important biological functions in a wide variety of processes. “Binders” that can specifically recognize a carbohydrate biomarker can be used for site specific delivery of therapeutic and imaging agents. Specific recognition of carbohydrates that mediate pathological processes has the potential to be used as a way to develop novel types of therapeutic agents. Recent years have seen a tremendous amount of work in developing carbohydrate “binders,” with boronolectins showing special promises. It is almost a certainty that new diagnostic and therapeutic agents will come out in the not too distant future that rely on carbohydrate recognition.

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