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# Biomimetic Macromolecules for Macrophage Targeting and Modulation

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# BIOMIMETIC MACROMOLECULES FOR MACROPHAGE TARGETING AND MODULATION

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**BIOMIMETIC MACROMOLECULES FOR MACROPHAGE TARGETING AND MODULATION**

**JOSHUA WHITED**

ABSTRACT

Carbohydrate recognition has come to the forefront of biological aiming to uncover the mechanisms of physiological and pathological processes. Cell surface glycans are involved in processes including cellular adhesion, cell signaling, and immune response. A new approach for profiling cell surface glycans has great potential for a wide range of biomedical applications. Lectins have been conventionally used to determine the structure and function of glycoproteins, however, their numbers are still restricted compared to the number of glycan structures. Boronic acid has proven a remarkable small molecule capable of binding diols in aqueous solution. This interaction indicates boronic acid derived molecules may serve as lectin mimetics for profiling and targeting cell surface glycans. In the first part of this dissertation study the specific binding site of boronic acid to individual pyranosides was confirmed followed by the synthesis and evaluation of protein-boronic acid conjugates as lectin mimetics.

3-aminophenylboronic acid was conjugated to gluco-, manno- and galactopyranosides, followed by methylation, both under basic conditions. Based on a specific permethylation product for the carbohydrate, boronic acid specificity towards 1,2 and 1,3 diol configurations was confirmed by $^{1}H$, $^{13}C$ NMR, and mass spectrometry. As a result, unique binding profiles were observed for each pyranoside. Next, bovine serum albumin (BSA)-

PBA conjugates were synthesized in a density controlled affording multivalent lectin mimetics. The resultant BSA-PBA conjugates were characterized by SDS-PAGE and MALDI-TOF MS. Cell surface glycan binding capacity was confirmed by a competitive lectin assay examined by flow cytometry.

Macrophages (Mϕ) express lectins as receptors for specific immune responses. Synthetic glycans are candidates for targeting cell surface lectins and for immunomodulation applications. In the second part of this dissertation, novel *N*-glycan polymers were synthesized and their immunomodulation effects were examined. *N*-linked glycopolymers were synthesized *via* cyanoxyl-mediated free radical polymerization (CMFRP). Then, their cytotoxicity and cell activation abilities against RAW 264.7 cells were examined. As a result, *N*-glycan polymers showed no cytotoxicity at a concentration of 1,250 mg/mL except the *N*-α2,6-sialolactosyl polymer, which proved cytotoxic at 1250 μg/mL. *N*-α2,3-sialolactosyl polymer showed the strongest activity for inducing cell surface marker expression compared to controls, indicating high Mϕ modulation activity.

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# ABBREVIATIONS

| | |
|---|---|
| 3-PBA: | 3-aminophenylboronic acid |
| 3D: | Three dimensional |
| ARS: | Alizarin Red S |
| Au-NP: | Gold nanoparticles |
| BA: | Boronic acid |
| BCL: | Boronic acid containing lectin mimetics |
| BCP: | Boronate containing polymer |
| BSA-PBA: | Bovine serum albumin-boronic acid |
| BSA: | Bovine serum albumin |
| CD: | Cluster of differentiation |
| CLR: | C-type lectin receptors |
| CMFRP: | Cyanoxyl-mediated free radical polymerization |
| COSY: | Correlation spectroscopy |
| CRD: | Carbohydrate recognition domain |
| DMEM: | Dulbecco's Modified Eagle Medium |
| E-: | Endothelial |
| EDC: | *N*-(3-(dimethylamino)propyl)-*N*-ethylcarbodiimide hydrochloride |
| FBS: | Fetal Bovine Serum |
| FITC: | Fluorescein isothiocyanate |
| FMG: | Fucoidan mimetic glycopolymer |
| Fuc: | Fucose |
| Gal: | Galactose |

| | |
|---|---|
| GalNAc: | *N*-acetylgalactosamine |
| Glc: | Glucose |
| GlcA: | Glucoronic acid |
| GlcNAc: | *N*-acetylglucosamine |
| GMs: | Glycomimetics |
| HMBC: | Heteronuclear multiple bond correlation |
| HPLC: | High pressure liquid chromatography |
| HSQC: | Heteronulcear single quantum coherence |
| L-: | Leukocyte |
| Lac: | Lactose |
| MAA: | Maackia amurensis |
| MAL: | Maackia amurensis leukoagglutinin |
| Man: | Mannose |
| MES: | 2-(*N*-morpholino)ethane sulfonic acid buffer |
| MHC: | Major histocompatibility complex |
| mRNA: | Messenger RNA |
| MS: | Mass spectrometry |
| MTT: | 3-(4, 5-di-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide |
| Mϕ: | Macrophage |
| Neu5Ac: | *N*-acetylneuraminic acid |
| Neu5Gc: | *N*-glycolylneuraminic acid |
| NMR: | Nuclear magnetic resonance |
| NOESY: | Nuclear overhauser effect spectroscopy |

| | |
|---|---|
| P-: | Platelet |
| PAMAM: | Poly(amidoamine) |
| PAMP: | Pathogen associated molecular patterns |
| PBS: | Phosphate-buffered saline |
| PTMs: | Post translational modifications |
| RNA: | Ribonucleic acid |
| SA: | Sialic acid |
| SELEX: | Systematic evolution of ligands by exponential enrichment |
| Ser: | Serine |
| Siglecs: | Sialic acid binding Ig-like lectins |
| SNA: | Sambucus nigra |
| TAFIa: | Thrombin activatable fibronolysis inhibitor |
| TBS: | Tris-buffered saline |
| TFA: | Trifluoroacetic acid |
| Thr: | Threonine |
| Xyl: | Xylose |

# CHAPTER I

INTRODUCTION

## 1.1 Glycans, glycosylation and glycomics

Following the completion of the Human Genome Project in 2003, research entered a new post genomics era. Down from first predictions of over 100,000 protein encoding genes to now 20,000-25,000 it has become clear that the number of proteins in a cell is significantly higher than predicated solely by gene code translation to messenger ribonucleic acid (mRNA).[1-3] The diversity of expressed proteins is controlled by varying types of mRNA produced from individual genes. These proteins perform a vast array of functions, many of which are facilitated through co- and post-translational modifications (PTM) before acquiring full or sometimes multiple functionalities.[4-6] Glycosylation, perhaps the most complicated and varied type of PTM, is the attachment of oligosaccharide moieties to the protein.[7-9] The complex character and varying attachments of each glycoform are substantial enough to dictate protein function and property.[10-13] Heterogeneity of glycans arises from the non-template driven nature of glycan biosynthesis, lack of proof reading machinery, and a large number of tissue specific

transferases and enzymatic glycosidases. Collectively, these justify the exponential number of patterns seen because of differing composition and branching. The affect is ambiguous, adding to both cellular individuality and function, while at the same time making glycomics a challenging area because of its complexity in comparison to both protein and nucleic acids.[14-16] Thus there is an urgent to need to detect, identify, and characterize glycans in a rapid manner.

### 1.1.1 Glycan structure

There are 10 monosaccharides used by mammals in enzymatic glycosylation processes. These are glucose (Glc), galactose (Gal), mannose (Man), sialic acid (SA), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), fucose (Fuc), xylose (Xyl), glucuronic acid (GlcA), and iduronic acid (Figure 1).[17] The Golgi complex in eukaryotic cells is of particular importance, in processing both glycoproteins and glycolipids, because it contains a set of glycosylation enzymes responsible for elongation and branching of saccharide chains.[18,19] Different types of glycoproteins contain a shared pattern of glycosylation that can be divided into three major categories. *O*-linked glycans are typically linked to either Serine (Ser) or Threonine (Thr) residues, *N*-linked glycans linked to Asparagine residues and finally glycosaminoglycans that are also Ser or Thr anchored but remain linear instead of branched.[20,21] Lipid glycosylation involves gangliosides which are composed of a glycosphingolipid with one or more SAs attached.[22,23] Equally important are cytoplasmic glycosylations, which happen outside of the Golgi secretory pathway, and they have been found to play important roles in PTM and regulation such as in cell viability and nutrient sensors.[24-26]

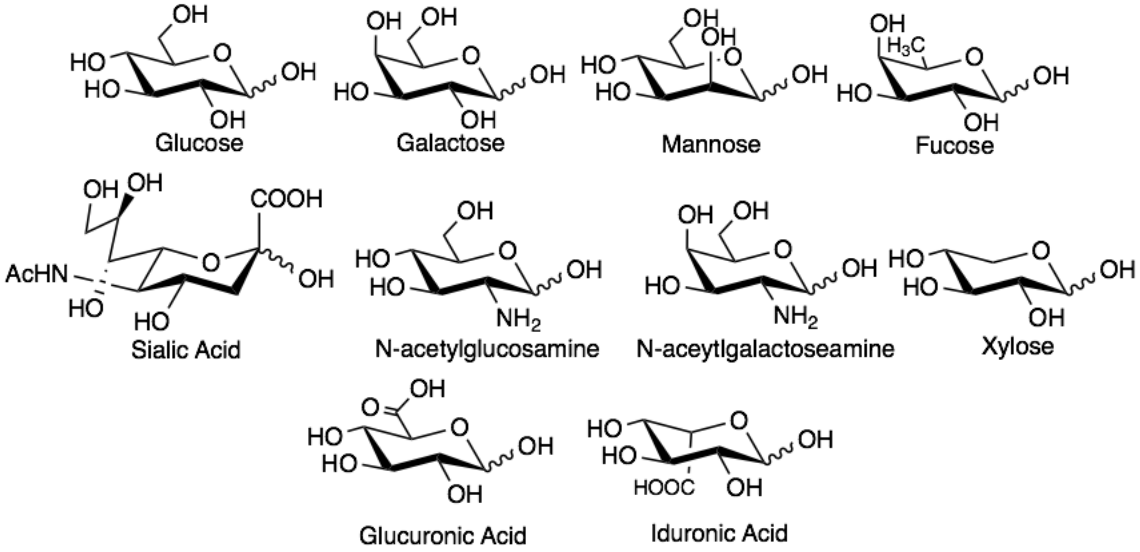



Figure 1: Monosaccharides used in glycosylation.

### 1.1.2 Glycosylation function/importance

Glycosylation products, comprised of glycoproteins, glycolipids and proteoglycans, play key roles in a wide variety of biological processes including cellular adhesion, cell signaling, cell-cell communication, and the immune response.[27-29] For example, changes in glycosylation on Asn in Activated Thrombin Activatable Fibrinolysis Inhibitor (TAFIa) has been shown to be a main contributor to the biochemical activity of TAFIa.[30] In addition, immune system responses can be directed by various specific site recognition mechanisms such as the recognition of key linkages on sialyl Lewis X ($sLe^x$).[31] In a similar manner, the body is able to recognize self from non-self and initiate appropriate immune responses through the discrimination of glycans expressed by pathogens compared to those of host cells.[32,33] For example, glycans and glycoconjugates comprise part of a vast array of pathogen associated molecular patterns (PAMP), which once recognized and bound can initiate phagocytosis by Mϕs to rid the body of invading organisms.[34-36] Furthermore, glycosylation plays a significant part in cellular receptor activation and signal transduction pathways. As evidence of this, research has shown regulation of fucose specific transferases during embryonic neuronal development can be altered with development of *O*-fucose derivatives.[37] The aforementioned examples only highlight some of the different areas of importance as far as glycosylation is concerned emphasizing the need for glycan sensing and study.

Given the importance of glycosylation in normal physiological functions, it is not hard to understand the dysfunction and disease that could result from abnormalities in certain glycosylation patterns. For example, the congenital disorders of glycosylation in children, refer to a large number of syndromes including severe morphogenic and metabolic defects

associated with general failure to thrive, most of which have been linked to distinct steps in glycan formation.[38-40] Defects in protein catabolism are also caused by glycosylation abnormalities. More specifically, in the case of I cell disease, failure to produce the mannose 6-phosphate modification on *N*-glycans in the Golgi results in a loss of signal transduction necessary for trafficking of hydrolases to the lysosome leading to a storage disorder.[41,42]

Glycans have additionally been recognized as biomarkers for certain types of pathological conditions such as cancer.[43-46] Increased expression and/or modifications to typical linkages can occur such as *O*-acetylation can either disappear, as in colon cancer, or become more prominent as in the case of melanomas and basal cell carcinomas.[47-49] Overexpression of particular glycans may also enhance the masking effect of abnormalities inhibiting the immune response. Even with well documented glycosylation related diseases, the discovery process is more serendipitous than well planned.[15] Common methods for protein separation are not able to catch small variations in glycosylation, making the development of such tools vital to a better understanding glycosylation and its implications.

### 1.1.3 Traditional tools and methods for glycosylation studies

Traditional analytical approaches for determination of unique primary glycan structures include but are not limited to tandem high pressure liquid chromatography (HPLC), mass spectrometry (MS),[50-52] and nuclear magnetic resonance (NMR).[53,54] Even with the power and proven versatility of these traditional methods they still suffer from their individual drawbacks. These include time consuming optimization, experience needed to perform experiments, and large sample amounts in the case of NMR. In addition

to the methods/instruments themselves, the ability to remove saccharides from bioconjugates and purify them for characterization is a difficult science. Because of glycan heterogeneity and the time consuming nature of sample preparation, systematic analysis of a large number of complex glycans demands a more efficient approach.

Lectin microarrays have also see widespread use in glycosylation research over the past few years.[55,56] Essentially, lectins are immobilized onto an array surface and analytes with fluorescent tags are applied to the microarray. After washing any unbound analytes from the array the fluorescent signals are analyzed and a specific glycosylation pattern is obtained. Using this method, information such as glycosidic linkages and monosaccharide content can be ascertained. Even with lectin microarrays widespread use, lectins themselves suffer from one fundamental drawback and that is the number of available lectins. There are hundreds of thousands of glycan and glycoconjugate combinations but only about 40 easily available commercial lectins. Another drawback to using lectins is the cost of using biological generated lectins greatly outweigh the cost of chemically synthesized lectins.

### 1.2 Sialic acid

The surfaces of all vertebrate cells in nature are comprised of a variety of glycoconjugates including glycoproteins, proteoglycans and glycolipids forming the cellular glycocalyx.[57,58] SA moieties are typically located at the terminal position of these glycoconjugates are known as “decorations” providing extreme structural and functional diversity (Figure 2).[59] The two major SA core structures are 2-keto-3-deoxynononic-acid and neuraminic acid sharing nine carbons and differing at the C5-position ( Figure 3). The variety of substituents on carbon 4, 5, 7, 8 and 9 generate a diverse family of more

than 50 structurally distinct molecules, which can be attached to underlying sugars, commonly via α-2,3 or α-2,6 linkage to Gal, via α-2,6 linkage to GalNAc or GlcNAc, or via α-2,8 linkage to another SA.[60-62] *N*-acetylneuraminic acid (Neu5Ac) is the most frequent form followed by *N*-glycolylneuraminic acid (Neu5Gc) and *O*-acetylated derivatives. Among mammals, humans are a known exception in their lack of Neu5Gc, due to an inactivation mutation in a hydroxylase, which modifies CMP-Neu5Ac to CMP-Neu5Gc.[63,64]

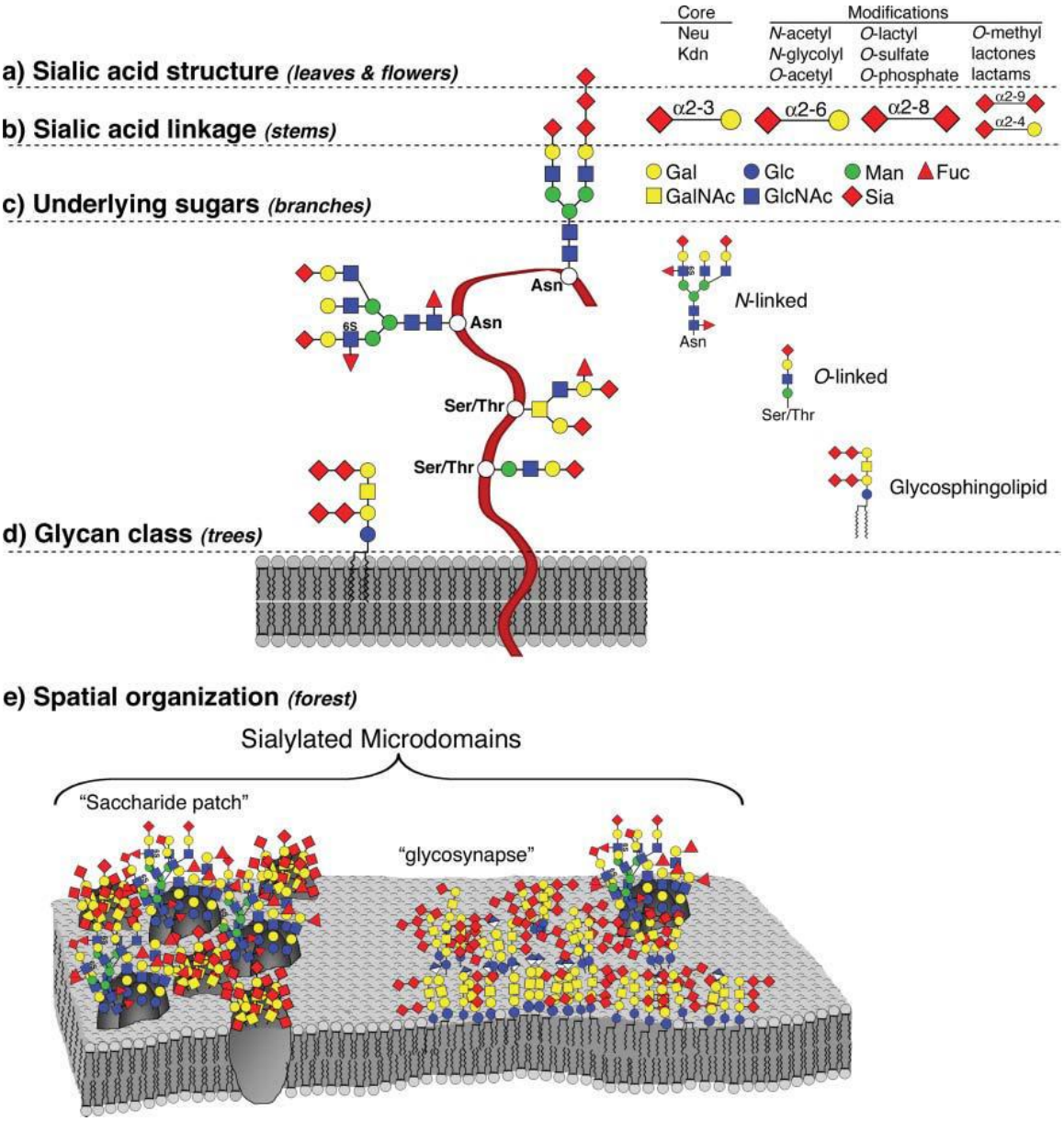
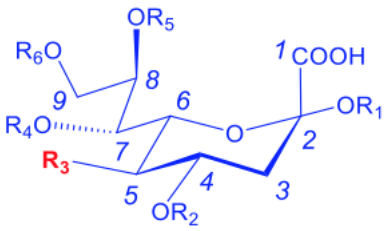



Figure 2: Complexity of SA layer on the cell surface. Adapted from reference 61




$R_1$ = OH in free SA; alpha linkage to Gal (3/6), GalNAc (3/6), or SA (8/9)
$R_2$ = H, or acetyl
$R_3$ = *N*-accetyl (Neu5Ac), *N*-glycolyl (Neu5Gc), hydroxyl (KND), or amino (Neu)
$R_4$ = H, or acetyl
$R_5$ = H, acetyl, methyl, sulfate, or SA
$R_6$ = H, acetyl, lactyl, phosphate, sulfate, or SA

Figure 3: Diversity of SA structures and linkages.

Given SAs terminal location and remarkable diversity in structure, glycosidic linkages, and underlying glycan chains, it is not surprising that SAs act as intermediaries in a wide variety of physiological and pathological processes. These processes include immunomodulatory effects, hormonal responses, signal transduction, tumor progression, cell adhesion and protection.[59, 65-67] SAs negative charge and hydrophilicity allow it to act as an anti-recognition agent, masking recognition sites such as Gal residues, antigenic proteins and receptor molecules.[68,69] SAs duality allows it to act simultaneously as a recognition site for a particular subset of proteins called lectins. Lectins are glycan recognition proteins that lack any enzymatic activity on their substrate. Angata et al. proposed to group lectins into three categories: pathogenic lectins, endogenous lectins, and lectins from other sources such as plants and protostomes.[62]

### 1.3 Lectins

As previously mentioned, proteins capable of interaction with carbohydrates are termed lectins. These types of non-covalent interactions occur widely throughout nature and include carbohydrate specific enzymes and antibodies formed as part of an immune response to carbohydrate antigens. The term lectin originates from Latin meaning "to select" or "choose" owing to their high specificity.[70-72] Lectins are defined as a class of carbohydrate binding proteins that can recognize and bind carbohydrates reversibly but without any catalytic activity.[73] Lectins exist in most organisms ranging from viruses and bacteria to plants and animals. Concanavalin A, the first purified lectin, was isolated by Sumner in 1919, who also demonstrated its carbohydrate specificity.[74] It was later determined that lectins could play a crucial role in blood group identification.[75] Currently, lectins are widely used to investigate cell-cell recognition events as well as the study of

carbohydrates on the cell surface.

Each lectin molecule is typically multivalent having two or more carbohydrate binding sites. Because of this feature, interactions involving cell surface glycans, or glycoconjugates, can result in the cross linking of cells resulting in precipitation. Indeed, this phenomenon is referred to as agglutination and is routinely used for the detection and characterization of cell surface glycoconjugates. As a method of control, these agglutination processes can be inhibited by the addition of those saccharides that each lectin shows affinity towards. Lectin affinity for monosaccharides is generally considered weak ($K_d$ = mM range), however highly selective, meaning, lectins specific for Man do not react with Glc, nor do those specific for Gal bind Glc. Cooperative multivalent interactions of lectins towards oligosaccharides changes their dissociation constant to the $K_d$ = μM range, while lectin-monosaccharide interactions remain unchanged.[76,77] However, the selectivity of lectins for monosaccharides is not always so high.

Lectin-glycan binding affinity is quite structure dependent and saccharides with different chemical compositions but similar topographic properties can bind to the same lectin.[78] In contrast, different lectins for the same saccharide could focus on separate regions of the saccharide structure. However, once bound by lectins, specific conformation of the glycan is locked and fixed and the rotational freedom around glycosidic linkage is reduced. Thus, lectins bind saccharides in a reversible and specific way, but they all have cross-reactivity issues to some extent.[78,79] Subsequently, some lectins are glycosylated and endogenous lectins could interact with the glycans on the surface of the lectin probe.[80] In conclusion, lectins high specificity and participation in physiological processes such as cell adhesion, cell trafficking and signal transduction make them among the most widely used

tools in glycan research despite their drawbacks.

### 1.3.1 Animal lectins

So far, three major classes of natural glycan binding proteins (GBPs) have been identified in mammalian systems and consist of 4 subgroups: 1) the S-type lectins; 2) the C-type lectins; 3) the P-type lectins; and 4) I-type lectins.[78] The S-type lectins, called galectins, are found inside the cytoplasm, the nucleus, on the cell surface, and outside the cell. The galectins are defined by their selective affinity towards Gal containing ligands on the cell surface.[81-83] More than ten mammalian galectins have been identified as well as other from such sources as birds, amphibians, and fish.[84] Galectins mediate such physiological processes such as cellular communication, inflammation, development, and differentiation.[85-87]

The C-type lectins are so named because of their dependence on $Ca^{2+}$ for proper function. C-type lectins can be divided further into three subclasses: endocytic lectins, collectins and selectins.[88] Endocytic lectins are transmembrane proteins with an extracellular carbohydrate recognition domain (CRD) specific for the Gal/GalNAc moiety.[89,90] In contrast, collectins are soluble proteins with a cysteine-rich amino terminal and a CRD carboxyl terminal.[91,92]

P-type lectins are distinguished from all other lectins by their ability to recognize phosphorylated Man residues. P-type lectins play an essential role in intracellular trafficking and the generation of functional lysosomes by directing newly synthesized lysosomal enzymes bearing the mannose 6-phosphate signal to lysosomes.[60, 93]

The I-type lectins, also called selectins or siglecs, are comprised of three cell surface glycoproteins called Endothelial (E-), Leukocyte (L-) and Platelet(P-) selectins which

participate in many cell-cell interactions in immunity, hemostasis, and inflammation. E-selectins recognize SAs on leukocytes and mediate the adhesion of leukocytes to activated endothelial cells.[94] P-selectins also mediate adhesion to SA containing ligands on leukocytes, migrating quickly in response to various stimuli.[95] Lastly, L-selectins can recognize sialylated ligands within the endothelium and facilitate migration of cells into the lymphatic system.[96]

### 1.3.2 Plant lectins

Plant lectins are like animal lectins and exhibit high specificity and some can distinguish isomeric glycans within identical glycan compositions. For example, *Sambucus nigra agglutinin* (SNA) binds to SA linked to either Gal or GalNAc via α-2,6 linkage and *Maackia amurensis* (MAA) recognizes α-2,3 linked SA.[97] Most plant lectins belong to the legume family and their specificity is owed to highly conserved binding sites consisting of four invariant amino acids: aspartic acid, asparagine, a glycine, and an aromatic amino acid or leucine. The binding site architecture is typically derived from four loops designated A, B, C, and D.[98] Loop A and B consists of the aspartic acid and glycine residues which are invariable and asparagine and the hydrophobic area make up loop C. Loop D, however, is highly variable having differences in length, sequence, and conformation meaning there are additional interactions and specifications for monosaccharides. What can be noted, is that loop D often exhibits identical properties among similar lectins. For example, loop D is identical in size for all Man specific lectins.[99]

### 1.3.3 Sialic acid specific lectins and their applications

Cell surface SAs, sialoglycans, and their modifications can be detected by the use of lectins in what can be referred to as bio-affinity recognition; the exploitation of biological

sample affinities. As previously mentioned, SA-specific lectins are particularly helpful because of their ability to discriminate between complex sialylated glycans and glycoconjugates on the cell surface. So far, fluorescently labelled lectins have been widely used to analyze the sialoglycoconjugates in histochemistry, lectin-microarrays, flow cytometry, and microscopic analysis (Table 1).

Lectin-histochemical staining offers detailed information about the occurrence and distribution of SA residues in tissues and in different types of biological samples.[100] Recently, lectin-histochemical staining has been used to study cancer, in which aberrant expression of sialoglycoconjugates is thought to play an important role in cancer progression.[101,102] SNA and Maackia amurensis leukoagglutinin (MAL), with their ability to discern between α2,6-linked and α2,3-linked SA residues respectively, have been effectively used for biochemical and histochemical analyses of sialoglycoconjugates.[103] Studies were first conducted comparing the expression of α2,6-linked SAs by SNA-digoxigenin staining of histological sections[104] followed by the use of biotinylated MAL in the investigation of the pathological significance of sialylation in colorectal cancer and gastric cancer.[105,106] In these studies, the sialoglycoproteins in gastric cancer tissues were analyzed using MAL in combination with 2-dimensional electrophoresis. Various MAL-positive sialoglycoproteins were detected in cancer tissues in comparison to healthy individuals suggesting the MAL-positive sialoglycoproteins detected in gastric cancer tissues have high molecular weights and may contain different numbers of α2,3-linked SA residues in the glycan moiety.[106] Recently, the clinical pathological feature of several types of sialylated glycoconjugates in colorectal cancer have been compared in regards to their expression levels using biotinylated MAL, Sambucus sieboldiana, and MAA.[107] It was

found that α2,3-sialylated type 2 chain (NeuAcα2,3Galβ1,4GlcNAcβR) structures were predominantly expressed in colorectal tissues associated with malignant transformation, in particular, with lymphatic spread of distal colorectal adenocarcinomas.

Table 1: SA-specific binding lectins.

| ***Lectin (Origin)*** | ***Specificity*** | ***References*** |
|---|---|---|
| ACG (*Agrocybecylindracea*) | Neu5Ac(α2,3)Gal, β-Gal | [108] |
| MAH (*Maackia amurensis hemagglutinin*) | Neu5Ac(α2,3)Gal | [109-111] |
| MAL (Maackia *amurensis leukoagglutinin*) | Neu5Ac(α2,3)Gal | [111-115] |
| MPA (*Macrophomina phaseolina agglutinin*) | Neu5Ac(α2,3)Gal | [116] |
| PSA (*Polyporussquamosus*) | Neu5Ac(α2,6)Gal | [117-118] |
| PVL (*Psathyrellavelutina*) | Neu5Ac(α2,3)Gal, GalNAc | [119] |
| SCA (*Sambucus canadensis*) | Neu5Ac(α2,6)Gal/GalNAc | [120] |
| SNA (*Sambucus nigra*) | Neu5Ac(α2,6)Gal/GalNAc | [121,122] |
| SSA (*Sambucus sieboldiana*) | Neu5Ac(α2,6)Gal/GalNAc | [123] |
| Saracin (*Saracaindica*) | Neu5Ac(α2,6/3)Galα1-4GlcNAc | [124] |
| TJAL (*Trichosanthes japonica*) | Neu5Ac(α2,6)Gal/GalNAc, HSO3(-)-6Galα1-4GlicNAc | [125] |
| WGA (*Wheat germ agglutinin*) | Neu5Ac, GlcNAc(β1,4)GlcNAc | [126,127] |
| *ML-1 (Viscum album)* | Neu5Ac(α2,6)Galα1-4GlcNAc | [128] |

Flow cytometry is a powerful tool for the analysis of individual cells within heterogeneous populations. In 2002, Lin et al. examined the effect of α2,6-sialylation on the adhesion properties of breast carcinoma cells with different levels of an expressed sialyltransferase ST6Gal-I.[129] It was discovered by using lectins to quantify cell surface sialylation that cellular adhesion of tumor cells was regulated by α2,6-sialylation expression levels. In another study, the expression levels of α2,3-linked SA residues were measured in human gastric adenocarcinoma cell lines by using fluorescein isothiocyanate (FITC)-labeled MAL indicating a high level α2,3-linked SAs is associated with the metastatic potential of human gastric cancer.[130]

Both flow cytometry and confocal microscopy have also shown cell surface α2,3-linked SAs were predominant under normal culture conditions and changed slightly upon activation with atorvastatin, while α2,6-linked SA abundance was minimal under normal conditions, increasing significantly increased upon activation with atorvastatin (Figure 4).[131] These results provide a closer look at the role SAs play in biological functions of the cells upon activation.

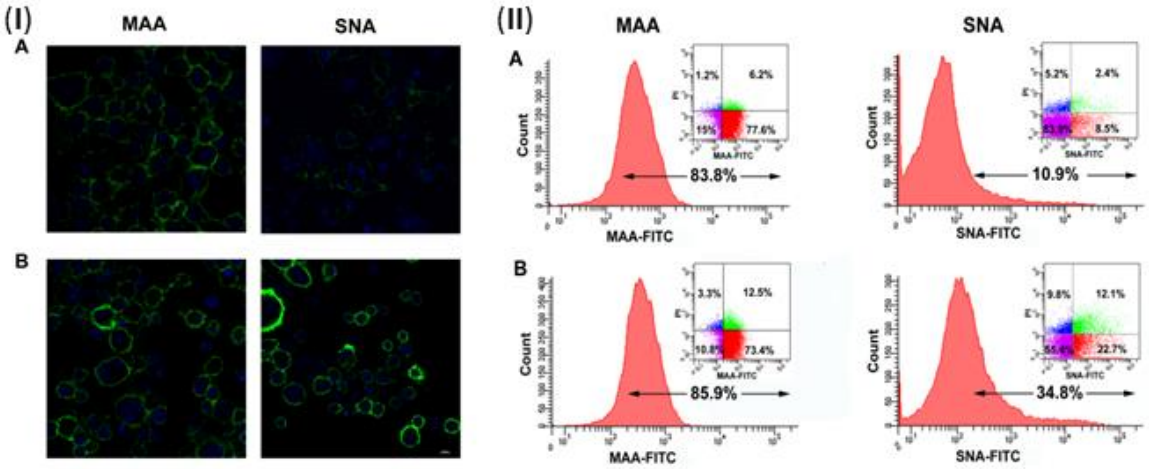



Figure 4: Confocal microscopy analysis of cell surface SAs. **(I)** (**A**) Raw 264.7 cells at the normal culture condition stained with MAA-FITC (10 μg/mL) and SNA-FITC (20 μg/mL), respectively. DAPI was used to stain nuclei. (**B**) Raw 264.7 cells treated with 20 μM atorvastatin for 24 h followed by staining with lectins and DAPI. The scale bar represents 10 μm. (**II**). Determination of cell surface SAs by flow cytometry: (**A**) Raw 264.7 cells at normal condition stained with MAA-FITC (10 μg/mL) and SNA-FITC (20 μg/mL), respectively. PI staining was used to distinguish living cells and dead cells. (**B**) Raw 264.7 cells were treated with 20 μM atorvastatin for 24 h then stained with lectins and PI. Adopted from reference 131 with permission from Oxford University Press.

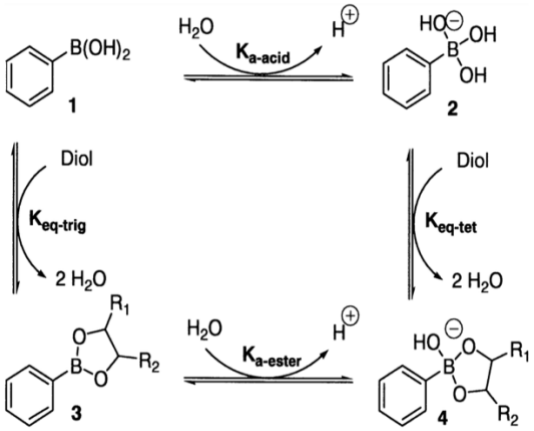
## 1.4 Boronic acid

As outlined above, glycomics requires ongoing investigation into novel tools to support and complement current biological approaches. In an ongoing effort to use biology driven methods, there has been a concerted effort to develop artificial sensors for carbohydrates. These synthetic and artificial sensors, termed lectin mimetics, will undoubtedly find their place in glycosylation research as well as clinical diagnostic and therapeutic applications. In general, there exists two methods when it comes to artificial sensor design strategy: boronic acid based and non-boronic acid based. The nature of biological research and clinical diagnostics makes water a primary requisite for carbohydrate sensing methods. This effectively eliminates non-boronic acid based mimetics, as most only function under an aprotic solvent relying primarily on hydrogen bonds along with hydrophobic and ionic interactions.[132,133] In contrast, boronic acid-based lectin mimetics work well in aqueous media and rely on strong reversible interactions between boronic acids and accessible diol moieties.[134]

### 1.4.1 Boronic acid chemistry

Boronic acid is widely acknowledged by chemists for its ability to react with 1,2 and 1,3 diols in aqueous media through reversible boronate ester formation (Scheme 1).[135-137] To discuss boronic acid-carbohydrate complexation there are several fundamental aspects that must be understood. Boronic acid, using phenylboronic acid as an example (Scheme 1, **1**), acts as a Lewis acid accepting a lone pair of electrons from a protic solvent such as water (Scheme 1, **2**). These electrons cause the hybridization state to change from $sp^2$ to $sp^3$. The second step is boronic acid's reaction with a diol to form a boronate ester (Scheme 1, **3**). At this point, the boronate ester is still considered acidic and can react with a protic

solvent molecule, releasing a proton, and forming the negatively charged tetrahedral boronate complex (Scheme 1, **4**). Lastly, it must be understood that because of the inherent acidity of both the boronic acid and its corresponding ester, binding can be affected by pH and individual pKa values. Most of the time ideal binding involves using a pH that falls in between the pKa of the boronic acid and that of the diol.[138] There are also additional factors that may play a role in the unique binding profiles of boronic acids including solvent system, buffer composition, temperature, and steric hindrance.[139]




Scheme 1: Binding of phenylboronic acid with a diol.

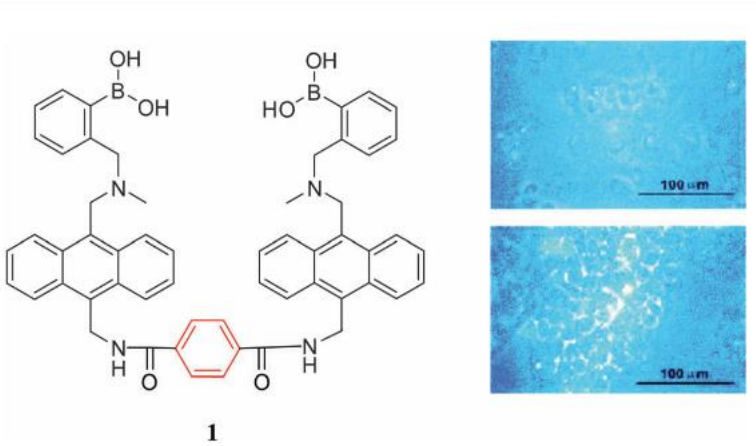
In conclusion, the basic chemistry of boronic acid and some fundamental features of the boronic acid-diol binding process have been discussed. Hopefully, this provides a good foundation for the rational design and synthesis of boronic acid-based carbohydrate sensors.

### 1.4.2 Molecular boronic acid as a carbohydrate sensor

`Due to the recent boom in glycobiology and glycomics, carbohydrate research has been recognized an area of enormous untapped biological potential. The diversity and complexity of glycan structures, together with their crucial role in many physiological or pathological processes, requires the development of new tools that allow for high affinity and specificity recognition events. Since their recognition over a century ago, boronic acid has been exploited extensively as chemo/biosensors in the detection of carbohydrates.[136, 139-141] Now, the boronic acid-diol interaction may be one of the most widely used single pair functional groups in the design of sensors and binders.[79, 142] Boronic acid sensors are typically, an organic molecule conjugated to a fluorophore used to analyze a signal generated after interaction with the analyte of interest. Although glycans and glycoconjugates differ on a macroscopic and microscopic scale, the main difference is the spatial arrangement and orientation of hydroxyl groups. Because of the various configurations, it can be difficult to detect analytes with good selectivity using a unimolecular model. For this reason, more than one boronic acid group has been introduced into molecules to afford multivalency, mimicking molecular recognition in biological systems.[143,144] Multivalent interactions require the existence of more than one binding site on both the receptor and analyte. This provides a synergism between multiple weak interactions resulting in a higher affinity. For example, in 2002, Wang et al., designed and

synthesized a series of fluorescent bisboronic acid sensors by changing the linker between the bisboronic moieties for the detection of $sLe^x$. Among the synthesized compounds was on that showed a strong affinity for $sLe^x$ in comparison to the control which was not labeled in the experiment.[145]

In conclusion, molecular boronic acid derivatives have seen widespread use and proven their versatility in glycomics research. One of the most difficult challenges is the design of a proper scaffold to orient the boronic acid moiety in the proper manner to afford the desired specificity and selectivity. Unimolecular boronic acid-diol interactions are too weak by themselves and multivalent molecules show much higher promise regarding higher affinity and selectivity. As a complement to the time-consuming method of de novo design and template directed synthesis, combinatorial libraries may help to overcome the limited availability of carbohydrate sensors and lectin mimetics. In an extension of this research, other groups have begun to attach boronic acid to larger molecules, offering an increase in number of available binding sites, altered orientation and spatial arrangement and have coined the term "boronolectin", referring to all boronic acid derived carbohydrate sensors.[79, 139, 146]




**1**

Figure 5: Structure of compound 1 and fluorescent labeling study. $sLe^x$ expressing HepG2 cells (bottom) and non-expressing $sLe^x$ COS-7 cells (top). Adopted from reference 134 with permission from The Royal Society of Chemistry.

## 1.5 Boronolectins

One of the biggest challenges in designing carbohydrate sensors is the construction of the three-dimensional framework to obtain the desired affinity and specificity toward a given glycan. Using polymers, dendrimers, proteins, and other larger molecules has afforded not only an increase in multivalency but also provides functional groups for complementary interactions. In addition, using these types of molecules also provides opportunities to perform combinatorial work while permitting the use of various macromolecular conformations.

### 1.5.1 Polymer based boronolectins

Boronate containing polymers (BCPs) have gained increasing attention over the last ten years ranging from water soluble synthetic polymers, to micelles, and polymeric brushes.[147,148] Because of their affinity towards saccharides in aqueous solutions BCPs have been widely used and studied for sugar sensing, drug delivery, pharmaceutical assays, purification, and recently for gene transfection.[149] Since natural lectins had previously showed promising activity as mucosal adhesive reagents, it seemed advantageous to some groups to use BCPs as an replacement.[150-152] Despite previously discussed drawbacks to lectins and lectin-mimetics, BCPs recognition of polysaccharides of mucin in aqueous solutions has been proven and multiple materials have been developed. For example, contact lenses containing BCPs on their surface were prepared by free radical polymerization.[153] The affinity of the polymer-coated lenses to mucin was proven by an enzyme-linked lectin assay: biotinylated jacalin, a Gal-binding lectin, was found to adsorb to the mucin-treated lenses as expected. This allows the contacts to bind mucins with the

tear film mitigating dry eye, while providing additional protection again bacterial, chemical and physical invasion.[153]

### 1.5.2 Dendrimer based boronolectins

Dendrimers are typically highly symmetric monodisperse molecules that comprise a subset of highly branched polymers. While the simplest linear chain BCPs have their own set of unique qualities, dendrimeric boronic acid functionalized molecules create an additional type of multivalent molecule. The Liu group for example, proposed a synthetic model for the enrichment of trace glycoproteins using magnetic nanoparticles as a solid support for the generation of multiple generation Poly(amidoamine) (PAMAM) dendrimers (generation 4.0, G4, 64 surface amino groups) functionalized with boronic acid (Figure 6). PAMAM dendrimers were chosen as the main scaffold due to its unique properties, plentiful functional groups, and easy modification. Notably, the magnetic core is not involved in the binding with glycoproteins. Using 3 representative glycoproteins with molecular masses of 44, 80, and 150 kDa, and each with its own carbohydrate makeup, they compared the binding strength between nanoparticles and each glycoprotein as well as the dendrimeric model to non-dendritic nanoparticles. The results of the experiment showed a synergistic quality of the dendrimer based molecules enhancing avidity towards glycoproteins 3-4 times higher than the boronate affinity of single binding events. As proposed, the improved binding strength allowed higher efficiency of low concentration glycoproteins as well as being tolerant of competing glycans.[154] Based on this work it can be said that this strategy can be applied to other types of boronic acid and other types of solid supports, thereby making combinatorial chemistry more resourceful.

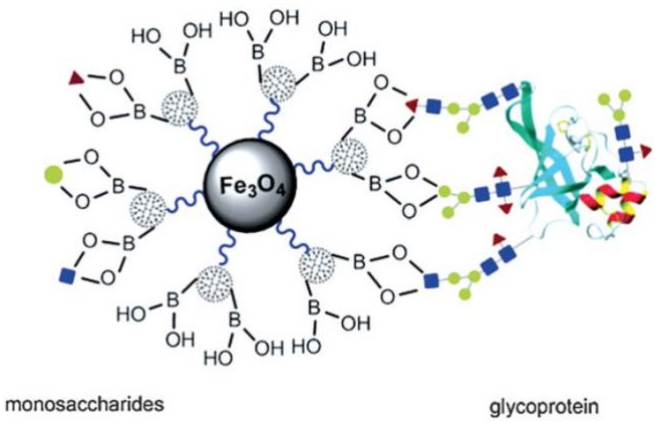



Figure 6: Boronic acid functionalized magnetic nanoparticles. Adopted from reference 154 with permission from MDPI.

### 1.5.3 Nucleic acid based boronolectins

In an ongoing effort to increase the number of boronic acid derivatives, there has been extensive research into the use of nucleic acid products. For example, the Anslyn group developed unmodified ribonucleic acid (RNA) to selectively tune a small molecule carbohydrate receptor[155] and the Yu group developed RNA aptamers for $sLe^x$ detection.[156]

Aptamers are single-stranded oligonucleotides able to bind small molecules, macromolecules or whole cells.[157] Anslyn's group successfully demonstrated the capability to use unmodified RNA aptamers to modify the selectivity of a boronic acid based receptor. Initially, a bis-boronic acid derivative (Figure 7,**1**) was known to preferentially bind citrate at $K_d = 5.5 \times 10^{-6}\ M^{-1}$ compared to tartrate at $K_d = 7.1 \times 10^{-6}\ M^{-1}$. After immobilization onto agarose beads (Figure 7, **2**), the boronic acid derivatives were subjected to an RNA pool in the presence of tartrate followed by systematic evolution of ligands by exponential enrichment (SELEX). Fundamentally, SELEX is a method that relies on the high affinity of a binding ligand to recognize high affinity RNA from a randomized pool of RNA. Enrichment performed over multiple "rounds", results in the ability to separate bound RNA from unbound RNA.[158] As a result, tartrate binding was found to increase when the RNA-receptor was involved. In the presence of one of the 7 aptamers tested, the $K_d$ for citrate changed to $>3 \times 10^{-3}\ M^{-1}$ and the $K_d$ for tartrate changed to $2.1 \times 10^{-4}\ M^{-1}$, or in other words a 14-fold higher biding constant for tartrate over citrate. In comparison, in the absence of the aptamer the receptor had a 1.3-fold higher preference for citrate over tartrate.[155]

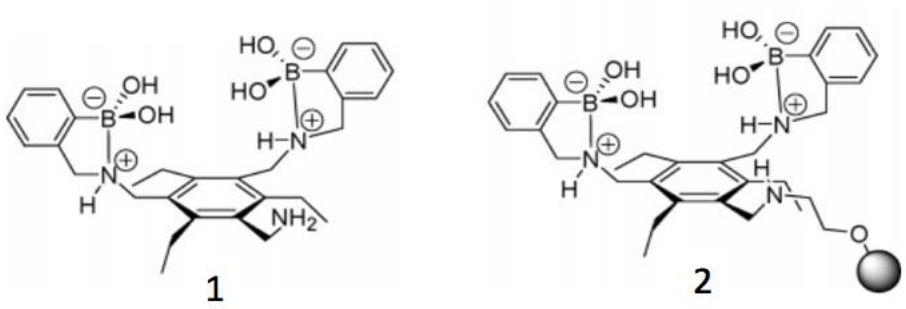



Figure 7: Bis-boronic acid, and its immobilized form. Adopted from reference 155 with permission from American Chemical Society.

### 1.5.4 Protein based boronolectins

Protein based boronolectins have recently been investigated for use as intracellular probes and therapeutic agents. One major challenge is the ability to direct proteins or therapeutic agents to the site of action. Based on carbohydrate sensing abilities this challenge could possibly be overcome using boronic acid derivatized proteins. It is well known that certain cancers over express SA and the targeting of such has been exploited in recent years.[159,160]

This section will focus on the use of boronic acid to extend the half-life of a drug while mitigating detrimental effects and acting as a glycan sensor. Among the list of medications for treating diabetes, insulin therapy remains the number one option for both palliative and preventative purposes.[161-163] One drawback to the use of insulin is that treatment is self-administered. This inevitably leads to dosage control issues leading to hypoglycemia that can be fatal. By minimizing the frequency of self-administration, the number of hypoglycemic incidents would undoubtedly decrease. Long acting insulin variants as opposed to native unmodified or fast acting types could reduce self-administration frequency by what can be considered tunable pharmacokinetics.[164,165]

The Anderson group, has succeeded in creating what could be called a "smart" long acting insulin, responsive to Glc, based on a boronic acid modified insulin. Multiple derivatives of human insulin were investigated and the best performing derivative could lower blood Glc levels and provide glycemic control compared to native insulin. The rate of hypoglycemia was reduced compared to native insulin as well. These studies were done using a human insulin derivative without BA as a positive control.[146, 166,167]

## 1.6 Glycopolymers as glycomimetics

In contrast to studying lectins and lectin mimetics is another area of research concerned with explaining the appealing selectivity of protein-glycan interactions. In this regard, glycopolymers, typically polymers with glycan pendant groups, have been extensively explored as multivalent carbohydrate for probing carbohydrate-protein interactions in an effort to gain a better understanding of their underlying mechanisms.[168] For example, glycopolymers can act as agonists or antagonists for understanding the molecular mechanisms of many biological processes, and also provide tremendous opportunities for therapeutic applications. In addition, glycopolymers can serve as potential receptors for biochip/biosensor development, which can be used for understanding carbohydrate-protein interaction, substrate specificity of carbohydrate-processing enzymes, antibody profiling, biomarkers, and pathogen and toxin identification applications. Therefore, design and synthesis of glycopolymers has become a very important research field, where significant efforts are needed to develop advanced glyco-polymeric architectures with improved performance.[169]

The precision in the design of synthetic glycopolymers - including chain composition, monomer sequence and architecture - has vital importance when it comes to mimicking the chemical and biological functions of glycoproteins. In addition, the glycan attachment to the polymer backbone is essential for its performance but has been paid little attention until now. So far, most glycans were attached to the polymer backbone through *O*-linked spacers or *N*-reductive amination-linked spacers, however, neither is a native glycan-amino acid linkage on glycoproteins, which may be a reason for the lower performance of glycopolymers.[170]

The synthesis of glycopolymers is still challenging since it often requires multistep synthetic routes. A procedure in the synthesis of glycopolymers begins with the synthesis of a glycomonomer with a functional group at the anomeric position of the saccharide that can take part in subsequent polymerization reactions. Alternatively, the attachment of glycosyl derivatives to the pre-synthesized polymer is often used. Protection and deprotection of hydroxyl groups on the saccharide moieties are often necessary in either glycomonomer or glycopolymer synthesis requiring multistep synthesis and purification processes and is both costly and time consuming. Therefore, ongoing research into straightforward synthesis of glycopolymers void of multistep synthesis and purification techniques and without the need of protecting groups is greatly needed.

One example of the use of glycopolymers as a glycomimetic (GM) is demonstrated by Oomen et al. The group successfully synthesized gold nanoparticles (Au-NP) coated with a fucose rich sulfated polysaccharide (fucoidan) mimetic glycopolymer (FMG) resembling heparin, that exhibited differential cytotoxicity toward colon cancer cells (HCT116) and were non-toxic to mouse fibroblast cells (NIH3T3). The FM glycopolymer alone was non-cytotoxic to either cell type indicating the FMG-Au-NP preferential induction of apoptosis in colon cancer cells. In conclusion, this experiment showed fucoidan GMs increased the cytotoxicity of Au-NPs towards HCT116 cells while remaining non-cytotoxic themselves.[171]

## 1.7 Aims and contributions of this dissertation

The aim of this dissertation research is to synthesize new boronic acid containing lectin mimetics (BCL) as well as glycopolymers for use as GMs. Ideally, the BCLs should bind the abundant and often terminal SA residues on the cell surface while both BCLs and GMs

should elicit a cellular response. It was determined that a combinatorial library would be the best and most efficient approach eliminating the need for extraneous planning and design of materials. The work described in this dissertation consists of the following three key parts:

**A. Investigating the regioselectivity of boronic acid**

A one-pot method for derivatization and methylation of glycans with 3-aminophenylboronic acid (PBA) under basic conditions has been investigated. Due to the specificity of PBA, a site-specific permethylation proceeds for galactopyranoside, mannopyranoside, and glucopyranoside as confirmed by $^{1}H$, $^{13}C$ NMR, and MS. This research proposes both a novel and viable method for glycan analysis and elucidates specificity of PBA.

**B. Boronic acid derived proteins as lectin mimetics**

The utility of BSA-PBA conjugates as successful glycan recognition molecules, to be used as lectin mimetics, has been demonstrated. The conjugates can be synthesized in a density controlled manner using traditional *N*-(3-(dimethylamino)propyl)-*N*-ethylcarbodiimide hydrochloride (EDC) coupling techniques affording amide derivatives from carboxylic acid residues within the BSA protein structure. Additionally, the boronic acid conjugated proteins were immobilized onto maleimide functionalized silica gel via thiol–maleimide interactions and used to study the sugar binding specificity of several sugars by Alizarin Red S (ARS) displacement assay. Evaluation of biocompatibility using a 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed no effect on cell viability after 24 hours. Lastly, cellular studies confirm binding of BSA-PBA conjugates to the cell surface based on the inhibition of lectin-FITC binding. These lectin

mimetics will provide an important tool for future glycomics and biosensor research and applications.

### C. Glycopolymers as glycomimetics

A straightforward synthesis of *N*-glycan polymers *via* acryloyl-glycosylamine and direct polymerization from free saccharides in aqueous conditions without protection/deprotection steps has been demonstrated. These glycopolymers have shown low levels of cytotoxicity based on multiple methods. In addition, immunomodulatory responses have been studied and significant differences seen based on the glycopolymer used in comparison to controls.

In conclusion, the weak and therefore reversible nature of boronic acid binding makes it a prolific source of molecular recognition events, especially when combined with polymeric materials. Conversely using GMs to study carbohydrate sensing may help to better understand the mechanisms that act as a driving force, while at the same time may elicit their own responses needing further investigation. Research efforts in both areas deal with the difficulty of structure complexity and the methods are now limited due to the natural number of each of these materials. This places a significant amount of pressure on the carbohydrate community to develop more bio-mimetics with versatility, affinity, and specificity in mind.

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# CHAPTER 2

## REGIOSELECTIVITY OF BORONIC ACID-GLYCAN BINDING (CARBOHYDRATE PERMETHYLATION CHARACTERISTICS IN THE PRESENCE OF BORONIC ACID)

### 2.1 Introduction

Due to the recent boom in glycobiology and glycomics, carbohydrate research has been recognized an area of enormous untapped biological potential. The diversity and complexity of glycan structures, together with their crucial role in many physiological or pathological processes, requires the development of new tools that allow for high affinity and specific recognition events. Since their recognition over a century ago, boronic acid has been exploited extensively as chemo/biosensors in the detection of carbohydrates.[1-4] Now, the boronic acid-diol interaction may be one of the most widely used single pair functional groups in the design of sensors and binders.[5,6] However, a clear understanding of the underlying chemical mechanisms regarding regioselectivity and binding site architecture is not well understood.

PBA was used as a glycan-binding reagent to determine its specific binding specificity and identify binding locations of gluco-, manno-, and galactopyranoside. The use of pyranosides (six membered rings) as a key recognition moiety was done for two reasons. First, pyranosides are known to generally have weak binding when compared to furanosides (five membered rings). Since the cell surface is mostly comprised of hexopyranosides it would follow that the studied sugars mimic these structures. Secondly, because the cell surface glycans are linked, most of them have non-reducing ends, which are represented by a methyl group on the pyranosides. It was clear from the beginning that if the use of boronic acid receptors is to mature into a general approach for saccharide recognition, new boronic acids with pyranoside-binding capability was required for the preparation of boronate conjugates. In this study, a one-pot method was developed for the determination of regioselectivity of PBA regarding glycopyranoside binding. PBA was conjugated to gluco-, galacto- and mannopyranosides followed by methylation, both reactions taking place under basic conditions (Scheme 2). Based on boronic acid specificity towards cis-diol configurations, a unique permethylation pattern was observed for each glycan and was characterized by 1D/2D NMR and mass spectrometry.

## 2.2 Materials and methods

Unless otherwise noted, all solvents and reagents were purchased from commercial sources and were used as received. Solvents were purified by standard methods prior to use. Deionized water with a resistance of 18MΩ $cm^{-1}$ was used as a solvent in all reactions and purification experiments. $^{1}H$ and $^{13}C$ NMR spectra were recorded at room temperature with a Bruker AV400 MHz spectrometer and DMSO was used as deuterated solvent. TLC analysis was performed on silica gel plates and column chromatography was conducted

using silica gel (mesh 230− 400), both of which were obtained from Silicycle, Ultrapure silica gels (Quebec, CA).

### 2.2.1 Conjugation and permethylation

Each pyranoside (Scheme 2, **1a-c**) (100 mg, 0.5 mmol) and PBA (80 mg, 0.5 mmol) was dissolved in $NaHCO_3$ buffer (2 mL, 100mM, pH = 8.6) and stirred overnight at room temperature. The solution containing product (Scheme 2, **3**) was washed three times with MeOH to remove buffer salts and dried by lyophilization.

Permethylation of (Scheme 2, **3**) was carried out by adding dry sodium hydroxide (NaOH) and iodomethane ($CH_3I$). NaOH (50% w/v, 200µL) to high pressure liquid chromatography (HPLC) grade MeOH (400 µL) to prepare the base. Anhydrous dimethyl sulfoxide (DMSO, 4 mL) was added and the mixture centrifuged at 2000 rpm for 1 min before removing the supernatant and the procedure repeated 4 additional times. The DMSO/NaOH slurry (1 mL) was added to the dry conjugated product followed by 600 µL of base and 150 µL $CH_3I$. The sample was vortexed for 10 minutes and washed 3 times with 2 mL $NaHCO_3$ buffer. The organic layer was discarded and the aqueous layer was dried by vacuum.

Cleavage of the PBA from the derivatized glycan (Scheme 2, **4)** was performed by dissolution in 0.3% formic acid (1:1, $H_2O$/MeOH) for 2 hours and dried by vacuum affording a crude mixture containing product (Scheme 2, **5**). Scheme 1 outlines the general procedure performed.

### 2.2.2 Purification

Following removal of the solvent, crude product (Scheme 2, **5**) was purified by silica gel chromatography (Ethyl acetate (A) / Hexanes (B) = 4:1, v/v) to afford pure permethylated derivatives in approximately 40% yield.

**Purification of methyl-β-D-glucopyranoside (5a):** Prepared as described for the general procedure. Yield: 43% (3.6 mg); linear A-B gradient from 80%-100% of A; $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ: 5.1385-5.1238 (d, 1H, 1 x OH-4), 4.5594-4.5297 (t, 1H, OH-6), 4.1689-4.1495 (d, 1H, H-1), 3.6876-3.6675(dd, 1H, H-6), 3.6580-3.6382 (dd, 1H, H-6'), 3.4766 (s, 3H, 3-$OCH_3$), 3.4190 (s, 3H, 2-$OCH_3$), 3.4096 (s, 3H, 1-$OCH_3$), 3.1953-3.1350 (m, 1H, H-4), 3.1350-3.0761 (m, 1H, H-5), 3.0173-2.9733 (t, 1H, H-3), 2.7868-2.7447 (dd, 1H, H-2); ESI-MS: $[M-H]^-$ calcd for $C_9H_{18}O_6$, 221; found, 221.

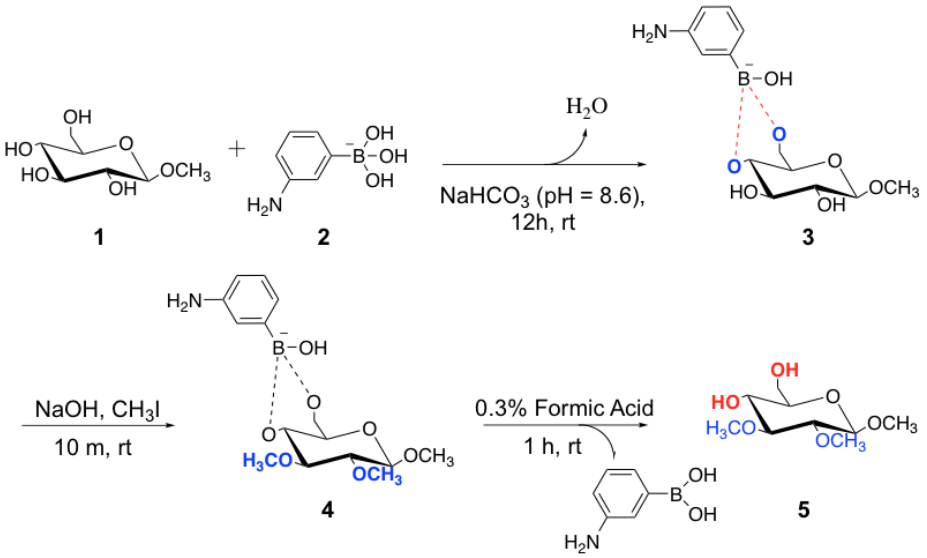
**Purification of methyl-β-D-galactopyranoside (5b):** Prepared as described for the general procedure. Yield: 38.6% (3.2 mg); linear A-B gradient from 80%-100% of A; $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ: 4.8708-4.8554 (d, 1H, 1 x OH-4), 4.5839-4.5718 (d, 1H, OH-3), 4.0965-4.0772 (d, 1H, H-1), 3.5633-3.5429 (t, 1H, H-5), 3.5284-3.4711 (m, 3H, 1 x H-3, 1 x H-6, 1 x H-6'), 3.4139 (s, 3H, 2-$OCH_3$), 3.3751 (s, 3H, 3-$OCH_3$), 3.3547-3.3385 (m, 1H, H-4), 3.2633 (s, 3H, 6-$OCH_3$), 3.0073-2.9640 (dd, 1H, H-2); ESI-MS: $[M-H]^-$ calcd for $C_9H_{18}O_6$, 221; found, 221.

**Preparation of methyl-α-D-mannopyranoside (5c):** Prepared as described for the general procedure. Yield: 33.5% (2.8 mg); linear A-B gradient from 80%-100% of A; $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ: 4.8974-4.8869 (d, 1H, 1 x OH-2), 4.7758-4.7586 (d, 1H, OH-3), 4.4665-4.4628 (d, 1H, H-1), 3.5766 (t, 1H, H-2), 3.5294-3.4977 (m, 2H, 1 x H-3,

1 x H-6), 3.4008 (s, 3H, 6-$OCH_3$), 3.3881-3.3475 (m, 1H, H-6), 3.2858 (s, 3H, 4-$OCH_3$), 3.2261 (s, 3H, 1-$OCH_3$), 3.1962-3.1486 (t, 1H, H-5); ESI-MS: $[M-H]^-$ calcd for $C_9H_{18}O_6$, 221; found, 221.

## 2.3 Results and discussion

A novel method was used to determine the regioselectivity of BA in one-pot fashion under basic conditions. First, unique glycopyranosides, simulating physiological glycans were conjugated to PBA in aqueous media under basic conditions. Following conjugation, a permethylation reaction utilizing NaOH, $CH_3I$, and DMSO was used to methylate reactive hydroxyl groups. Following the permethylation reaction PBA was cleaved from the sugar affording two hydroxyl groups previously unable to undergo methylation. Following purification by chromatography, purified products were analyzed by NMR and MS (Scheme 2, 5a-c).




Scheme 2: General synthetic procedure. (1) Conjugation of methyl-D-glycosides with PBA (2) to form boronate conjugate (3) based on diol recognition. Permethylation with NaOH and $CH_3I$ to form (4) followed by cleavage of PBA with formic acid affording identifiable binding locations on each pyranoside (5). (Methyl $\beta$-D-glucopyranoside is shown as a representative glycoside for the procdure. Methyl $\beta$-D-galactopyranoside and Methyl α-D-mannopyranoside were also used following the same procedure).

### 2.3.1 Mass spectroscopy

Permethylation has been widely used to increase sensitivity and stability of glycans for characterization by MS. In this study, MS successfully confirmed permethylation products without the need for purification. The mass spectra for each pyranoside was obtained in negative electrospray ionization mode because of an amplified signal compared to positive mode. As seen in Figure 8, the derivatized product of methyl β-D-glucopyranoside shows an m/z ratio of 221$[M-H]^-$ , indicating the addition of two methyl groups (-$CH_2$ = 14) compared to the expected value of 271 $[M-H]^-$ for the fully permethylated derivative or 193 $[M-H]^-$ for the starting material. This corresponds to boronic acid's specificity towards 1,2 and 1,3 diols and of the possible five potential binding sites, only two were observed to react, each corresponding to a particular glycan.

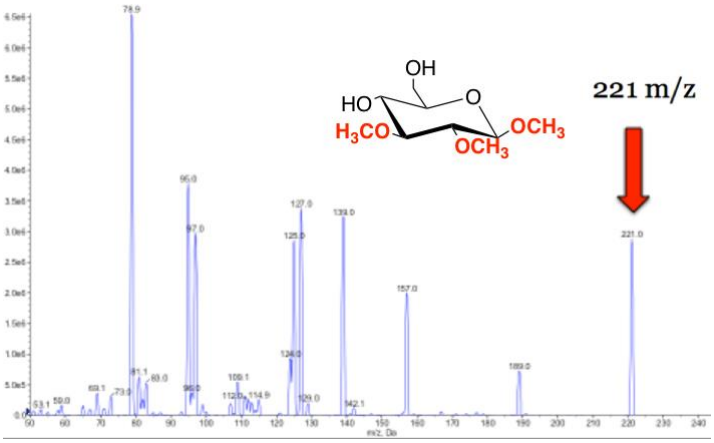



Figure 8: Representative ESI/MS chromatogram of Methyl $\beta$-D-glucopyranoside (5a).

### 2.3.2 Nuclear magnetic resonance

NMR characterization was carried out using 1D and 2D experiments including $^{1}H$, $^{13}C$, Correlation Spectroscopy (COSY), Nuclear Overhauser Effect Spectroscopy (NOESY), Heteronuclear Single Quantum Coherence (HSQC), and Heteronuclear Multiple Bond Correlation (HMBC). Characterization of the glucopyranoside product was used as a representation of the general method of characterization. Briefly, 3-5 mg of purified product was dissolved in DMSO-$d_6$ and subjected to NMR spectroscopy. Figure 9, shows the $^{1}H$ NMR comparison of glucopyranoside before and after derivatization. More specifically, the appearance of two $–CH_3$ peaks between 3.4-3.5 ppm in Figure 2B, in comparison to Figure 2A, confirms both BA conjugation as well as success methylation of reactive protons. Figure 10B, $^{13}C$ NMR further confirms the addition of two methyl groups with a total of three methyl peaks near 60 ppm compared to only one present in the Figure 3A. With the presence of free hydroxyl groups at Figure 11A at 4.6 and 5.1 ppm the $^{1}H$ NMR spectra gives correlations in the COSY to the protons at carbons 4 and 6, allowing the determination of methyl groups to be on carbons 1, 2, and 3. NOESY was used to further confirm peak assignments by showing spatial correlation between protons (Figure 11). The arrows at 2.7, 3.0, 4.2 ppm indicate correlations between protons directly attached to carbons 1, 2, and 3 and the three corresponding methyl groups between 3.4-3.5 ppm.

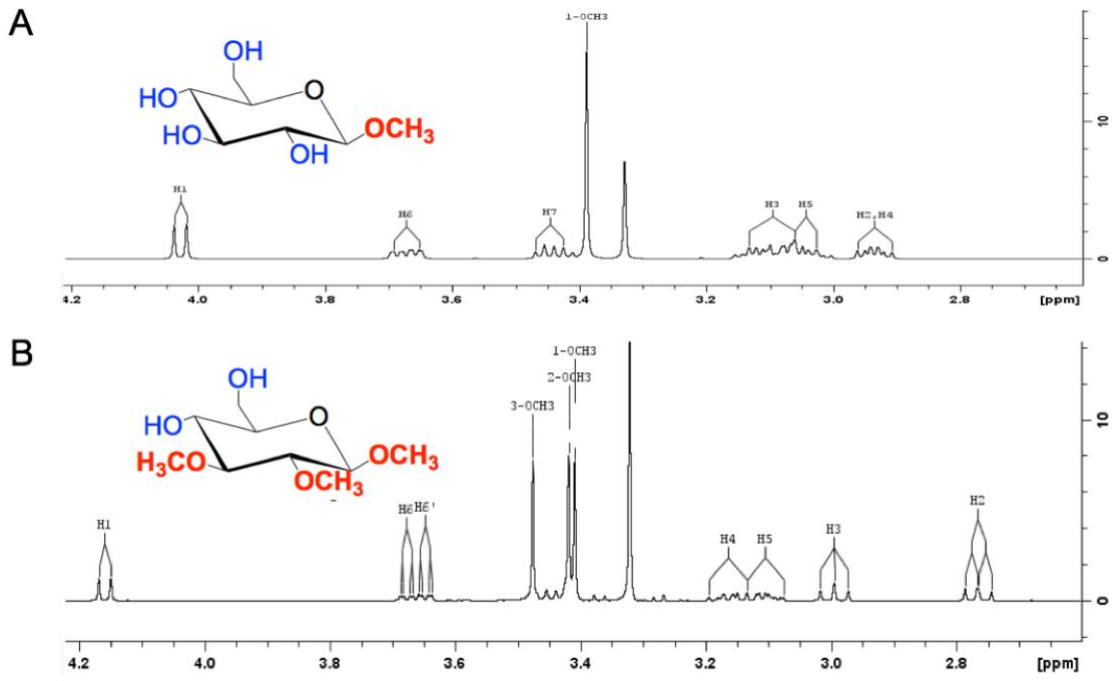



Figure 9: $^{1}$H NMR, DMSO-d$_6$, Methyl $\beta$-D-glucopyranoside (5a). **A**) Methyl -$\beta$-*O*-glucopyranoside starting material. **B**) Glucopyranoside after conjugation to PBA followed by permethylation.

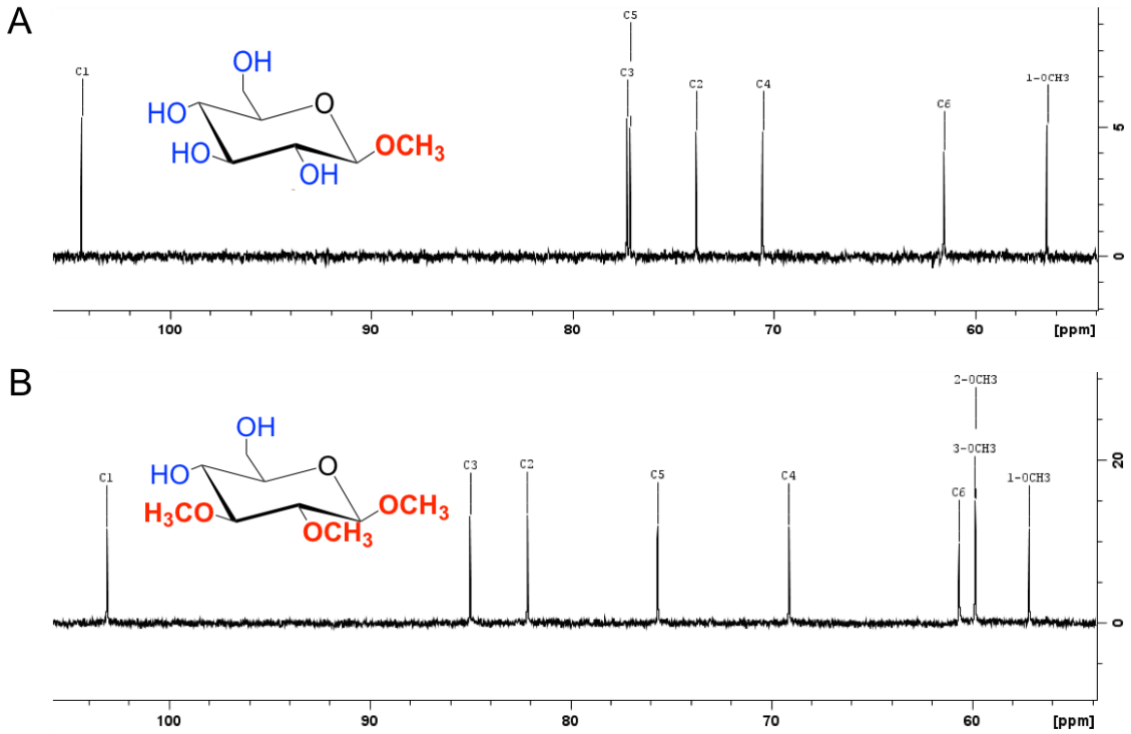



Figure 10: $^{13}C$ NMR, DMSO-$d_6$, Methyl $\beta$-D-glucopyranoside (5a). **A**) Methyl -$\beta$-*O*-glucopyranoside starting material. **B**) Glucopyranoside after conjugation to PBA followed by permethylation

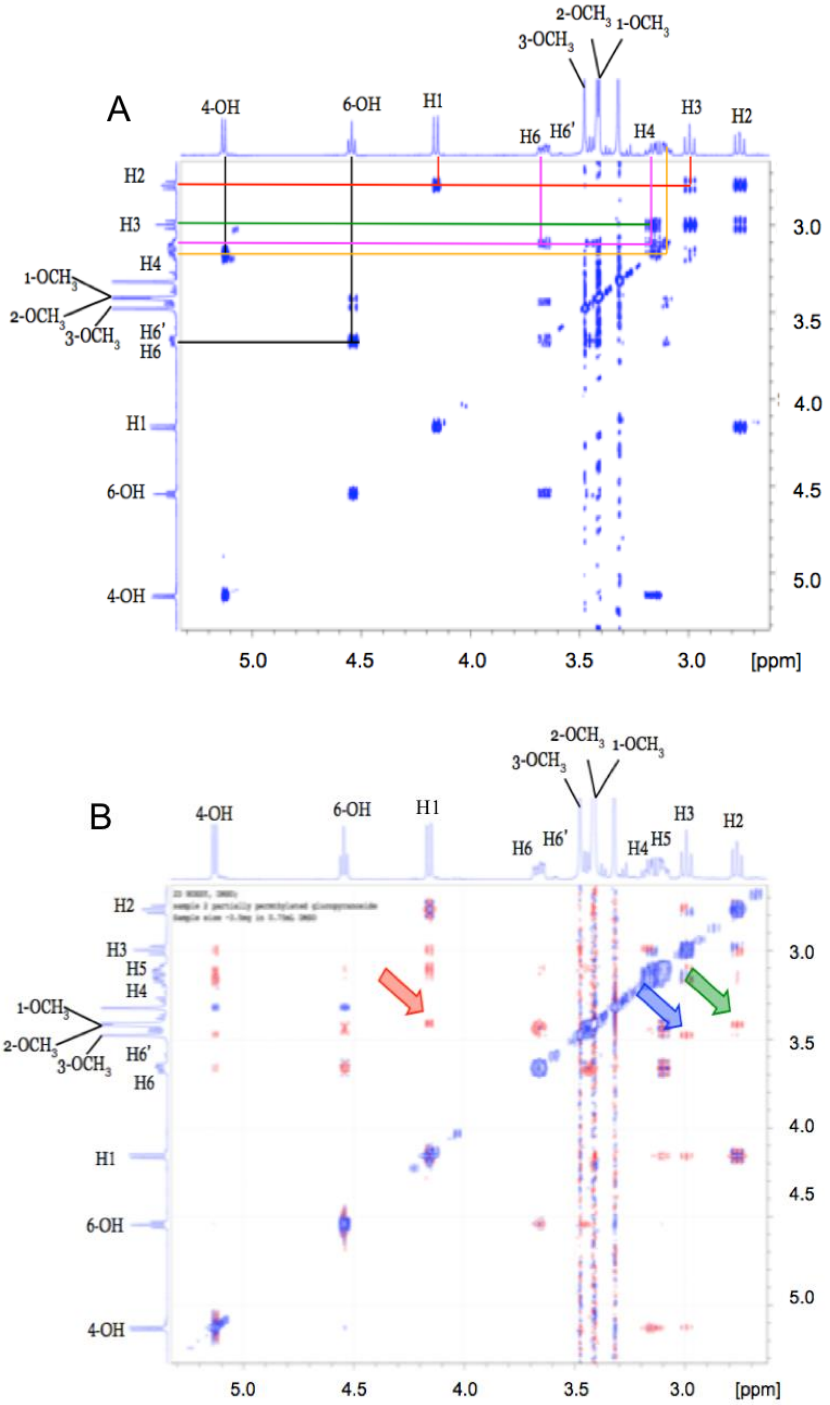



Figure 11: COSY and NOESY spectra for proton and methyl group assignment. **A)** COSY assignment of $^{1}H$ peaks for product 1a. **B)** NOESY spectra indicating methylated hydroxyl groups on the 1, 2, and 3 carbons for Methyl $\beta$-D-glucopyranoside (5a).

## 2.4 Conclusion

In conclusion, a one-pot method for derivatization and methylation of glycans with PBA under basic conditions has been demonstrated. Due to the unique specificity of PBA, a site-specific permethylation proceeds for gluco- galacto- and manno- pyranosides and PBA conjugation couples with permethylation results in unique profiles characterizable through basic NMR and mass spectroscopy techniques. PBA was shown to bind to each sugar in the following manner; methyl $\beta$-D-glucopyranoside at carbons 4 and 6, methyl $\beta$-D-galactopyranoside at carbons 3 and 4, and lastly methyl α-D-mannopyranoside at carbons 2 and 3 (Figure 12). This research proposes both a novel and viable method for glycan analysis and shows both the regioselectivity of PBA under basic aqueous conditions and well as its stability during permethylation procedures.

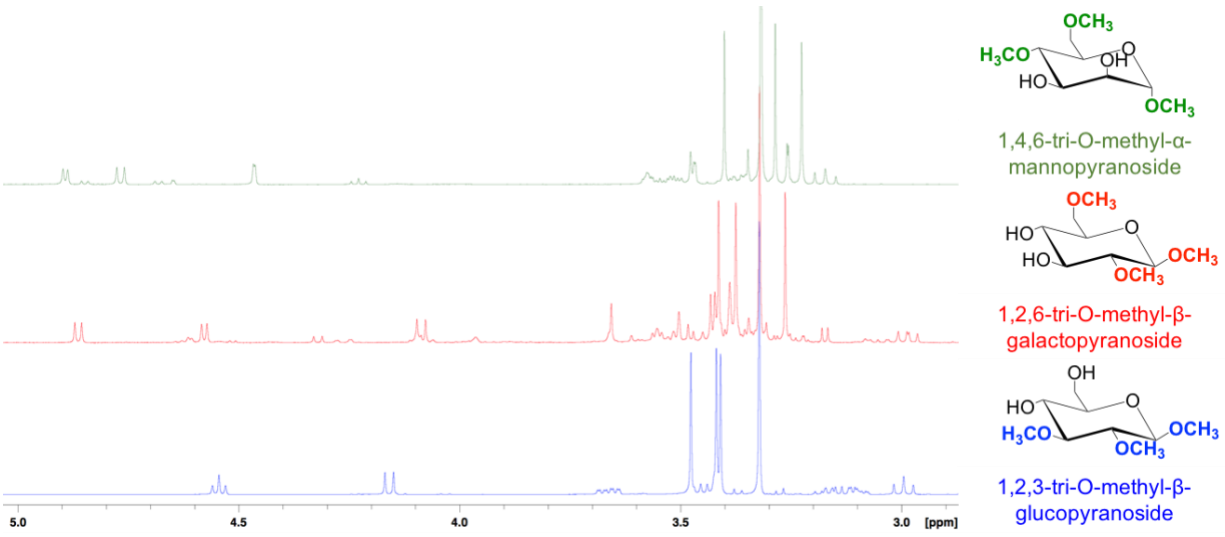



Figure 12: $^{1}$H NMR comparison of methyl pyranoside products.

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# CHAPTER 3

# SYNTHESIS AND EVALUATION OF BORONIC ACID CONTAINING MACROMOLECULES AND LECTIN MIMETICS

## 3.1 Introduction

Carbohydrate recognition is a crucial event in many biological processes.[1] For example, cell surface glycans, existing as glycoproteins, glycolipids or proteoglycans are involved in a variety of biological processes including cellular adhesion, cell signaling, cell-cell communication, and immune response.[2-4] Cell surface glycans are highly associated with diseases development, such as inflammation and cancers.[5,6] Over expression of cell surface glycan SAs is confirmed in cancer cells compared to normal control.[7] Therefore, sensitive profiling of cell surface glycans is highly demanded for basic glycomic advancement, clinical diagnostics, and therapeutic applications. In fact, the large diversity and complexity of glycan structures together with their crucial role in many physiological or pathological processes require the development of new techniques for analyses. Lectins are carbohydrate-binding protein having at least one non-catalytic domain that binds reversibly to a specific carbohydrate.[8] Due to their specificity, they have been largely employed to

identify cell surface glycans and glycoconjugates. Fluorescently labeled lectins have been widely used as intracellular and extracellular labels for cellular glycan profiling.[9-11] Further, lectin arrays using lectins as probes are well established to determine the specific glycan markers among different cell populations.[12] In addition, lectins are also used for cellular targeting, showing promise in multiple applications including the targeting of apoptotic and autophagic pathways useful in anticancer therapies.[13] Although natural lectins are very important tools for glycoscience research and application, they also have major limitations such as difficulty to produce, instability toward rigorous use, high cost and lack of availability.[14] Another limiting factor is low binding affinity and specificity leading to poor sensitivity for analytical assays, because rarely is any glycan found at high abundance in a biological sample.[15] In an effort to increase the binding specificity and affinity, clustered or linked lectins were explored as multivalency is typically regarded as one of the most important aspects in glycan-protein interactions.[16,17]

Boronic acids react with 1,2 and 1,3 diols of saccharides in aqueous media through a reversible boronate ester formation.[18-20] This interaction has been explored for potential application in analysis of glycans. So far, boronic acid containing ligands have been employed as artificial carbohydrate receptors,[21] membrane transport agents,[22] and cell surface carbohydrate recognition ligands.[23] It has been reported that phenylboronic acid can selectively bind to the glycerol side chain of SAs under physiological conditions, and the complex is stabilized through coordination of the amide NH or CO located at the C-5 position of SAs.[24] This anomalous binding profile enables significantly increased binding efficiency of boronic acid to SAs in comparison to other sugars, which strongly suggests an innovative molecular targeting platform for selective recognition of cell surface SA

residues of both glycoproteins and glycolipids. Taking advantage of the specific and reversible binding of SAs residues to boronic acid in neutral pH, a potentiometric method employed a boronic acid-modified gold electrode was developed for cell surface SAs as well as free SAs.[25] The detection method was based on the change in charge density of the electrode, due to the binding of SA residues. This method enabled a label-free, living cell operative, and real-time manner cytology, and was successfully applied to differentiate the degree of tumor metastasis through the detection of cell-membrane SAs.[26] In this study, we designed protein-PBA conjugates as lectin mimetics. Specifically, bovine serum albumin (BSA)-PBA conjugates were synthesized in a density controlled manner by targeting both aspartic and glutamic acids to afford the lectin mimetics with multivalent PBA as multivalence is a key factor for glycan-protein binding of both specificity and affinity. The resultant BSA-PBA conjugates were characterized by SDS-PAGE, MALDI-TOF MS. Further, its cell surface glycan binding capacity was confirmed by competitive lectin assay examined by flow cytometry.

## 3.2 Materials and methods

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Deionized water was used as a solvent in all procedures. PBA, ARS, BSA, EDC, MTT, and maleimide functionalized silica beads, Sephadex G-25 were purchased from Sigma-Aldrich (St. Louis, MO). Glc, Methyl β-*O*-glucopyranoside, Gal, Methyl *β*-*O*-galactopyranoside, Man, Methyl-α-*O*-mannopyranoside, SA, and Lac were purchased from Sigma-Aldrich (St. Louis, MO). MAA-FITC and SNA-FITC were purchased from Bio-World (Dublin, OH). 2-α-*O*-Methyl glycoside of Neu5Ac was synthesized as a literature method.[27]

### 3.2.1 BSA-PBA conjugation

BSA (100 mg, 1.5 μmol) and PBA (30 mg, 200 μmol) were dissolved in 5 mL of 0.05 M 2-(*N*-morpholino)ethane sulfonic acid buffer (MES; pH 6.0.). To this mixture, EDC (15 mg, 80 μM) was added at constant stirring and allowed to react overnight at room temperature. Then, the reaction mixture was subjected to Sephadex G-25 column with dd$H_2O$ as elute for purification and lyophilized. BSA-PBAs of different densities were prepared using same procedure as above by changing the ratios of BSA to PBA. Characterization was carried out by SDS-PAGE using both Coomassie blue and ARS as stains and imaged on a Typhoon 9410 Variable Mode Imager.

### 3.2.2 Matrix-assisted laser desorption ionization/time-of-flight mass spectrometry

The high purities and expected structures of the conjugated BSA-PBA derivatives were verified by MALDI-TOF MS using a Bruker Autoflex III MALDI-TOF mass spectrometer. The sample was applied onto the target plate using the dry droplet technique, in which 1 μL sample containing 1μg total protein in 0.1% trifluoroacetic acid (TFA) was mixed with 1 μL matrix (20 mg/mL sinappic acid in 0.1% TFA, 40% acetonitrile). The spots were dried at room temperature to allow sample crystallization prior to insertion into the instrument and the spectrum was obtained in linear mode.

### 3.2.3 BSA-PBA immobilization

BSA-PBA (45 mg, 2 mM) and maleimide functionalized silica beads (250 mg) were dissolved in phosphate buffered saline (PBS, 3 mL, 0.1 M, pH 7.4) and reacted for four hours at room temperature. The reaction mixture was then centrifuged and washed with

PBS three times to remove unreacted BSA-PBA. The same procedure was used for immobilization of all BSA-PBA conjugates as well as unmodified BSA.

### 3.2.4 Alizarin Red S binding assay

BSA-PBA modified silica beads (15 mg) were incubated with ARS (400 μM) in PBS (1 mL, 0.1 M, pH 7.4) for 30 min at room temperature and centrifuged to remove unreacted ARS. The beads were then centrifuged and washed with 0.1 M PBS (pH 7.4) three times to remove unreacted or loosely bound ARS followed by incubation of these silica beads with 100 mM sugar solutions (Glc, Methyl β-*O*-glucopyranoside, Gal, Methyl β-*O*-galactopyranoside, Man, Methyl α-*O*-mannopyranoside, Neu5Ac, 2-α-*O*-Methyl glycoside of Neu5Ac, and Lactose) in 0.1 M PBS (1 mL, pH 7.4) for 30 min at room temperature, respectively. Supernatant containing displaced ARS was removed after centrifugation and subjected to UV-vis spectroscopy. The absorbance of each well was measured on a microplate reader (Molecular Devices Spectrometer Plus 384) at 570 nm.

### 3.2.5 Cell culture

Raw 264.7 cells (ATCC**)** were cultured using DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptavidin at 37 °C in a humidified 5% CO2 atmosphere. Subculture was performed when the cells had 80 - 90% confluence using trypsin-EDTA.

### 3.2.6 MTT assay

The biocompatibility of the BSA-PBA conjugates was measured by MTT assay. RAW 264.7 cells were seeded in 96 well plates at a density of 0.5 x $10^4$ cells/well in medium and incubated for 24 hours at 37 $^oC$ w/ 5% $CO_2$. After 24 hours, the medium was removed and

new medium, supplemented with BSA-PBA conjugates, at varying concentrations (0.062, 0.125, 0.25, 0.5 and 1 μM). After an additional 24 hours, the cell medium was again removed and 100 μL of 5 mg/mL MTT solution was added to each well, and the plates was incubated for 4 hours. The MTT solution was removed and 100 μL of 100% DMSO per well was added to solubilize the precipitate and the plates were shaken for 10 min. The absorbance of each well was measured on a microplate reader at 570 nm.

### 3.2.7 Flow cytometry

Raw 264.7 cells were seeded at 4 x $10^5$ cells/well (5 mL tubes) and treated with BSA-PBA conjugates for 90 min. The cells were then washed 3 times with cold phosphate buffered saline (PBS; 0.2 mL, pH 7.4) and suspended in 50 μL PBS solution (pH 7.4) containing MAA-FITC (10 μg/mL) or SNA-FITC (10 μg/mL). After incubation for 30 min at room temperature, the cells were washed with cold PBS (pH 7.4) 3 times and resuspended in 500 μL of the same buffer for flow analysis. A minimum of 10,000 cells were measured each time. The fluorescence intensity of FITC-labeled lectins was subtracted from the intensity of the cell-lectin complex. All experiments were carried out in triplicate and spectra were obtained on a BD FACSCanto II Flow Cytometer.

## 3.3 Results and discussion

Considering PBA's ability to reversibly bind 1,2 and 1,3 diols in aqueous media along with BSA's well tolerated and widespread application, BSA-PBA conjugates were synthesized *via* EDC coupling. In this instance, amidation of carboxylic acids in BSA was followed by conjugation to amine moieties presented by PBA in the presence of EDC dissolved in MES buffer (Figure 13). Conjugation was followed by purification on Hi-Trap

Sephadex G-25 column by using $H_2O$ as eluent. Different densities of BSA-PBA conjugates were synthesized by altering the ratio of PBA to BSA.

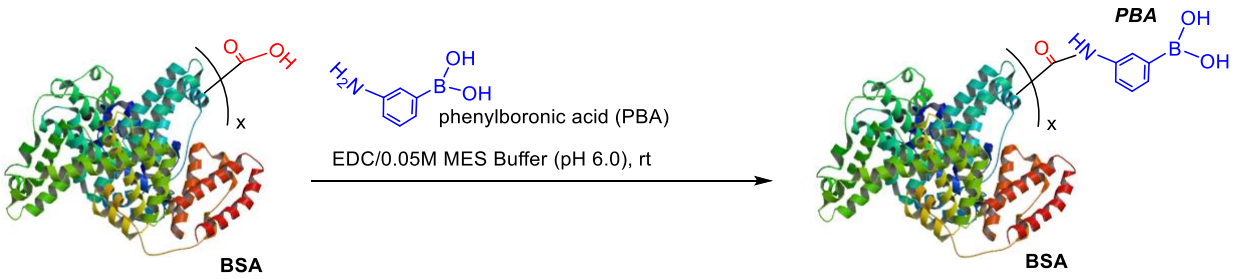

Figure 13: Synthesis of BSA-PBA conjugates

### 3.3.1 SDS-PAGE characterization

The resultant BSA-PBA conjugates were characterized by SDS-PAGE gel with Coomassie blue and ARS staining assay. First, BSA-PBA conjugates were characterized by SDS-PAGE, where the BSA-PBA conjugates showed an increase in molecular weight (Figure 14A). ARS binds to BA in a 1 to 1 ratio and a dramatic change in both color and fluorescent emissions can be monitored. Therefore, the BSA-PBA conjugates characterized on SDS-PAGE were stained with ARS. As shown in Fig. 1B, clear yellowish spots of all three BSA-PBA conjugates were observed, but not for unmodified BSA due to the lack of ARS binding. In the same respect, only bound ARS is fluorescent and can be seen in Figure 14C. This finding not only confirms binding of ARS to BA but also eliminates the possibility of non-specific binding. These data confirm that BSA-PBA conjugates were successfully synthesized.

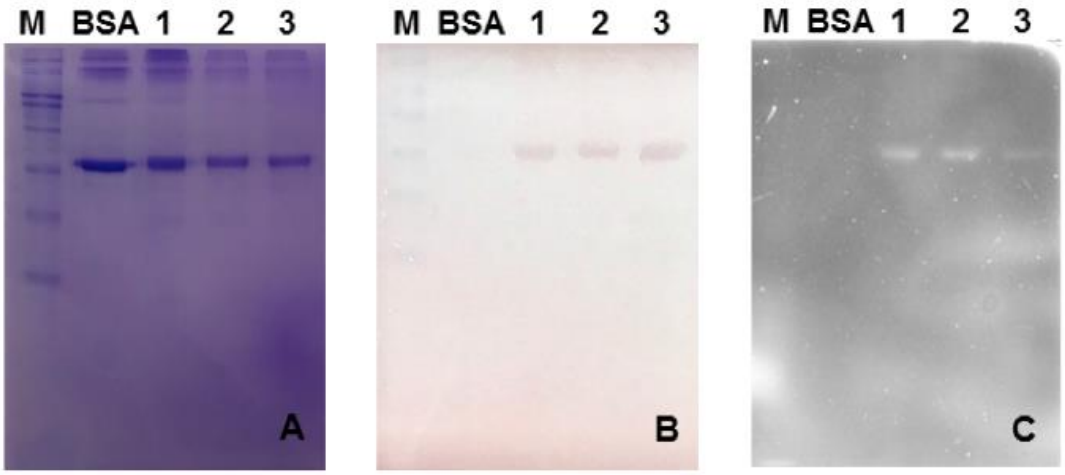


Figure 14: SDS-PAGE Characterization of BSA-PBA conjugates. **A)** Coomassie Blue staining; **B**) ARS staining (**C**) Fluorescent image of SDS-PAGE with ARS staining. M: Protein Molecular Weight Marker, BSA: Bovine Serum Albumin, 1: BSA-PBA1, 2: BSA-PBA2, 3: BSA-PBA3.

### 3.3.2 MALDI-TOF analysis

MALDI-TOF MS was used to characterize the BSA-PBA conjugates using a sinapic acid matrix with a Bruker Autoflex III mass spectrometer. The mass spectrum acquired for unmodified BSA showed the typical profile of a non-glycosylated, non-functionalized protein. The molecular ion peak was broadly dispersed over 66,430 Daltons due to an isotopic effect relative to the size of the protein (Figure 15A). The m/z ratio increases and peak position shifts to the right as BSA functionalized with PBA and the amount of shift is directly correlated to the amount of PBA conjugated to BSA (Figure 15A (2, 3, 4)). As a result, three BSA-PBA conjugates were obtained with the PBA/BSA ratio increase in the reactions, affording BSA-PBA1 determined to have 5 PBA functionalities, BSA-PBA2 to have 10 PBA and BSA-PBA3 to have 15 PBA functionalities. The MALDI-TOF MS spectrum of BSA (1) and BSA mixed with PBA in different ratios but without EDC (Figure 15B (2-4)) showed no molecular weight change, indicating no PBA conjugation occurred.

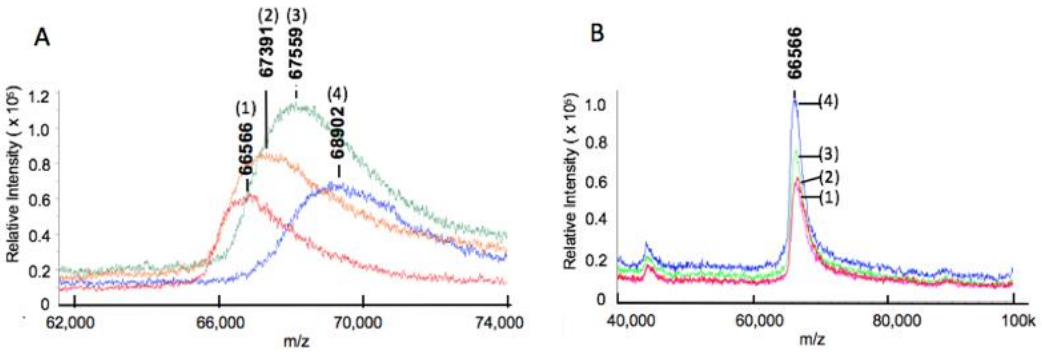


Figure 15: Characterization of BSA-PBA conjugates by MALDI-TOF MS. **A)** MALDI-TOF MS spectrum of BSA (1), BSA-PBA1 (2), BSA-PBA2 (3) and BSA-PBA3 (4) obtained in different ratios of BA in the presence of EDC; **B)** MALDI-TOF MS spectrum of BSA (1) and BSA (2-4) mixtures.

### 3.3.3 Glycan binding

The carbohydrate binding capacity of BSA-PBA conjugates was examined by ARS displacement assay, which has been used extensively to quantify boronic acid and carbohydrate binding capabilities.[28-30] Briefly, ARS shows a color change from red to yellow when bound to BSA-PBA and shifts the UV absorption wavelength from 520 to 460 nm in PBS (pH 7.4). When adding a (0.1 M) fructose, the fructose-boronic acid complex forms, releasing ARS with the color changing from yellow back to red and the wavelength shifting back to 520 nm (Figure 16B).

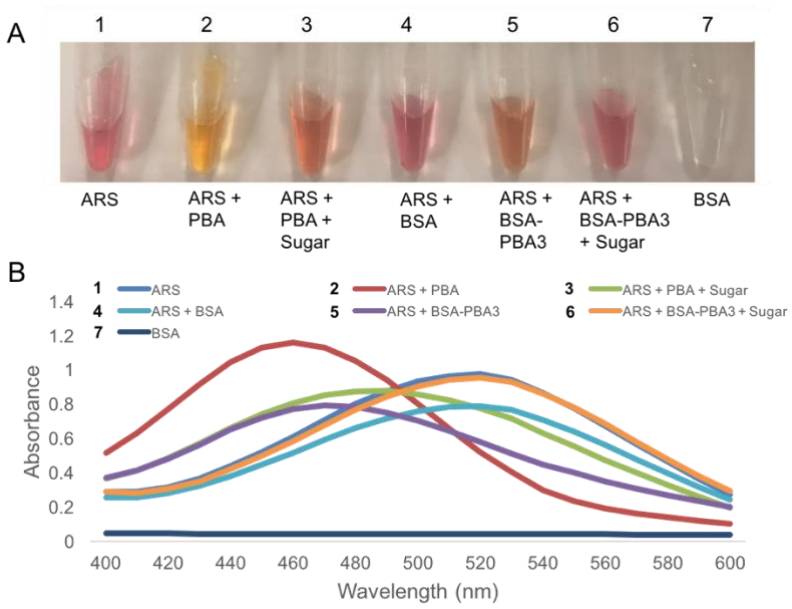


Figure 16: ARS displacement assay in PBS (pH 7.4) buffer. **A)** ARS solutions after incubation with PBA and BSA-PBA conjugates then fructose: (1) ARS, (2) ARS mixed with free PBA, (3) ARS mixed with free PBA, then with fructose, (4) ARS mixed with free BSA, (5) ARS mixed with BSA-PBA, (6) ARS mixed with BSA, then with fructose, (7) BSA. **B)** US spectra of ARS solutions after incubation with PBA or BSA-PBA conjugates then fructose: (1) ARS, (2) ARS mixed with free PBA, (3) ARS mixed with free PBA, then with fructose, (4) ARS mixed with free BSA, (5) ARS mixed with BSA-PBA, (6) ARS mixed with BSA-PBA, then with fructose, (7) BSA.

### 3.3.4 Glycan specificity

Silica beads have been widely used as small, rigid particles for high performance affinity chromatography as and is capable of withstanding high flow rates and/or pressures. Recently, surface functionalized silica beads have received wide spread attention for affinity chromatography applications.[31,32] In the present study, BSA-PBA conjugates were immobilized onto silica beads and their respective carbohydrate binding affinity and specificity were investigated. First, BSA-PBA was dissolved in PBS (pH 7.4). This BSA-PBA solution was added to commercially available maleimide functionalized silica beads and allowed to thiol- maleimide coupling react for 4 hours at room temperature. Then, the unreacted BSA-PBA was removed by washing the silica beads with PBS (pH 7.4) 3 times to afford the BSA-PBA functionalized silica beads (SB-BSA-PBA). Then, the resultant SB-BSA-PBA was characterized by ARS binding assays comparing to BSA modified silica beads and un-treated maleimide functionalized silica beads as well. As a result, SB-BSA-PBA incubated with ARS solution showed strong ARS binding compared to unmodified silica beads and BSA modified silica beads incubated with the same ARS solution (Data not shown).

Next, the SB-BSA-PBA was used to evaluate the carbohydrate binding affinity and specificity by ARS displacement assay. First, SB-BSA-PBA (15 mg) was incubated with ARS (0.1 μM) in PBS (pH 7.4) at room temperature for 30 min. After centrifugation, the supernatant containing unreacted ARS was removed by pipet and the silica beads were washed three times with PBS (pH 7.4). Among the BSA-PBA conjugates used, the BSA-PBA3 modified silica beads displayed the highest binding of ARS as it has higher density of PBA compared to BSA-PBA1 and BSA-PBA2 (Figure 17). The BSA modified silica

beads and silica gel beads alone showed no ARS released and were subtracted as controls during the experiment. To support this evidence, ARS displacement by the introduction of a high concentration (0.1 M) of saccharides was investigated. This method has been used in the past to determine multiple facets of PBA binding to include saccharide kinetics, affinity, and specificity.[33] The ARS bound SB-BSA-PBA was incubated with free monosaccharides and their O-methyl glycosides solution (0.1 M) in PBS (pH 7.4) at room temperature for 30 min to displace the bound ARS from the SB-BSA-PBA. *O*-methyl glycosides of all mimic native form of sugars in glycan chains linked *via O*-glycosylation. The displaced ARS was subjected to UV-vis spectroscopy, and the corresponding absorbance data was in direct correlation with the amount of ARS released from the SB-BSA-PBA, which is the indicator of the binding affinity and specificity of BSA-PBA conjugates to free monosaccharides and their *O*-methyl glycosides. As a result, BSA-PBA3 conjugates with the highest density of PBA showed highest binding capacity for both free monosaccharides and their *O*-methyl glycosides compared to BSA-PBA1 and BSA-PBA2 modified silica beads (Figure 17). However, there was no significant difference was observed regarding specificity for both monosaccharides and their *O*-methyl glycosides. This result indicates that the SB-BSA-PBA binding is dependent on the number of PBAs but it is unknown based on this assay if the saccharide truly makes a difference.

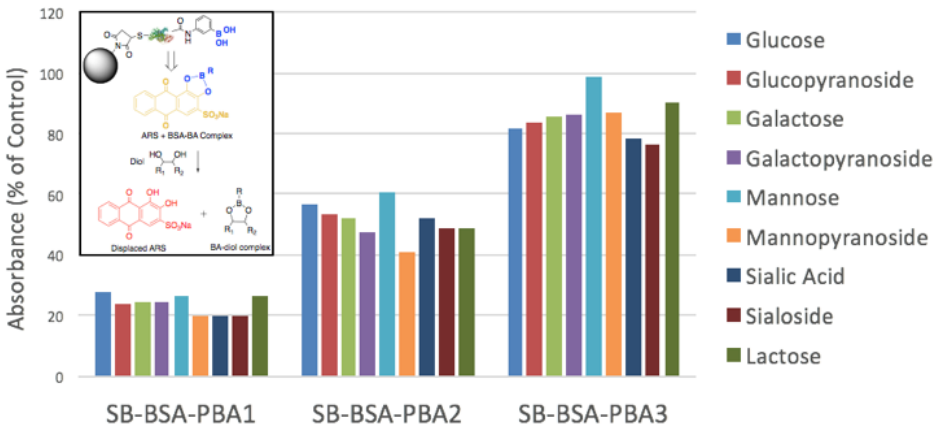


Figure 17: Carbohydrate binding capacity of BSA-PBA.
ARS displacement assay immobilized on silica gel beads: BSA-PBA conjugates were immobilized onto maleimide-functionalized silica gel, incubated with ARS, followed by washing and incubation with monosaccharides and their methyl glycoside. Absorbance at 520 nm was recorded for the free ARS replaced by sugars from the beads (Sialic acid: *N*-acetylneuraminic acid, Sialoside: 2-*O*-Methyl-α-D-*N*-acetylneuraminic acid).

### 3.3.5 Biocompatibility

The relative cytotoxicity of BSA-PBA conjugates towards Raw 264.7 cells were estimated by an MTT viability assay. Briefly, Raw 264.7 cells were seeded into 96-well plates with at 1 x $10^4$ density per well in 200 µL of medium. After 24 h of incubation, the culture medium was removed and replaced with 200 µL of some medium containing serial dilutions of BSA-PBA conjugates. The cells were grown for another 24 h. Then, 200 µL of 0.5 mg/mL MTT assays stock solution, in phenol red free medium, was added to each well. After incubating the cells for 4 h, the medium having unreacted dye was removed carefully. The obtained purple formazan crystals were dissolved in 100 µL per well SDS-HCl and the absorbance was measured at 570 nm. As a result, no apparent cytotoxicity to Raw 264.7 cells was observed up to a concentration of 1 µM for all three BSA-PBA conjugates. Instead, an increased level of cell proliferation was observed for higher concentrations of the three BSA-PBA conjugates (Figure 18).

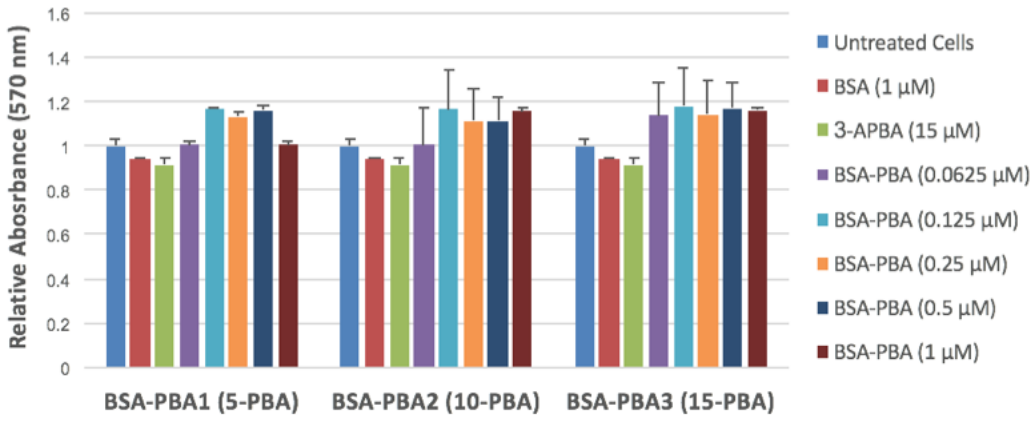


Figure 18: MTT assay to assess cell viability.
Raw 264.7 cells were incubated with BSA, PBA, and BSA-PBA conjugates for 24 h at 37 $^{o}$C. The error bars represent one standard deviation of the averages cell percent viability (n=3).

### 3.3.6 Cell surface targeting

SAs are found linked to Gal residues on the cell surface by either α-2,3 or α-2,6 linkages.[34] In this study, the binding capacity of BSA-PBA conjugates to cell surface SA was determined *via* competitive inhibition of the binding of lectins that specifically recognize SA by flow cytometry analysis (Figure 19). First, Raw 264.7 cells were incubated with BSA-PBA conjugates for 90 minutes, followed by incubation with MAA-FITC and SNA-FITC, which specifically bind to α-2,3 and α-2,6 linked SAs, respectively.[35] From the flow cytometry study, MAA-FITC showed very strong binding compared to SNA-FITC on the cell surface of Raw 264.7 cells cultured under the normal condition. And, it was apparent that BSA-PBA could inhibit binding of MAA-FITC by blocking available binding sites with respect to the number of PBA residues available from BSA-PBA for interaction. SNA-FITC on the other hand showed a drastic decrease in binding when incubated with BSA-PBA1 cells followed by an increase in fluorescent intensity with BSA-PBA2 and BSA-PBA3, which have high PBA content. This inverse relationship may be due to PBA location is different in these three BSA-PBA conjugates and its ability to bind cell surface glycans may different, thereby having different inhibition capacity for SNA binding. It is also possible that BSA-PBA2 and BSA-PBA3 may induce more expression of α-2,6 linked SAs, resulting in an increase in the fluorescent intensity of the SNA-FITC. We plan to quantify cell surface expression of Mϕ upon incubation with these lectin mimetics in our future immunomodulation study in detail. To explain the reduction in fluorescent intensity caused by the BSA control, it is possible the BSA may limit this effect showing true 100% lectin binding.

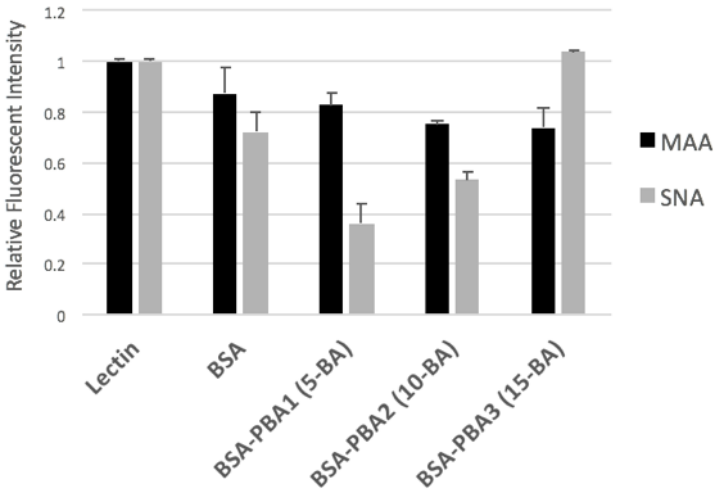


Figure 19: Cell surface SA binding capacity of BSA-PBA lectin mimetics. Raw 264.7 cells were incubated with BSA, BSA-PBA conjugate (5 μg/mL), followed with MAA and SNA lectin (10μg/mL) respectively. The error bars indicate one standard deviation of the averaged fluorescent intensity (n=3).

## 3.4 Conclusion

BSA-PBA conjugates were successfully synthesized and evaluated as lectin mimetics for glycan recognition has been demonstrated. The conjugates were synthesized in a density controlled manner using traditional EDC coupling techniques affording amide derivatives from carboxylic acid residues within the BSA protein structure. The BSA-PBA conjugates were immobilized onto maleimide functionalized silica gel *via* thiol–maleimide interactions and used to study the sugar binding specificity of several sugars by ARS displacement assay. Evaluation of biocompatibility using an MTT assay showed no effect on cell viability after 24 hours. Lastly, cellular studies confirm binding of BSA-PBA conjugates to Raw 264.7 cells cell surface SA based on the inhibition of lectin-FITC binding. These lectin mimetics have more favorable properties compared to natural lectin in terms of toxicity and inherent immunogenicity and will provide an important tool for future glycomics, biosensor and immunomodulation research and applications.

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# CHAPTER 4

# SYNTHESIS AND EVALUATION OF GLYCOPOLYMERS AS BIOMIMETIC GLYCOLIGANDS FOR MACROPHAGES TARGETING

## 4.1 Introduction

Mϕ cells play significant roles in immune system responses.[1] They are the first cells to recognize and engulf foreign substances (antigens), breaking them down and presenting the smaller proteins to T-lymphocytes, functioning as antigen presenting cells. Mϕs also express cytokines to help regulate the activity of lymphocytes and are differentially activated into different functional subtypes in response to antigen stimuli or cytokines present in the microenvironment.[2] They differentiate, or polarize, into classically (M1) or alternatively (M2) activated cells, both representing phenotypic extremes of Mϕ differentiation.[3,4] In particular, control of Mϕ phenotypic balance from proinflammatory M1 to reparative M2 is a choice of investigators to optimize the host response. Therefore, modulating Mϕs offers an opportunity to augment or inhibit specific immune functions and thus contribute to the discovery of novel therapeutic applications.

Mϕs express various lectins as receptors for modulating specific immune responses.[5,6] In particular, two major classes of lectins in innate immunity are promising targets: (i) C-type lectin receptors (CLRs), which are calcium-dependent carbohydrate binding receptors and (ii) SA binding Ig-like lectins (Siglecs), which are mainly expressed by immune cells. CLR receptors recognize carbohydrate moieties present on pathogens, but also bind to altered carbohydrate structures found on apoptotic, cancerous, and necrotic cells.[5, 7,8] Man receptor (Cluster of Differentiation 206, CD206) and DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) also known as CD209 are CLRs widely expressed on the surface of Mϕs and both recognize and bind to Man type carbohydrates commonly found on viruses, bacteria, and fungi. This binding interaction activates phagocytosis.[9,10] On the other hand, Siglecs recognize SAs as ligands and are expressed on human leukocytes in a cell-type restricted manner that regulates cellular adhesion, antigen uptake and signaling.[11,12] Siglec-1's (CD169) specific expression, makes it an attractive target for delivering antigens to tissue Mϕs *via* Siglec-1-mediated endocytosis.[13,14] Siglec-7 is expressed on Mϕs as well and based on its restricted expression, it has been proposed as an attractive target for cell-targeted therapies directed towards myeloid cells.[11, 15] Therefore, glycan binding by CLRs and Siglecs can be exploited for immunotherapy and the design of glycan-based therapeutics will inspire new biotechnological approaches to effectively tune immunological processes in cancer and infectious disease.[16-18]

Glycans are often presented in a multivalent fashion on either pathogens or repetitive protein sequences expressed on the cell surface and their interactions with receptors depends on multivalent recognition events.[19] In addition, CLRs and Siglecs show

multimerized expression on the cell surface. Therefore, to create the ultimate targeting ligand, a high affinity glycan must be combined with highly multivalent presentation.[20] In this regard, glycopolymers, typically polymers with glycan pendant groups, have been extensively explored as multivalent carbohydrates for probing carbohydrate-protein interactions in an effort to gain a better understanding of their underlying mechanisms.[21] For example, glycopolymers can act as agonists or antagonists for understanding the molecular mechanisms of many biological processes, and also provide tremendous opportunities for therapeutic applications. Therefore, glycopolymers may serve as active glycoligands for targeting Mϕs to initiate specific immune responses.[22] The precise design of synthetic glycopolymers has vital importance when it comes to mimicking the chemical and biological functions of glycoconjugates, as well as providing enhanced biological activities. Design and synthesis of glycopolymers has become a very important research field, where significant efforts are highly needed to develop advanced glyco-polymeric architectures with improved performance.[23]

The precision in the design of synthetic glycopolymers, including chain composition, monomer sequence and architecture, has vital importance in mimicking the chemical and biological functions of glycoproteins.[24] In addition, the glycan attachment to the polymer backbone is essential for its performance but has been paid little attention until now. So far, most glycans were attached to the polymer backbone through *O*-linked spacers or *N*-reductive amination-linked spacers, however, neither is a native glycan-amino acid linkage on glycoproteins, which may be a reason for the lower performance of glycopolymers.[25] The *N*-glycans are mostly found in natural glycoproteins, where the sugar molecule is attached to a nitrogen atom of an Asn residue in a protein. Previously, a straightforward

synthesis of *N*-glycan polymers for the development of high-affinity and selective ligands was developed.[25] In this study, *N*-sialylglycan polymers that closely mimic the natural *N*-sialylglycan polymers were proposed for targeting Mϕs Siglecs.

A micropillar/microwell chip platform facilitating miniaturized three-dimensional (3D) cell cultures and high-throughput biochemical and cell-based assays has been developed.[26] In this study, the immunomodulation effect of the *N*-sialylglycan polymers on mouse Mϕ RAW 264.7 cells on the micropillar/microwell chip platform was investigated. Specifically, an assay was established on the micropillar/microwell chip platform chip to streamline the evaluation of dose-dependent cytotoxicity and cell activation simultaneously, which allows one to examine the expression level of cell surface markers and the cytokine secretion profiles on 3D-cultured Mϕs in a high-throughput fashion. The micropillar chip coupled with the microwell chip can be used to investigate immune cell responses to external stimuli, cell staining, and imaging, thus allowing multiplexed, miniaturized, 3D immune cell-based assays in high throughput fashion alternative to conventional *in vitro* glycoarrays and multi-well plate platforms

## 4.2 Methods and materials

All solvents and reagents were purchases from commercial sources and were used as received. Lactose (Lac) was purchased from Sigma (USA). CMP-Neu5Ac was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). α2,3-Sialyltransferase, α2,6-silayltransferase were granted by Prof. Peng George Wang at Georgia State University. Deionized water with a resistance of 18MΩ $cm^{-1}$ was used as a solvent in all polymerization reactions and dialysis experiments. Dialysis was performed with cellulose membranes with a molecular weight cutoff of 3kDa with water as solvent. $^{1}H$ and $^{13}C$ NMR spectra were measures at

room temperature with a Bruker AV400 MHz spectrometer and $D_2O$ or $CD_3OD$ was used as deuterated solvent.

### 4.2.1 Synthesis and characterization of *N*-glycan polymers

**Synthesis of glycosylamines *via* Likhosherstov method** (Scheme 3): The preparation of glycopolymers began with synthesis of glycosylamines prior to polymerization. A solution of Lac (5.0 mmol) and ammonium bicarbonate (395.0 mg, 5.0 mmol) in 20 mL of aqueous ammonia was kept in an oil bath at 42 °C for 40 h and then was freeze-dried. The dried product was directly subjected to the next reaction without purification.

**Acrylation**: After amination, the dry lactosylamine (**2)** was dissolved in 60 mL of $CH_3OH$-$H_2O$ (1:1, V/V) and 3.0 g of $Na_2CO_3$ was added. The mixture was cooled in an ice bath for 30 min and then a solution of 1.28 mL of acryloyl chloride in 7 mL of THF was added dropwise. After that, the reaction mixture was stirred at 0 $^oC$ for another 1 hour. The organic solvents were evaporated under reduced pressure and the remaining aqueous layer was lyophilized to dryness.

***N*-(Prop-2-enoyl)-$\beta$-D-lactosylamine**: Glycomonomer (**3)** was purified by silica gel column chromatography eluted with $CHCl_3$-$CH_3OH$ (70: 30). $^1H$ NMR ($D_2O$, 400 MHz) δ: 6.30 (1H, d, 4.4), 6.29 (1H, d, 6.8), 5.85 (1H, dd, 6.8, 4.4), 5.05 (1H, d, 9.2), 4.44 (1H, d, 8.0), 3.42-3.91 (12H, m); $^{13}C$ NMR ($D_2O$, 100 MHz) δ: 169.4, 129.5, 129.4, 102.9, 79.3, 77.9, 76.5, 75.4, 75.1, 72.6, 71.6, 71.0, 68.6, 61.1, 60.0.

**Synthesis of *N*-lactosyl glycopolymer** (**4**) (Scheme 2): 4-Chloroaniline (4.34 mg, 0.034 mmol) and sodium nitrite ($NaNO_2$, 2.82 mg, 0.04 mmol) were dissolved in 1.2 mL of $H_2O$-THF (1: 1) in a three-necked flask. The mixture solution was cooled in an ice bath for 30 min and then 12 µL of $HBF_4$ solution (48 wt%, 0.092 mmol) was added to react for

1 hour. Then, a degassed mixture of *N*-(prop-2-enoyl)-β-D-lactospyranosylamine (**3**, 134.7 mg, 0.34 mmol), acrylamide (96.6 mg, 1.36 mmol) and NaOCN (3.0 mg, 0.046 mmol) dissolved in 0.8 mL of $H_2O$ was added into the flask containing the diazonium salt. The reaction solution was kept in an oil bath to react for 20 hours at 60 °C. The resultant mixture was dialyzed against deionized water for 2 days to remove the impurity and then freeze-dried to yield glycopolymer (143.1 mg, 61.9%). The molecular weight was about 13284.2 as determined by $^1$H NMR spectrum.

**Enzymatic synthesis of sialyllactose *N*-glycan polymer** (**5, 6**) (Scheme 5): Lactosyl glycopolymer (**4,** 10.0 mg, 0.75 μmol) and CMP-Neu5Ac (3.6 mg, 5.66 μmol) were dissolved in 800 μL of Trix-HCl buffer (pH 8.35). To the mixture solution was added 1.0 U α2,6-sialyltransferase and the mixture solution was kept in a water bath to react for 17 hours at 37 °C. The resultant α2,6-sialylloctose glycopolymer (**5**) was purified by dialysis against deionized water for 2 days. The same protocol was applied to synthesize and purify the α2,3-sialylloctose glycopolymer (**6**). The resultant sialyllactose glycopolymers were characterized by $^1$H NMR spectra.

**Characterization of sialyllactosyl glycopolymer:** The resultant sialylglycopolymers were characterized by $^1$H NMR spectra as well (Figure 20). The successful sialyation of *N*-Lac copolymer (**4)** was confirmed by the signals of protons from Neu5Ac (1.95 ppm, $CH_3$-Neu5NAc and 2.68 ppm, $H_{3eq}$-Neu5Ac), the degree of sialyation and the polymer length as well were calculated also using the $^1$H NMR spectra by comparing the integration value of proton signals from aromatic protons (7.31 ppm and 7.16 ppm), anomeric protons (4.98 ppm and 4.40 ppm) of Gal and Glc, C3-equatorial proton (2.70 pm) of Neu5Ac and methyl and methylene protons of polymer backbone. As a result, more than 90% enzymatic

sialylation was obtained for both α2,3-sialylation and α2,6-sialylation of *N*-Lac copolymer (Figure 20, **C,D**).

### 4.2.3 Biocompatibility

Dose-dependent cytotoxicity of the glycopolymers against 3D-cultured RAW 264.7 cells was evaluated on the micropillar/microwell chip platform. Briefly, the micropillar chip containing 60 nL of RAW 264.7 cells was sandwiched with the microwell chip containing 950 nL of synthetic glycopolymers, including *N*-lactosyl polymer, *N*-α2,3-sialolactosyl polymer, and *N*-α2,6-sialolactosyl polymer, at a concentration range of 0, 10, 50, 250, 1250, and 6250 μg/mL (dissolved in DMEM) for 24 hours, then rinsed with 0.9% saline solution, and stained with calcein AM and ethidium homodimer-1 for 1 hour. Fluorescent cell images were acquired from the chip using the S+ scanner, and dosage response curves were obtained.

### 4.2.4 Cell surface targeting

Next, the immune response to RAW 264.7 cells was examined on the chip platform. The micropillar chip containing 3D-cultured RAW 264.7 cells (60 nL) was sandwiched with the microwell chip containing 950 nL of 1250 μg/mL glycopolymer and incubated at 37°C and 5% $CO_2$ for 24 hrs. The treated cells were rinsed with Tris-buffered saline (TBS) twice for 10 min each and fixed in a mixture of ice-cold methanol and acetone (at 1:1 ratio) for 10 min at room temperature. After rinsing with TBS, cells were incubated with blocking buffer for 1 hour at room temperature, and stained with 0.01 mg/mL of the FITC-labelled primary antibodies (rat anti-mouse CD40, CD1d, CD80, and I-Ad) at 4°C overnight. Finally, the cells were stained with 1 μg/mL DAPI for 30 min, and rinsed with TBS twice for 10 min each. After drying the micropillar chips for at least 3 hours in the dark,

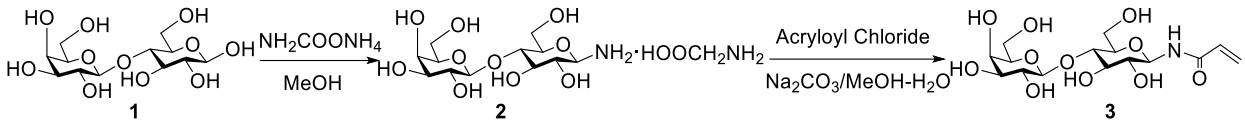
fluorescent images were acquired from the chip using the S+ scanner for fluorescent extraction and data analysis to detect the changes of cell surface biomarkers.

## 4.3 Results and discussion

In this study, *N*-lactosyl glycopolymer was synthesized from free glycan *via* glycosylamine intermediates followed by acrylation and polymerization *via* cyanoxyl-mediated free radical polymerization (CMFRP). The synthetic method is facile with no protection and deprotection used in either glycomonomer or glycopolymer synthesis. Then, *N*-α2,3-sialolactosyl glycopolymer and *N*-α2,6-sialolactosyl glycopolymer were synthesized *via* enzymatic reaction with sialyltransferase. The enzymatic glycosylation was reported as a highly efficient synthesis method with nearly 100% product yield. Finally, cytotoxicity and Mϕ targeting effect of the *N*-glycan polymers on RAW 264.7 cells were investigated.

### 4.3.1 Synthesis of *N*-(Prop-2-enoyl)-*β*-D-lactosylamine (3)

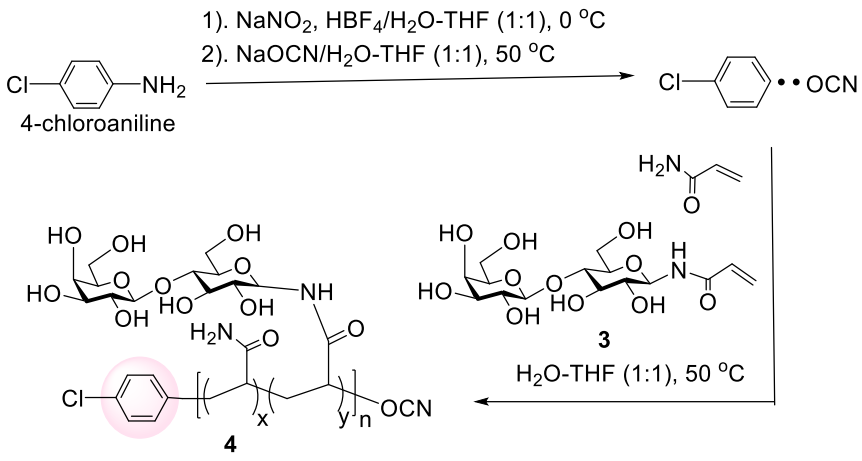
A practical protocol for the selective amination of unprotected sugar derivatives was introduced by Likhosherstov et al., which used ammonium carbamate in methanol.[27] In this method, with ammonium carbamate, the carbamic acid salt of glycosylamine formed as a white precipitate from the reaction solution, which allows easy purification from a crude carbohydrate preparation. In addition, carbamic salt formation prevents hydrolysis and glycosylamine-dimer formation. After obtaining the glycosylamine salt from free sugars, the *N*-acryloyl group was introduced by adding acryloyl chloride to the glycosylamine salts in methanol-water and in the presence of $Na_2CO_3$ at 0 $^oC$, followed by the removal of excess acryloyl chloride and sodium carbonate to yield *N*-acryloyl-lactosylamine as glycomonomers for *N*-glycan polymers synthesis (Scheme 3).




Scheme 3: Facile syntheses of glycosylamines. Likhosherstov method, and *N*-acryloyl derivatives as glycomonomers.

### 4.3.2 Synthesis of *N*-lactosyl glycopolymer (**4**)

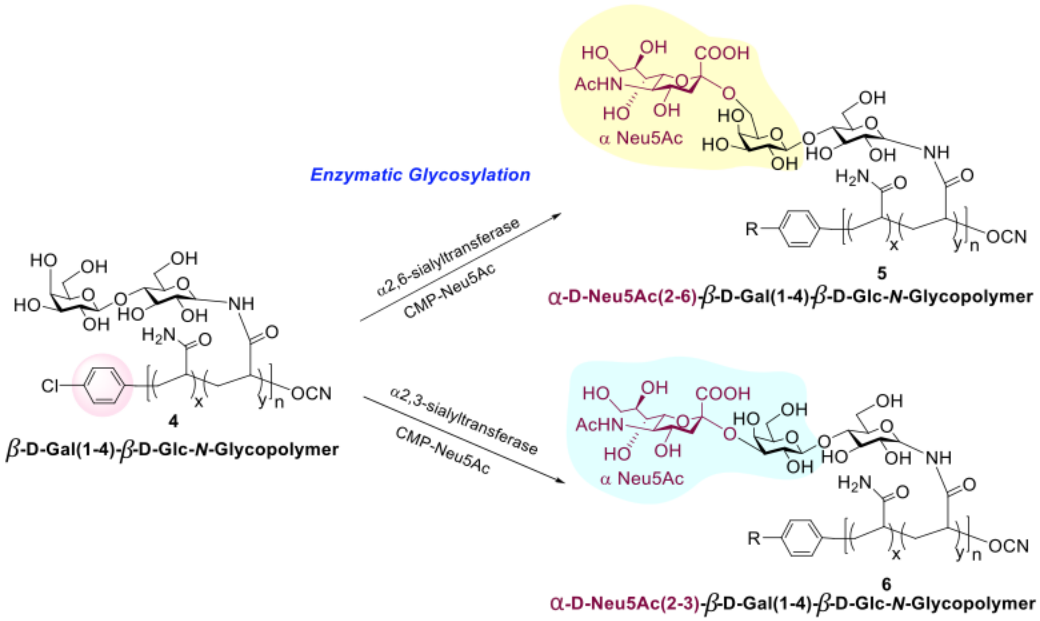
CMFRP is a straightforward approach for synthesizing glycopolymers in high yield with low polydispersity (PDI < 1.5). Polymerization can be conducted in aqueous solution and is tolerant of a broad range of functional groups excluding protection and deprotection steps often used in other methods. *N*-lactosyl glycopolymer (**4**) was synthesized *via* CMFRP in one-pot fashion (Scheme 4), in which, 4-chloroanaline was used as initiator for the copolymerization of *N*-acryloyl-lactosylamine and acrylamide. Initially, cyanoxyl radicals were generated by an electron-transfer reaction between cyanate anions from a sodium cyanate aqueous solution and aryl-diazonium salts prepared *in situ* through a diazotization reaction of arylamine in water. In addition to cyanoxyl persistent radicals, aryl-type active radicals were simultaneously produced, and only the latter species was capable of initiating chain growth.




Scheme 4: Synthesis of *N*-glycan glycopolymer *via* CMFRP.

### 4.3.3 Syntheses of *N*-sialylglycan polymers

The enzymatic glycosylation was reported as an efficient synthesis of oligosaccharides, without protection and in high anomeric control. In the past decade, enzymatic sialylation was explored to synthesize various sialyloligosaccharides, in which the glycosidic linkage between the SA and the acceptor carbohydrate is extremely controlled by the type of sialyltransferase selected. In the present study, the transfer of SA residue from CMP-Neu5Ac to 3- and 6-positions of terminal Gal of *N*-Lac copolymer (**4**), by enzymes α2,3-sialyltransferase and α2,6-sialyltransferase, were investigated to afford sialyllactose-containing *N*-glycopolymer α2,6 Sialylglycopolymer (SGP) (**5**) and α2,3 SGP (**6**), respectively (Scheme 5). The polymers were characterized NMR spectra by comparing the integration value of proton signals allowing a determination as to the degree of sialyation and the polymer length (Figure 20).




Scheme 5: Enzymatic synthesis of sialyl *N*-glycan glycopolymers from *N*-Lac glycopolymer.

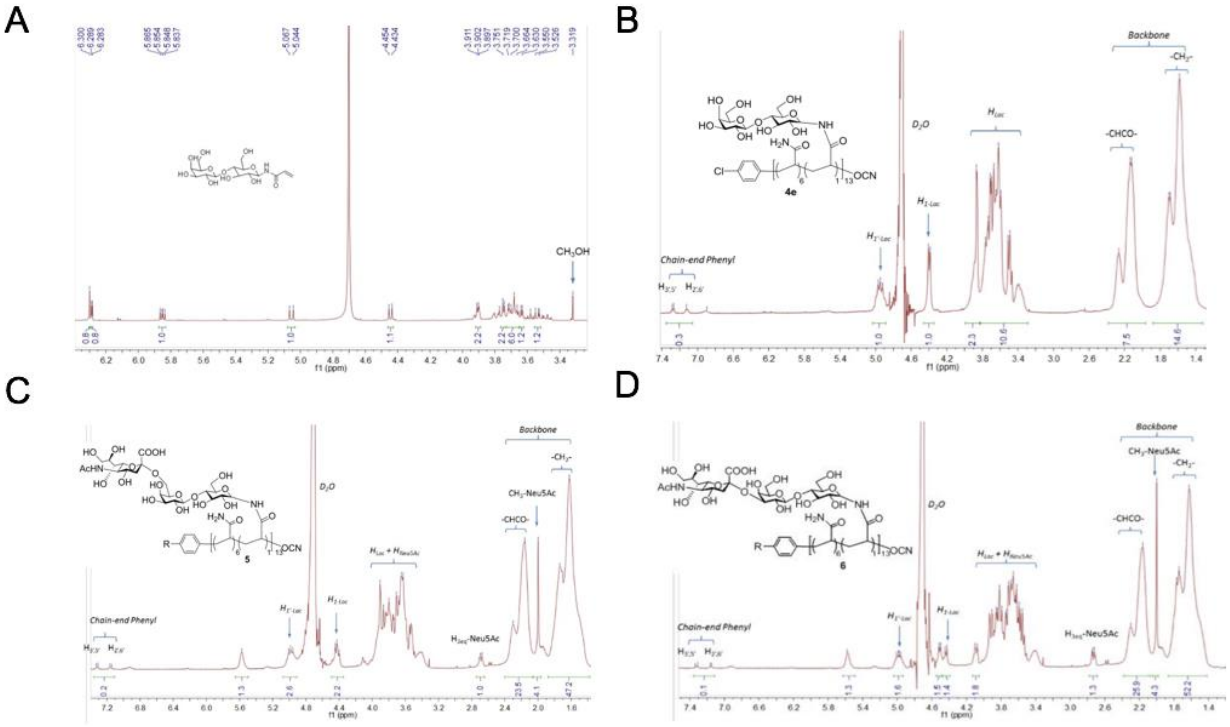



Figure 20: NMR spectra of lactosylamines and glycopolymer derivatives. **A**) $^{1}$H NMR spectrum of *N*-(prop-2-enoyl)-B-D-lactopyranosylamine in $D_2O$. **B**) $^{1}$H NMR spectrum of lactosyl glycopolymer in $D_2O$. (**C**) $^{1}$H NMR spectrum of α2,6-sialyllactose glycopolymer in $D_2O$. (**D**) $^{1}$H NMR spectrum of α2,3-sialyllactose glycopolymer in $D_2O$.

### 4.3.2 Biocompatibility

As a result of cytotoxicity assays, all *N*-glycan polymers were shown to be non-cytotoxic against 3D-cultured RAW 264.7 cell at a concentration of 1,250 mg/mL except for *N*-α2,3-sialolactosyl polymer (**4**), which was cytotoxic at 1250 μg/mL (Figure 21). Therefore, further evaluations of immune cell modulation with glycopolymers were performed at 1250 μg/mL.

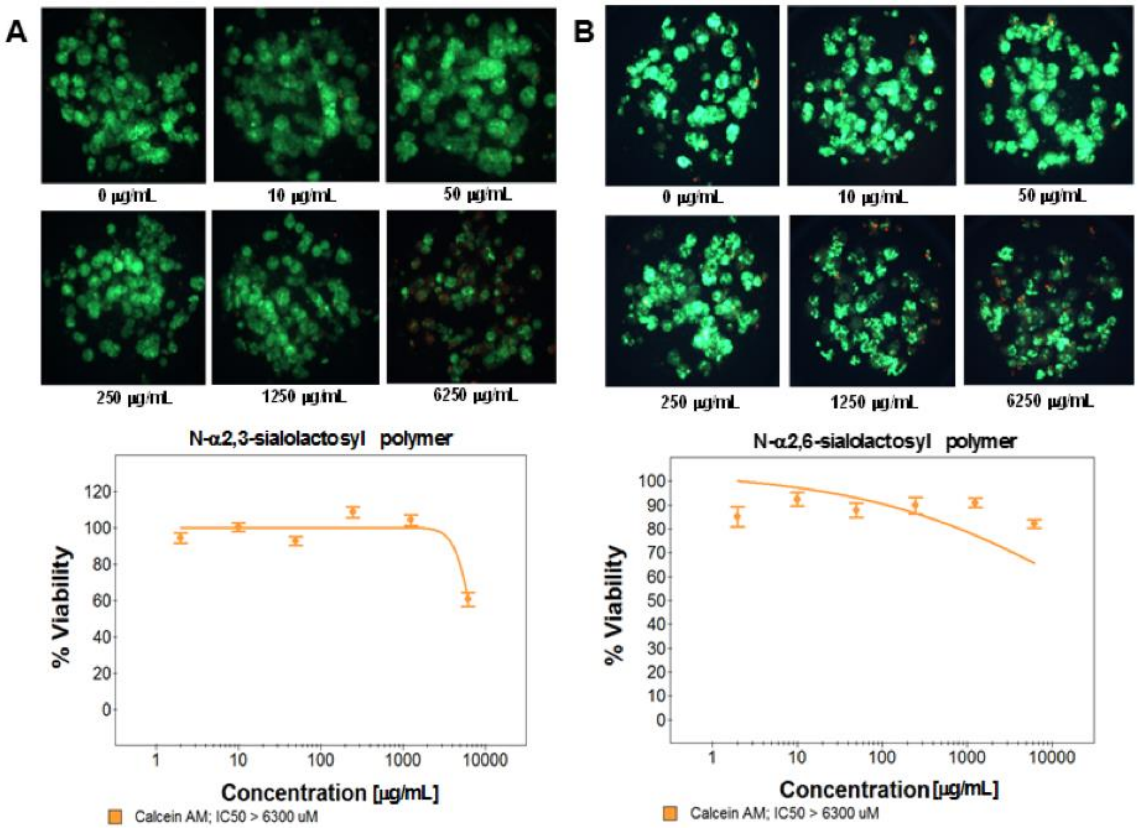



Figure 21: Cytotoxicity of *N*-glycan polymers against 3D-cultured RAW 264.7 cells.Dose-dependent cytotoxicity of *N*-α2,3-sialolactosyl polymer (**A**) and *N*-α2,6-sialolactosyl polymer (**B**) against RAW 264.7 cells have been demonstrated. The compounds were incubated with the cells for 24 hours on the chip. Green dots represent live RAW 264.7 cell spheroids stained with 1 µM calcein AM and 1 µM ethidium homodimer-1.

### 4.3.3 Cell surface targeting

Cell surface biomarker changes including CD40, CD1d, CD80, and IAd (major histocompatibility complex, MHC) were measured via immunofluorescent assay. These biomarkers have been studied extensively and used as a model for immunomodulatory effects as well as cell differentiation. Each of these biomarkers is located on various types of antigen presenting cells and their levels of expression are highly impactful on the immune response. Typically, upregulation of these unique receptors increases the cell's level of activation, leading to an increased immune response. By monitoring their levels of expression, we can observe their level of activation and identify GM compounds that induce a desired effect as well as clarify our understanding of carbohydrate involvement in the cell defense.

Next, cell surface binding of glycopolymers with RAW 264.7 cells was examined by measuring surface biomarker changes. As a result, CD40, CD80, CD1d, and MHC II were induced mostly with the *N*-sialyllactosyl polymers (**4 and 5)** (Figure 22) as compared to DMEM and LPS as negative and positive controls. In particular, *N*-α2,3-sialolactosyl polymer (**4**) showed the strongest induction of all four cell surface markers, indicating increased Mϕ modulation activity. This is correlated well with precedent reports that Mϕ receptors have a preference to binding glycan ligands with the Neu5Acα2-3Galβ1 sequence.[28] In addition, *N*-α2,6-sialolactosyl polymer (**5**) showed strong activity for inducing MHC II cell surface marker expression.

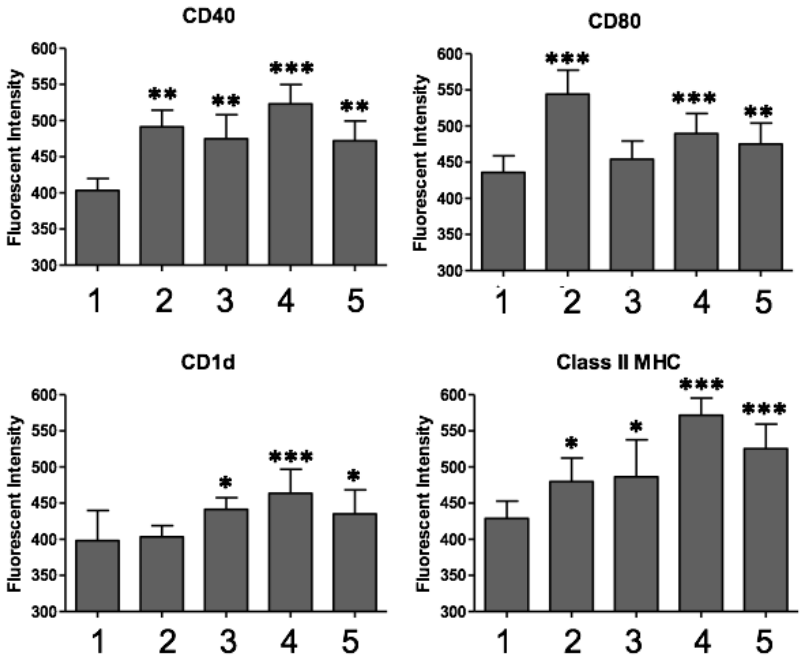



Figure 22: Expression of cell surface markers after treatment with glycopolymers.
Fluorescent intensity of CD40, CD80, CD1d, and Class II MHC. The numbers indicate as follows: (**1**) for growth medium control, (**2**) for LPS, (**3**) for *N*-lactosyl polymer, (**4**) for *N*-α2,3-sialolactosyl polymer, and (**5**) for *N*-α2,6-sialolactosyl polymer, all 3D-cultured RAW 264.7 cells on the micropillar chip. (* for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$).

## 5.1 Conclusion

SA-terminated oligosaccharides expressed on the cell surface are involved in multiple types of cellular communication, cell adhesion and other signaling pathways. Therefore, synthetic SA-containing oligosaccharides may serve as biomimetic agents to help understand the underlying mechanisms of signaling such as in immunomodulation. A straightforward synthesis of *N*-glycan polymers *via* acryloyl-glycosylamine and direct polymerization from free saccharides, all in aqueous conditions, without protection/deprotection steps has been demonstrated. The present simple and efficient synthetic method can be applied to synthesize glycomonomers and glycopolymers from any free saccharide, either purified natural *N*-linked and *O*-linked oligosaccharides or synthetic *N*-linked and *O*-linked oligosaccharides, for a variety applications.

The cytotoxicity and immunomodulation effect of novel biomimetic *N*-sialylglycan polymers was tested for cytotoxicity and immunomodulation activity on the chip platform in high throughput fashion. The *N*-sialylglycan polymers had a significant immunomodulatory effect on 3D-cultured RAW 264.7 cells. The *N*-α2,3-sialolactosyl polymer was non-cytotoxic to RAW 264.7 cell at a concentration of 1,250 mg/mL, while *N*-α2,6-sialolactosyl polymer showed toxicity at 1250 μg/mL. In addition, *N*-α2,3-sialolactosyl polymer **(4)** showed stronger activity for inducing CD40, CD80, CD1d, and MHC II cell surface markers expression, indicating a high Mϕ modulation activity.

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# CHAPTER 5

## SUMMARY

The major contributions of the work in this dissertation were the successful synthesis of a both boronic acid derived lectin mimetics as well as glycopolymers as GMs. One of the biggest challenges in designing carbohydrate sensors is the construction of the three-dimensional framework to obtain the desired affinity and specificity toward a given glycan. Using polymers, dendrimers, proteins, and other larger molecules has afforded not only an increase in multivalency but also provides functional groups for complementary interactions. In addition, using these types of molecules also provides an opportunity to perform combinatorial chemistry without the need for extraneous planning and design of a limited number of materials.

First, traditional approaches for studying cell surface glycans often employ the use of lectins, which recognize glycans with high specificity. However, the application of lectins is limited to their isolation from nature and limited number in comparison to glycan combinations. For this reason, BSA-PBA conjugates were synthesized in a density controlled manner by targeting both aspartic and glutamic acids to afford the lectin

mimetics with multivalent PBAs as multivalency is a key factor for glycan-recognition in both specificity and affinity. The resultant BSA-PBA conjugates were characterized by SDS-PAGE and MALDI-TOF MS. Mϕ cell surface glycan binding capacity was characterized by a competitive lectin assay examined by flow cytometry and an MTT assay showed biocompatibility. These novel lectin mimetics have the potential to find widespread use in both therapeutic and diagnostic applications as they can be wittingly modified, altering specificity and capacity.

Glycopolymers as GMs were synthesized to work opposite that of the lectin mimetics in that they were to be bound by cell surface receptors rather than binding to cell surface carbohydrates. A straightforward synthesis of *N*-glycan polymers from free glycans *via* glycosylamine intermediates followed by acrylation and polymerization *via* CMFRP in one-pot fashion. No protection and deprotection techniques were used in either glycomonomer or glycopolymer synthesis. A typical synthetic procedure for *N*-glycan polymers from free monosaccharide and disaccharide, Glc, Gal, Man, GlcNAc and Lac, was demonstrated. In addition, enzymatic sialylation of the Lac-containing *N*-glycan polymers and their immunomodulation effects on RAW 264.7 cells were investigated by microchip/microwell cytokine assay.

In summary, the work accomplished was a systematic preliminary study of the ability of lectin-mimetics and GMs ability to interact with RAW 264.7 cells. BSA-PBA conjugates were seen to bind to terminal SA residues based on flow cytometry and GMs were shown to elicit specific cellular response based on the polymer structure. Further investigation into immunomodulatory effects may require more effort concerning cytokine assay development. Also, more derivatives with varying functional group density as well

as orientation could be used to elicit different and even unique responses while helping to understand underlying mechanisms for specificity and affinity.

# CHAPTER 6

## FUTURE PERSPECTIVE

The Mϕ cell surface expresses a dense layer of glycans often terminated with SAs.[1] Due to their terminal position and properties, SAs are involved in the modulation of immune responses of Mϕs such as host-pathogen recognition, migration, and antigen presentation among other non-immune related processes. For example, a family of > 15 SA binding immunoglobulin superfamily lectins or Siglecs are expressed on a variety of cell types, but mainly on immune cells.[2] Siglecs are cell surface transmembrane proteins thought to regulate immune responses *via* intracellular signaling domains. They bind SAs in various contexts and, depending on the Siglec and the interacting partner, can suppress or promote cellular activation, inflammation or apoptosis.[3] Thus, targeting Mϕ cell surface glycans with lectins have great potential for modulating Mϕs to engineer immune responses toward a desired outcome, such as anticancer immunotherapy. However, a disadvantage of using natural lectins in immunotherapy is their inherent toxicity and immunogenicity.[4] Lectin mimetics like BSA-PBA conjugates may solve this problem. In this dissertation, the

Mϕ cell surface SA binding capacity of newly synthesized BSA-PBA conjugates were investigated by competitive lectin binding assays examined by flow cytometry and their biocompatibility with RAW 264.7 cells was investigated using an MTT assay. Further, their immunomodulation activity should be examined by quantifying cytokine release upon treating Raw 264.7 cells with BSA-PBA conjugates. These novel lectin mimetics are expected to find a wide range of applications in glycomics and biomedical research and development applications.

## 6.1 Immunomodulation of macrophages with lectin mimetics and glycopolymers quantifying cytokine release and cell surface marker changes of macrophages

Glycomic analysis seeks to understand glycans and glycoconjugates and how they react and alter the environment around them. More specifically, sialylated glycoconjugates take part in many biological processes, from intracellular signaling to organ development and tumor growth.[5] However, a clear understanding of the underlying mechanisms governing glycan-protein communication is not well understood, and even less, how they are involved in physiological and pathological processes. Our current studies have shown these lectin mimetics have more favorable properties compared to natural lectin in terms of toxicity and inherent immunogenicity and will provide an important tool for future glycomics, biosensor and immunomodulation research and applications. To understand the underlying molecular mechanism of cell modulation and reveal new functions of boronic acid derived lectin mimetics, it would be valuable to determine the exact changes in cytokine expression as well as cell surface marker changes.

ELISA based assays are the primary technique for characterizing cytokine expression based on easy quantification upon activation. More importantly, the high sensitivity of

these types of assays require small amounts of cells, compounds, and reagents. This approach generates “high quality” information on compound effects that will be vital in designing and discovering immune cell-targeting and modulating glycoligands for immunotherapy applications. This study only investigated four cytokines as a model, and the investigation of more cytokines will undoubtedly give a deeper understanding of cellular mechanisms. However, traditional methods lack the ability for high throughput analysis and reagent amounts could be reduced further, eliminating even more excessive and costly materials.

In addition, cell surface marker changes are typically identified using fluorescent antibody techniques and suffer from the same drawbacks as ELISA based assays. In this study, only four cell surface markers were measured as a model to demonstrate the applicability of glycopolymers as cellular modulators. Many more cell surface markers could be studied further elucidating cellular communication and signaling mechanisms. As carbohydrate roles in cellular communication come to the forefront of biological research, the need for an automated, high throughput, 3-D analysis technique becomes increasingly necessary and will offer the best glimpse of protein-carbohydrate interactions and the roles they play in both therapeutic and diagnostic applications.

## 6.2 Investigation of additional macromolecular structures

In this study, macromolecules were used as a basis for glycan recognition based on an increase in multivalency and therefore and increase in affinity. Multiple types of multivalent macromolecular structures have been studied including nucleotides, proteins, dendrimers, and linear polymers and have had varying results based on their individual architecture.[6,7] In addition, to the macromolecular “skeleton” structure, the change in

number of boronic acids, their orientation, and even boronic acid substituents has major implications in the unique abilities of each synthesized molecule. To compensate for natural lectins' limited numbers and immunogenicity, a larger number of synthesized lectin mimetics with varying capabilities can increase glycan recognition and cellular immunomodulation as well as mitigate drawbacks. Two primary methods exist for this type of advancement: combinatorial and specific molecular design. It is my belief that the best approach given the current situation is to develop a combinatorial library of additional macromolecule structures based on linear polymers and branched dendrimers. The techniques for derivatization are already standard lab practice, yet the number of potential derivatives means they have not all been studied for glycan recognition and/or cellular modulation. As the need for progression in glycomics research becomes more widespread, lectin- and glyco- mimetics will inevitably become increasingly more valuable.

## 6.3 References


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