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Design, Synthesis and Characterization of Chain-End Functionalized Glyco-Polymer for Efficient Proteomic Analysis

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DESIGN, SYNTHESIS AND CHARACTERIZATION OF CHAIN-END FUNCTIONALIZED GLYCO-POLYMER FOR EFFICIENT PROTEOMIC ANALYSIS

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DESIGN, SYNTHESIS AND CHARACTERIZATION OF END-FUNCTIONALIZED GLYCO-POLYMER FOR EFFICIENT PROTEOMIC ANALYSIS

SATYA NANDANA NARLA

ABSTRACT

Carbohydrates, especially, cell surface carbohydrates act as receptors for a variety of ligands such as proteins and thereby play significant roles in physiological and pathological processes, including cellular recognition and communication, bacterial and viral infection, and tumor metastasis. As such, binding interactions of carbohydrates and proteins have provided a starting point for the development of concept of isolation and probing of proteins and cells in biological research and novel diagnostic agents as well as new therapeutic applications. In this thesis study, oriented and multivalent carbohydrate macromolecules were designed and developed based on a chain-end functionalized carbohydrate-containing polymer (glycopolymer) for efficient protein analysis. Firstly, *O*-cyanate chain-end functionalized glycopolymer was synthesized *via* cyanoxyl-mediated free-radical polymerization (CMFRP) in one-pot fashion. This glycopolymer was tested for its oriented glyco-marcroligand formation and its glyco-affinity capturing and glycanarray application by combining *O*-cyanate chain-end functionalized glycopolymer with commercially available amine-functionalized silica gel and glass slide

via isourea bond formation, respectively. In the second study we demonstrated a new type of glycan microarray, namely, oriented and density controlled glyco-macroligand microarray based on end-point immobilization of glycopolymer that was accompanied with boronic acid (BA) ligands in different sizes as detachable "temporary molecular spacers". The spaced glycopolymer microarray showed enhanced lectin binding compared to non-spaced one. Third, a chemoenzymatic synthesis of chain-end functionalized sialyllactose-containing glycopolymers with different linkages was performed and their oriented immobilization for glycoarray and SPR-based glyco-biosensor applications were studied. Specifically, *O*-cyanate chain-end functionalized sialyllactose-containing glycopolymers were synthesized by enzymatic α2,3- and α2,6-sialylation of a lactose-containing glycopolymer and characterized by ^{1}H NMR. They were printed onto amine-functionalized glass slides for glycoarray formation. Specific protein binding activity of the arrays were confirmed with α2,3- and α2,6-sialyl specific binding lectins together with inhibition assays. In addition, sialyllactose glycopolymers were immobilized on amine-modified SPR chip to study the specific binding activity of lectins and influenza viral hemagglutinins (HA). These sialyloligo-macroligand derivatives combined with oriental and density controlled glycan array and SPR-based glyco-biosensor are closely to mimic 3D nature presentation of sialyloligosaccharides and will provide important high-throughput tools for virus diagnosis and potential anti-viral drug candidates screening application.

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ABBREVIATIONS

FRP	Free-Radical Polymerization
ROP	Ring-Opening Polymerization
ROMP	Ring Opening Metathesis Polymerization
CMFRP	Cyanoxyl Mediated Free-Radical Polymerization
CLRP	Controlled/Living Free-Radical Polymerization
NMP	Nitroxide Mediated Controlled Free-Radical Polymerization
ATRP	Atom Transfer Radical Polymerization
RAFT	Reversible Addition-Fragmentation Chain Transfer
DNA	Deoxyribonucleic acid
SPR	Surface Plasmon Resonance
BSA	Bovine serum Albumin
PD	Polydispersity
FITC	Fluorescein isothiocyanate
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween 20

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
NMR	Nuclear Magnetic Resonance
IR	Infrared
GP	Glycopolymer
GP-SG	Glycopolymer-Silica gel
PNA	*Arachis hypogae*
Con A	*Concanavalin A*
WGA	*Wheat germ agglutinin*
EDC	Ethyl(Dimethylaminopropyl) Carbodiimide
NHS	N-Hydroxysuccinimide
DSPE	1, 2-disteroyl-sn-glycero-3-phosphoethanolamine
PEG	Polyethylene Glycol
HPA	*Helixpomatia agglutinin*
BA	Boronic Acid
RCAI	*Ricinus communis*
AA	Acryl amide
GM	Glycomonomer

MES	2-(N-morpholino)ethanesulfonic acid
ARS	Alizarin Res S
BSA-BA	Bovine Serum Albumin – Boronic Acid
Lyz-BA	Lysozyme – Boronic Acid
PA-BA	Polyacrylamide – Boronic Acid
APBA	Amino Phenyl Boronic Acid
HA	Hemagglutinin
NA	Neuraminidase
MAA	*Macckia amurensis*
SNA	*Sambucus nigra*
SGP	Sialyllactose Glycopolymer
CMP-Neu5Ac	Cytidine-5′-monophospho-N-acetylneuraminic acid
SL	Sialyl Lactose

CHAPTER I

INTRODUCTION

1.1 Introduction

Carbohydrates envelop almost all living cell surfaces, mostly in the form of glycoconjugates such as glycoptoteins, glycolypids and proteoglycans. These carbohydrates on cell surface are playing significant roles in many biological processes, such as, cell-cell signaling,[1-2] immune recognition events,[3] pathogen host interaction,[4] tumor metastasis,[5] tissue growth and repair,[6] etc (Fig 1). Therefore, studying carbohydrate binding interaction has provided a starting point for understanding the molecular mechanisms that carbohydrate related and development of new therapies,[7] protein and cell isolation,[8] and targeted drug and gene delivery[9] concept in biomedical research and applications. It has been known that the carbohydrate-protein interactions are significantly enhanced by multivalent carbohydrate ligands referred as "cluster glycosidic effect".[10] In the past decades, variety multivalent carbohydrate ligands such as glycopolymers, namely polymers with carbohydrate pendent groups, have been

extensively explored for different applications.[14-22] Moreover, it is accepted that synthetic glycopolymers mimic functions of naturally occurring polysaccharides[23-24] and were employed for binding cell-surface receptors.[25] The strength and selectivity of binding interactions between multivalently displayed carbohydrates and targets are likely to depend on the density and relative spatial arrangement of the carbohydrate residues of glycopolymers.[25] Recently, the potential utility of glycopolymers in bio- and immunochemical assay, as biocapture reagents and for microarray applications has been demonstrated by the addition of functional anchor groups either as a pendant to the polymer backbone or at the chain end. Particularly, the chain-end groups of the polymer are the focus for bio-functionalization, as they allow for a direct one-to-one attachment[26-27] and also facilitate site specific[16] and oriental immobilization on solid surface.[28] This molecular recognition is especially exploited on surfaces for the development of biosensors, chip-based bioassay, or cell-adhesion studies[29] as they mimic the three dimensional display of glycans on the cell surface.

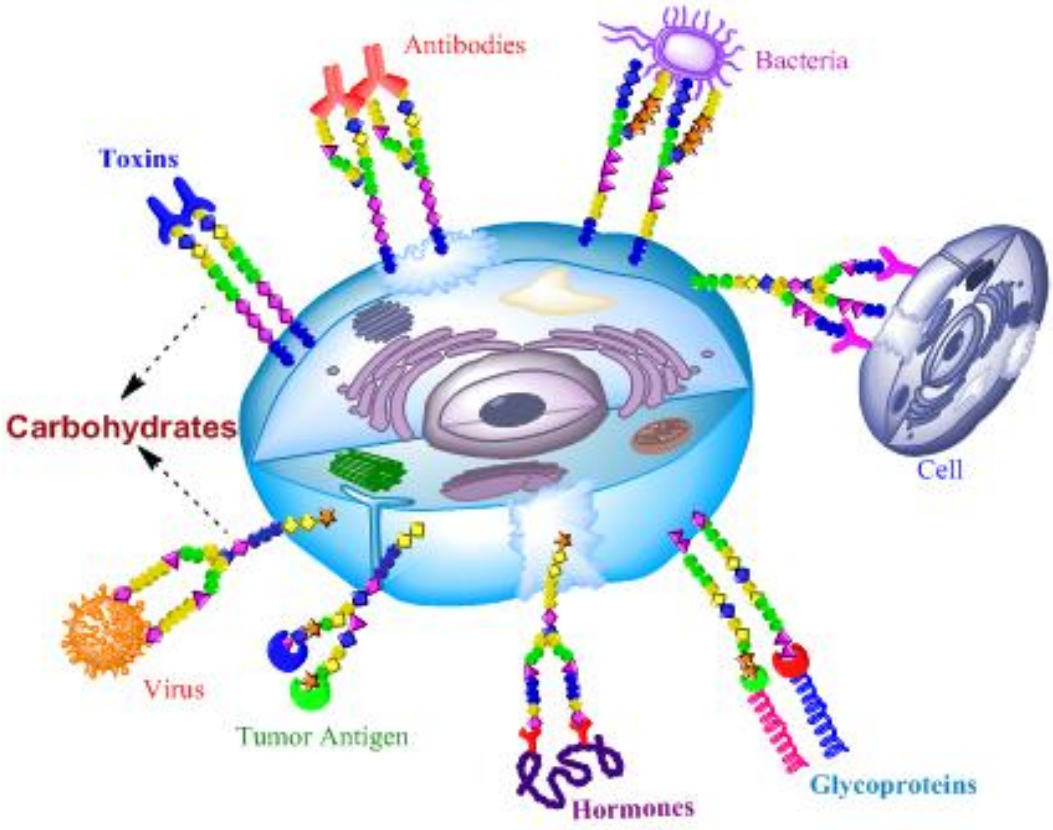

Figure 1. Cell Surface Carbohydrates: protein–carbohydrate interactions at the cell surface mediating biological processes.

1.2 Chain-end functionalized glycopolymers

Glycopolymers carrying pendant sugar moieties are either synthesized by direct polymerization of carbohydrate-containing monomers with protection group or without protection group, or by the post polymerization glyco-conjugation of synthetic polymers.[30] The polymerization can be carried out by a range of polymerization techniques. This includes: free-radical polymerization (FRP),[31] living anionic polymerization,[32] ring-opening polymerization (ROP),[33] ring opening metathesis polymerization (ROMP),[34] cyanoxyl mediated free-radical polymerization (CMFRP),[35]

and controlled/living free-radical polymerization (CLRP) which, encompasses nitroxide mediated controlled free-radical polymerization (NMP),[36] atom transfer radical polymerization (ATRP),[37] reversible addition-fragmentation chain transfer (RAFT)[38] polymerization. During the past decade, various glycopolymers with diverse chain-end functional groups like aldehyde, azide, biotin, *N*-hydroxysuccinamide, malimide, pyridine disulfide, and *O*-cyanate have been synthesized for various applications. ROMP,[39-41] CMFRP,[28, 42-43] ATRP,[44-47] and RAFT[48-52] have been employed for the synthesis of chain-end functionalized glycopolymers. The syntheses and applications of the above stated chain-end functionalized glycopolymers was summarized as in Table 1.

Table 1. Chain-end functionalized glycopolymers and their syntheses and biomedical applications

Chain End	Carbohydrate	Synthesis	Application	Reference
Fluorescein	3,6-*O*-disufo-Gal	ROMP and post conjugation	L-selectin binding on cell surface	*39*
COOH	3,6-*O*-disufo-Gal	ROMP and post conjugation	Surface immobilization	*40*
Biotin	Chondrotin Sulfate	ROMP and post conjugation	Surface Immobilization	*41*
Biotin	Lact	CMFRP	Surface immobilization	*42*
Biotin, COOH, NH2, Hydrazide	Lact, *O*-sufo-Lact	CMFRP	Protein modification	*43*

O-Cyanate	Lact.	CMFRP	Surface immobilization Microarray	*44*
Azide	Glc	ATRP	Protein modification	*45*
Biotin	GlcNAc	ATRP	Surace immobilization	*46*
Malimide	Man	ATRP	Protein modification	*47*
Pyridine disulfide	GlcNAc	ATRP	Protein modification	*48*
Biotin	Gal	RAFT		*49*
Biotin	Glc, Lact	RAFT and post conjugation	Surface immobilization	*50*
Alkyne	GalNAc	RAFT and post conjugation	Microarray	*51*
Biotin	GlcNAc, Man	RAFT	Surface immobilization	*52*
Biotin	Glc, Gal, Man,Fuc,Rham, Xyl, Lact,6α-mannobiose Panose, GlcNAc, GalNAc, *N*-acetyllactosamine, Antigen H, SialylLacNAc, Lacto-N-fucopentanose II, Sialyl Lewis X	RAFT and post conjugation	Microarray	*53*

The two significant features of these chain-end functionalized glycopolymer are multivalency, which aids for high affinity and specificity for bimolecular recognition and

chain-end functional group that facilitates oriental immobilization of glycopolymer on to solid surfaces to mimics cell surface carbohydrates. These glycopolymers were covalently attached to, proteins, viruses, nucleic acid, nanoparticles, silica gel beads and glass slides by chain-end functional group for protein modification and capturing applications (Fig 2). Amongst all applications summarized, capturing of carbohydrate binding protein by glycanarray technique is widely used and exhibits high potential for development as a high-throughput analytical tool for investigating biological processes engaged with carbohydrates. To date, many applications were demonstrated by chain-end functionalized glycopolymers with model proteins like lectins to exhibit their potential as an analytical tool for studying biological processes. Recent advances in living radical polymerization have provided methods to build chain-end functionalized glycopolymers with a whole range of biological recognition abilities.

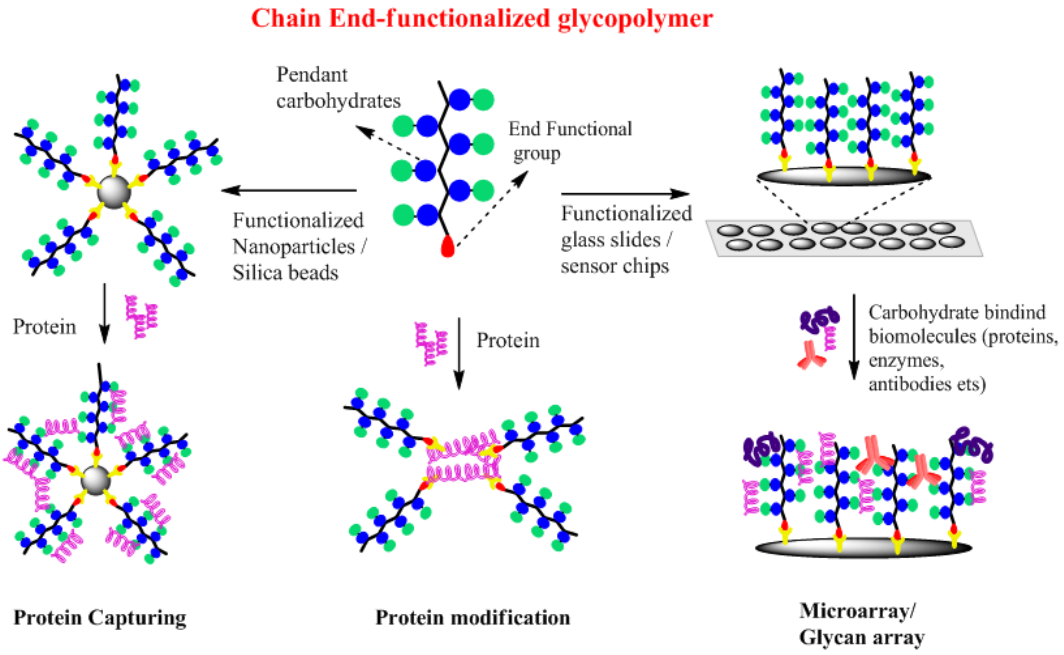

Figure 2. Biomedical applications of chain-end functionalized glycopolymers.

1.3 Glycanarray

Microarray techniques have been developed as an analytical tool for studying biological processes. DNA microarrays were first to be developed for analyzing mutation of genes, studying changes in the gene expression patterns in disease conditions and tracking the activities of genes at several times,[54-57] later protein microarrays have been developed as a high-throughput tool to study protein-protein interactions and to study protein expressions in normal and diseased states.[58-62] At first carbohydrate microarrays have been developed to study the interactions between carbohydrates and carbohydrate binding molecules like proteins,[63-64] antibodies,[65] pathogens[66] etc,. Unlike DNA and protein microarray development of carbohydrate microarrays courses more challenges like sugars must be displayed at the reducing end for being recognized by the carbohydrate binding molecules, the isolation and synthesis of carbohydrates are very challenging and moreover the interactions between carbohydrates and proteins is stronger and specific when the carbohydrates are present in a multivalent component (cluster effect). Initial microarrays were developed with monovalent carbohydrates or the oligosaccharides immobilized direly with the help of several linkers or functionalization of carbohydrates.[63-66] Later several multivalent carbohydrate molecules like glycodendrimers, glycopolymers, glycoliposomes, neoglycoproteins, glyconanoparticles etc were developed to achieve more specific and strong binding of proteins to carbohydrate microarrays. It is understood that not only the multivalency of the carbohydrates effect the binding specificity also the density and orientation of the carbohydrates present on the solid surface effects the interactions significantly i.e. the tight binding of proteins to carbohydrates on the array surface depends on multivalency

of carbohydrates with appropriate spacing and orientation of carbohydrates on the surface.

1.4 Surface Plasmon Resonance

Surface Plasmon resonance (SPR) imaging is a surface sensitive optical technique that is used for monitoring label free bio-molecular interactions in liquids.[66-67] It has several applications in the field of life sciences, electrochemistry, chemical vapor detection and food and environmental safety. Especially in the field of life sciences SPR is applied to determine the bio-molecular interactions and also to determine the association and dissociation kinetics between bio-molecules like protein-protein,[68-69] DNA-protein,[70-72] DNA-DNA,[73-74] antigen-antibody,[75-76] protein-carbohydrate[77-79] etc. Surface Plasmon Resonance is a phenomenon that occurs when polarized light hits a metal film at the interface of media with different refractive indices, in which light is focused onto a metal film through a glass prism and the subsequent reflection is detected (Fig 3). Briefly, the ligand is immobilized onto a gold chip and the analyte is passed over the ligand with continues flow. As the analyte binds the ligand, the addition of mass on the surface results in an increase in the refractive index and shift of angle from I to II (Fig 3). This change in the refractive index is measured in real time and plotted as response units versus time as sensorgram (Fig 3). SPR technique have several advantages like flexible to use with several bio-molecules, requires no radioactive or fluorescent labeled samples, very minute amount of sample required and also can study the molecular interactions in real-time. SPR also have some limitations like nonspecific interactions between sensor

surface and sample, background refractive index variations due to different buffers used in running buffer sample, sample temperature etc.[80]

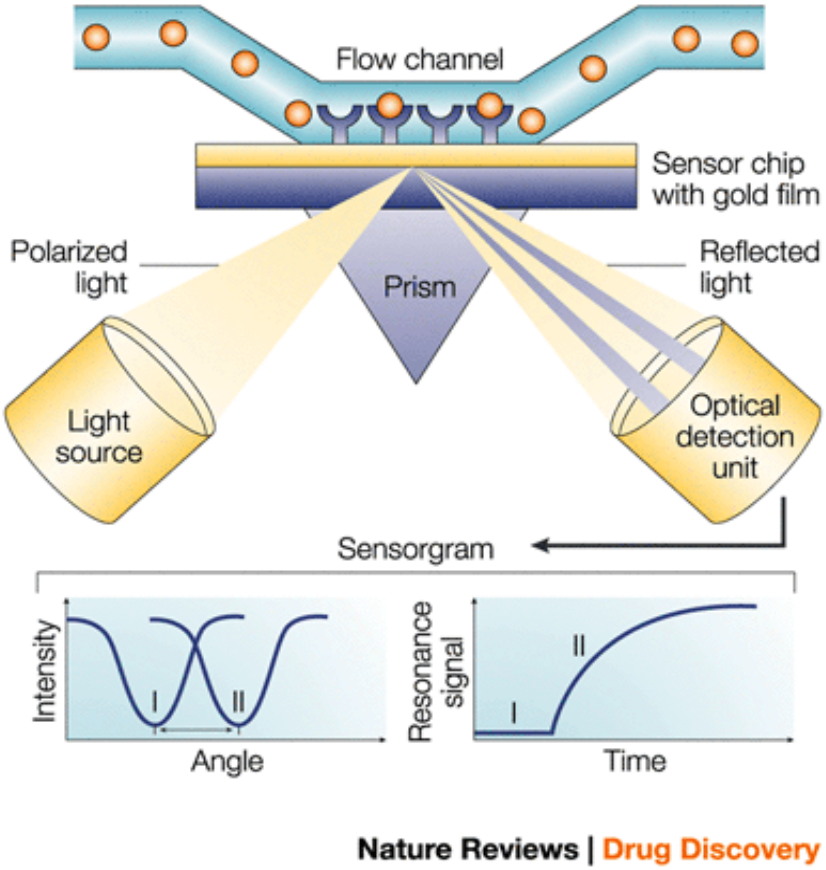

Figure 3: Surface Plasmon Resonance: Change in the refractive index with addition of mass onto the sensor chip, and shift of angle from I to II (lower left) plotted in real time as sensorgram (lower right). Cited from Nature Reviews Drug Discovery, 1,515-528

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CHAPTER II

ORIENTATED GLYCO-MACROLIGAND FORMATION BASED ON SITE-SPECIFIC IMMOBILIZATION OF *O*-CYANATE CHAIN-END FUNCTIONALIZED GLYCOPOLYMER

2.1 Introduction

Glycopolymers as a multivalent clustering of carbohydrate derivates have proven to be an effective tool in the study of carbohydrate-based cellular processes and show great potential in biomedical applications like glycomics, biotechnology, biosensors, and medicine. For example, glycopolymers have been explored for the development of therapeutic agents,[1] in bio and immunochemical assays,[2] and as biocapture reagents[3] applications. It has been found that the shape and size of glycopolymers[4] as well as the density and relative positioning of their glycan appendages[5] turned out to be of high importance regarding their effectiveness in bio-interactions. Therefore, a large variety of various glyco-homo- and (block) copolymers, linear or branched, are reported realizing

high control over the molar mass and the structure of the products.[6] Furthermore, surface-bound multivalent glycan ligands were shown to exhibit higher avidity to protein receptors compared to immobilized monomeric glycans.[7] We first reported a biotin chain-end functionalized glycopolymer for oriented glyco-surface engineering based on specific non-covalent interactions.[8-11] Since then, several groups had demonstrated the potential of biotin chain-end functionalized glycopolymers for bioconjugation applications.[12-16] Chain end functionalized glycopolymers were synthesized by the following techniques.

2.1.1 Synthesis of chain-end functionalized glycopolymer via ROMP

ROMP using a ruthenium-catalyst was frequently employed to synthesize glycopolymers in the early 1990s. ROMP allows glycomonomers to be polymerized in a controlled/living fashion, which will then enable the synthesis of block copolymers. The catalysts used in ROMP are tolerant to many functional groups, thus permits the synthesis with unprotected glycomonomer. In addition, the metal alkylidene catalysts could potentially allow the preparation of specifically end-labeled compounds used for detection in biological systems. Kiessling and co-workers first employed ROMP technique for synthesis of bifunctionalized glycopolymer containing an enol ether at one end and a masked carboxylic acid at the other end to which a fluorescein derivative was conjugated.[17] Recently, Linda C. Hsieh-Wilson et *al*. reported a synthesis of biotin chain-end functionalized glycopolymers that mimic the native-like, multivalent architecture found on chondroitin sulfate (CS) proteoglycans *via* ROMP.[18] Overall, ROMP combined with post-polymerization modification method provided the chain-end functionalized glycopolymers. Although this method often requires an excess of substrate as well as

some purification, it can still be useful. However, one may also argue that the toxic heavy metals used in ROMP could potentially contaminate the final polymers; hence the removal of these catalysts has to be scrutinized if the products are to be used in biomedical applications.[19]

2.1.2 Synthesis of chain end functionalized glycopolymer via ATRP

ATRP is a control radical polymerization technique that has been widely employed to prepare glycopolymers.[20] With this technique, glycopolymers of predictable molecular weights, narrow molecular weight distribution furthermore wide range of functional groups were synthesized. M. G. Finn co workers employed the Cu^{I}–catalyzed ATRP and azide-alkyne cycloaddition reactions together for the synthesis of chain-end functionalized glycopolymers and their attachment to a suitably modified viral protein scaffold.[21] Later, Geng et al. synthesized malimide chain-end functionalized glycopolymer by combining ATRP and Huisgen [2+3] cycloaddition.[22] In this study, bovine serum albumin (BSA) was employed as a single thiol-containing model protein to test the conjugation between thiol and malimide. A fluorescent tag rhodamine B dye was introduced into polymer back bone to improve the characterization of polymer protein conjugation and also for monitoring of their *in vitro* studies.

ATRP technique was also employed by Maynard and coworkers to synthesize a biotin chain-end functionalized glycopolymer with *N*-acetyl-D-glucoamines as pendent carbohydrates.[23] They synthesized both protected and deprotected glycomonomers and subjected them to polymerization, interestingly, by both ways well-defined glycopolymers with narrow polydispersity were achieved. Also different molecular

weight polymers by altering the initial monomer-to-initiator ratios ($[M]_o/ [I]_o$) were synthesized and the molecular weights (M_n) and polydispersity were determined by gel permeation chromatography. The same group later synthesized a pyridyl disulfide chain-end functionalized glycopolymer with the same *N*-acetyl-D-glucoamines as pendent carbohydrate.[24]

Overall, ATRP represents an excellent avenue to controlled polymerization of glycomonomers. The advantage of ATRP is the lower polymerization temperature. The temperature during polymerization is particularly crucial as glycopolymers and their monomers are usually unstable at temperatures higher than 120^{o}C.[25] A concern is the use of toxic Cu ions to control the polymerization, which demands the thorough purification of the glycopolymer if it is for biomedical application.

2.1.3 Synthesis of chain end functionalized glycopolymer via RAFT

The RAFT polymerization is carried out with thiocarbonylthio compounds (of the general formula Z-C(dS)-SR, known as RAFT agents), which reversibly react with growing radicals *via* chain transfer reactions. Consequently, chains undergo successive active/dormant cycles that minimize radical termination processes and lead to a simultaneous growth of all chains. The obtained polymer chains are characterized by the presence of R and Z groups from the RAFT agents at their α- and *ω*-ends, respectively. Then, the modification of the structure of the RAFT agent, that is, the R and Z groups, appears as a highly powerful means for introducing a molecule of interest at polymer chain ends. Therefore, RAFT is one of the promising techniques employed for the synthesis of glycopolymers with chain-end functional groups. Ravin Narain et al.

synthesized a series of glycopolymers using two different glycomonomers by RAFT technique; further these glycopolymers were evaluated for synthesis of glyconanoparicles.[26] Charreyre et al. reported a synthesis of biotin chain-end functionalized glycopolymer - poly(NAM-*co*-GalAm) *via* RAFT technique using biotin-CTA.[27]

Direct polymerization of sugar-containing monomers has been proven to be successful; modification of preformed polymers using saccharide-containing reagents offers an alternative synthetic route. Betrozzi et al. have synthesized an orthogonally end functionalized glycopolymer *via* RAFT and post-functionalization with saccharides.[28] They synthesized mucin mimetic glycopolymer, with one end terminal bearing a surface attachment element and the other end set for conjugation of a reporter group.

Using RAFT technique, the same group later reported a synthesis of biotin-terminal poly(acryloyl hydrazide) (PAH) scaffold which could be conjugated to a range of reducing sugars from simple mono- and disaccharides to highly complex human milk and blood oligosaccharides.[29]

Overall, RAFT polymerization is a popular route for synthesizing glycopolymers. Reaction temperatures used in RAFT polymerization are usually between 60 to 70^{o}C, but also the synthesis of polymers at room temperature is possible. The robustness of this polymerization technique is reflected by the number of publications reporting the synthesis of glyopolymers in past decade by RAFT technique. Comparing to direct polymerization, the post-functionalization approach was demonstrated to be a convenient to produce libraries of glycopolymers with the same macromolecular architecture by attaching different sugar moieties to pre-formed polymer scaffolds. The drawback of the

post-functionalization is the unsure reaction yield of saccharide modification, which might lead to uncontrolled glycopolymers with different saccharide units in the polymer and low reproducibility as well.

2.1.4 Synthesis of chain end functionalized glycopolymer via CMFRP

CMFRP of unprotected glycomonomers can be conducted in aqueous solution, is tolerant of a broad range of functional groups including -OH, $-NH_2$, -COOH, and SO_3^- moieties, and can yield glycopolymers with low polydispersity (PDI < 1.5). CMFRP is a straightforward approach to synthesize chain-end functionalized glycopolymers using functionalized arylamine initiators.

In this study we designed a facile synthesis of amine and carboxylic **acid** chain-end functionalized glycopolymers *via* cyanoxyl mediated free radical polymerization (CMFRP) by using amine and carboxylic acid functionalized aryl amine as initiator, respectively.[11] Interestingly, all these glycopolymers have an *O*-cyanate group at the other chain-end, which might serves as a reactive chain-end group, but not being investigated yet. During the past decades, *O*-cyanate-based isourea bond formation has been proven to be a very useful tool in bioconjugate chemistry such as protein and antibody immobilization.[30-32] Therefore, we envisioned that the chain-end *O*-cyanate group of the glycopolymer provides an anchor for site-specific and covalent immobilization of the glycopolymer onto an amine surface *via* isourea bond formation and thereby facilitates an oriented glyco-macroligand formation (Fig 1). Herein, we report the oriented glyco-marcroligand formation and its glyco-affinity capturing and glycoarray application by

combining *O*-cyanate chain-end functionalized glycopolymer with commercially available amine-modified silica gel and glass slide *via* isourea bond formation, respectively. In this study, the multivalent lactose units serve as model affinity carbohydrate ligands for lectins[33] and cells surface carbohydrate such as GM3 for carbohydrate-carbohydrate interaction-based cell – cell intereactions.[34]

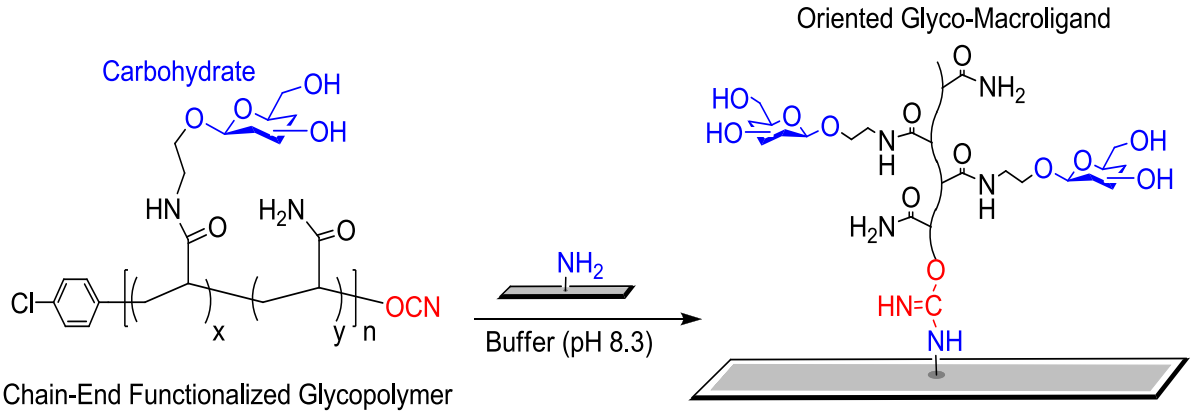


Figure 1. Chemical structure of *O*-cyanate chain-end functionalized glycopolymer and its oriented glyco-macroligand formation *via* isourea bond formation.

2.2 Experimental

2.2.1 Materials and methods

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Deionized water with a resistivity of 18 MΩcm was used as a solvent in all polymerization reactions. Glycomonomer, 2-*N*-Acryoyl-aminoethoxyl 4-*O*-(*β*-D-galatopyranosyl)-*β*-D-glucopyranoside (**3**) was synthesized as described in reported method.[11] Thin-layer chromatography (TLC) was performed on

Whatman silica gel aluminum backed plates of 250 μm thickness on which spots were visualized with UV light or by charring the plate after dipping in 15% H_2SO_4 in methanol. Dialysis was performed on cellulose membrane with a molecular weight cutoff of 3K with water as solvent. ^{1}H spectra were recorded at room temperature with a Varian INOVA 300 MHz spectrometer. In all cases, the sample concentration was 10 mg/mL, and the appropriate deuterated solvent was used as an internal standard. UV-vis absorbance spectra were recorded on Varian Bio50 UV-vis spectrometer. IR spectra were measured on Broker FT-IR spectrometer. Fluorescence spectra were measured on Hitachi F-7000 Fluorescence Spectrophotometer.

2.2.2 Synthesis of O-cyanate chain-end functionalized glycopolymer 4

In a three-necked flask, dissolved 4-chloroaniline (21.6 mg, 1.69 x 10^{-4} mol), sodium nitrite (14.1 mg, 2.04 x 10^{-6} mol) in a mixture of 2 mL water and THF (1:1, v/v). To the above mixture, added HBF_4 (66 mg, 7.51 x 10^{-4} mol) and allowed it to react for 30 min at 0°C under Ar atmosphere. Following this, a degassed mixture of 2-*N*-Acryoyl-aminoethoxyl 4-*O*-(β-D-galatopyranosyl)-β-D-glucopyranoside **3** (186 mg, 2.65 x 10^{-5} mol), acryl amide (210 mg, 2.65 x 10^{-4} mol) and NaOCN (55.2 mg, 8.49 x 10^{-4} mol) dissolved in 1 mL of water was added into the flask containing diazonium salt. The reaction solution was thus heated at 65°C for 16 hrs, and then was filtered to remove any precipitates. The resultant mixture is separated of any inorganic salts and impurities by dialysis against deionized water for 2 days at room temperature to afford glycopolymer **4** (248 mg). The conversion yield was about 60%, which was determined by weight for the resultant glycopolymer.

2.2.3 Quantification of carbohydrate in glycopolymer (Phenol-Sulfuric Acid Assay)

Phenol-sulfuric acid assay was used to quantify the lactose molecules attached on the glycopolymer **4**. To 0.5 mL of sugar solution, added 0.5 mL of 5% aq. phenol solution, 2.5 mL of H_2SO_4 (96%) and vortexed the mixture. Prepared the blank with distilled water replacing the sugar solution. Allowed above solutions to stand for 30 minutes at room temperature and measured the absorbance at 490 nm. A calibration curve of sugar (β-lactose) concentration versus absorbance (A) was plotted and the presence of sugar on the polymer was detected by measuring the absorbance of glycopolymer samples (0.1 moles, 0.5 moles and 1 mole).

2.2.4 Immobilization of glycopolymer 4 onto amine-modified silica gel through isourea bond formation and its affinity lectin capture

Glycopolymer **4** (8.5 mg, 1.21 x 10^{-3} mol) was added to 200 mg of amine functionalized silica gel beads (Sigma) in 2 mL of $NaHCO_3$ buffer (pH 8.3). The mixture was shaked for 4 hrs at room temperature and then was packed into a column. The column was washed with $NaHCO_3$ buffer (pH 8.3) followed by PBS buffer (pH 7.4). Lectin-FITC solution (*Arachis hypogae,* FITC-Labeled, 0.5 mg, 4.16 x 10^{-9} mol) in PBS buffer (pH 7.4) (1 mL) was loaded onto the column. The drained solutions were collected and preserved for further analysis. The column was then washed with PBS buffer (pH 7.4) to remove the unbounded lectin. Finally, the captured lectin was released by eluting with free lactose (1 M) and was analyzed by SDS-PAGE and quantified by fluorescence spectroscopy.

2.2.5 Oriented glycopolymer glycoarray via O-cyanate-based isourea bond formation

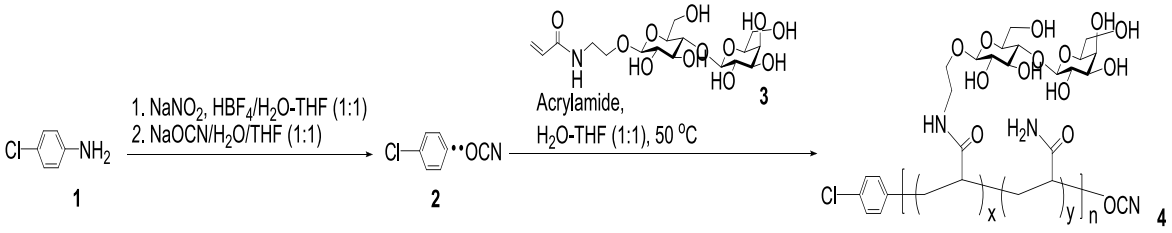
Glycopolymer microarrays were fabricated by microcontact stamping with MicroCaster Arrayer (Whatman). The MicroCaster array tool (spot size 500 μm diameter) was inked with a solution of glycopolymer (**4**, 1 mg/mL, 1.4 x 10^{-7} moles) in $NaHCO_3$ buffer (pH, 8.3). The MicroCaster tool was pressed onto amine functionalized glass slide (Xenopore, Co) for 10 min. The glass slide was incubated in a humidifier chamber for 4 hrs and then washed for 1 hr (3 times) with respective buffers followed by washing with PBS containing 0.2% tween 20 (PBST) to minimize nonspecific binding of proteins to the surface. Then the glass slides were incubated with lectin-FITC **(**Arachis hypogae, FITC-Labeled, Sigma**)** solution (0.2 mg/mL) in PBST (pH 7.4) buffer for 3 hrs followed by extensive washing with PBST buffer for 1 hr and subjected to fluorescent imaging by using Typhoon 9410 Variable Model Imager (Amersham Biosciences, USA). A control was prepared by incubating the glycopolymer printed glass slide with lactose (200 mM, PBS (pH 7.4)) pre-incubated lectin-FITC.

2.3 Results and discussion

2.3.1 One-pot synthesis and characterization of O-cyanate chain-end functionalized glycopolymer

The synthesis of glycopolymer with chain-end functionalized still poses significant challenges, including a requirement for serial protection/deprotection steps or further polymer derivatization after initial synthesis. In the present study, the *O*-cyanate chain-end functionalized glycopolymers were synthesized *via* our previously developed cyanoxyl free radical mediated copolymerization scheme in one-pot fashion (Scheme

1).[18] Briefly, 4-chloroanaline **1** was used as initiator for the copolymerization of acrylaminoethyl lactoside **3** and acrylamide. Specifically, 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. A series of *O*-cyanate chain-end functionalized glycopolymers of varying sugar density were prepared by altering glycomonomer (GM) and acrylamide (AA) in good conversion yield (60-70%) and low polydispersity (1.1 < Mw/Mn < 1.6) as described previously.[11]



Scheme 1. Synthesis of *O*-cyanate chain-end functionalized glycopolymer.

The chain-end *O*-cyanate functional group of glycopolymer **4** was confirmed by IR spectrum, in which *O*-cyanate absorption was observed at 2157 cm^{-1} (Fig 2A). It is also noteworthy that the *O*-cyanate group could be converted to a hydroxyl group by treatment with pyridine in water.[35] Therefore, the disappearing of *O*-cyanate absorption band at 2157 cm^{-1} after pyridine treatment of glycopolymer **4** (to **5**) confirmed the existing of *O*-cyanate chain-end group and complete hydrolysis of the *O*-cyanate group

(Fig 2B). By comparison of ^{1}H NMR of **4** and **5** (Fig 3), there was no apperent polymer structural change observed during the hydrolysis reaction.

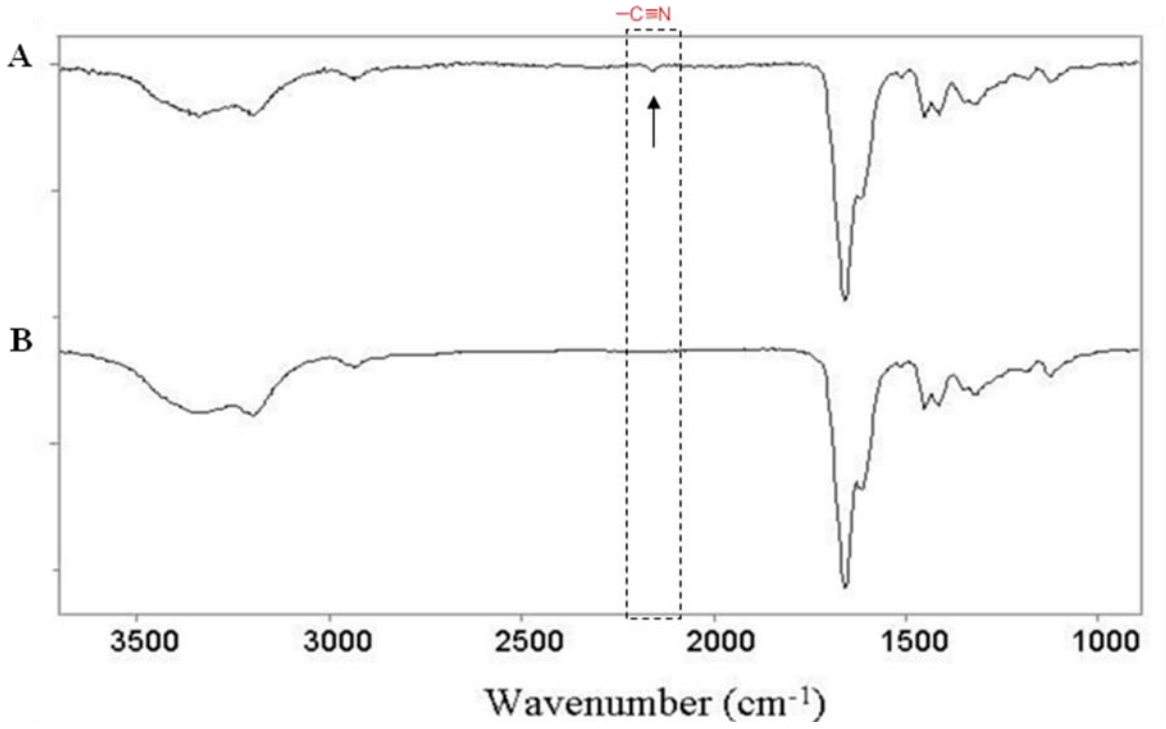


Figure 2. FT-IR spectra of *O*-cyanate chain terminated glycopolymer **4** (**A**) and hydroxy chain terminated glycopolymer **5** (**B**).

^{1}H NMR spectrum was used to calculate of polymer component content and length by using phenyl group at the polymer other chain terminal. As shown in ^{1}H NMR spectrum of glycopolymer **4** (Fig 3A), comparison of the integrated signal from the phenyl protons (7.04 ppm, $H_{2',6'}$ and 7.22 ppm, $H_{3',5'}$) with that due to the anomeric protons of lactose (4.36 ppm, $H_{1'\text{-Lact}}$ and 4.43 ppm, $H_{1\text{-Lact}}$), as well as that of the polymer backbone methine (2.10 ppm, CH) and methylene (1.55 ppm CH_2), indicated an average glycopolymer composition of 10 lactose and 70 acrylamide units. The molecular weight (Mn) was about 7,000 as determined by ^{1}H NMR. Furthermore, the grafted carbohydrate

on the glycopolymer **4** was quantified *via* the phenol-sulfuric acid method.[37] Briefly, concentrated H_2SO_4 was added directly into a solution of phenol and glycopolymer **4**. The mixture was then vortexed, and allowed to stand for 30 min at room temperature. The optical density was then recorded at 490 nm. As a result, there was an average of 8 lactose units per polymer (Fig 4) , which is close to that determined by the ^{1}H NMR above.

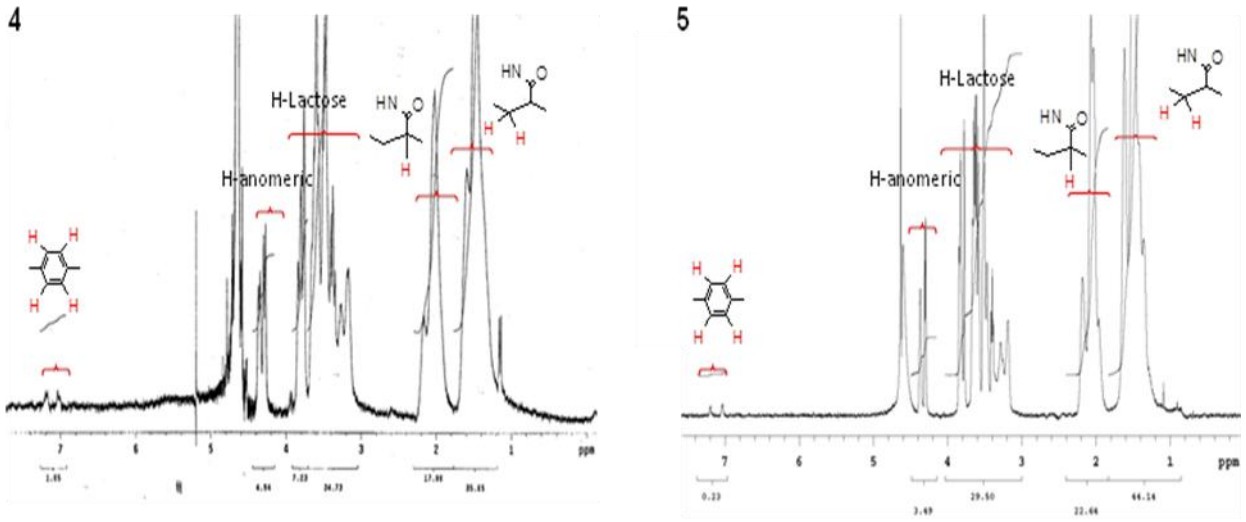


Figure 3. ^{1}H NMR spectra of *O*-cyanate chain terminated glycopolymer **4** (**A**) and hydroxyl chain terminated glycopolymer **5** (**B**) in D_2O.

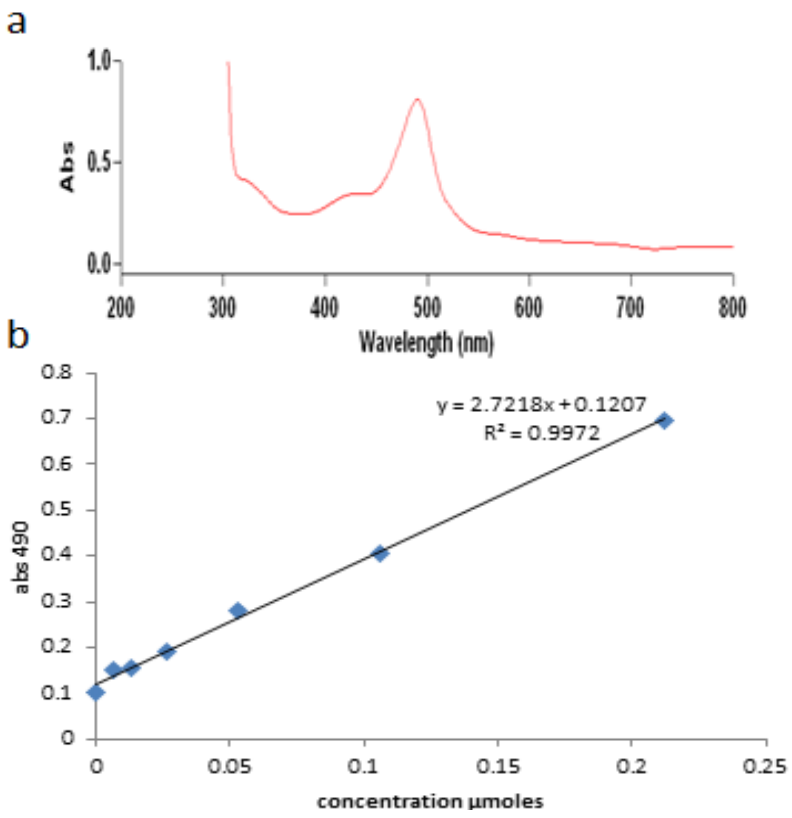


Figure 4. a.UV Absorbance spectrum of sugar-phenol conjugate b. A calibration curve of sugar (β-lactose) concentration versus absorbance (A)

2.3.2 Immobilization of glycopolymer onto silica gel via O-cyanate-based isourea bond formation and its affinity chromatography application

Silica gel has been widely used as small, rigid particle for high performance affinity chromatography since it is capable of withstanding the high flow rates and /or pressures. The introduction of functional groups on a silica surface to yield modified silica gel has received great attention for the development of functionalized silica gel for chromatography application.[37] In particular; methods such as covalent grafting of

polymers or coating with hydrophilic polymers have succeeded in passivating the silica surface and conferring specificity. Recently, silica materials containing covalently bound sugars have been explored for boron removal[38] and isolation and purification of lectins[39-40] applications. In the present study, oriented immobilization of multivalent carbohydrates onto silica gel surface was investigated for efficient and inexpensive isolation and purification of carbohydrate-binding proteins (Fig 5). Briefly, *O*-cyanate end-terminated glycopolymer **4** was immobilized onto the surface of silica gel by incubation of **4** with 3-aminopropyl-functionalized silica gel (Sigma) in sodium bicarbonate (pH 8.3) buffer solution at room temperature for 2 hrs and followed by thoroughly washing with *di* water three times. Control experiment with the hydroxyl chain-end functionalized glycopolymer **5** was conducted in the same scale. The amount of glycoplymer immobilized on the silica gel was determined by phenol-sulfuric acid method [37]. Briefly, phenol solution was added to a solution of glycopolymer-silica gel, and mixed. Then concentrated H_2SO_4 was added directly into the solution. The mixture was then vortexed, and allowed to stand for 30 min at room temperature and finally centrifuged. The optical density of the supernatant was then recorded at 490 nm. As a result, there was an average of 276 nmol of *O*-cyanate chain terminated glycoplymer **4** per gram of silica gel, while there was no detectable glycopolymer determined with hydroxyl chain terminated glycopolymer **5** treated silica gel. This result indicated specific immobilization of glycopolymer through isourea bond formation.

Once the glycopolymer-silica gel (GP-SG) was synthesized, its ability to capture lectin was evaluated as an adequate methodology to characterize and determine their potential applicability as a chromatographic support. The characterization was carried out

by packing the glycopolymer-silica gel in a column followed by saturating the column with a β-galactose-specific lectin (*Arachis hypogae,* FITC-Labeled, 0.5 mg, 4.16 x 10^{-9} mol, Sigma) in PBS (pH 7.4) buffer solution and followed by washing with PBS (pH 7.4) buffer to remove the unbounded lectin (Fig. 5A). Lectin capturing was confirmed with visualization of FITC-labeled lectin on the glycopolymer silica gel column under UV lamp (Fig 5B1), while raw silica gel showed little non-specific capturing of the lectin (Fig 5B2). Further, there was no capturing was observed for the non-specific lectin (glucose/mannose-specific Concavalin A) on the glycopolymer silica gel (data not shown). Next, eluting the column with 1 M free lactose in PBS (pH 7.4) buffer released the specific captured lectin on the glycopolymer-silica gel (Fig 5C1). A similar protocol was followed with raw silica that was thus established as a control assay (Fig 5C2). The lectin-capturing capacity was 13.9 nmol of lectin per mg of silica gel modified with glycopolymer, while 2.9 nmol of lectin per mg of raw silica gel as non-specific binding as determined by fluorescence intensity analysis. Finally, the specific captured lectin was confirmed with SDS-PAGE (Fig 6). Specifically, the captured lectin on glycopolymer modified silica gel was confirmed on protein staining gel (Gel A, lane 4) and silver staining gel (Gel B, lane 4), while no non-specific lectin (glucose/mannose-specific Concavalin A) on the glycopolymer silica gel was observed on protein staining gel (Gel A, lane 5) and silver staining gel (Gel B, lane 5). These results demonstrated the successful oriented glyco-marcroligand formation and its carbohydrate-binding protein purification and identification capacity.

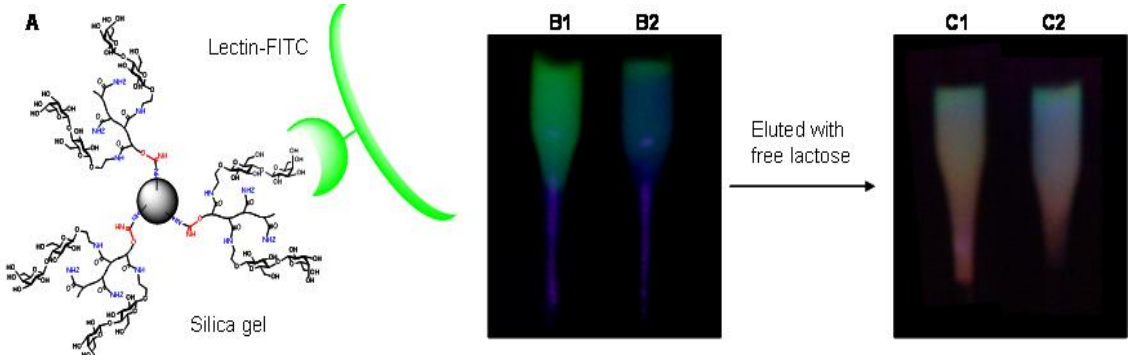


Figure 5. Illustration of glycopolymer immobilization onto silica gel and its lectin capturing onto glycopolymer-silica gel (**A**), Lectin capturing onto glycopolymer-silica gel column (**B1**) and raw silica gel (**B2**), after elution with free lactose (**C1** and **C2**).

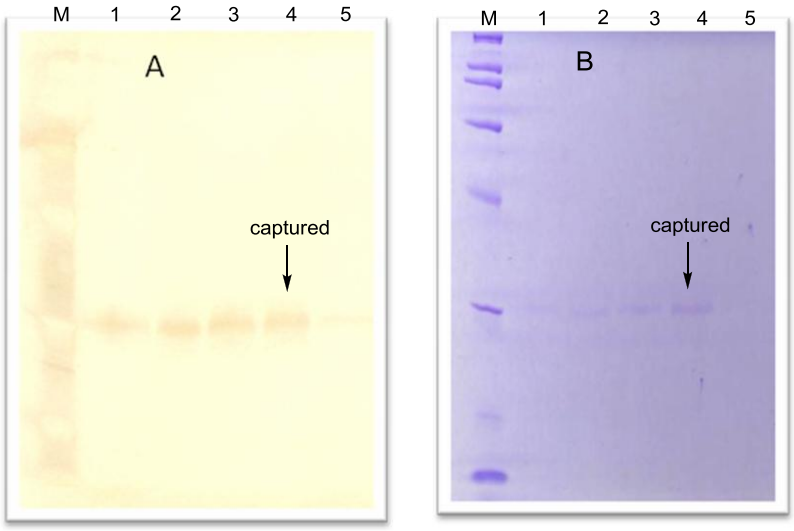


Figure 6. SDS-PAGE identification of unbound and released lectins from silica gel beads (**A**: silver staining; **B**: Protein staining): M: MW marker, 1: lectin alone, 2: unbound lectin from glycopolymer-silica gel, 3: unbound lectin from control raw silica gel, 4: captured lectin eluted from glycopolymer-silica gel, 5: non-specific lectin eluted from glycopolymer-silica gel.

2.3.3 Oriented glycopolymer glycoarray via O-cyanate-based isourea bond formation

Surface-bound multivalent glycan ligands showed higher avidity to protein receptors compared to immobilized monomeric glycans.[14] Inspired by this example, we sought to develop oriented glycopolymer array that spatially position the pendant glycans similarly to natural glycoproteins. Specifically, integration of such constructs into arrays may create a more physiologically authentic platform for probing glycan binding proteins. In the present study, glycopolymer microarray (spot size 500 μm diameter) was fabricated by microcontact stamping glycopolymer 4 onto amine functionalized glass slide (Xenopore, Co) in $NaHCO_3$ buffer (pH, 8.3) (Fig 7). The glass slide was incubated in a humidifier chamber for 4 hrs and then washed for 1 hr (3 times) with respective buffers followed by washing with PBS containing 0.2% tween 20 (PBST) to minimize nonspecific binding of proteins onto the surface. Then the glass slides were incubated with lectin-FITC (*Arachis hypogae,* FITC-Labeled, Sigma) solution in PBST buffer for 3 hrs followed by extensive washing with PBST buffer for 1 hr and finally subjected to fluorescent imaging. A control was prepared by incubating the glycopolymer arrayed glass slide with lactose (200 mM) pre-incubated lectin-FITC instead of free lectin. Fluorescence microscopy analysis of the surfaces revealed specific binding of lectin only to the immobilized glycopolymer (Fig 7B). The lectin binding to arrayed glycopolymer was inhibited in the presence of free lactose (Fig 7C), further confirming the specific lectin binding to the glycopolymer. Moreover, glycopolymer array with different concentration of glycopolymer showed concentration-dependent lectin binding to glycopolymer (Fig 7D). These observations indicated that arrayed glycopolymers can distinguish glycan-binding proteins based on their ligand specificity.

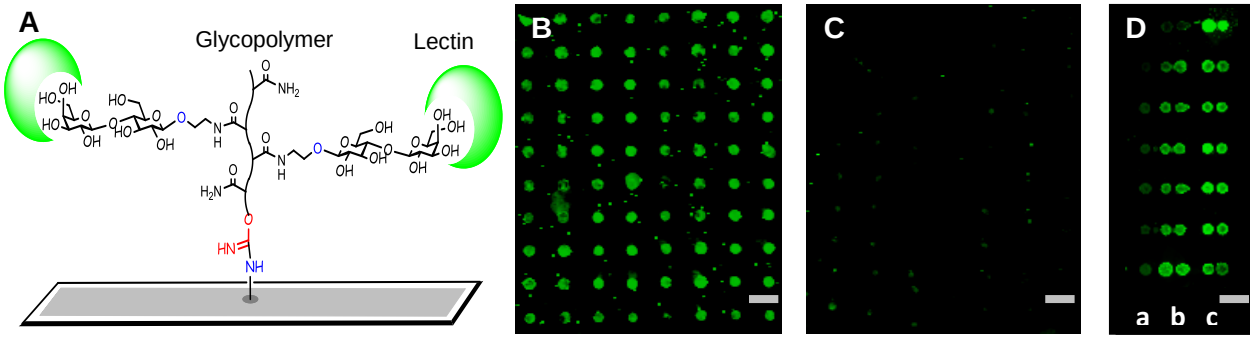


Figure 7. Glycopolymer microarray for probing specific glycan–protein interactions (**A**), fluorescent imaging of FITC-labeled lectin binding to microarrayed patterns of glycopolymers (**B**), control with lactose pre-incubated lectin-FITC (**C**) and with different concentration of glycopolymer in array (a: 0.001 mg/mL, b: 0.1 mg/mL and c: 0.5 mg/mL) (**D**). Bar size: 500μm.

2.4 Conclusions

An oriented glyco-marcroligand formation based on *O*-cyanate chain-end functionalized glycopolymer with commercially available amine modified silica gel and glass slide *via* isourea bond formation was demonstrated, respectively. The *O*-cyanate chain-end functionalized glycopolymer was synthesized *via* one-pot cyanoxyl mediated free radical polymerization. It is notable that the polymerization can be performed under aqueous condition and is tolerant of a wide range of monomer functionalities, including -OH, -COOH, $-NH_2$ and $-OSO_3^-$ groups.[18] Therefore, there is no protection and deprotection needed and thus is a straightforward approach.

Notably, multivalent lactose units serve as model ligands but can be changed to any specific carbohydrates for specific lectins or cells. The chain-end *O*-cyanate group of the polymer provides an anchor for site-specific and covalent immobilization of the

glycopolymer onto any amine surface *via* isourea bond formation in mild condition, which can be used for biosensor and glycoarray applications. In addition, the chain-end functionalizable glycopolymer can be used for polymer-protein conjugation as well.

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CHAPTER III

GLYCO-MACROLIGAND MICROARRAY WITH CONTROLLED ORIENTATION AND GLYCAN DENSITY

3.1 Introduction

Glycan microarray has become powerful high-throughput tool for examining binding interactions of carbohydrates with lectins, antibodies, cells, and viruses.[1] Recently, it has been applied to clinical antibody detection and profiling,[2] vaccine development,[3] biomarker discovery,[4] and drug screening[5] applications. Nevertheless, they are still not perfect for clinical applications and novel microarray designs are much needed to improve the performance of this technology. Two critical limitations prevent wide and potential applications of the glycan microarray technology. First, the detection is limited by restricted epitopes available for microarray fabrication from both synthesis and isolation from nature sources. Second, the features of glycan presentation on the microarray surface such as density and orientation of glycans have substantial effect on protein recognition related to both affinity and specificity. Conventional glycan microarrays were made by directly immobilizing glycans onto microarray surfaces by

either physical absorption or covalent conjugation, which directly mimics the multivalent display of carbohydrate epitopes on the cell surface. However, this two dimensional (2D) surface immobilization chemistry usually results in low signal intensity and substantial non-specific binding of target proteins due to an insufficient number of accessible glycans and the presence of surface-protein interactions. To overcome this limitation, recently, glycan density on the array surface has been recognized as very important feature of carbohydrate recognition and thus has been investigated extensively.[6-12]

A general strategy for modulating glycan density is to vary the concentration of the monovalent glycans in the printing solution that is being spotted onto a microarray slide. However, this approach is not generating variations in density in the molecular level and often yields inconsistent results. Particularly, often variations in density gradient over the spot are seen when the surface is not saturated with diluted printing solution. Alternatively, new strategies have been developed, in which multivalent glycoconjugates with varying density were first synthesized, and then printed onto a solid support to generate a microarray of multivalent glycans in different densities.

Another key feature of the glycan presentation in glycan microarray is the glycan orientation. Cell surface glycans are spatially displayed such as glycoprotein scaffolds with three-dimensional (3D) geometries. However, conventional glycan arrays present glycans on a 2D substrate and thus have limited activity. Early study confirmed that surface-bound multivalent glycan ligands show higher avidity to protein receptors compared to immobilized monomeric glycans.[13]

3.1.1 Glycodendrimer-Based Glycoarray

Pieters and coworkers have studied the binging of lectins to carbohydrates with respect to the effect of multivalency of carbohydrate moieties and the inter-binding-site distances of lectins[14-15] They primarily started with synthesized mannose based glycodendrimers and tested against two different lectins ConA and WGA[14] later enlarged their work by synthesizing five different glycodendrimers to evaluate a series of lectins.[15] The same phenomenon was observed with the other eight lectins that were tested against five different glycodendrimers, lectins with increase in inter–binding-site distance displayed decrease in the multivalency effect.

Wang and coworkers employed a different approach for construction of glycodendrimer microarray in their recent studies[16] by initially preparing a three dimensional dendrimeric platform followed by linking the carbohydrate to the dendrimers surface. The cluster effect was tested by spotting oligosaccharides dendrimers of all five generation which were further incubated with lectin and observed that with increase in the surface groups the fluorescent intensity also increase exhibiting the multivalency effect.

Overall, glycodendrimers were successfully synthesized for microarray applications and were qualified in the screening of carbohydrate-protein interactions through carbohydrate microarrays.

These glycodendrimers microarrays not only explored the protein-carbohydrate interactions but also reported how the multivalent carbohydrate display on the array surface aids the protein binding to carbohydrates in the aspects of both sensitivity and selectivity. From the reported glycodendrimers microarrays it is accepted that the

multivalency of the carbohydrates is well controllable by dendrimers but the spacing and orientation of the carbohydrates on the array surface are not yet organized to study their effect on protein binding to carbohydrates.

3.1.2 Neo-Glycoprotein-Based Glycoarray

Gildersleeve et al have developed a method for varying both the structure and density of the carbohydrates on the carbohydrate antigen microarray and evaluated the density dependent binding effects for tumor- binding lectins, monoclonal antibodies and serum antibodies.[5] For this approach they have conjugated carbohydrate to carrier protein like bovine serum albumin (BSA), in which the carrier protein served as both the multivalent scafold and the linker for immobilization on to glass slides. Carbohydrates were conjugated to free amine groups of BSA *via* reductive amination of oligosaccharides or by coupling the oligosaccharides with carboxylic acid *via* activation with EDC/NHS. To vary the density number of carbohydrates attached to the BSA were controlled.

Gildersleeve's group[17] synthesized several neo-glycoconjugated with variying carbohydrate density. They have observed that selectivity of lectins for particular carbohydrate over other carbohydrates declined at high density. Followed by this they evaluated the density –dependent binding properties of a set of monoclonal antibodies and determined that antibodies to the same carbohydrate antigen can recognize density in significantly different ways. Also they have tested the density-dependent binding properties of serum antibodies of 30 subjects, the results demonstrated that modulation of antigen density can be used to reveal differences in antibody populations between different subjects. Their results from the above study reveled that both structure and the

density of the carbohydrates significantly affect the affinity and selectivity to carbohydrate binding compounds. Gildersleeves et al proved that the density of the carbohydrates on the surface are significant in studying in carbohydrate binding ligands.

3.1.3 Liposome-based glycoarray

Our group recently investigated the application of azide-reactive liposome for efficient and chemically selective immobilization and microarray fabrication[18]. Azide reactive liposomes were synthesized with DSPE-PEG2000-triphenylphosphine, phospholipids (DSPE) and cholesterol rapid extrusion method. Azide reactive liposomes were immobilized on azide functionalized glass slides and later subjected to and lectin binding studies were conducted by incubating the liposome immobilized glass slide with lectin PNA and observed the lectin binding to lactosylated liposome and no binding observed to liposome with just anchor group.

Later this work was extended to study the glycolipid and protein interactions[19] by incorporating natural and synthetic glycolipids into liposomes, and immobilizing them onto an azide functionalized glass slide to test the binding affinities of lectins. These microarrays mimic the presentation of carbohydrates on the cell surface and the binding curves indicate that the liposomal microarrays with different glycolipids show specific density dependent binding capacity and affinity as well.

3.1.4 Glycopolymer-Based Glycoarray

Glycopolymers carrying pendant sugar moieties are either synthesized by direct polymerization of carbohydrate-containing monomers with protection group or without

protection group, or by the post polymerization glyco-conjugation of synthetic polymers.[20] In recent years glycopolymers have been synthesized by several techniques for various biomedical applications particularly glycopolymers with end functional group have been synthesized excessively for microarray applications. Chain-end functionalized glycopolymers demonstrated homogeneous immobilization of the glycopolymer *via* a single terminal anchor to yield an oriented and density controlled displaying of multivalent pendent carbohydrates.

Bertozzi's group synthesized a duel end functionalized mucin like glycopolymer[21] with a center mucin mimetic domain with N-actylgalactosemamine moieties, one terminal alkyne group as surface attachement element and a second terminal outfitted for conjugation of texas red as a fluorophore. By microcontact printing the alkyne terminal glycopolymers were immobilized onto azide functionalized surface in presence of a copper catalyst, which forms a stable triazol linkage to covalently immobilize the glycopolymer. As the glycopolymer was also functionalized with fluorescent tag Texas red on the other end the immobilization of polymer was confirmed by fluorescent microscopy. Also the ligand specificity of glycans of the polymer was tested by immobilizing non-fluorescent tag glycopolymer and testing the binding of Texas Red tagged *Helixpomatia agglutinin* (Texas Red-HPA) that recognizes α-GalNAc, the analysis of the surface by fluorescent microscopy revealed the specific binding of HPA only to α-GalNAc confirming the specific binding of glycans. The same group later, developed an approach synthesis of glycopolymers for microarray applications *via* ligation of reducing sugars to backbones carrying hydrazide groups and end functional group.[22] They arrayed a group of biotinylated glycopolymers onto streptavidin-coated

glass slides which were recognized by lectins specifically depending on their pendant glycans.

Recently, Linda C. Hsieh-Wilson et al. synthesized a biotin end functionalized glycopolymer that mimic chondroitin sulfate proteoglycans *via* ring-opening metathesis polymerization technique[23] which were employed for microarray and SPR applications. Both sulfated CS-E and nonsulfated CS-C glycopolymers were immobilized onto streptavidin coated glass slides by high-precision contact-printing robot which delivers a nanoliter volmes of glycopolymer onto glass slides. Polymer immobilized glassslides were tested for binding of specific monoclonal antibodies 2D11 and 2D5, which are selective for CS-E and CS-C glycopolymers respectively. And they also studied the binding of a growth factor, glial cell-derived neurotropic factors selective for sulfated epitopes, which showed higher selectivity for CS-E compared to CS-C by both microarray and SPR techniques.

Nevertheless, the density of the immobilized glycopolymer on the array surface is still uncontrollable, and thus, the possibility to access the multivalent glycans in parallel might be limited and thus does not facilitate maximum protein binding affinity and specificity. In this study, we studied an oriented and density controlled glycopolymer microarray formation based on end-point immobilization of glycopolymer combined with molecular spacing technique. Briefly, *O*-cyanate chain-end functionalized glycopolymer was pre-complexed with boronic acid ligands in different sizes and then immobilized onto amine-functionalized glass slide *via* isourea bond formation at high pH value (pH 10.3 buffer). Once the immobilization is complete, the spacer boronic acid ligands were released from the immobilized glycopolymers at a reduced pH value (pH 7.4 buffer)

condition so as to afford the oriented and density controlled glycopolymer microarray (Fig 1). This glyco-macroligand microarray platform will facilitate both affinity and specificity of protein binding and thus will provide a versatile tool for profiling glycan recognition for both basic biological research and practical applications.

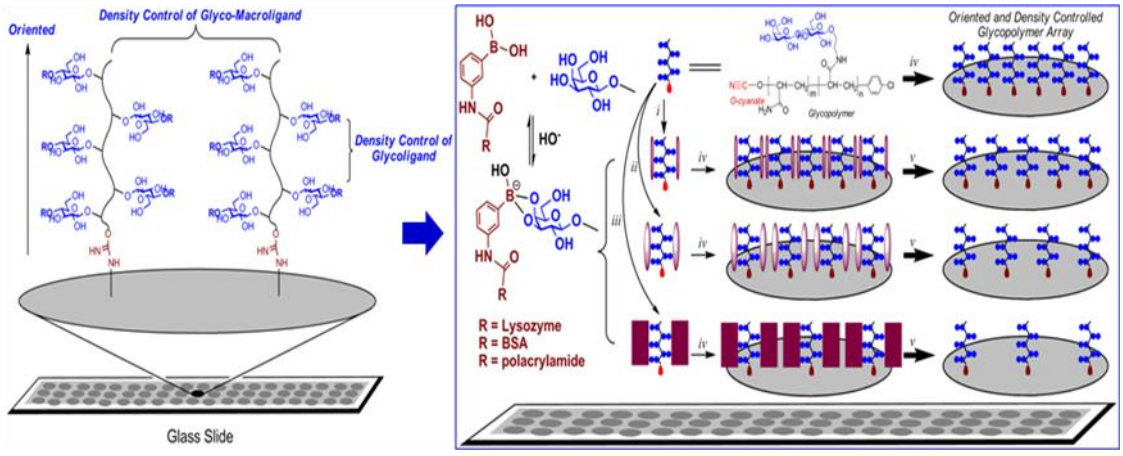


Figure 1. Schematic illustration of orientation and density controlled glycopolymer microarray formations based on end-point immobilization of glycopolymers that were acompanied with boronic acid (BA) ligands in different sizes as temporory molecular spacers followed by releasing the BA ligands from the immobilized glycopolymers. *Reaction conditions*: *i*. R_1-BA, $NaHCO_3$ buffer (pH 10.3), *ii*. R_2-BA, $NaHCO_3$ buffer (pH 10.3), *iii*. R_3-BA, $NaHCO_3$ buffer (pH 10.3), *iv*. $NaHCO_3$ buffer (pH 10.3), *v*. 1 mM Glucose, PBS buffer (pH 7.4)

3.2 Experimental

3.2.1 Materials and Methods

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Lysozyme, bovine serum albumin, amino phenyl boronic acid, FITC-PNA (*Arachis hypogae)* and alizarin red S were purchased from Sigma. lectin FITC-RCAI (*Ricinus communis)* was purchased from EY Laboratories. amine functionalized glass slides were purchased from Xenopore, Co.

3.2.2 Syntheses of O-cyanate chain-end functionalized glycopolymers

Glycopolymers were synthesized according to our previously reported method.[24] Briefly, in a three-necked flask, dissolved 4-chloroaniline (21.6 mg, 1.69 x 10^{-4} mol), sodium nitrite (14.1 mg, 2.04 x 10^{-6} mol) in a mixture of 2 mL water and THF (1:1, v/v). To the above mixture, added HBF_4 (66 mg, 7.51 x 10^{-4} mol) and allowed it to react for 30 min at 0°C under Ar atmosphere. Following this, a degassed mixture of 2-*N*-Acryoyl-aminoethoxyl 4-*O*-(*β*-D-galatopyranosyl)-*β*-D-glucopyranoside (186 mg, 2.65 x 10^{-5} mol), acryl amide (210 mg, 2.65 x 10^{-4} mol) and NaOCN (55.2 mg, 8.49 x 10^{-4} mol) dissolved in 1 mL of water was added into the flask containing diazonium salt. The reaction solution was thus heated at 65°C for 16 hrs, and then was filtered to remove any precipitates. The resultant mixture is separated of any inorganic salts and impurities by dialysis against deionized water for 2 days at room temperature to afford glycopolymer (248 mg). The conversion yield was about 60%, which was determined by weight for the resultant glycopolymer. Four kinds of glycopolymers with different ratios of pendant glycan and molecular weights (**1a, 1b, 1c** and **1d**) were obtained by using different ratios of glycomonomer (GM) to acryl amide (AA) and were characterized by ^{1}H NMR spectra (Table 1 and Figure 2).

3.2.3 Syntheses of boronic acid (BA) ligands

3.2.3.1 Synthesis of Lysozyme-BA: To 5 mL ice cold 0.05 M MES (pH 6), add 100 mg of lysozyme (6.9 μmoles), 20 mg of aminophenyl boronic acid (APBA) (130 μmoles) and 10 mg of EDC with constant stirring. The reaction was allowed to take place for 2 hrs at room temperature, then adjust the pH to 7 and let the reaction continue overnight at room temperature. Separate the salts and the unreacted APBA by centrifugation in 10 kDa cut-off filter tube for 30 mins. The resultant lysozyme-BA conjugate was characterized by SDS-PAGE and Alizarin Red S assay (ARS) assay (Fig. 5A).

3.2.3.2 Synthesis of BSA-BA: To 5 mL ice cold 0.05 M MES (pH 6), add 100 mg of BSA (1.5 μmoles), 20 mg of APBA (130 μmoles) and 10 mg of EDC with constant stirring. After 2 hrs, adjust the pH to 7 and let the reaction sit overnight at room temperature. Separate the salts and the unreacted APBA by centrifugation in 10 kDa cutoff filter tube for 30 mins. The resultant BSA-BA (BSA-BA) conjugate was characterized by SDS-PAGE and ARS assay (Figgure 5B).

3.2.4 Glycopolymer microarray formation and lectin binding assay

MicroCaster microarray tool (Whatman, spot size 500 μm diameter) was inked with a solution of glycopolymer *(1a, 4.8 X 10^{-5} mM)* in $NaHCO_3$ buffer (pH 10.3), then was pressed onto an amine functionalized glass slide (Xenopore, Co) at room temperature for 10 mins. The glass slide was then incubated in a humidifier chamber at room temperature for 4 hrs and then washed with $NaHCO_3$ buffer (pH 10.3) for 30 mins (3 times) to removed un-reacted glycopolymer, then followed by washing with 0.2% PBST for 30

min. The glass slides were then incubated with lectin-FITC (*Arachis hypogae),* FITC-Labeled, Sigma, 0.2 mg, 1.61 X 10^{-9} moles) PBS containing 0.2% Tween 20 (PBST) solution at room temperature for 3 hrs followed by extensive washing with PBST buffer for 30 mins. Finally, the glass slide was subjected to fluorescence imaging and the fluorescence intensity was recorded by using Typhoon 9410 Variable Model Imager (Amersham Biosciences, USA). In the same condition, glycopolymer microarrays with glycopolymer 1b, 1c, 1d were conducted, respectively.

3.2.5 Density controlled glycopolymer microarray formation and lectin binding assays

Glycopolymer (**1b**, 3 mg, 7.7 x 10^{-5} mmoles) was mixed with polyacrylamide-BA ligand (3 mg, 1.5 x 10^{-4} mmoles) in 3 mL of 10.3 pH $NaHCO_3$ buffer and was stirred at room temperature for 4 hrs. MicroCaster microarray tool (Whatman, spot size 500 µm diameter) was inked with the glycopolymer- boronic acidcomplex solution (1 mg/mL, 1:2 mol) and then was printed onto an amine functionalized glass slide in $NaHCO_3$ buffer (pH 10.3). The glass slide was then incubated in a humidifier chamber for 4 hrs and then washed with $NaHCO_3$ buffer (pH 10.3) 30 mins (3 times) to remove un-reacted glycopolymer and boronic acid ligands. Detachment of boronic acid ligand from the immobilized glycopolymer was performed by incubating the glass slide with 7.4 pH PBS buffer for 30 mins and followed by 7.4 pH PBS buffer with 1 mM glucose for 10 mins. Boronic acid ligands detached from the glycopolymer were confirmed by ARS assay (fig 7). The glass slide was washed with PBST buffer for 30 mins then incubated with lectin-FITC (Arachis hypogae, FITC-Labeled, Sigma, 0.2 mg, 1.61 X 10^{-9} moles) in 0.2% PBST solution at room temperature for 3 hrs, followed by extensive washing with PBST

buffer for 30 mins and subjected to fluorescent imaging and the fluorescence intensity was recorded as above. In the same condition, density controlled glycopolymer microarray formation of glycopolymer **1b** spaced with polyacrylamide-BA in different ratios (as shown in Figure 8) were prepared and their lectin binding were tested, respectively.

Similar protocol was followed for glycopolymer **1b** microarray formation spaced with other two boronic acid ligands conjugates (BSA-BA and Lyz-BA) in different ratios (Figure 8) and their lectin (*Arachis hypogae*) binding assays, respectively.

Similar protocol was followed for spaced glycopolymer **1b** microarray formation spaced with these three boronic acid ligands in different ratios and their lectin *Ricinus communis* (RCAI, FITC-labeled, EY Laboratories, Inc) binding assays, respectively (Figure 10).

3.2.6 Control experiments for spaced glycopolymer microarray formation

Glycopolymer (**1b**, 3 mg) was mixed with unmodified lysozyme (3 mg) in 3 mL of 10.3 pH $NaHCO_3$ buffer and was stirred at room temperature for 4 hrs. MicroCaster array tool (Whatman, spot size 500 µm diameter) was inked with the glycopolymer/BSA solution (1 mg/mL) and then was printed onto amine functionalized glass slide in $NaHCO_3$ buffer (pH 10.3). The glass slide was then incubated in a humidifier chamber for 4 hrs and then washed for with $NaHCO_3$ buffer (pH 10.3) 30 mins (3 times) The glass slide was then incubated with lectin-FITC (Arachis hypogae, FITC-Labeled, Sigma, 0.2 mg, 1.61×10^{-9} moles) in 0.2% PBST solution at room temperature for 3 hrs followed by extensive washing with PBST buffer for 30 mins and subjected to fluorescent imaging and the

fluorescence intensity was recorded as above. Same protocols were followed for glycopolymer treated with unmodified BSA, respectively (Figure 9).

Same protocols were followed for printing glycopolymer with hydroxyl chain end group and its complex with polyacrylamide-BA, respectively.

3.2.7 Surface Plasma Resonance (SPR) assay for immobilized glycopolymers spaced with boronic acid ligands

3.2.7.1 Glycopolymer immobilization onto SPR chip: A CM5 chip (GE health science) surface was activated with EDC/NHS 1:1 for 6 mins at 10 µL/min, treated with 70 µL of 0.1 M ethylenediamine in 8.5 pH 0.1 M borate buffer at 10 µL/min, followed by 70 µL of 1 M ethanolamine 8.5 pH 0.1 M borate buffer for 7 min at 10 µL/min. Once the amine modified surface was generated, glycopolymer (1b) was immobilized by flowing glycopolymer solution in 10.3 pH $NaHCO_3$ buffer (0.5 mg/mL) for 7 min at 10 µL/min followed by washing with 10.3 pH $NaHCO_3$ buffer for 5 min (flow cell 2). Immobilization of glycopolymer was confirmed by observing the increase in response from the base line.

3.2.7.2 Boronic acid ligands spaced glycopolymer immobilization onto SPR chip: CM5 chip (GE health science) was modified as mentioned earlier to create amine functionalization. Glycopolymer pre-modified with polyacrylamide-BA (0.5 mg/mL, 1:2 mol) in pH 10.3 $NaHCO_3$ buffer was flowed for 7 min at 10 µL/min. Immobilization of polyacrylamide-BA modified glycopolymer was confirmed by the increase in response from the base line, followed by washing with 10.3 pH $NaHCO_3$ buffer for 5 mins. Polyacrylamide-BA was released from the immobilized glycopolymer by flowing with

7.4 pH PBS buffer for 7 min. Same procedure was followed to immobilize lysozyme-BA pre modified glycopolymer (1b).

3.2.7.3 Lectin binding onto immobilized glycopolymer: The binding of lectin to immobilized glycopolymer was studied by flowing lectin (Arachis hypogae, Sigma). Different concentrations of lectin ranging from 0.125 nmoles to 2 nmoles in 7.4 pH PBST buffer were flowed through immobilized glycopolymer for 2 mins at 10 µL/min; and the amplification of response with respect to increase in lectin concentration was illustrated.

3.3 Results and Discussion

3.3.1 Glycopolymer microarray formation and lectin binding assay

In our previous study, we have demonstrated that *O*-cyanate chain-end functionalized glycopolymer could be immobilized onto amine-functionalized silica gel and glass slide surfaces *via* isourea bond formation.[24] To fully exploit the potential of the oriented immobilized glycopolymer, herein, we examined molecular control of both the density of glycan in the glycopolymer and the glycopolymer itself on the microarray surface. First, we examined the glycan density effect for lectin binding in the glycopolymer by synthesizing a series of glycopolymers with different ratios of pendant glycan and molecular weights. Four glycopolymers were synthesized by our previously reported method (Fig 2, Table 1).[24]

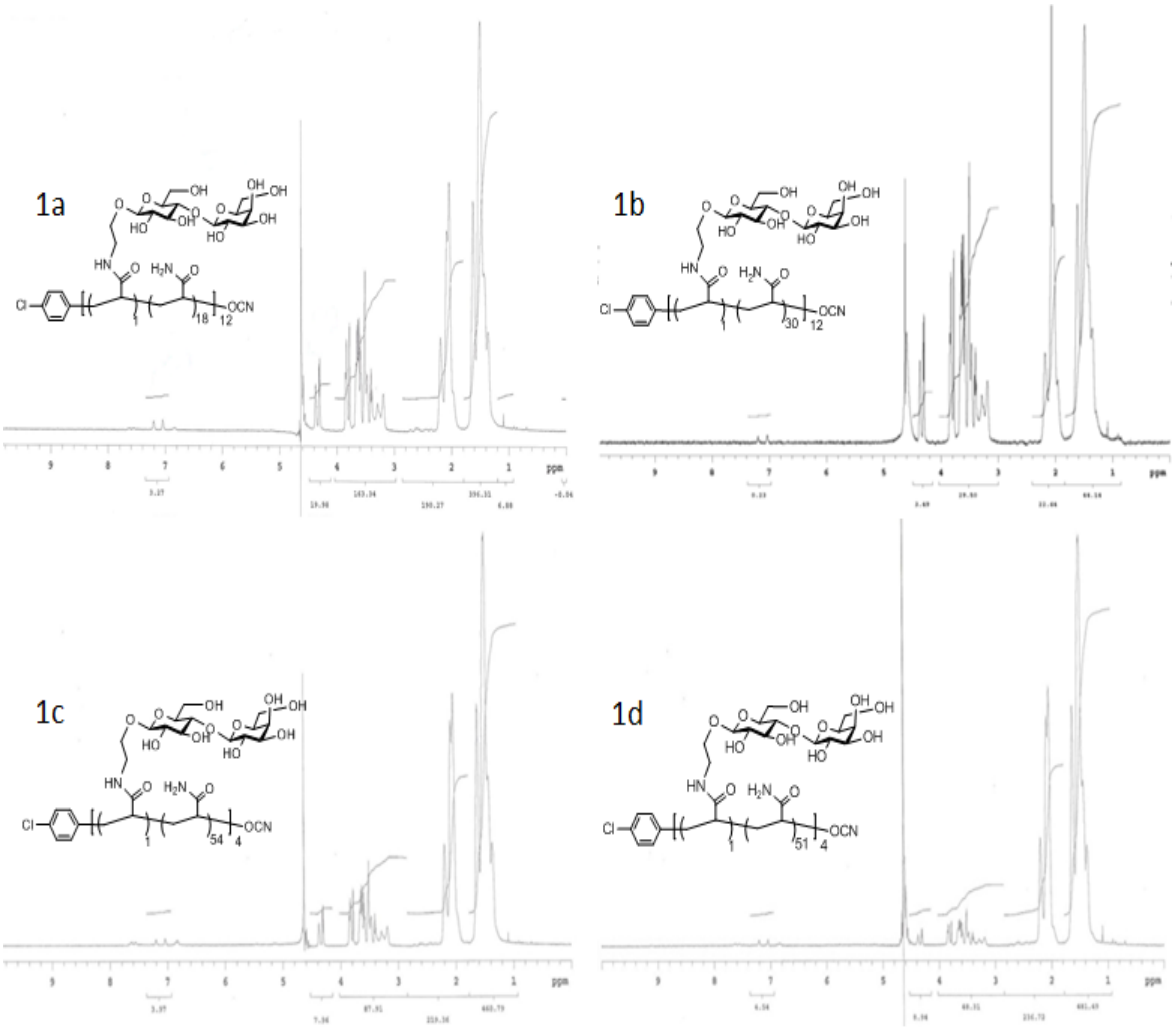


Figure 2 ^{1}H NMR spectrum of glycopolymer **1a** in D_2O**, 1b** in D_2O**, 1c** in D_2O**,1d** in D_2O

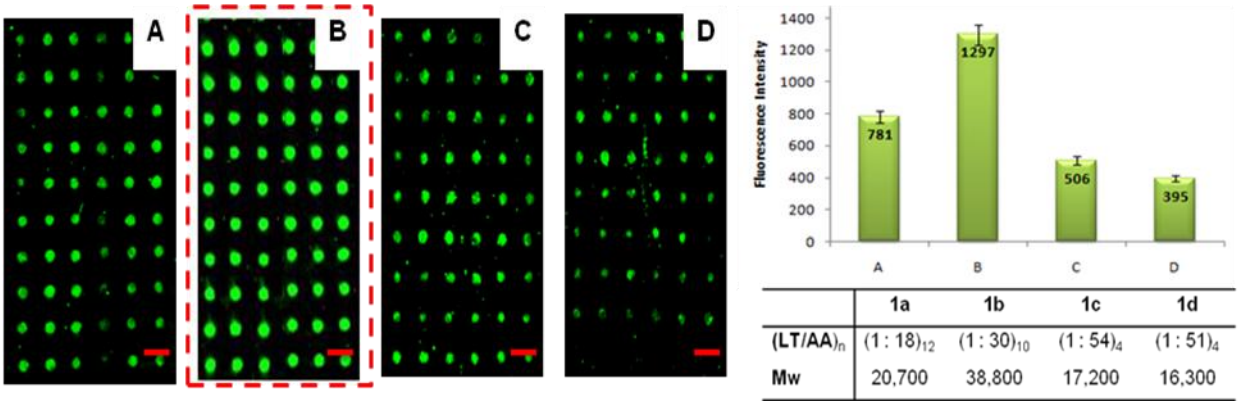
Table 1. *O*-cyanate chain-end functionalized glycopolymers of different densities

Glycopolymer	GM/AA[a]	GM/AA[b]	Mn(g/mol)[c]
1a	1/1	$(1/18)_{12}$	20,700
1b	1/10	$(1/30)_{12}$	38,800
1c	1/20	$(1/54)_4$	17,200
1d	1/50	$(1/51)_4$	16,300

a. Ratio of GM and AA added. *b. Ratio of* GM and AA determined by ^{1}H NMR. *c.* Molecular weight determined by ^{1}H NMR analysis (D_2O).

Then, glycopolymer microarrays (spot size about 500 μm diameter) were fabricated by microcontact stamping (MicroCaster) of glycopolymers onto amine functionalized glass slides (Xenopore Co.) in $NaHCO_3$ buffer (pH 10.3). The glass slides were incubated in a humidifier chamber for 4 hrs and then washed for 30 mins (3 times) with respective buffers followed by washing with PBST to minimize the nonspecific binding of proteins onto the surface. Then, the glass slides were incubated with lectin-FITC (*Arachis hypogae*, FITC-labeled, Sigma) solution in PBST buffer for 3 hrs, followed by extensive washing with PBST buffer for 30 mins, and are finally subject to fluorescence imaging. As shown in Fig 3, glycopolymer (**1b**) with 1 to 30 ratio of lactoseand acryl amide (LT/AA) showed the highest level of lectin binding (Fig 3B) compared to the glycopolymers with 1 to 18 (**1a**) (Fig 3A), 1 to 54 (**1c**) (Fig 3C) and 1 to 51 (**1d**) (Fig 3D) ratio of lactose and acryl amide. These results indicated that the glycan

density in the polymer has impact for the lectin binding. We chose glycopolymer **1b** for our continued density controlled glycopolymer microarray study below.



	1a	1b	1c	1d
$(LT/AA)_n$	$(1:18)_{12}$	$(1:30)_{10}$	$(1:54)_4$	$(1:51)_4$
Mw	20,700	38,800	17,200	16,300

Figure 3. Fluorescence images of glycopolymer microarrays with different glycan density after incubation with lectin (*Arachis hypogae*, FITC-labeled, Sigma). **A**: glycopolymer **1a** (LT/AA, 1:18), **B**: glycopolymer **1b** (LT/AA, 1:30), **C**: glycopolymer **1c** (LT/AA, 1:54), **D**: glycopolymer **1d** (LT/AA, 1:51). LT: Lactose, AA: Acrylamide. Bar size: 500 µm.

3.3.2 Syntheses and charecterization of boronic acid (BA) ligands

Three macro-boronic acid ligands (**2**), lysozyme-BA (**2a**, Mw: about 15 kDa), BSA-BA (**2b**, Mw: about 70 kDa) and polyacrylamide-BA (**2c,** Mw: about 10 kDa) conjugates were designed and synthesized as spacing molecules to vary the immobilized glycopolymer density since they have different molecular sizes and BA conjugation density on it can be variable. Lysozyme-BA **2a** and BSA-BA **2b** conjugates were synthesized by amidation of carboxylic acid groups in lysozyme and BSA with aminophenyl boronic acid in the presence of EDC, respectively. Polyacrylamide-BA

conjugate **2c** was prepared as per our previously reported method.[25] Lysozyme-BA and BSA-BA conjugates were characterized by SDS-PAGE. As shown in Figure 4, the comassie staining clearly showed large molecular weight shifts for the protein-BA conjugates compared to unmodified BSA and lysozyme (Fig 4A). Interestingly, the gel stained with Alizarin Red S. (ARS) in PBS buffer (pH 7.4) showed similar molecular weight shifts for the conjugates but the color of the bands is brown comparing to yellow band of the unmodified lysozyme and BSA. The ability of the boronic acid ligands to specifically bind with carbohydrate was characterized by ARS-binding assay.[26] Qualitative determination of boronic acid conjugation to both BSA and Lysozyme were performed by Alizarin Red S assay. Briefly, Alizarin Red S (ARS) exhibits change in fluorescence intensity and color by the addition of boronic acid. As shown in Figure 5, ARS (a) showed a color change from pink to yellow when bound to boronic acid (b) and also a shift in wavelength from 510 nm to 460 nm by UV absorption in PBS (pH 7.4) buffer; and by adding free galactose, galactose -boronic acid complex was formed to release ARS indicated by a color change from yellow to pink (c) with a shift in wavelength to 510 nm from 460 nm. The same pattern of fluorescence intensity and color change was observed by both BSA-BA and Lysozyme-BA conjugates as shown in Figure 5A: ARS plus Lyz (d) no color change, ARS plus Lyz-BA (e) color change from pink to yellow and ARS-Lyz-BA plus galactose (f) color change back to pink, Figure 5B: ARS plus BSA (g) no color change, ARS plus BSA-BA (h) color change from pink to yellow and ARS-BSA-BA plus galactose (i) color change back to pink. (Fig 5).

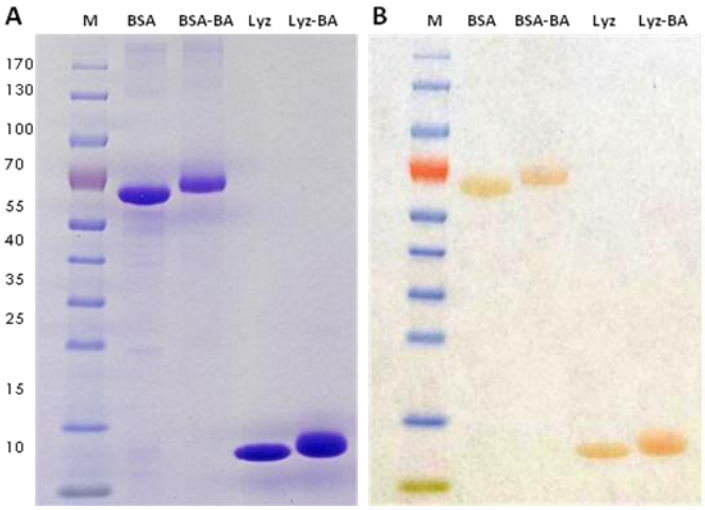


Figure 4. SDS-PAGE characterization of BSA-BA and lysozyme-BA conjugates: **A**. stained with coomassie blue, **B**. stained with ARS in PBS buffer (pH 7.4) for 30 mins.

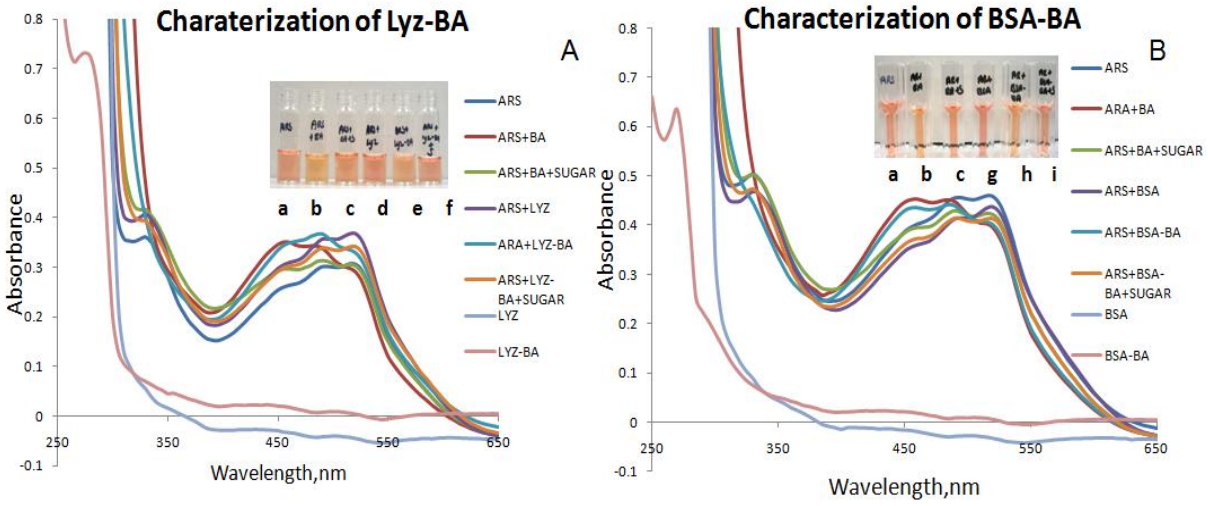


Figure 5. ARS assay of Lysozyme-BA (**A**) and BSA-BA (**B**) monitored by UV-Vis Spectroscopy

Also quantitative determination of boronic acid in BSA-BA and Lysozyme-BA conjugates was performed by monitoring ARS assay through fluorescence spectroscopy.

Solutions of BSA-BA and Lysozyme-BA were added to 1 mM ARS solution in PBS (pH 7.4) buffer and fluorescence intensity was recorded at 378 nm. From the calibration curve made with standard ARS and APBA below (Fig 6), the ratio of BA and protein obtained was determined as shown in Table 2.

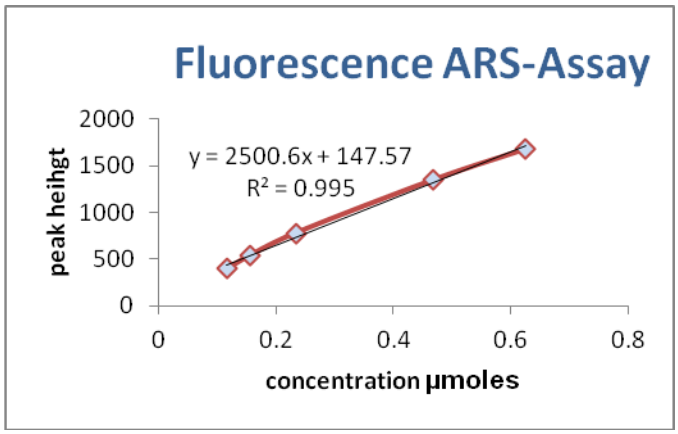


Figure 6. Calibration Curve of boronic acid by ARS assay monitored by Fluorescence Spectroscopy

Table 2. Quantification of BSA-BA and Lyz-BA conjugates from ARS assay by fluorescence spectroscopy

Protein-BA	**Protein/APBA[a] (mole)**	**Protein/BA[b](mole)**
Lyz-BA	1/33	1/4
BSA-BA	1/87	1/54

a. Ratio of protein and APBA added. b. Ratio of BA and protein determined from ARS assay.

3.3.3 Density controlled glycopolymer microarray formation and lectin binding assays

Molecular imprinting technique has been recognized as a powerful tool for the preparation of synthetic polymers with molecular sized cavities that are capable of molecular recognition.[27-28] This technique has been used for chromatographic separations,[29] solid phase extraction,[30] binding assay[31] and sensors,[32-33] and drug delivery[34] applications. Boronic acid-carbohydrate complex formation is a pH-dependent reversible process and has been used for the preparation of molecularly imprinted polymers targeting sugars.[35-38] In the present study, we envisioned that the reversible interaction between boronic acid and carbohydrate can be used to modulate the density of immobilized glycopolymer on the microarray surface by using boronic acid ligands in different sizes as "temporary spacing molecules". Briefly, the glycopolymer was pre-complexed with boronic acid liands. These assemblies were then printed onto the microarray surface, in which the boronic acid ligands temporarily occupy space between the immobilized glycopolymers. Once the immobilization was complete, the spacer boronic acid ligands are removed leaving empty space behind and thus afforded density controlled glycopolymer microarray. The key factor for this innovative fabrication of density controlled glycopolymer microarray is the compatible conditions, first the basic condition for both boronic acid and carbohydrate complex formation and *O*-cyanate-based isourea bond formation immobilization; second, neutral condition for removal of spacer boronic acid ligands from the microarray surface (Fig 1).

The *O*-cyanate chain end functionalized lactose-containing glycopolymer **1b**, which showed the highest lectin binding above, was used as a model glyco-macroligand for oriented and density controlled glycopolymer microarray formation.

The general process for oriented and density controlled glycopolymer microarray formation includes three steps as shown in Fig 1. First, prior immobilization, the complexes formation between glycopolymer (**1b**) and spacing broronic acid ligands (**2**) were conducted by mixing **1b** and **2** in $NaHCO_3$ buffer (pH, 10.3) for 4 hrs at room temperature. Next, MicroCaster array tool (Whatman, spot size 500 μm diameter) was inked with the solution of glycopolymer-boronic acid ligand complex in $NaHCO_3$ buffer (pH, 10.3), then was pressed onto an amine functionalized glass slide (Xenopore, Co) for 10 mins at room temperature. The glass slide was then incubated in a humidifier chamber for 4 hrs at room temperature. Finally, the spacing boronic acid ligands were detached from the immobilized glycopolymer by incubating the glass slides in PBS buffer (pH 7.4) solution in the presence of high concentration glucose (1 mM) as well, and followed by washing with PBS buffer (pH 7.4) for 30 mins (3 times) so as to afford the expected oriented and density controlled glycopolymer microarrays. ARS assay was also used to confirm the boronic acid ligands detached from the immobilized glycopolymer, in which PBS buffer solution containing ARS turned to brown color from red color upon falling in of detached boronic acid ligands from the immobilized glycopolymers on the microarray surface (Fig 7).

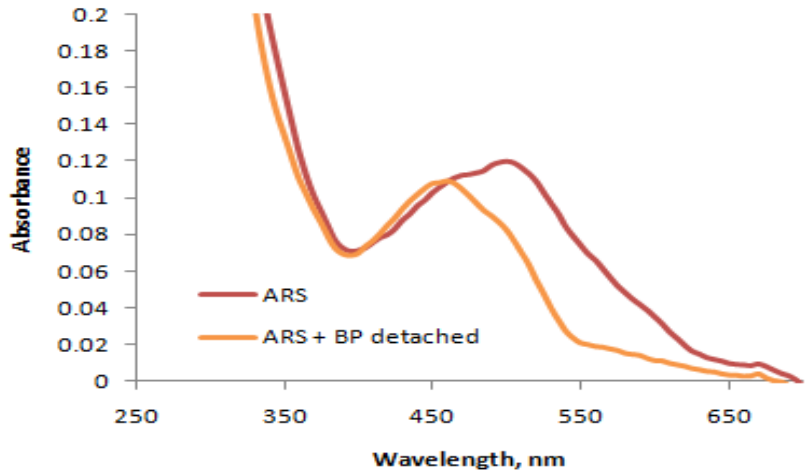


Figure 7. UV spectra of ARS and ARS upon boronic acid ligand detached from the glass slide in PBS buffer (pH7.4). BP: polyacrylamide-boronic acid

For protein binding, the resultant glycoarray glass slides were washed with PBS containing 0.2% tween 20 (PBST) first to minimize nonspecific protein binding, then incubated with *β*-galactose specific lectin (*Arachis hypogae* (PNA) FITC- Labeled, Sigma) solution in PBST buffer (pH 7.4, 0.2 mg/mL) for 3 hrs at room temperature followed by extensive washing with PBST buffer for 1 hr. The slides then were subjected to fluorescence imaging. As results, all spaced glycopolymer microarrays showed enhanced lectin binding compared to non-spaced one (Fig 8). Among them, the polyacrylamide-BA spaced glycopolymer showed the highest enhanced lectin binding (Fig 8A), while BSA-BA spaced glycopolymer and lysozyme-BA spaced glycopolymer showed moderately enhanced lectin binding (Fig 8B and 8C). Significantly, glycopolymer microarrays spaced with boronic acid ligands in different sizes showed different levels of lectin bindings (Fig 8A, 8B, 8C). Specifically, the polyacrylamide-BA spaced glycopolymer showed its highest level of lectin binding with the glycopolymer/polyacrylamide-BA ratio of 1 to 6 (mol) (Fig 8A3), while the lysozyme-

BA spaced glycopolymer showed its highest level of lectin binding with the glycopolymer/lysozyme-BA ratio of 1 to 2 (mol) (Fig 8B2), and BSA-BA spaced glycopolymer showed its highest lectin binding with the glycopolymer/BSA-BA ratio of 1 to 0.6 (mol) (Fig 8C3), both of which are lower than that of polyacrylamide-BA spaced glycopolymer microarray (Fig 8A3). These results indicated that glycopolymer microarrays with different densities can be made with different boronic ligands in different ratios and hence revealed relevant protein bindings.

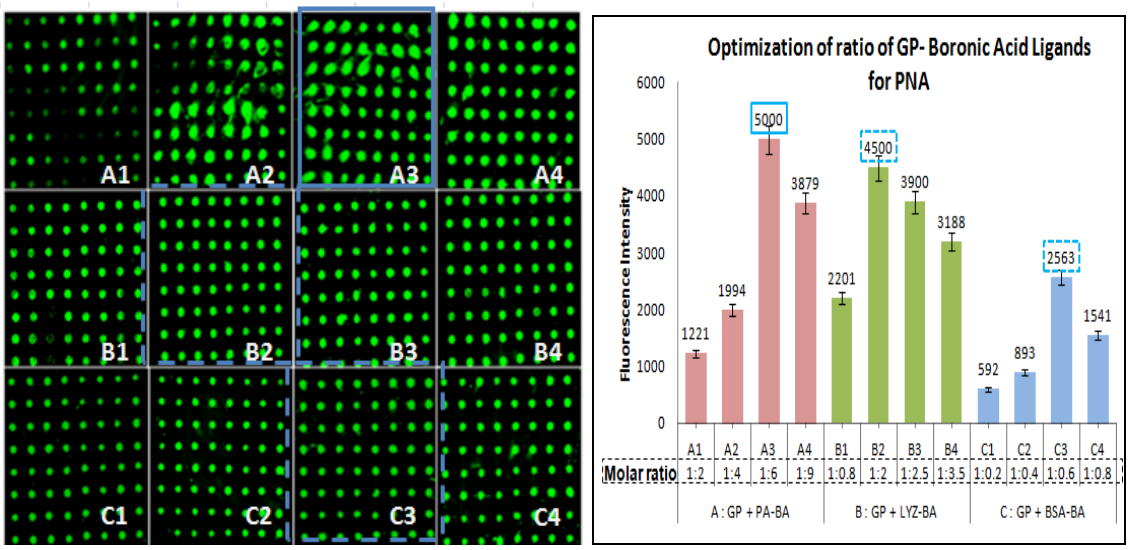


Figure 8. Fluorescence images of density controlled glycopolymer microarrays spaced with boronic acid ligands in different molar ratios after incubation with lectin (*Arachis hypogae* (PNA), FITC-Labeled, Sigma) and their fluorescence intensities: **A1-A4**. glycopolymer spaced with polyacrylamide-BA in different ratios, **B1-B4**. glycopolymer spaced with lysozyme-BA in different ratios, **C1-C4**. glycopolymer spaced with BSA-BA in different ratios. Bar size: 500 µm. GP: glycopolymer, PA-BA: polyacrylamide-BA, LYZ-BA: lysozyme-BA.

It should be noted that both the glycopolymer and the boronic acid ligands have multi-binding sites and thus could afford a mixture of conjugates or form aggregates due to the multivalent interactions. In current study, low concentrations (mg/mL) of both polymers were used and no aggregates were observed.

The spaced glycopolymer microarrays formations were reproduced with the highest lectin binding (Fig 9A-C). In addition, control experiments for glycopolymer microarray formation with unmodified lysozyme and BSA showed no enhanced lectin binding compared to glycopolymer alone microarray (Fig 9C1, 9C2, 9D). On the other hand, no glycopolymer microarray formed when the chain end *O*-cyanate group was converted to hydroxyl group in the glycopolymer[16] (Fig 9C3 and 9C4). Overall, these results indicated the successful oriented and density controlled glycopolymer microarray formation and that the immobilized glycopolymer density had a substantial effect on its lectin recognition.

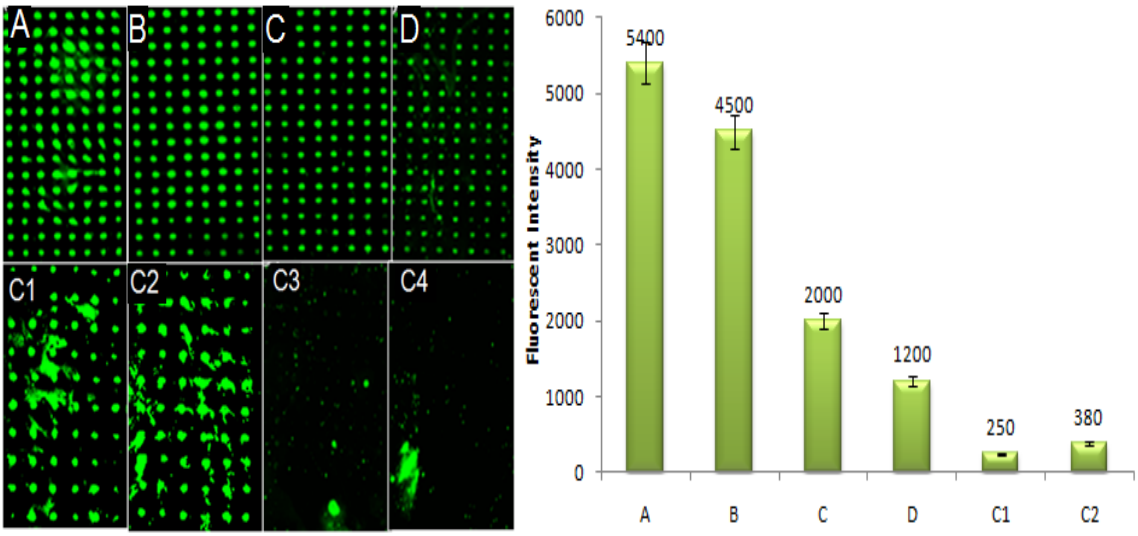


Figure 9. Fluorescence images of density controlled glycopolymer microarrays after incubation with lectin (*Arachis hypogae*, FITC-Labeled, Sigma) and their fluorescence intensities: **A.** glycopolymer spaced with polyacrylamide-BA (1:6 mol), **B**. glycopolymer spaced with lysozyme-BA (1:2 mol), **C**. glycopolymer spaced with BSA-BA (1:0.6 mol), **C1**. glycopolymer treated with lysozyme (1:2 mol), **C2**. Glycopolymer treated with BSA (1:0.6 mol), **C3**. glycopolymer-OH, **C4**. glycopolymer-OH treated with polyacrylamide-BA (1:6 mol), and **D**. glycopolymer alone. Bar size: 500 μm.

Next, the oriented and density controlled glycopolymer microarrays above were also tested for lectin *Ricinus communis* (RCAI, FITC-labeled, EY Laboratories, Inc) binding, which recognizes *β*-galactose too. Interestingly, different from lectin *Arachis hypogae* bindings above, RCAI showed stronger bindings to polyacrylamide-BA spaced glycopolymer with the glycopolymer/polyacrylamide-BA ratio of 1 to 4 (mol) (Fig 10A2) and lysozyme-BA spaced glycopolymer with the glycopolymer/lysozyme-BA ratio of 1 to 2 (mol) (Fig 10B2), and BSA-BA spaced glycopolymer showed stronger lectin binding with the glycopolymer/BSA-BA ratio of both 1 to 0.4 and 1 to 0.6 (mol) (Fig 10C2 and

10C3). Both lectin *Ricinus communis* and lectin *Arachis hypogae* have four binding sites and about 120 kDa molecular weight. However, they prefer different spacer distance and glycan density. This different binding preference might be due to different location geometry of their binding sites in the protein or the protein conformation. Overall, these observations indicated that arrayed glycopolymers with controlled ligand density facilitate optimized protein binding, which will be important for studying glycan-protein interaction, such as assessing multivalent glycans in parallel in a microchip format.

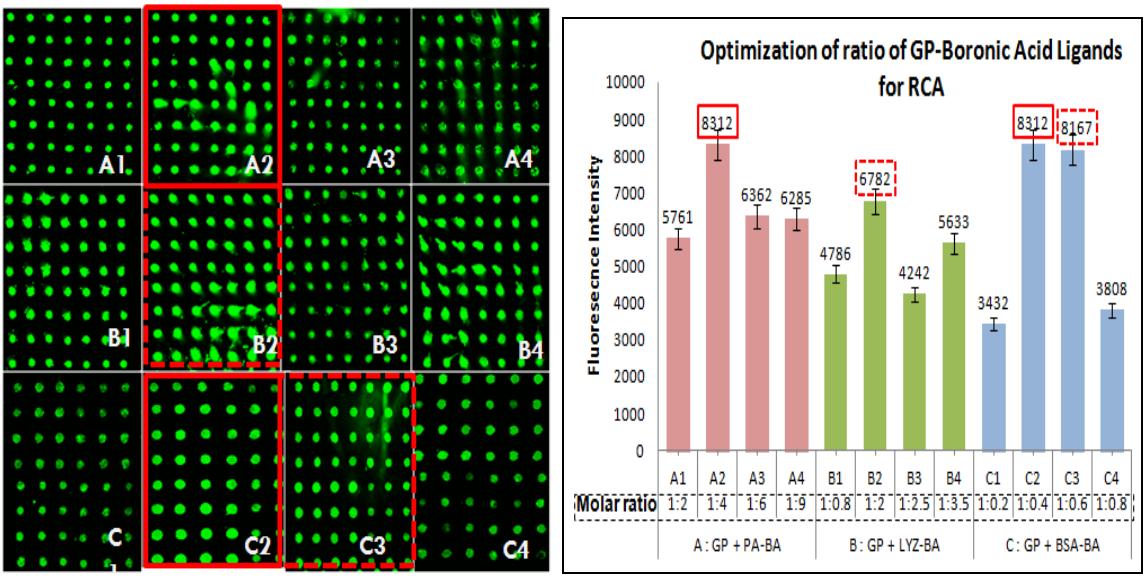


Figure 10. Fluorescence images of density controlled glycopolymer microarrays spaced with boronic acid ligands in different molar ratios after incubation of with lectin *Ricinus communis* (RCAI), FITC-labeled, EY Laboratories, Inc) and their fluorescence intensities: **A1-A4**. glycopolymer spaced with polyacrylamide-BA in different ratios, **B1-B4**. glycopolymer spaced with lysozyme-BA in different ratios, **C1-C4**. glycopolymer spaced with BSA-BA in different ratios. Bar size: 500 µm. GP: glycopolymer, PA-BA: polyacrylamide-BA, LYZ-BA: lysozyme-BA.

3.3.4 Surface Plasma Resonance (SPR) assay for boronic acid ligands imprinted glycopolymer

The interaction of lectin with oriented multivalent glycans in different densities was also investigated with surface plasmon resonance (SPR). The major advantages of this assay are that it is a label free assay and monitors binding in real time. Three different density controlled immobilized glycopolymers were made onto CM5 chip (GE health science), in which the active ester NHS was first converted to amine surface by reacting with ethylene diamine and followed by *O*-cyanate-based glycopolymer immobilization similarly as described for glycopolymer microarray formation above. Briefly, CM5 chip was treated with EDC/NHS first, then with ethylene diamine in borate buffer (pH 8.5), followed by 1 M ethanolamine in borate buffer (pH 8.5) to quench all NHS on the chip surface. Once the amine modified surface is formed, glycopolymer, glycopolymer/lysozyme-BA, glycopolymer/polyacrylamide-BA complexes (5 mg/mL, $NaHCO_3$ buffer (pH 10.3) were flowed over the chip for 7 mins at 10 µL/min and followed by washing with $NaHCO_3$ buffer (pH 10.3) for 5 mins, respectively. Finally, flowing PBS (pH 7.4) buffer for 7 mins 10 µL/min to remove the boronic acid ligands afforded the oriented and density controlled glycopolymer on the SPR chip surface. Binding of lectin to the immobilized glycopolymers were assessed by flowing lectin (*Arachis hypogae*, Sigma) over the chip at various concentrations (0.125 nM, 0.25 nM, 0.5 nM, 1 nM, 2 nM.) and recording the SPR sensorgrams (10 µL min^{-1}, 25 °C). As shown in Figure 11, glycopolymer spaced with different boronic acid ligands showed different level of lectin bindings. Among them, the polyacrylamide-BA spaced glycopolymer showed the highest lectin binding (Fig 11A, track 3), while glycopolymer

alone showed the least lectin binding (Fig 11A, track 1). These results are consistent with glycoarray results above (Figure 11B). These results further demonstrated that the density of the multivalent glycan displaying plays a critical role for enhancing their interactions with proteins.

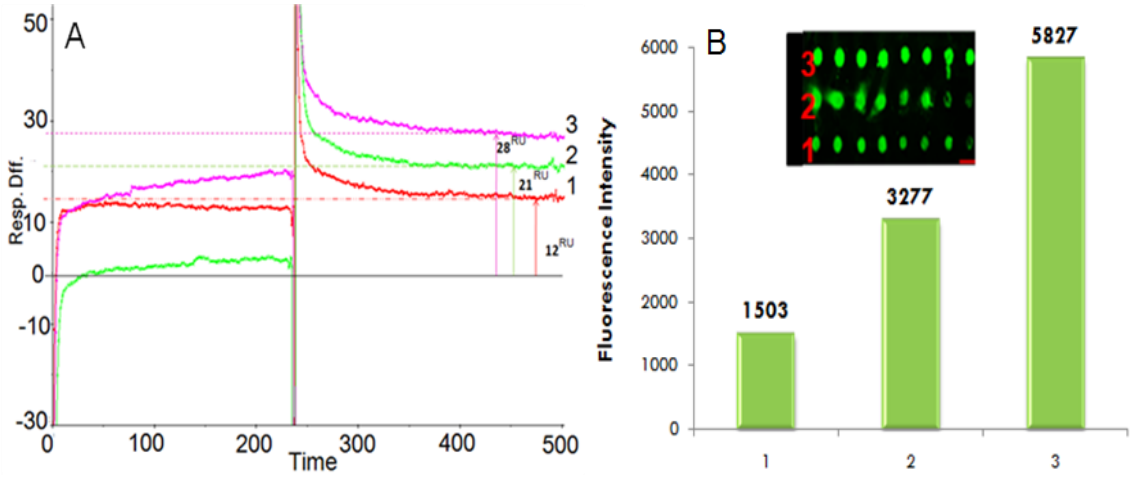


Figure 11. Specific binding of lectin (*Arachis hypogae*, FITC-labeled, Sigma) onto oriented and density controlled glycopolymers on SPR surface (**A**) and microarray (**B**): 1. Glycopolymer, 2. Glycopolymer spaced with lysozyme-BA (1:2 mol), 3. Glycopolymer spaced with polyacrylamide-BA (1:6 mol). Bar size: 500 µm.

3.4 Conclusion

We have demonstrated an oriented and density controlled glycopolymer microarray formation based on end-point immobilization of glycopolymer that was spaced with boronic acid ligands of different sizes. Our studies confirmed that end-point immobilized glycopolymers with density control of both glycan in the polymer and glycopolymer on the microarray surface can effectively engage glycan-binding proteins and function as novel glycan microarray format. Lectins that bind the same glycan are affected by the

glycopolymer density in unique ways. The reported well-defined glycopolymer microarray in both orientation and glycan density can be utilized for various biological analyses such as profiling the glycan–protein interactions and clinical antibody detection and profiling, vaccine development, biomarker discovery, and drug screening applications.

3.5 References

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CHAPTER IV

IMMOBILIZED SIALYLOLIGO-MACROLIGAND AND ITS PROTEIN BINDING SPECIFICITY

4.1 Introduction

Sialic acids are a family of 9-carbon containing acidic monosaccharides, often terminate oligosaccharide structures of cell surface glycoconjugates such as glycoproteins and glycolipids and are involved in many biological recognition systems. For example, sialic acid expressed on respiratory epithelial cell surface is involved in influenza virus infection in both virus hemagglutinin-based attaching[1] and neuraminidase-based detaching[2] processes. The mechanisms by which influenza virus recognizes sialic acid have therefore been very important targets to design anti-influenza agents.[3] To date, two effective neuraminidase inhibition-based anti-influenza drugs, zanamivir and oseltamivir, have substantially improved antiviral therapy for influenza infection.[4-5] On the other hand, site-specific blocking of the viral hemagglutinin binding to host cell receptors with

sialic acid derivatives has been intensively investigated for antiinfluenza drug development.[6]

Influenza virus contains about 350-400 HA trimers[7] and 50 NA tetramers[8] on its surface, which facilitate strong binding affinity through multivalent interactions with the sialic acid receptors on the host cell surface. Previous efforts using sialic acid-bearing macromolecules provided a proof of the concept of multivalent hemagglutinin inhibition.[6] However, the monosaccharide sialic acid cannot account for the molecular determinant of virus receptor-binding specificity in the context of the whole sialyloligosaccharide receptor. Recently, it has been known that the host cell binding specificity and affinity of influenza viruses depend on not only sialic acid but also the penultimate sugar and the linkage of sialic acid to the penultimate sugar in the receptor. For example, Siaα2,6Gal is indispensable receptor for the human-to-human spread of the influenza virus, while Siaα2,3Gal is the receptor for avian influenza virus infection.[9-10] Therefore, sialyoligosaccharide is likely the choice as an active ligand for specific and stronger influenza HA binding and thereby a multivalent sialyloligosaccharide would be rational influenza HA inhibitors. During the past decade, several sialyloligosaccharide-containing macromolecules have been demonstrated to inhibit influenza virus attachment to target cells and suppress the virus-mediated hemagglutination and neutralize virus infectivity in cell culture.[11]

Glycopolymers, namely polymers with carbohydrate pendent groups, have been extensively explored for different biological and biomedical applications.[12-13] It is generally accepted that glycopolymers can mimic the functions of naturally occurring glycoconjugates[14-15] and have been employed as cell-surface binding receptors.[16] The

strength and selectivity of binding interactions between multivalently displayed carbohydrates and targets are likely to depend on the density and relative spatial arrangement of the carbohydrate residues on the glycopolymers. Recently, the potential utility of glycopolymers in bio- and immunochemical assays as biocapture reagents and for microarray applications have been demonstrated by adding functional anchor group either as a pendant to the polymer backbone or at the chain end.[17] Particularly, the chain-end groups of glycopolymers are the focus for bio-functionalization, as they allow for a direct one-to-one attachment[18-19] and also facilitate site specific[20] and oriented immobilization on solid surfaces.[21] This molecular recognition pattern is especially exploited on solid surfaces for the development of biosensors, chip-based bioassays, and cell-adhesion studies[22] as they mimic the three dimensional (3D) display of glycans on the cell surface. Recently, Bertozzi's group[23] and our group[24] reported oriented glycopolymer microarrays based on end-point immobilization of glycopolymers to mimic 3D natural cell surface glycan displaying. In these approaches, the densities and orientations of the glycan ligands are determined by the polymer structure rather than by features of the underlying microarray surface. These parameters should therefore be more controllable than in the case with conventional 2D glycan microarrays. In our previous study, *O*-cyanate chain-end functionalized glycopolymer provides an anchor for site-specific and covalent immobilization of the glycopolymer onto an amine surface *via* isourea bond formation and thereby facilitates an oriented glyco-macroligand formation.[24] Herein, we report a chemoenzymetic synthesis of *O*-cyanate chain-end functionalized sialyllactose-containing glycopolymers and their oriented sialyloligo-marcroligand formation for glycoarray and glyco-biosensor applications (Fig 1).

Particularly, *O*-cyanate chain-end functionalized sialyllactose-containing glycopolymer was immobilized onto amine-modified glass slide and SPR chip *via* isourea bond formation, respectively. This oriented sialyloligo-macroligand microarray and SPR-based glyco-biosensor platform are expected to facilitate both affinity and specificity of protein binding and thus will provide a versatile tool for profiling glycan recognition for both basic biological research and practical applications.

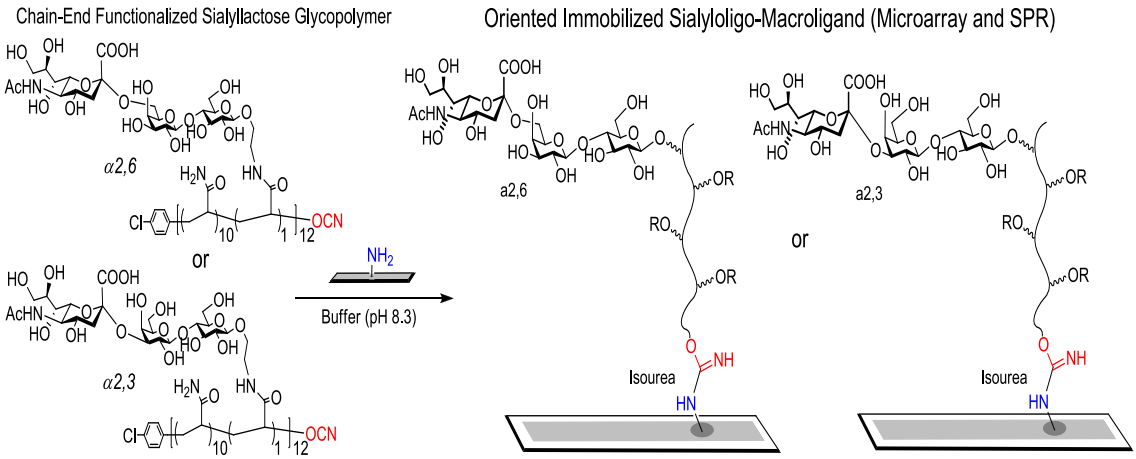


Figure 1. Chemical structure of *O*-cyanate chain-end functionalized sialyllactose-containing glycopolymers and their oriented immobilization *via* isourea bond formation for microarray and SPR applications.

4.2 Experimental

4.2.1 Materials and methods

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. CMP-Neu5Ac, α2,3-sialyltransferase and α2,6-

sialyltransferase were purchased from Sigma. FITC-labeled MAA (*Macckia amurensi*) and SNA (*Sambucus nigra*) were purchased from Bioworld (Dublin, OH). Avain HA (A/Anhui/1/2005(H5N1)) and human HA (A/Brisbane/10/2007(H3N2)) were purchased from Sino Biological (China). *O*-Cyanate chain-end functionalized lactose-containing glycopolymer was synthesized as previously reported.[24]

4.2.2 Enzymatic synthesis of sialyllactose glycopolymers (SGPs)

O-Cyanate chain-end functionalized lactose-containing glycopolymer (40 mg, 1 μmole, 10 μmoles of lactose) was dissolved in 2 mL of buffer [Triton X-100 (0.5%) + HEPES (0.10 M) + $MgCl_2$ (5 mM) + $MnCl_2$ (2 mM) + $ZnCl_2$ (5 x 10^{-5} M), pH 7.4]. To the dissolved polymer, CMP-Neu5Ac (6.5 mg, 100 μmoles), calf alkaline phosphate (10 U), bovine serum albumin (0.8 mg), and α2,3-sialyltransferase (0.5 U) were added and the mixture was stirred gently at room temperature for two days (48 hrs). The resultant α2,3-sialyllacotose glycopolymer (α2,3SGP) was purified by gel-filtration with Sephadex G-25 column (eluted with water) and the fractions containing SGP were confirmed with phenol sulfuric acid assay. The same protocol was employed to synthesize α2,6-sialyllacotose glycopolymer (α2,6SGP) by using α2,6-sialyltransferase, the *O*-cyanate chain-end functionalized lactose-containing glycopolymer and CMP-Neu5Ac. The resultant SGPs were characterized by ^{1}H NMR spectrometry.

α2,6SGP (**4**): Molecular Weight: 17,600 (determined by ^{1}H NMR) ; ^{1}H NMR (D_2O, 400 MHz) δ(ppm): 7.21, 7.06 (H of phenyl), 4.36, 4.33 (H_1 of Gal and H_1 of Glc), 3.85-3.36 (H of Gal and Glc), 3.28-3.20 (H of -OCH_2CH_2O-, linker), 2.56 (H_{3eq} of Neu5Ac), 2.20-

1.94 (-CH- of polymer backbone), 1.89 (CH_3 of Neu5Ac), 1.70-1.30 (-CH_2- of polymer backbone).

α2,6SGP (**5**): Molecular Weight: 17,600 (determined by ^{1}H NMR); ^{1}H NMR (D_2O, 400 MHz) δ(ppm): 7.20, 7.04 (H of phenyl), 4.36, 4.33 (H_1 of Gal and H_1 of Glc), 3.95-3.40 (H of Gal and Glc), 3.28-3.18 (H of -OCH_2CH_2O-, linker), 2.60 (H_{3eq} of Neu5Ac), 2.20-1.94 (-CH- of polymer backbone), 1.89 (CH_3 of Neu5Ac), 1.70-1.30 (-CH_2- of polymer backbone).

4.2.3 Sialyllactose glycopolymer microarray fabrication

MicroCaster microarray tool (Whatman, spot size 500 μm diameter) was inked with a solution of *α2,3SGP* in $NaHCO_3$ buffer (pH 10.3, *2.1 x 10^{-2} mM*), then was pressed onto amine functionalized glass slides (Xenopore, Co) at room temperature for 10 min. The glass slides were then incubated in a humidifier chamber at 37 ^{o}C for 4 h and then washed with $NaHCO_3$ buffer (pH 10.3) (30 min, 3 times) and followed by washing with 0.2% PBST buffer (pH7.4). Then the array glass slides were incubated with lectin MAA-FITC (*Macckia amurensis*, 1.4 x 10^{-3} mM, Bioworld) in 0.2% PBST buffer (pH 7.4) at room temperature for 3 h and followed by extensive washing with 0.2% PBST buffer (pH 7.4) for 30 min and finally subjected to fluorescent imaging. As control experiments, the α2,3SGP printed glass slides were incubated with lectin SNA-FITC (*Sambucus nigra*,1.3 x 10^{-3} mM, Bioworld), PNA-FITC (*Arachis hypogaea*, 1.6 x 10^{-3} mM, Sigma), MAA-FITC (0.2 mg, 1.4 x 10^{-3} mM, Bioworld) that was pre-incubated with α2,3-sialyllactose (1.5 x 10^{-2} mM, V-Labs), α2,6-sialyllactose (1.5 x 10^{-2} mM, V-Labs) and free sialic acid (3.2 x 10^{-2} mM, Rose Scientific ltd) in 0.2% PBST buffer (pH 7.4) at room temperature

for 2 h, respectively, and followed by extensive washing with 0.2% PBST buffer (pH 7.4) for 30 min and finally subjected to fluorescent imaging, respectively.

The same protocol was used for α2,6SGP microarray fabrication and followed by incubation with lectin SNA-FITC (1.3×10^{-3} mM). As control experiments, the α2,6SGP printed glass slides were incubated with lectin MAA-FITC (1.3×10^{-3} mM), lectin SNA-FITC (1.4×10^{-3} mM) that was pre-incubated with α2,6-sialyllactose (1.5×10^{-2} mM), α2,3-sialyllactose (1.5×10^{-2} mM) and free sialic acid (3.2×10^{-2} mM), PNA-FITC (*Arachis hypogaea*, 1.6×10^{-3} mM, Sigma) in 0.2% PBST buffer (pH 7.4) at room temperature for 2 h, respectively, and followed by extensive washing with 0.2% PBST buffer (pH 7.4) for 30 min and finally subjected to fluorescent imaging, respectively.

4.2.4 SPR analysis of specific MAA and SNA binding to immobilized α2,3SPG and α2,6SGP

The specific binding of lectin to immobilized SGP was analyzed with SPR with a BI 2000 biosensor system (BI Biosensing Instruments). To prepare the sensing surface, the commercial SPR gold chip (BI Biosensing Instuments) was rinsed with piranha solution of sulfuric acid and hydrogen peroxide (1:1, v/v), followed by water and ethanol (3 times) and dried under nitrogen. The dried gold chip was incubated with 1 mg/mL of cystamine solution in ethanol at 4 ˚C overnight and followed by washing with ethanol and water to remove weakly adsorbed cystamine to generate amine functionalized gold chip surface. Next, α2,3SGP was covalently immobilized onto the amine functionalized gold chip surface by flowing the polymer solution (2 mg/mL, $NaHCO_3$ buffer (pH 10.3)) at a flow rate of 10 μL/min for 600 sec and repeated at least 3 times until the surface is saturated

with the polymer, $NaHCO_3$ buffer (pH 10.3) was used as a running buffer. Then a serial of solution of lection MAA in concentration ranging from 0.5 μM to 10 μM in PBS running buffer (pH 7.4) were injected over the immobilized α2,3SGP at a flow rate of 20 μL/min for 2 min (120 sec, association phase). For regeneration of the surface, 0.1 M HCl (pH 1.5) was injected at a flow rate of 20 μL/min for about 30 sec and sometimes repeated twice to remove bound lectin completely. The association and dissociation constants of the lectin binding to immobilized SGP were determined by standard BI 2000 (scrubber) evaluation software. For the experiments with α2,6SGP, the same surface modification procedure was followed as above. For lectin SNA binding, the same procedure was used as above except different concentrations ranging from 1 μM to 10 μM used.

4.2.5 Competitive binding assay of lectin to immobilized SGP

Both α2,3SGP and α2,6SGP were immobilized on the gold sensor chip by following the same procedure as above. Solutions of MAA and SNA were prepared in PBS buffer (pH 7.4, 10 μM) and were pre-incubated with a series of α2,3-sialyllactose and α2,6-sialyllactose in concentrations ranging from 0.075 nM to 7.5 nM, respectively. As control, MAA and SNA (10 μM) were pre-incubated with high concentration (7.5 nM) of 2,6-sialyllactose and 2,3-sialyllactose, respectively. These pre-incubated MAA and SNA were injected over the immobilized α 2,3SGP and α2,6SGP at a flow rate of 20 μL/min for 3 min (180 sec, association phase), respectively. For regeneration of the surface, 0.1 M HCl (pH1.5) was injected at a flow rate of 20 μL/min for 30 sec. The response of the binding was calculated by standard BI 2000 (scrubber) evaluation software.

4.2.6 Hemagglutintin (HA) binding to immobilized SGP

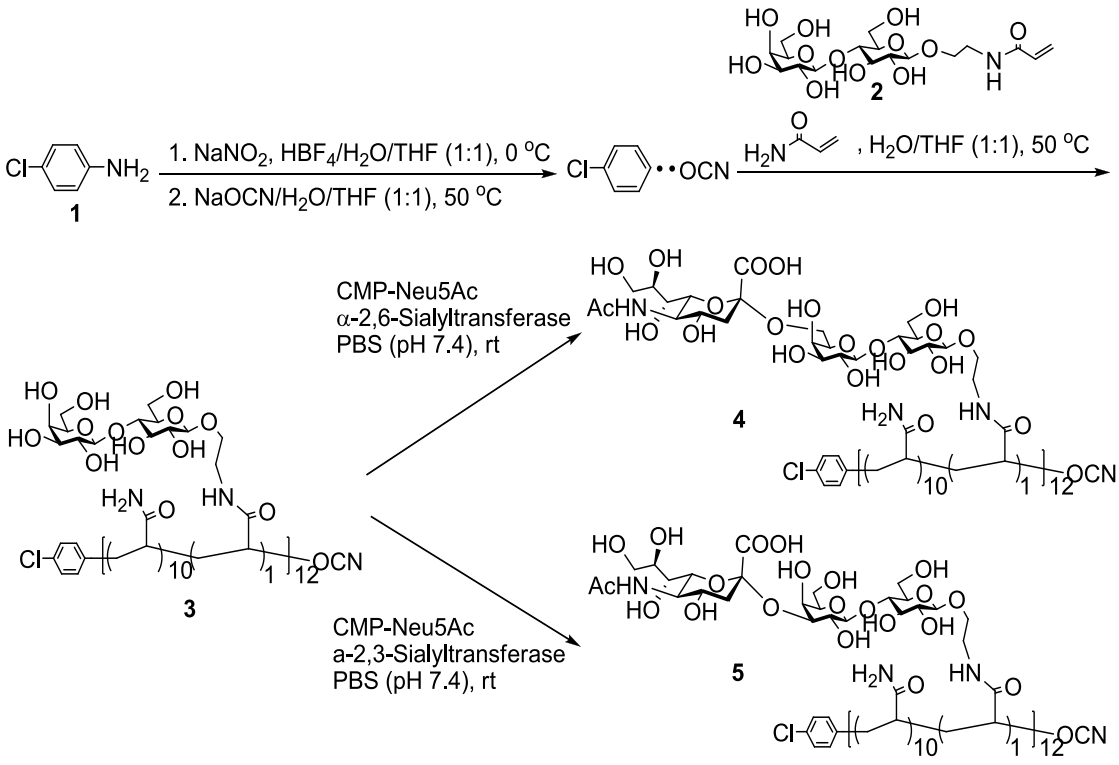
Both α2,3SGP and α2,6SGP were immobilized on the gold sensor chip following the same procedure as above. Solutions of H5N1 HA (A/Anhui/1/2005(H5N1)) and H3N2 HA (A/Brisbane/10/2007(H3N2)) (Sino Biological, China) were prepared in PBS buffer (pH 7.4 pH, 420 nM), then were injected over the two immobilized α2,3SGP and α2,6SGP at a flow rate of 20 μL/min for 180 sec, respectively. As control, both H5N1 HA and H3N2 HA were pre-incubated with α2,3-sialyllactose (PBS buffer (pH 7.4), 5 x 10^{-6} mM) and α2,6-sialyllactose (PBS buffer (pH 7.4), 5 x 10^{-6} mM) for 30 min, and then injected over immobilized α2,3SGP and α2,6SGP, respectively. 100 mM NaOH was injected for regeneration of surface between each HA injections. The association and dissociation constants of the protein to immobilized SGP were determined by standard BI 2000 (scrubber) evaluation software.

4.3 Results and Discussion

4.3.1 Synthesis and characterization of O-cyanate chain-end functionalized sialyllactose glycopolymers

Recently, a variety of methods have been developed for the synthesis of chain-end functionalized glycopolymers for potential biomedical applications such as protein modification and microarray fabrication applications.[17] We have demonstrated that cyanoxyl mediated free-radical polymerization is a straightforward approach to synthesize *O*-cyanate chain-end functionalized glycopolymer that could be immobilized onto amine functionalized silica gel beads *via* isourea bond formation and amine functionalized glass slide for oriented glycopolymer microarray fabrication

applications.[24] Particularly, the polymerization could be conducted in aqueous solution, tolerant of a broad range of functional groups including -OH, -NH_2, -COOH, and SO_3^- moieties, and yield glycopolymers with low polydispersity (PDI < 1.5). In this study, an *O*-cyanate chain-end functionalized lactose-containing glycopolymer (**3**) was synthesized as described in our previous report,[24] and then enzymatic sialylation of the terminal galactose (Gal) pending on the polymer was investigated for synthesizing *O*-cyanate chain-end functionalized silallylactose-containing glycopolymer. The enzymatic approach for transferring sialic acid from CMP-Neu5Ac to the non-reducing end of the carbohydrate acceptor was extensively reported.[25] The glycosidic linkage between the sialic acid and the acceptor carbohydrate is extremely controlled by the type of sialyltranferase selected. The transfer of sialic acid residue from CMP-Neu5Ac to 3- and 6-positions of terminal Gal of lactose glycopolymer (**3**), by enzymes α2,3-sialyltransferase and α2,6-sialyltransferase afforded silallylactose-containing glycopolymer α2,6SGP (**4**) and α2,3SGP (**5**), respectively (Scheme 1). The resultant SGPs were characterized by ^{1}H NMR spectra. The successful sialyation of lactose glycopolymer was confirmed by the signals of protons from Neu5Ac (1.95 ppm, CH_3-Neu5NAc and 2.60 ppm, H_{3eq}-Neu5Ac), the degree of sialyation and the polymer length as well were calculated also using the ^{1}HMR spectra by comparing the integration value of proton signals from aromatic protons (7.21 ppm and 7.06 ppm), anomeric protons (4.36 ppm and 4.33 ppm) of Gal and Glc, and C3-equatorial proton (2.60 pm) of Neu5Ac as shown in Figure 2. By comparing the integrated signal from the anomeric protons of Gal and Glc (4.36 ppm and 4.43 ppm,) with that of the C3-equatorial proton of Neu5Ac (2.60 ppm, H_{3eq}-Neu5Ac), the percentages of both sialyalations were more than 90%.



Scheme 1. Chemoenzymatic synthesis of *O*-cyanate chain-end functionalized sialyllactose-containing glycopolymers *via* cyanoxyl mediated free radical polymerization followed by enzymatic sialylation.

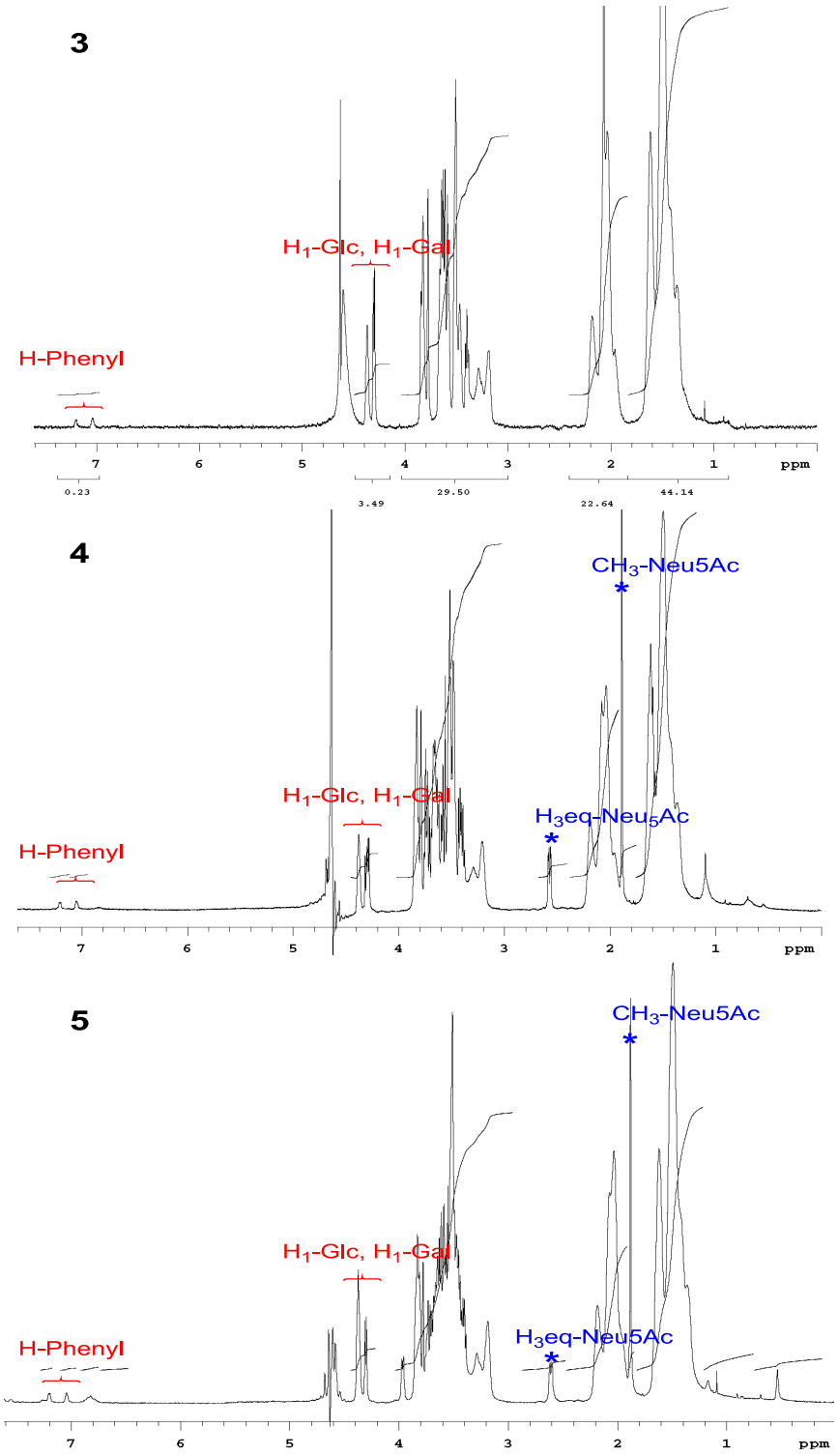


Figure 2. ^{1}H NMR spectra of glycopolymers in D_2O: A. lactose glycopolymer (**3**), B. α2,6-sialyllactose glycopolymer **(4)**, C. α2,3-sialyllactose glycopolymer **(5)**.

4.3.2 SGP Microarray fabrication and its specific lectin binding

SGP-based microarrays were fabricated by printing *O*-cyanate chain-end SGP onto amine functionalized glass slide. Briefly, α2,3SGP and α2,6SGP were printed (sport size 500 μm diameter) on amine functionalized glass slides (Xenopore Co) with MicroCaster in $NaHCO_3$ buffer (pH 10.3). The glass slides were incubated in humidifying chamber at 37^{o}C for 4 h and then washed with $NaHCO_3$ buffer and followed by washing with PBS buffer (pH 7.4) containing 0.2% tween 20 (PBST) in order to minimize nonspecific binding of proteins onto the glass surface later. Next, the specific lectin binding ability of the SGP-based microarray was performed. The resultant α2,3SGP and α2,6SGP printed glass slides were incubated with respective FITC-labeled lectins, *Macckia amurenisis* (MAA) and *Sambucus nigra* (SNA) (Bioworld) in PBST buffer (pH 7.4) followed by extensive washing with PBST buffer (pH 7.4) and finally the glass slides were subjected to fluorescent imaging, respectively. Lectin MAA that has affinity for α2,3-linked sialic acid showed binding to α2,3SGP (Fig 3Ia), while lectin SNA which has affinity for α2,6-linked sialic acid showed no binding to α2,3SGP (Fig 3Ib). To confirm the specific binding, competitive inhibition assays were performed as by incubating the α2,3SGP arrayed glass slides with lectin MAA that was pre-incubated with α2,6-siallylactose, α2,3-siallylactose and free sialic acid, respectively. As a result, MAA pre-incubated with α2,3-sialyllactose showed no binding to α2,3SGP (Fig 3Id), while MAA pre-incubated with α2,6-siallylactose and free sialic acid still showed lectin binding to α2,3SGP (Fig 3Ic and 3Id). On the other hand, lectin MAA showed no binding to α2,6SGP (Fig 3IIa), while lectin SNA showed binding to α2,6SGP (Fig 3IIb). Same pattern of inhibition assays were performed by incubating α2,6SGP arrayed glass slides with SNA that was

pre-incubated with α2,6-sialyllactose, α2,3-sialyllactose and free sialic acid, respectively. As a result, SNA pre-incubated with α2,6-sialyllactose showed no binding to α2,6SGP (Fig 3IIc), while SNA PI with α2,3-siallylactose and free sialic acid still showed lectin binding to α2,6SGP (Fig 3IId and 3IIe). In addition, in order to determine the quantitative sialyation of lactose glycopolymer (**3**), both α2,3SGP and α2,6SGP printed glass slides were incubated with lectin PNA that has specificity to α-galactose on the starting glycopolymer (**3**). As a result, no apparent PNA binding was observed for both α2,3SGP and α2,6SGP printed glass slides (Fig 3If and 3IIf), which indicated that there were no lactose left on the glycopolymer after enzymatic sialylation. Overall, these observations indicate that arrayed SGPs are capable of distinguishing different glycan-binding protein based on their sialic acid linkage to the galactose in the SGPs.

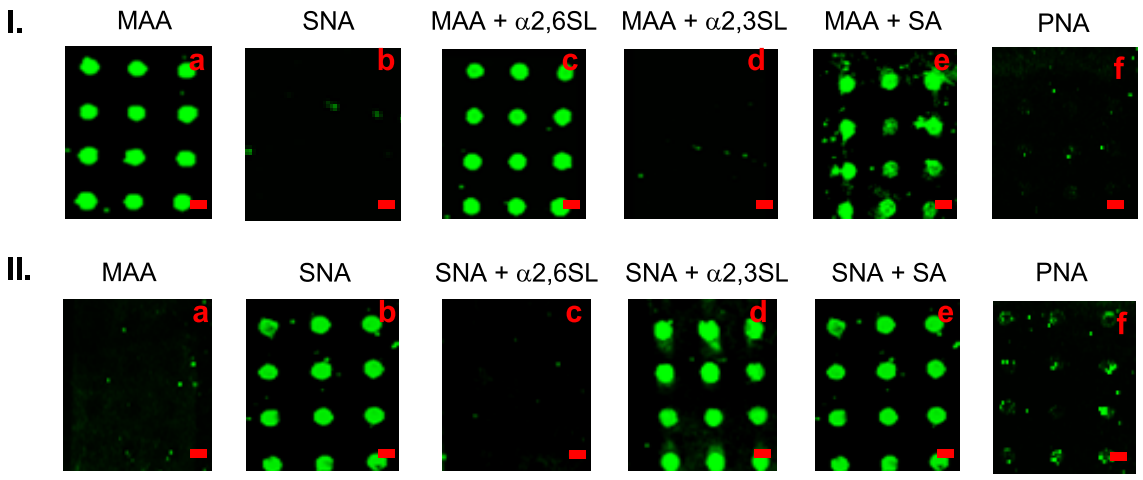


Figure 3. Fluorescence images of α2,3SGP and α2,6SGP microarrays: I. α2,3SGP array incubated with FITC-labeled lectin, *a*. MAA, *b*. SNA, *c*. MAA pre-incubated with α2,6-sialyllactose (α2,6SL), *d*. MAA pre-incubated with α2,3-sialyllactose (α2,3SL), *e*. MAA pre-incubated with free sialic acid (SA), and *f*. PNA; II. α2,6SGP array incubated with FITC-labeled lectin, *a*. MAA, *b*. SNA, *c*. SNA pre-incubated with α2,6SL, *d*. SNA pre-incubated with α2,3SL, *e*. SNA pre-incubated free SA, and *f*. PNA. Bar size: 500 µm. [MAA: *Macckia amurensis*, SNA: *Sambucus nigra,* PNA: *Arachis hypogaea*]

4.3.3 Surface Plasmon Resonance (SPR) analysis of lectin binding to immobilized SGP

The interaction of lectin with oriented immobilized SGPs was also investigated with SPR (BI 2000, Biosensing Instrument). The major advantages of SPR assay are that it is a label free assay and monitors the binding in real time. Initially, the gold sensor chip was functionalized with amine by treating the sensor chip with cysteamine in ethanol overnight, the monolayer formation on the sensor chip was confirmed by increase in the resonance units upon modification of the chip (data not shown). Next, α2,3SP was

covalently immobilized onto the amine modified sensor chip at a flow rate of 5 μL/min in $NaHCO_3$ buffer (pH 10.3) in flow cell one for 10 min while the flow cell two was used a control to subtract the non-specific binding to the surface. Glycopolymer immobilization was repeated until there is no further immobilization observed at fourth time (Fig 4A1-4). Then, a various concentrations of MAA in PBS buffer (pH 7.4) were injected over the α2,3SGP modified sensor chip at a flow rate of 20 μL/min for 2 min. A typical sensogram was obtained as increased binding of lectin to the SGP was observed with increasing lectin concentration (Fig 5A and 5A1). In contrast, only a negligible binding was observed when lectin SNA was injected over α2,3SGP (Fig 6A2), verifying the specific binding of SGP to its specific lectin. Similar experiments were performed with α2,6SGP immobilization onto the chip surface (Fig 4B1-4) and its lectin binding monitored by SPR. As a result, specific SNA binding to immobilized α2,6SGP was observed as shown in Figure 5B, 5B1 and Figure 6A1, while a negligible binding was observed when lectin MAA was injected over α2,6SGP (Fig 6B2). The dissociation constant (k_d) values for MAA and SNA for their respective SGP were determined. Interestingly, MAA and SNA have similar association constant (k_a) values for α2,3SGP and α2,6SGP, respectively (Table 1). However, the k_d were relatively different, the k_d of SNA from α2,6 SGP was observed to be almost 14 fold higher than that of MAA from α2,3SGP. This indicated that the binding of MAA to α2,3SGP is stronger when compared to that of SNA to α2,6SGP.

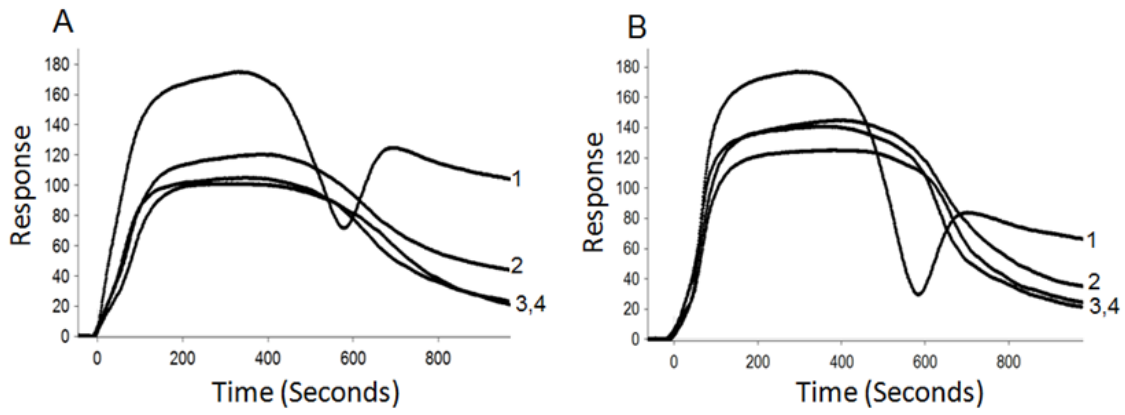


Figure 4. SPR sensorgrams of SGP immobilization: **A**. α2,3SGP immobilization, **B**. α2,6SGP immobilization. **1**. First Injection, **2**. Second injection, **3**. Third injection, **4**. Fourth injection.

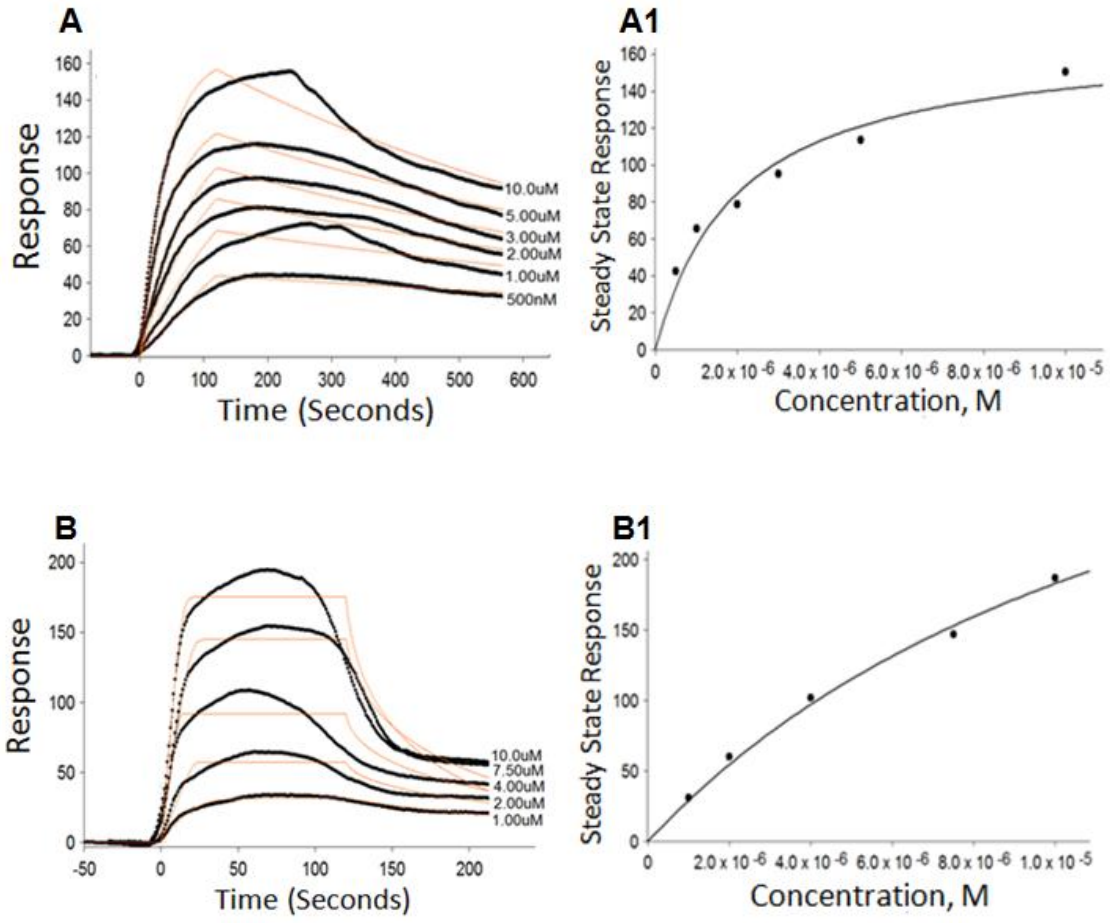


Figure 5. SPR sensorgrams of lectin binding onto SGP: **A**. different concentrations of MAA binding to α2,3SGP; **A1**. Steady state binding response of MAA to α2,3SGP; **B**. different concentrations of SNA binding to α2,6SGP; **B1**. Steady state binding response of SNA to α2,6SGP.

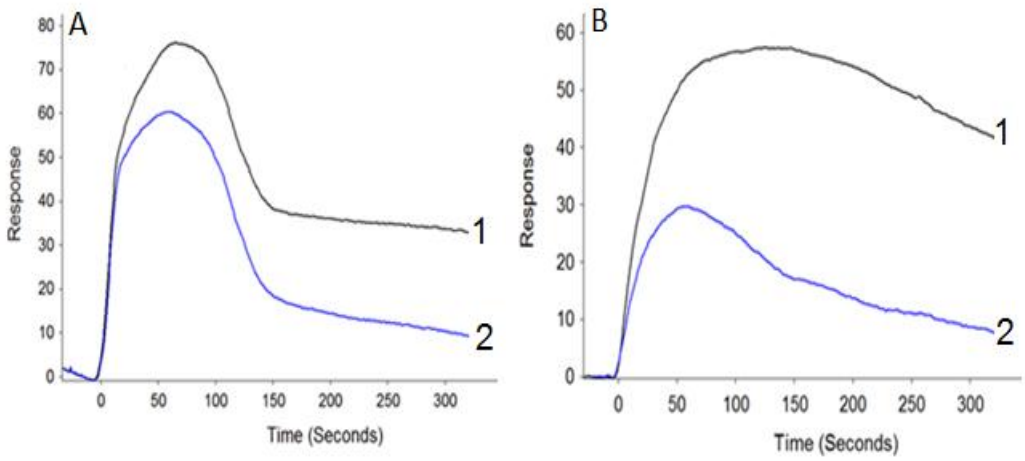


Figure 6. SPR sensorgrams of lectin SNA (**A**) and MAA (**B**) binding to SGP: **A1**. SNA (5 μM) binding to immobilized α2,6SGP; **A2**. SNA binding to immobilized α2,3SGP; **B1**. MAA (5μM) binding to immobilized α2,3SGP; **B2**. MAA binding to immobilized α2,6SGP.

Table 1. Binding kinetics of lectins to immobilized SGPs in SPR assays

Glycopolymer	Lectin	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
α2,3SGP	MAA	4.39×10^{3}	9.08×10^{-4}	2.06×10^{-7}
α2,6SGP	SNA	4.69×10^{3}	1.25×10^{-2}	2.60×10^{-6}

4.3.4 Competitive binding assay of lectin to immobilized SGP

Next, competitive binding assays were conducted to confirm the specific lectin binding to immobilized SGP by pre-incubating the lectin with free ligands. Briefly, the SGP were immobilized onto SPR chip as described above. The lectin was pre-incubated with different concentrations of sialyllactose (α2,3SL for MAA and α2,6SL for SNA) in

PBS buffer (pH 7.4) for 30 min before injecting over the immobilized SGP. It was observed that by increasing the concentration of sialyllactose (from 0.75 nM to 7.5 nM) the binding of lectin to immobilized SGP is reduced significantly as shown in Figure 7Aa-b for α2,3SGP and Figure 7Ba-b for α2,6SGP, whereas when the lectins were pre-incubated with non specific sialyllactose i.e. MAA with α2,6SL (Fig 7A-e) and SNA with α2,3SL (Fig 7B-e), there was no inhibition observed. Even in higher concentration of 7.5 nM, there is no inhibition in lectin binding to their corresponding SGP when compared to the lectins pre-incubated with specific sialyllactose (Fig 7A1and B1). This was due to the fact that sialyllactoses saturate the binding sites of the lectin, causing its reduced binding to the immobilized SGP and so further confirms the fact that the α2,3SGP and α2,6SGP show specifically binding activity to their corresponding protein, respectively.

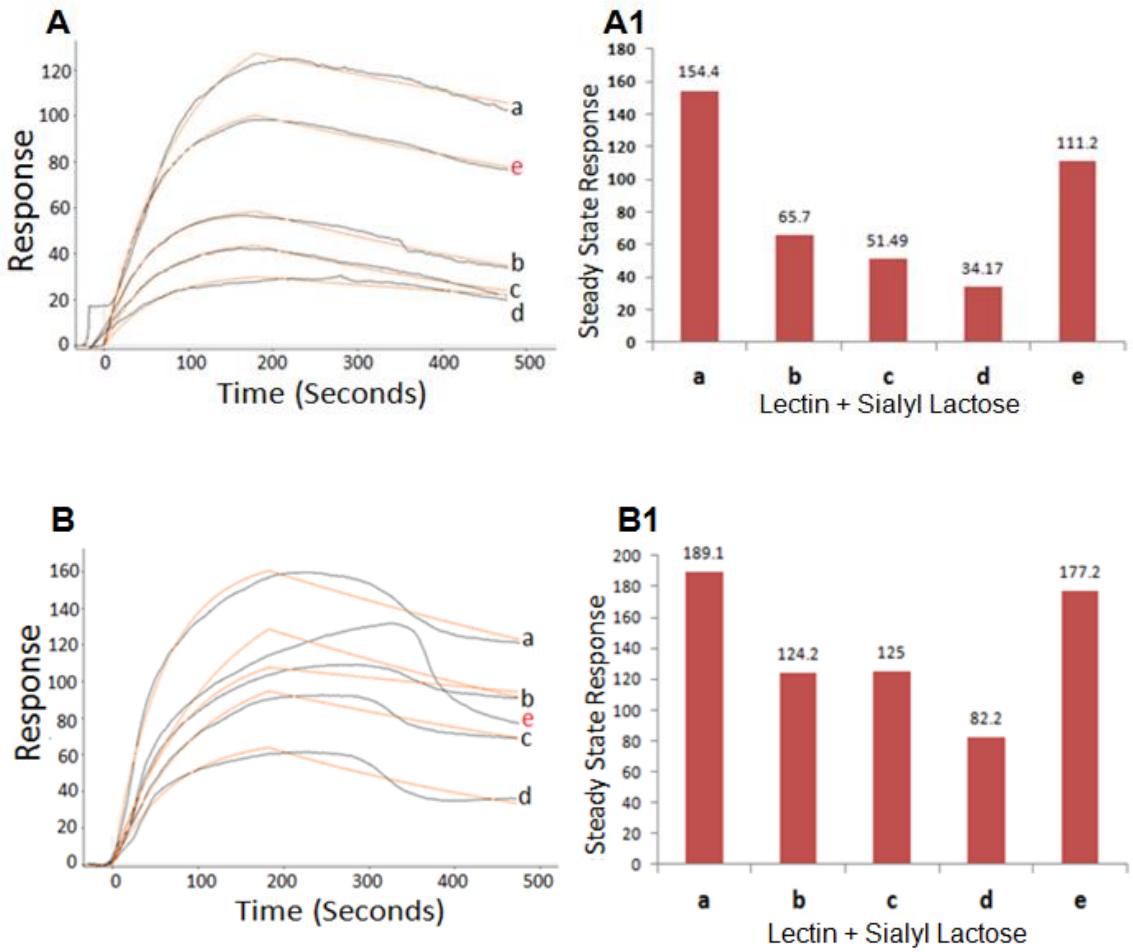


Figure 7. SPR sensorgrams of lectin binding to SGP: **A**. Lectin binding to α2,3SGP, MAA pre-incubated with α2,3SL in concentration of ***a***. 0.075 nM, ***b***. 0.15 nM, ***c***. 0.25 nM, ***d***. 0.75 nM, ***e***. MAA pre-incubated with 0.75 nM of α2,6SL; **A1**. Steady state response of lectin binding to α2,3SGP; **B**. Lectin binding to α 2,6SGP, SNA pre-incubated with α2,6SL in concentration of ***a***. 0.075 nM, ***b***. 0.15 nM, ***c***. 0.25 nM, d 0.75 nM, ***e***. SNA pre-incubated with 0.75 nM of α2,3SL; **B1**. Steady state response of lectin binding to α2,6SGP.

4.3.5 Hemagglutinin binding to immobilized SGP monitored by SPR

With the binding specificity confirmed for immobilized SGPs, we next examined their specific binding to influenza viral hemagglutinins (HAs) of avian H_5N_1 (A/Anhui/1/2005(H5N1)) and human H_3N_2 (A/Brisbane/10/2007(H3N2)) with SPR technique, which bind to α2,3SGP and α2,6SGP, respectively. Human and avian HA in PBS buffer (pH 7.4) were injected over immobilized α2,3SGP and α2,6SGP, respectively. As a result, avian HA showed stronger binding (response units) to α2,3SGP (Fig 8Aa) than α2,6SGP (Fig 8Bd), and avian HA binds to α2,3SGP much stronger so that there is no dissociation observed from α2,3SGP (Table 2). Also, when avian HA was pre-incubated with both α2,6-sialylactose and α2,3-sialylactose, the binding affinity of HA decreased rapidly compared to HA alone (Fig 8Ab, 8Ac), however, the dissociation of avian HA pre-incubated with α2,3-sialylactose was 2 fold higher than that of avian HA pre-incubated with α2,6-sialylactose (Table 2) indicating that avian HA binds strongly to α2,3-linked sialic acid. On the other hand, human HA showed higher binding (response units) to α2,6SGP (Fig 8Ba) than α2,3SGP (Fig 8Ad), the dissociation of human HA from α2,3SGP is 25 fold higher than that from α2,6SGP (Table 2), indicating that human HA detached more rapidly from α2,3SGP and binds strongly to α2,6SGP. When human HA was pre-incubated with both α2,6-sialylactose and α2,3-sialylactose, the binding affinity of human HA decreased rapidly compared to human HA alone (Fig 8Bb, 8Bc), however, the dissociation of human HA pre-incubated with 2,6-sialylactose was 5 fold higher than that of human HA pre-incubated with α2,3-sialylactose (Table 2) indicating that human HA binds strongly to α2,6-linked sialic acid. Overall, these observations

indicated that avian influenza virus HA has higher affinity to α2,3-linked sialic acid and human virus to α2,6-linked sialic acid of the immobilized SGP.

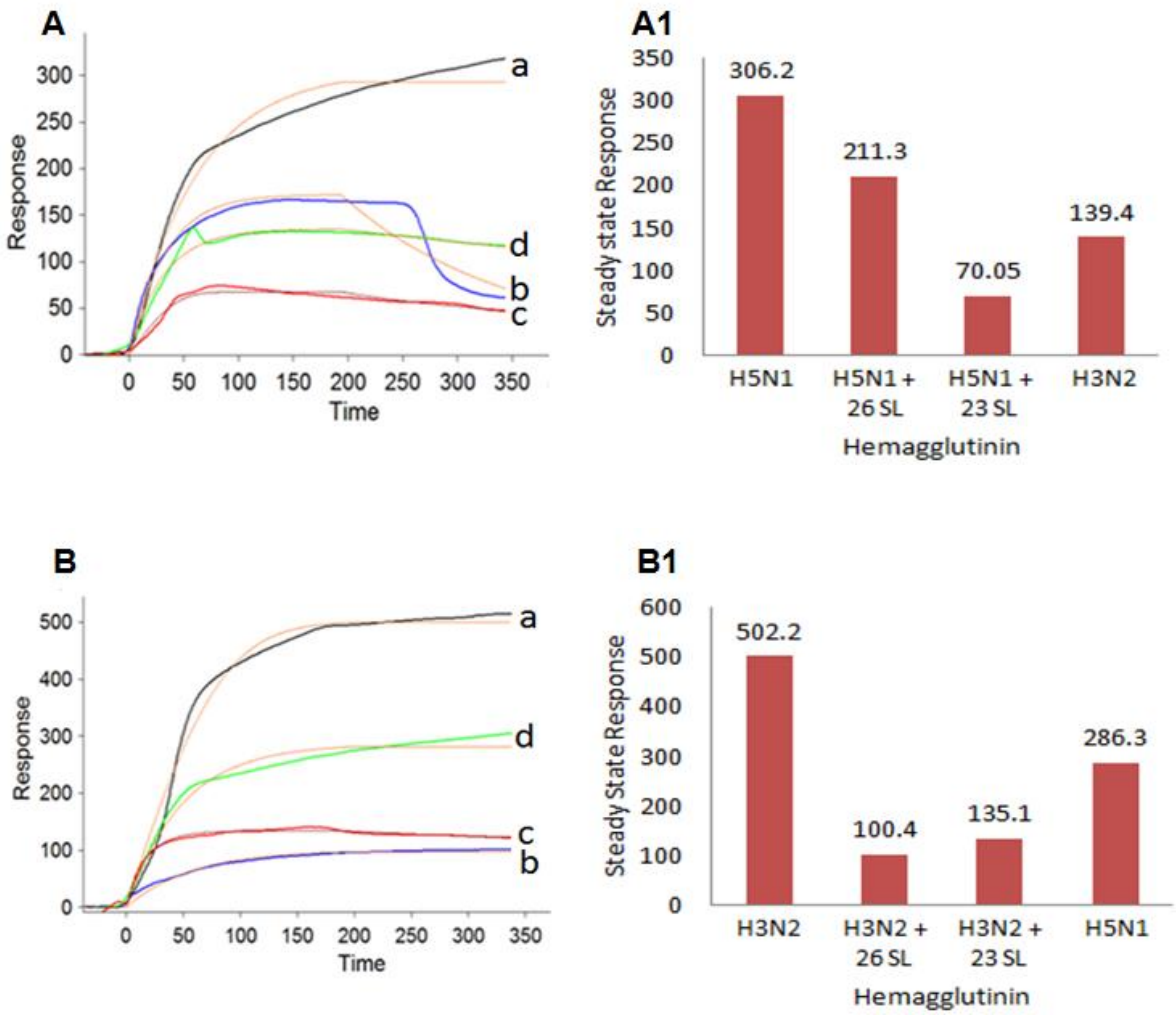


Figure 8. SPR sensorgrams of HA binding to SGP: **A**. HA binding to α2,3SGP, ***a***. avian HA, ***b***. avian HA pre-incubated with α2,6SL, ***c***. avian HA pre-incubated with α2,3SL, ***d***. human HA; **A1**. Steady state response of HA binding to α2,3SGP; **B**. HA binding to α2,6SGP, ***a***. human HA, ***b***. human HA pre-incubated with α2,6SL, ***c***. human HA pre-incubated with α2,3SL, ***d***. avian HA; **B1**. Steady state response of HA binding to α2,6SGP.

Table 2. HA binding to immobilized SGP monitored by SPR

Glycopolymer	HA type	k_d (s^{-1})	Response (RU)
α2,3SGP	H5N1	nd	307.2
	H5N1 + α2,3SL	6.5×10^{-3}	212.4
	H5N1 + α2,6SL	3.7×10^{-3}	69.84
	H3N2	1×10^{-3}	138.2
α2,6SGP	H3N2	4×10^{-5}	502.2
	H3N2 + α2,3SL	2×10^{-4}	100.4
	H3N2 + α2,6SL	7×10^{-4}	135.1
	H5N1	6×10^{-5}	286.3

(nd: not detectable)

4.4 Conclusion

A chemoenzymatic synthesis of *O*-cyanate chain-end functionalized sialyllactose-containing glycopolymers was developed *via* one-pot cyanoxyl mediated free radical polymerization followed by enzymatic sialylation. The chain-end *O*-cyanate group of the polymer provides an anchor for oriented and covalent immobilization of the glycopolymer onto amine surface *via* isourea bond formation in mild condition, which were used for glycoarray and biosensor applications. The specific binding activity of the arrays were confirmed with α2,3- and α2,6-sialyl binding lectin together with inhibition assays. Further, SPR-based glyco-sensor showed specific binding activity for lectins and influenza hemagglutins (HA) as well. These oriented sialyloligo-macroligand derived

glycoarray and SPR-based glyco-sensor are closely to mimic 3D nature presentation of sialyloligosaccharides and will provide important high-throughput tools for virus diagnosis and potential anti-viral drug candidates screening applications. In addition, the chain-end functionalizable glycopolymers can be used for polymer-protein conjugation as well.

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CHAPTER V

SUMMARY

As carbohydrates present on the cell surfaces act as biomolecular recognition markers for a variety of important biological functions, including cell communications, cell adhesion, fertilization, differentiation and immune response through specific interactions with proteins, it is essential to carefully choose carbohydrate epitopes and study the carbohydrate-protein interactions. Glycans structured into multivalent ligands in the form of linear polymers, have potential microarray application to study the carbohydrate binding molecules. Also the low affinity and specificity which is typical for monomeric carbohydrate-protein interactions are noticeably enhanced when carbohydrate components are present as multivalent ligands like glycopolymers. Besides carbohydrate microarrays processes a wide variety of potential applications in glycomics including rapid determination of binding profile of carbohydrate-binding proteins, detection of specific antibodies for disease diagnosis, characterization of carbohydrate-cell recognition events and the high-throughput screening of inhibitors to prevent carbohydrate-protein interactions. On the other hand surface Plasmon resonance

technique has several advantages in studying the bio-molecular interaction in real-time with a minute amount of label free samples.

In this thesis study, oriented and multivalent glycomacroligands were designed and developed based on a chain-end functionalized carbohydrate-containing polymer (glycopolymer) for efficient proteomic applications.

First, the *O*-cyanate chain-end functionalized glycopolymer was synthesized *via* one-pot cyanoxyl mediated free radical polymerization technique and there is no protection and deprotection steps needed and thus is a straightforward approach. The chain-end *O*-cyanate group of the polymer provides an anchor for site-specific and covalent immobilization of the glycopolymer onto any amine surface *via* isourea bond formation in mild condition. An oriented glyco-marcroligand formation based on *O*-cyanate chain-end functionalized glycopolymer with commercially available amine modified silica gel and glass slide *via* isourea bond formation was demonstrated, respectively.

Second, an oriented and density controlled glyco-marcroligand array formation was demonstrated by end-point immobilization of glycopolymer imprinted with boronic acid ligands in different sizes. Briefly, *O*-cyanate chain-end functionalized glycopolymer was pre-modified by phenylboronic acid-lysozyme, BSA and polyacrylamide ligands and then immobilized onto amine-functionalized glass slide *via* isourea bond formation at pH 10.3, followed by releasing the phenylboronic acid ligands at pH 7.4, respectively. Glycoarray and SPR results confirmed the same trend of density-dependent binding of lectins. Imprinted glyco-marcroligand showed more lecting binding than non-imprinted one, and the phenylboronic acid-polyacrylamide imprinted glyco-marcroligand showed highest lectin binding.

Third, a chemoenzymatic synthesis of *O*-cyanate chain-end functionalized sialyllactose-containing glycopolymers was developed *via* one-pot cyanoxyl mediated free radical polymerization followed by enzymatic sialylation. The chain-end *O*-cyanate group of the polymer provides an anchor for oriented and covalent immobilization of the glycopolymer onto amine surface *via* isourea bond formation in mild condition, which were used for glycoarray and biosensor applications. The specific binding activity of the arrays were confirmed with α2,3- and α2,6-sialyl binding lectin together with inhibition assays. Further, SPR-based glyco-sensor showed specific binding activity for lectins and influenza hemagglutins (HA) as well.

The orientally immobilized multivalent glyco-macroligands could be used for will provide important high-throughput tools for virus diagnosis and potential anti-viral drug candidates screening applications. In addition, the chain-end functionalizable glycopolymers can be used for polymer-protein conjugation as well.

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CHAPTER VI

FUTURE PERSPECTIVES

Overall, in this study, oriented multivalent glycopolymer has been developed and could be used to study carbohydrate-protein interactions and thus are expected to constitute a core strategy of rapidly and sensitively identifying carbohydrate binding proteins. Also the immobilized sialyated glycopolymer was used to study the binding affinities of both avian and human influenza viral recombinant hemagglutinin proteins. In future, the potential of the oriented glyco-macroligad for analyzing influenza viral hemagglutinin should be investigated by mass spectrometry technique. Mass spectrometry has served an important role in the characterization of a range of viral and bacterial pathogens over several decades.[1-6] It has been recruited to characterize viral proteins,[7-9] their posttranslational modifications[10] from disulphide bonds to glycosylation[11-12] follow mutations in these proteins[13] that result from replication errors or the trading of genetic material from one strain to another, and assess viral structure through studies of protein interactions and assemblies[14-16] and the binding of antiviral

drugs.[17] Mass spectrometry offers advantages for characterizing the influenza virus in terms of its genetic character, viral protein character, antigenicity, and for the quantitation of viral proteins and antiviral drugs.[18]

In future, both α,2-3 and α,2-6 linked sialylglycopolymers will be immobilized onto silica gel for HA and its proteolysis product isolation. For this study HA will be digested and followed by isolation with oriented glyco-macroligand on silica gel and subsequent mass spectrometry analysis so as to seek the sialic acid-binding residue, conserved residue and peptide segments that can be used to subtype the influenza virus. On the other hand, proteolysis of whole virus or gel-recovered influenza antigens[19-20] combined with mass spectrometry provides a means with which to investigate these proteins and subtype strains where the hemagglutinin surface antigens will be studied. With the efficient influenza virus capture surfaces with receptor selectivity established above, rapid isolation and identification of real influenza virus will be conducted.

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