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Immobilized Sialyloligo-Macroligand and Its Protein Binding Specificity

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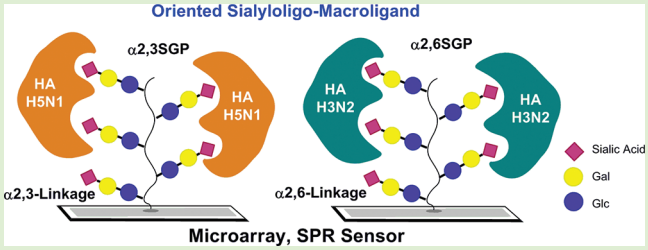
ABSTRACT: We report a chemoenzymatic synthesis of chain-end functionalized sialyllactose-containing glycopolymers with different linkages and their oriented immobilization for glycoarray and SPR-based glyco-biosensor applications. Specifically, *O*-cyanate chain-end functionalized sialyllactose-containing glycopolymers were synthesized by enzymatic α2,3- and α2,6-sialylation of a lactose-containing glycopolymer that was synthesized by cyanoxyl-mediated free radical polymerization. ^{1}H NMR showed almost quantitative α2,3- and α2,6-sialylation. The *O*-cyanate chain-end functionalized sialyllactose-containing glycopolymers were printed onto amine-functionalized glass slides via isourea bond formation for glycoarray formation. Specific protein binding activity of the arrays was confirmed with α2,3- and α2,6-sialyl specific binding lectins together with inhibition assays. Further, immobilizing *O*-cyanate chain-end functionalized sialyllactose-containing glycopolymers onto amine-modified SPR chip via isourea bond formation afforded SPR-based glyco-biosensor, which showed specific binding activity for lectins and influenza viral hemagglutinins (HA). These sialyloligo-macroligand derived glycoarray 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 antiviral drug candidates screening applications.



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 an indispensable receptor for the human-to-human spread of the influenza virus, whereas Siaα2,3Gal is the receptor for avian influenza virus infection.[9,10] Therefore, sialyoligosaccharide is likely the choice for an active ligand for specific and stronger influenza HA binding and thereby multivalent sialyloligosaccharides 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

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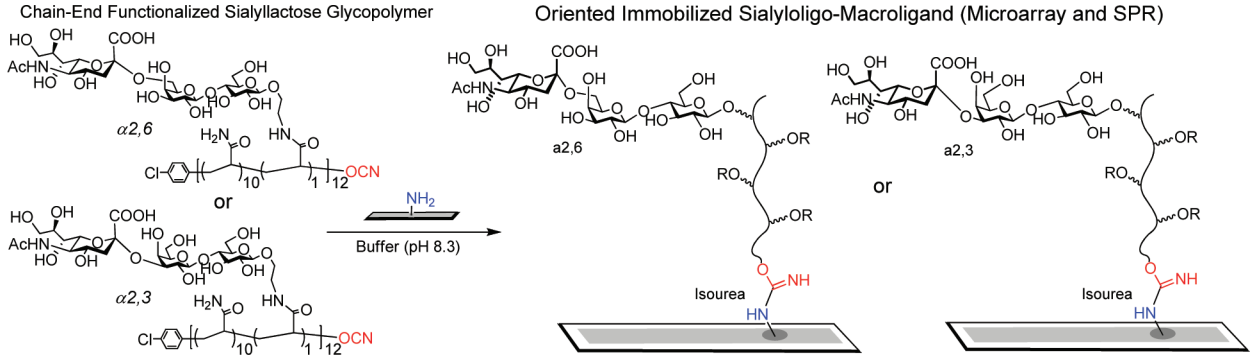

Figure 1. Chemical structure of *O*-cyanate chain-end functionalized sialyllactose-containing glycopolymers and their oriented immobilization via isourea bond formation for microarray and SPR applications.

glycopolymers are the focus for biofunctionalization, 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 (Figure 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.

EXPERIMENTAL SECTION

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]

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) + $ZnCl_2$ (5×10^{-5} M), pH 7.4]. To the dissolved polymer were added 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), and the mixture was stirred gently at room temperature for two days (48 h). The resultant α2,3-sialyllacotose glycopolymer (α2,3SGP) was purified by gel-filtration with a 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_2− 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_2− of polymer backbone), 1.89 (CH_3 of Neu5Ac), 1.70−1.30 (−CH_2− of polymer backbone).

Sialyllactose Glycopolymer Microarray Fabrication. Micro-Caster microarray tool (Whatman, spot size 500 μm diameter) was inked with a solution of α2,3SGP in $NaHCO_3$ buffer (pH 10.3, 2.1×10^{-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 °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*, 1.4×10^{-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*, 1.3×10^{-3} mM, Bioworld), PNA-FITC (*Arachis hypogaea*, 1.6×10^{-3} mM, Sigma), and MAA-FITC (0.2 mg, 1.4×10^{-3} mM, Bioworld) that was preincubated with α2,3-sialyllactose (1.5×10^{-2} mM, V-Laboratories), α2,6-sialyllactose (1.5×10^{-2} mM, V-Laboratories), and free sialic acid (3.2×10^{-2} mM, Rose Scientific ltd) in 0.2% PBST buffer (pH 7.4) at room temperature for 2 h, respectively, 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 preincubated 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), and 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

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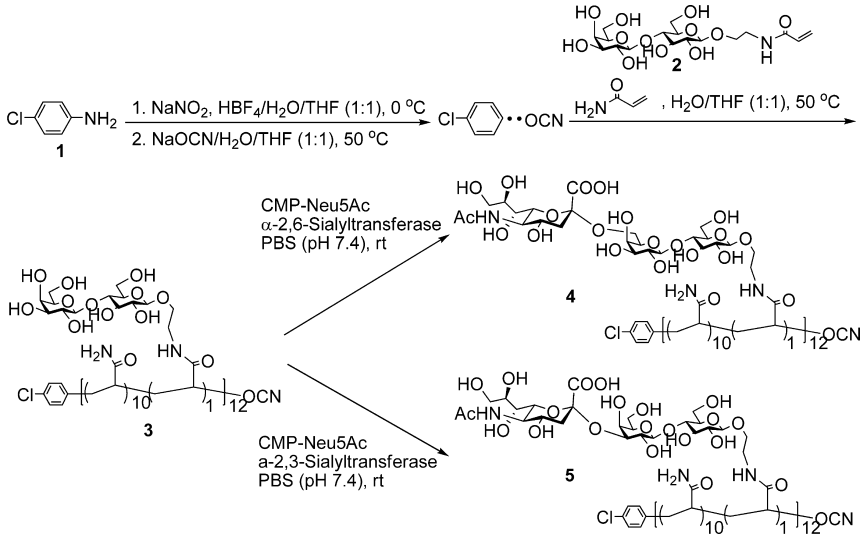
Scheme 1. Chemoenzymatic Synthesis of *O*-Cyanate Chain-End Functionalized Sialyllactose-Containing Glycopolymers via Cyanoxyl Mediated Free Radical Polymerization Followed by Enzymatic Sialylation



(pH 7.4) for 30 min and finally subjected to fluorescent imaging, respectively.

SPR Analysis of Specific MAA and SNA Binding to Immobilized α2,3SGP 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 s 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 s, 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 s 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.

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 preincubated with a series of α2,3-sialyllactose and α2,6-sialyllactose in concentrations ranging from 0.075 to 7.5 nM, respectively. As control, MAA and SNA (10 μM) were preincubated with high concentration (7.5 nM) of 2,6-sialyllactose and 2,3-sialyllactose, respectively. These preincubated MAA and SNA were injected over the immobilized α2,3SGP and α2,6SGP at a flow rate of 20 μL/min for 3 min (180 s, 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 s. The response of the binding was calculated by standard BI 2000 (scrubber) evaluation software.

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) and then were injected over the two immobilized α2,3SGP and α2,6SGP at a flow rate of 20 μL/min for 180 s, respectively. As a control, both H5N1 HA and H3N2 HA were preincubated with α2,3-sialyllactose (PBS buffer (pH 7.4), 5×10^{-6} mM) and α2,6-sialyllactose (PBS buffer (pH 7.4), 5×10^{-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.

RESULTS AND DISCUSSION

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 nonreducing 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

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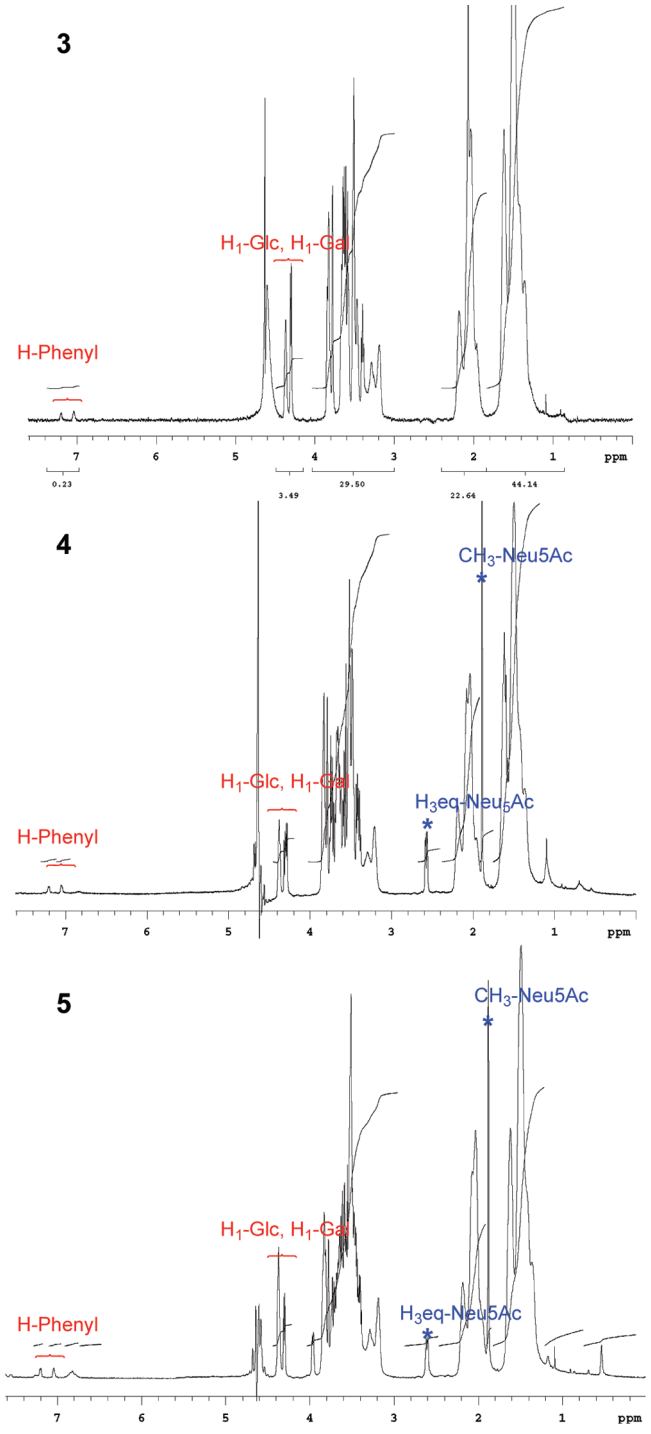

Figure 2. ^{1}H NMR spectra of glycopolymers in D_2O: (A) lactose glycopolymer (**3**), (B) α2,6-sialyllactose glycopolymer (**4**), and (C) α2,3-sialyllactose glycopolymer (**5**).

enzymes α2,6-sialyltransferase and α2,3-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), and 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 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%.

SGP Microarray Fabrication and Its Specific Lectin Binding. SGP-based microarrays were fabricated by printing *O*-cyanate chain-end SGP onto an 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 °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. Lectin MAA that has affinity for α2,3-linked sialic acid showed binding to α2,3SGP (Figure 3Ia), whereas lectin SNA which has affinity for α2,6-linked sialic acid showed no binding to α2,3SGP (Figure 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 preincubated with α2,6-siallylactose, α2,3-siallylactose, and free sialic acid, respectively. As a result, MAA preincubated with α2,3-sialyllactose showed no binding to α2,3SGP (Figure 3Id), whereas MAA preincubated with α2,6-siallylactose and free sialic acid still showed lectin binding to α2,3SGP (Figures 3, panels Ic and Id). On the other hand, lectin MAA showed no binding to α2,6SGP (Figure 3IIa), whereas lectin SNA showed binding to α2,6SGP (Figure 3IIb). The same pattern of inhibition assays were performed by incubating α2,6SGP arrayed glass slides with SNA that was preincubated with α2,6-sialyllactose, α2,3-sialyllactose, and free sialic acid, respectively. As a result, SNA preincubated with α2,6-sialyllactose showed no binding to α2,6SGP (Figure 3IIc), whereas SNA PI with α2,3-siallylactose and free sialic acid still showed lectin binding to α2,6SGP (Figures 3, panels IId and IIe). 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 β-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 (Figure 3, panels If and IIf), 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.

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

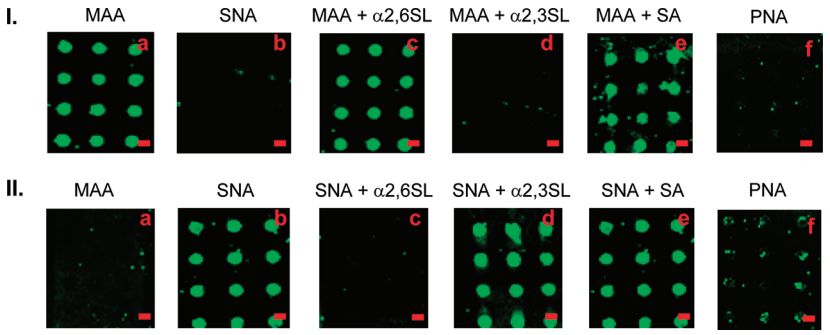

Figure 3. Fluorescence images of α2,3SGP and α2,6SGP microarrays: I. α2,3SGP array incubated with FITC-labeled lectin, a. MAA, b. SNA, c. MAA preincubated with α2,6-sialyllactose (α2,6SL), d. MAA preincubated with α2,3-sialyllactose (α2,3SL), e. MAA preincubated with free sialic acid (SA), and f. PNA;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

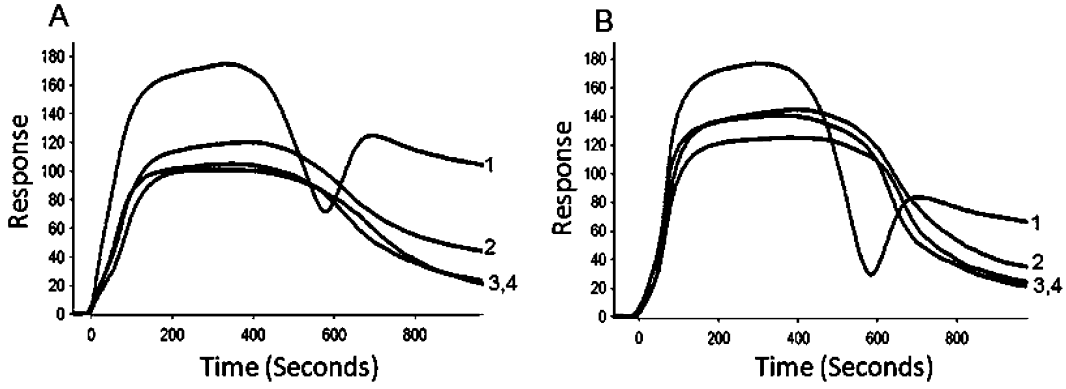

Figure 4. SPR sensorgrams of SGP immobilization: A. α2,3SGP immobilization, B. α2,6SGP immobilization. 1. First Injection, 2. Second injection, 3. Third injection, 4. Fourth injection.

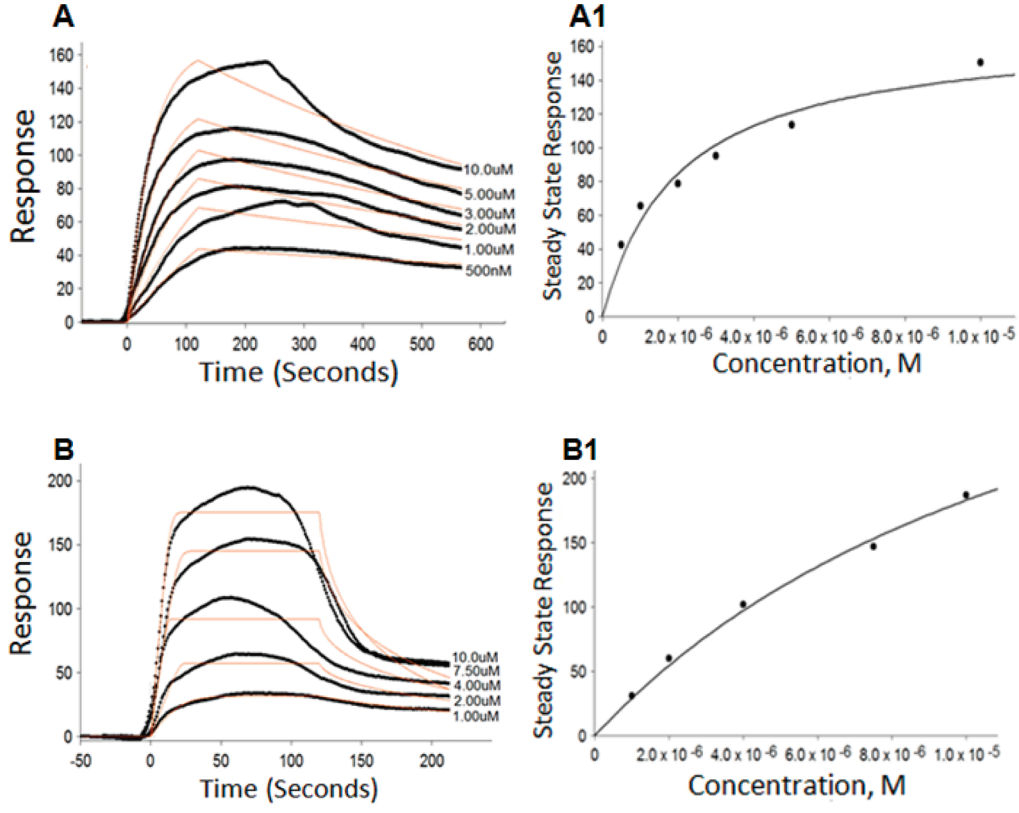

Figure 5. SPR sensorgrams of lectin binding onto SGP: A. different concentrations of MAA binding to α2,3SGP; A1. Steady state binding response of MAA to α2,3SGP; B. different concentrations of SNA binding to α2,6SGP; B1. Steady state binding response of SNA to α2,6SGP.

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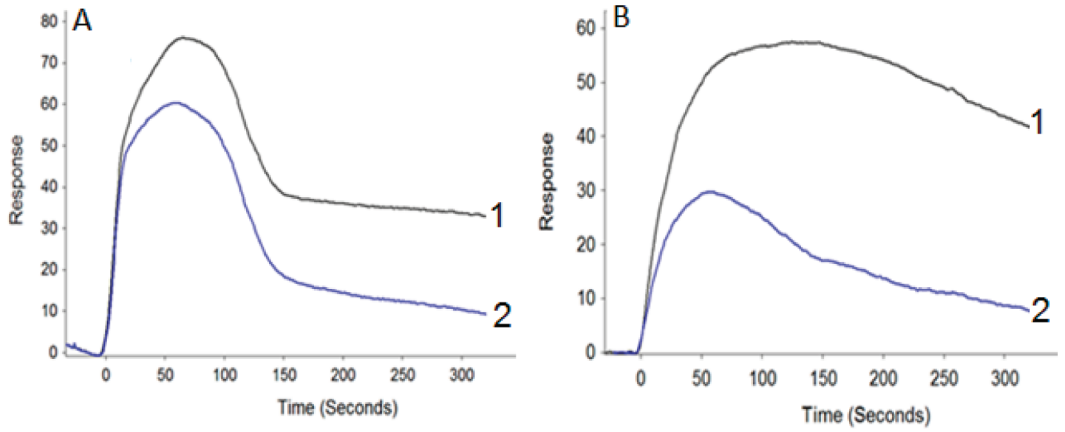

Figure 6. SPR sensorgrams of lectin SNA (A) and MAA (B) binding to SGP: A1. SNA (5 μM) binding to immobilized α2,6SGP; A2. SNA binding to immobilized α2,3SGP; B1. MAA (5 μM) binding to immobilized α2,3SGP; B2. MAA binding to immobilized α2,6SGP.

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}

confirmed by increase in the resonance units upon modification of the chip (data not shown). Next, α2,3SGP 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 nonspecific binding to the surface. Glycopolymer immobilization was repeated until there is no further immobilization observed at fourth time (Figure 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

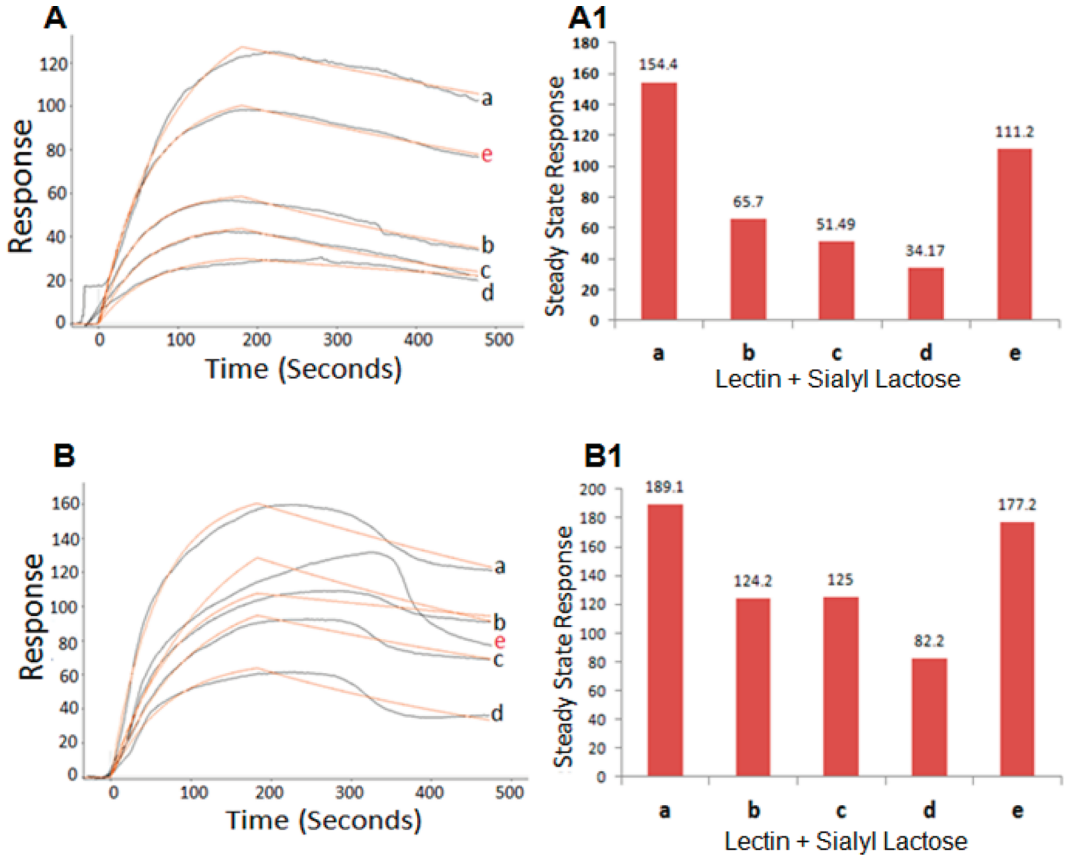

Figure 7. SPR sensorgrams of lectin binding to SGP: A. Lectin binding to α2,3SGP, MAA preincubated with α2,3SL in concentration of a. 0.075 nM, b. 0.15 nM, c. 0.25 nM, d. 0.75 nM, e. MAA preincubated with 0.75 nM of α2,6SL; A1. Steady state response of lectin binding to α2,3SGP; B. Lectin binding to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

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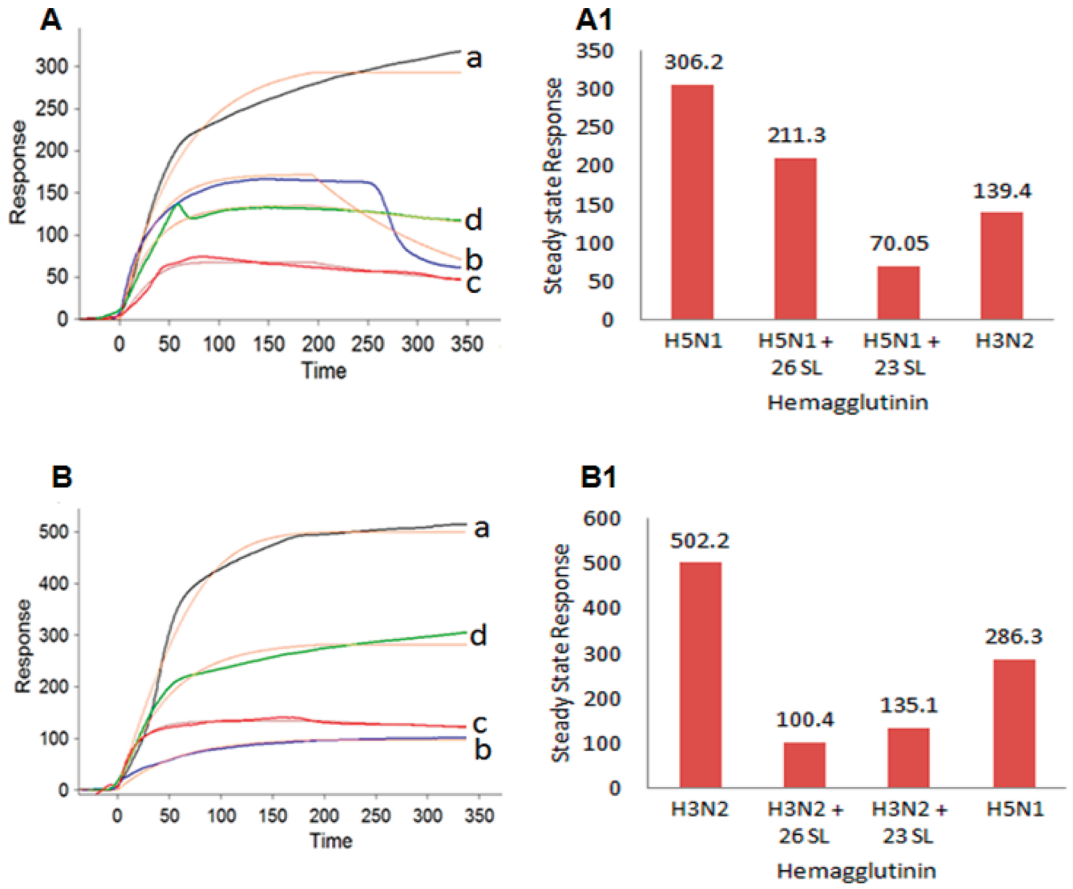

Figure 8. SPR sensorgrams of HA binding to SGP: A. HA binding to α2,3SGP, a. avian HA, b. avian HA preincubated with α2,6SL, c. avian HA preincubated with α2,3SL, d. human HA; A1. Steady state response of HA binding to α2,3SGP; B. HA binding to α2,6SGP, a. human HA, b. human HA preincubated with α2,6SL,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

of lectin to the SGP was observed with increasing lectin concentration (Figure 5, panels A and A1). In contrast, only a negligible binding was observed when lectin SNA was injected over α2,3SGP (Figure 6A2), verifying the specific binding of SGP to its specific lectin. Similar experiments were performed with α2,6SGP immobilization onto the chip surface (Figure 4B1−4) and its lectin binding monitored by SPR. As a result, specific SNA binding to immobilized α2,6SGP was observed as shown in Figures 5, panels B and B1, and 6A1, while a negligible binding was observed when lectin MAA was injected over α2,6SGP (Figure 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.

Competitive Binding Assay of Lectin to Immobilized SGP. Next, competitive binding assays were conducted to confirm the specific lectin binding to immobilized SGP by preincubating the lectin with free ligands. Briefly, the SGP were immobilized onto a SPR chip as described above. The lectin was preincubated 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 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 preincubated with nonspecific sialyllactose, i.e., MAA with α2,6SL (Figure 7A-e) and SNA with α2,3SL (Figure 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 preincubated with specific sialyllactose (Figure 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.

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 H5N1 (A/Anhui/1/2005(H5N1)) and human H3N2 (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 (Figure 8Aa) than α2,6SGP (Figure 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 preincubated with both α2,6-sialylactose and α2,3-sialylactose, the binding affinity of

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Table 2. HA Binding to Immobilized SGP Monitored by SPR

glycopolymer	HA type	k_d (s^{-1})	response (RU)
α2,3SGP	H5N1	nd[a]	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

[a]nd: not detectable.

HA decreased rapidly compared to HA alone (Figure 8, panels Ab and Ac), however, the dissociation of avian HA preincubated with α2,3-sialylactose was 2 fold higher than that of avian HA preincubated 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 (Figure 8Ba) than α2,3SGP (Figure 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 preincubated with both α2,6-sialylactose and α2,3-sialylactose, the binding affinity of human HA decreased rapidly compared to human HA alone (Figure 8, panels Bb and Bc), however, the dissociation of human HA preincubated with 2,6-sialylactose was 5 fold higher than that of human HA preincubated 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.

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 antiviral drug candidates screening applications. In addition, the chain-end functionalizable glycopolymers can be used for polymer−protein conjugation as well.

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Notes

The authors declare no competing financial interest.

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