Note

An α2,3-Linked Sialylglycopolymer as a Multivalent Glycoligand against Avian and Human Influenza Viruses

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Abstract: A glycopolymer bearing α2,3-linked sialyltrisaccharides was synthesized by living radical polymerization using a glycomonomer prepared by a protecting-group-free process, direct azidation of the free sialyllactose, and subsequent azide-alkyne cycloaddition. The prepared glycopolymer with pendant 3′-sialyllactose moieties strongly interacted with both avian and human influenza viruses analyzed by the hemagglutination inhibition assay and the quartz crystal microbalance method.

Key words: sialyloligosaccharide, protecting-group-free, click chemistry, RAFT polymerization, glycocluster effect, influenza virus

Oligosaccharides on the cell surface play significant roles in many important cellular recognition processes, including cell growth regulation, differentiation, adhesion, cancer cell metastasis, cellular trafficking, inflammation by bacteria and viruses, and immune response. It is well known that influenza viruses have two spike glycoproteins on their surface, hemagglutinin (HA) and neuraminidase. Sialic acid (SA) is an important monosaccharide for human health due to its functional groups, including hydroxy, carboxy, and acetamido groups. HA binds to the SA moiety of glycoconjugates on the host cell surface, and this bound HA acts as a functional receptor to initiate infection. Avian and human influenza virus HAs mainly bind to α2,3- and α2,6-linked sialylgalacto oligosaccharides, respectively.[1)2)3)] Although saccharide-protein interactions are generally weak, these interactions are amplified by multivalent forms of saccharides. Such multivalent saccharides give rise to the "glycocluster effect"[4)5)] that underlies various biological processes, including the interaction between HA and sialyloligosaccharides. Glycopolymers are synthetic polymers with multivalent pendant saccharides and have received much attention as artificial glycoclusters in diverse fields such as polymer science, material science, and biology.[6)7)8)9)10)11)] Glycopolymers amplify the saccharide

signals of natural ligands, as well as of glycodendrimers, glyconanoparticles, and glycoconjugates. The synthesis of glycomonomers to obtain glycopolymers is laborious and requires a multistep process, including the protection of hydroxy groups on the saccharide moieties, activation of the anomeric position, introduction of a polymerizable group, and removal of the protecting groups. Furthermore, when oligosaccharides with a SA moiety are used as starting materials, a more laborious and time-consuming process is required to synthesize glycomonomers because the carboxy group on the SA moiety must be protected.

Little has been reported regarding the synthesis of glycopolymers bearing sialyloligosaccharides. Kobayashi *et al.* reported the synthesis of sialyltrisaccharide-bearing polystyrene by radical polymerization using a styrene derivative carrying a saccharide moiety, starting from a mixture of free α2,3- and α2,6-linked sialyllactoses.[12)] However, most of them are enzymatic methodology conducted by sialyltransferase to attach the SA moiety onto the saccharide moieties of the polymer backbone.[13)14)15)16)17)] Another method to obtain glycopolymers bearing sialyloligosaccharides is post-attachment of sialyloligosaccharide moieties by chemical reaction onto the polymer backbone.[18)19)] The development of a simpler and more efficient synthetic method for glycopolymers that can be applied to free sialyloligosaccharides is desirable for the preparation of glyco-monomers and -polymers from free saccharides. In this paper, we report the protecting-group-free synthesis of glycomonomers carrying α2,3-linked sialyltrisaccharides from free corresponding sialyllactose via the direct synthesis of β-glycosyl azide and copper-catalyzed azide-alkyne cycloaddition (CuAAC).[20)] Furthermore, we followed the reversible addition-fragmentation chain transfer (RAFT) polymerization reaction[21)22)] using the resulting glycomonomer. In ad-

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Abbreviations: HA, hemagglutinin; SA, sialic acid; CuAAC, copper-catalyzed azide-alkyne cycloaddition; RAFT, reversible addition-fragmentation chain transfer; HI, hemagglutination inhibition; QCM, quartz crystal microbalance; 3′SALac, 3′-sialyllactose; DMC, 2-chloro-1,3-dimethylimidazolinium chloride; DIPEA, *N,N*-diisopropylethylamine; CuSO₄, copper(II) sulfate pentahydrate; AscNa, L-ascorbic acid sodium salt; TBTA, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)-methyl]amine; 6′SA-Lac, 6′-sialyllactose; Lac, lactose; AAm, acrylamide; V-70, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); BTSE, 2-(benzylsulfanylthiocarbonylsulfanyl) ethanol; BSA, bovine serum albumin.
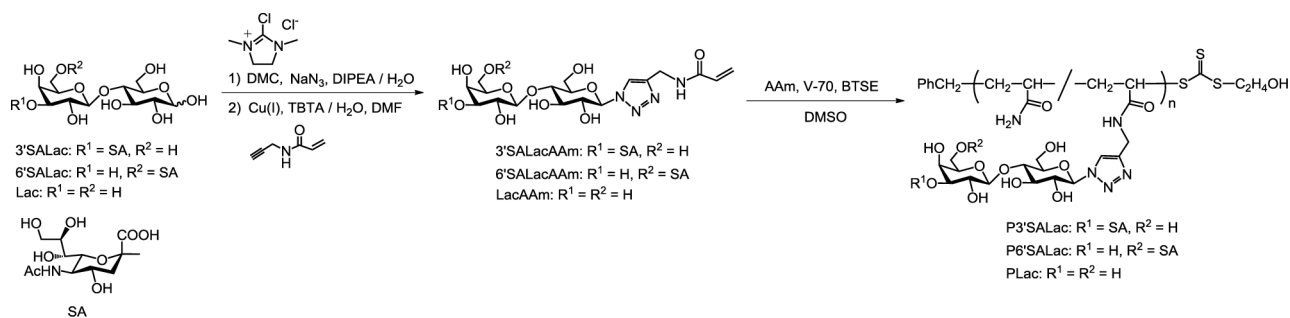
Fig. 1. Protecting-group-free synthesis of glyco-monomers and -polymers from free saccharides.

Table 1. Synthesis of glycopolymers by RAFT polymerization.[a]

| Glycopolymer	Glycomonomer	Molar ratio of glycomonomer /AAm	Conv. (%)[b]	Yield (%)[c]	$M_{n(NMR)}$ (g mol^{-1})[b]	$M_{n(GPC)}$ (g mol^{-1})[d]	M_w/M_n[d]	Saccharide unit ratio in polymer (%)[b]
P3´SALac	3´SALacAAm	1/9	88	63	24400	18100	1.22	7.9
P6´SALac	6´SALacAAm	1/9	76	76	19000	15800	1.10	6.4
PLac	LacAAm	1/9	71	71	12000	9600	1.12	7.2

[a] RAFT polymerization reactions were carried out under molar ratio of total monomer/BTSE/V-70 = 150/1/0.2 in DMSO at 35 °C for 24 h. [b] Determined by ^{1}H NMR. [c] Isolated yield by dialysis. [d] Determined by GPC.

dition, we investigated the binding properties of the glyco-polymer against avian and human influenza viruses using the hemagglutination inhibition (HI) assay and the quartz crystal microbalance (QCM) method.

A synthetic procedure for glycopolymer from free 3´-sia-lyllactose (3´SALac) via direct anomeric azidation, subse-quent CuAAC, followed by RAFT polymerization, is shown in Fig. 1. The β-glycosyl azide was directly synthe-sized from free saccharide using 2-chloro-1,3-dimethylimi-dazolinium chloride (DMC), sodium azide, and *N,N*-diiso-propylethylamine (DIPEA) in water at 0 °C. The resulting β-glycosyl azide was reacted in aqueous DMF at room tem-perature with *N*-propargyl acrylamide in the presence of a catalytic amount of copper(II) sulfate pentahydrate (Cu-SO$_4$), L-ascorbic acid sodium salt (AscNa), and tris[(1-ben-zyl-1*H*-1,2,3-triazol-4-yl)-methyl]amine (TBTA). The acrylamide derivative carrying triazole-linked 3´SALac was successfully obtained starting from free 3´SALac with-out using protecting-groups. The ^{1}H NMR spectra of the glycomonomer having 3´SALac showed signals due to tria-zole, vinyl, and anomeric protons at 8.1, 6.2, and 5.7 ppm, respectively (Fig. 2a). The signals due to two 3-position protons and methyl protons of the acetamido group on the SA moiety were observed at 2.7, 1.7, and 2.0 ppm. Other glycomonomers having 6´-sialyllactose (6´SALac) and lac-tose (Lac) were synthesized using the same protecting-group-free process, starting from the corresponding free saccharides.[23] The glycomonomers were subjected to RAFT copolymerization with acrylamide (AAm) in DMSO at 35 °C to obtain glycopolymers using 2,2´-azobis(4-me-thoxy-2,4-dimethylvaleronitrile) (V-70) and 2-(benzylsulfa-nylthiocarbonylsulfanyl) ethanol (BTSE) as an initiator and a chain transfer agent, respectively (Table 1). RAFT poly-merization was conducted at lower temperature using V-70 because sialyllinkages are generally unstable and the SA moiety is easy cleaved at higher temperatures. Polyacryla-mides bearing 3´SALac, 6´SALac, and Lac (P3´SALac, P6´SALac, and PLac) were obtained in good yields after dial-ysis. All glycomonomers provided the desired glycopoly-mers with low dispersity (M_w/M_n < 1.22). The saccharide unit ratio in the product polymers was slightly lower (around 7 %) than the glycomonomer ratio in the feed (10 %). The ^{1}H NMR spectra of the glycopolymer P3´SA-Lac showed the triazole, phenyl, and anomeric protons of 3´SALac, and the polymer backbone signals, at 8.1, 7.2, 5.7, and 2.3−1.3 ppm, respectively (Fig. 2b). The signals due to two 3-position protons and the methyl protons of the acet-amido group on the SA moiety were observed at 2.7, 1.7, and 2.0 ppm, respectively, supporting the presence of SA moieties on the polymer.

Next, we investigated the binding properties of the gly-copolymers against avian and human influenza viruses us-ing the HI assay (Fig. 3). Interestingly, P3´SALac strongly bound with both the avian influenza virus A/duck/Hong Kong/313/4/1978 (H5N3) and the human influenza virus A/Memphis/1/1971 (H3N2). The minimum concentration of P3´SALac required to obtain a positive result against both the avian and human influenza viruses was 2.5×10^{-4} g/mL. No activity was observed against the avian influenza virus with P6´SALac and PLac, which lacked the α2,3-linked sialylgalacto moiety. Fetuin is a blood protein con-taining oligosaccharides having α2,3- and α2,6-linked sia-lylgalacto residues at the nonreducing ends of the saccha-ride chains.[24)25)26] The minimum concentration of fetuin re-quired to obtain a positive HI result against the avian influ-enza virus was 6.3×10^{-4} g/mL. P6´SALac and fetuin had activity against the human influenza virus, and the mini-mum concentration required to obtain a positive result was 2.5×10^{-4} and 7.8×10^{-5} g/mL, respectively. P3´SALac bound to the human influenza virus at the same level as did P6´SALac, although it is well known that the α2,3-sialylga-lacto moiety is a ligand for avian influenza virus. When
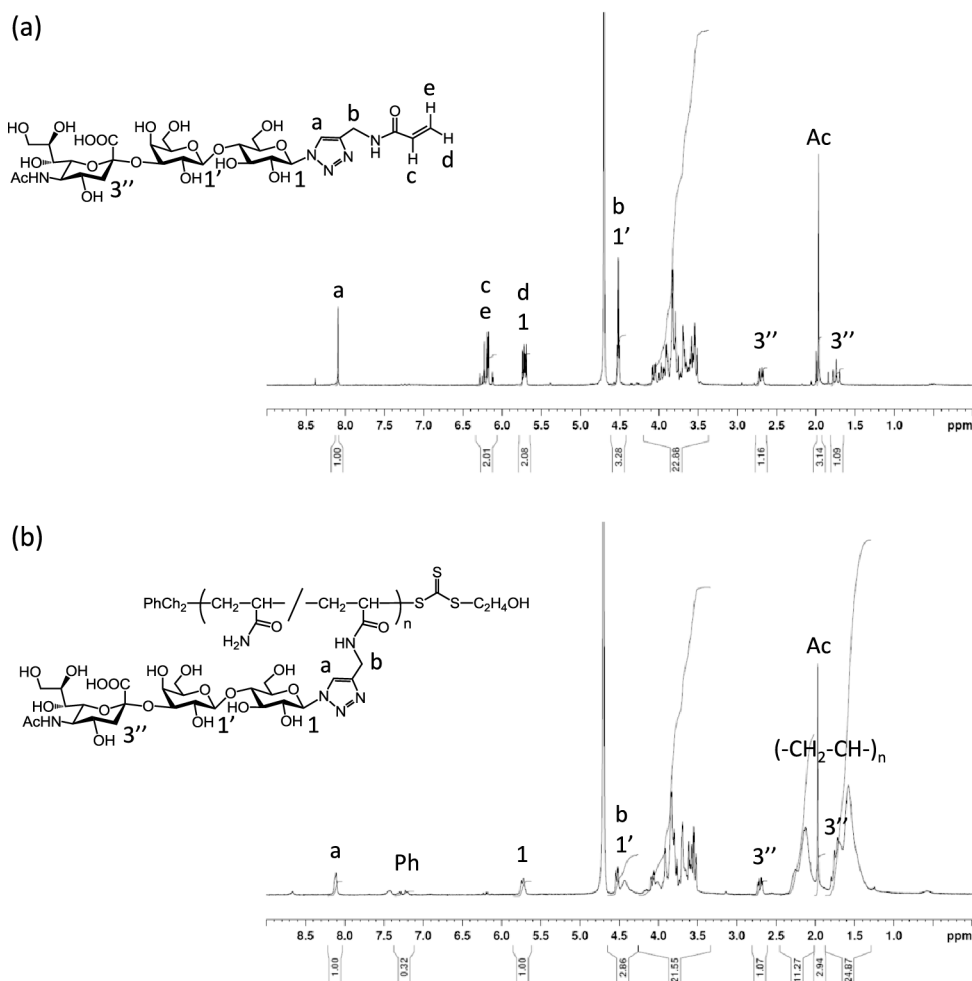
Fig. 2. ^{1}H NMR spectra of (a) the glycomonomer 3′SALacAAm, and (b) the glycopolymer P3′SALac, in D_2O.

free sialyllactoses were tested (1.0×10^{-3} g/mL), no activities were observed against both influenza viruses (data was not shown). These results suggested that the weak interaction between the α2,3-sialylgalacto moiety and the human influenza virus is amplified by the multivalent forms of saccharides. In our previous report, a glycopolymer bearing 3′SALac, which was synthesized by post-click chemistry using glycosyl azide and the degree of polymerization was lower than that of P3′SALac in this study, had no activity against both avian and human influenza viruses, suggesting that the length of polymer backbone and the number of saccharide moiety in polymer affected the binding affinity with influenza viruses.[19]

QCM binding assays using the glycopolymers and HAs were conducted using gold-coated QCM sensor chips. The thiol-terminated glycopolymers, which were prepared by reducing with sodium borohydride, were immobilized at a concentration of approximately 100 ng/cm^2 on a gold-coated QCM sensor tip via Au-S bond formation, then subjected to a binding assay with HAs in PBS. The addition of either avian HA (H5N3) or human HA (H3N2) to the P3′SALac-immobilized QCM sensor chip resulted in observable interaction (Fig. 4a) and the values of the association constant (K_a) were on the order of 10^7 M^{-1} (avian, 1.0×10^7 M^{-1}; human, 3.1×10^7 M^{-1}). No decrease in frequency was observed when BSA was added to the glycopolymer-immobi-
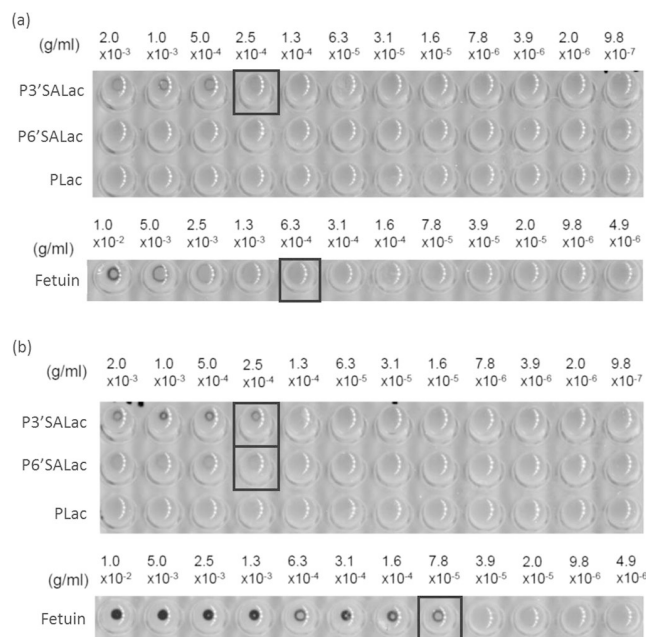
Fig. 3. HI assays of the glycopolymers against influenza viruses. (a) The avian influenza virus A/duck/Hong Kong/313/4/1978 (H5N3). (b) The human influenza virus A/Memphis/1/1971 (H3N2). The squares show the minimum concentration required for HI activity.

(a)

(b)
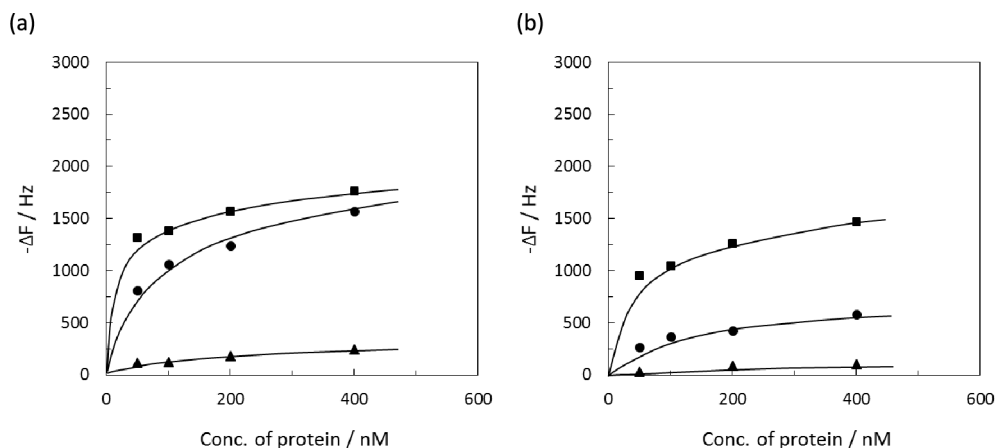
Fig. 4. QCM analyses of the interaction between glycopolymers and proteins.
(a) P3′SALac. (b) P6′SALac. ●, Avian HA (H5N3); ■, Human HA (H3N2); ▲, BSA.

Table 2. K_a values of interaction with HAs by QCM.

Glycopolymer	Avian HA[a] (M^{-1})	Human HA[b] (M^{-1})
P3′SALac	1.0×10^7	3.1×10^7
P6′SALac	7.1×10^6	2.2×10^7

[a]A/duck/Hong Kong/313/4/1978 (H5N3). [b]A/Brisbane/10/2007 (H3N2).

lized QCM sensor chip. It was previously reported that the K_a value for the binding between free saccharide and protein is on the order of 10^3 M^{-1}.[27] In contrast, the interaction of P6′SALac with human HA was stronger ($K_a = 2.2 \times 10^7$ M[-1]) than with avian HA ($K_a = 7.1 \times 10^6$ M[-1]) (Fig. 4b). Table 2 summarizes the K_a values of interaction with HA on the glycopolymer-immobilized QCM sensor chip. These results supported the result from the HI assay: P3′SALac strongly interacted with both avian and human influenza viruses, whereas P6′SALac strongly interacted with the human influenza virus. Although the human influenza virus mainly binds with an α2,6-linked sialylgalacto moiety, the interaction between an α2,3-linked sialylgalacto moiety and the human influenza virus has been reported.[14)28)29)30)31)] These particularly higher K_a values between glycopolymers and HAs are attributed to the glycocluster effect, where saccharide-protein interactions are amplified by the multivalency of the saccharides in the glycopolymers.

In conclusion, we succeeded in synthesizing glycopolymers bearing α2,3-linked sialyllactose from free saccharide without any protection of the saccharide hydroxy and carboxy groups by direct anomeric azidation and CuAAC, followed by RAFT polymerization. The glycopolymer bearing α2,3-linked sialyllactose strongly interacted with avian and human influenza viruses with a high K_a value on the order of 10^7 M^{-1}. This result indicated that multivalent sialyloligosaccharides amplified saccharide-protein interactions due to the multivalent forms of saccharides. This finding suggests that glycopolymers bearing sialyloligosaccharides will contribute to the development of various biomaterials that mimic natural glycoconjugates, as well as to the development of biomedicines and of biosensors for viruses and toxins.

EXPERIMENTAL

Materials. Free 3′SALac and 6′SALac were purchased from Nagara Science Co., Ltd. (Gifu, Japan). AAm was used after recrystallization from chloroform/methanol = 10/3. *N*-Propargyl acrylamide was synthesized using acryloyl chloride and propargylamine in the presence of triethylamine according to the literature.[32] A chain transfer agent, BTSE, was synthesized using 2-mercaptoethanol, carbon disulfide, and benzyl bromide according to the literature.[33] Avian influenza virus strain, A/duck/Hong Kong/313/4/1978 (H5N3), and its HA were propagated and purified as described previously.[34] Human influenza virus strain, A/Memphis/1/1971 (H3N2), was propagated and purified as described previously.[35] Human influenza virus HA, A/Brisbane/10/2007 (H3N2), was purchased from Sino Biological Inc. (Beijing, China). All other reagents were commercially available and were used without further purification.

Measurements. NMR spectra were recorded using a Bruker BioSpin AV-300 spectrometer. ESI mass spectra were recorded using a Bruker Daltonics microTOF Q-III spectrometer. GPC measurements were conducted using a system consisting of a JASCO PU-2089 pump, a CO-2065 column oven, an RI-2031 refractive index detector, and a Shodex OHpak SB-804 HQ (8.0 × 300 mm) column. 20 mM phosphate buffer (pH 7.0) was used as the eluent at a flow rate of 0.5 mL/min at 30 °C. Pullulan samples were used as standards.

Synthesis of 3′SALac-N₃. DMC (161 mg, 0.952 mmol) was added to a mixture of 3′SALac (192 mg, 0.293 mmol), DIPEA (502 μL, 2.93 mmol), and sodium azide (190 mg, 2.93 mmol) in water (1.2 mL), and the resulting mixture was stirred for 1 h at 0 °C. After concentration of the reaction mixture under reduced pressure and addition of DMF, the solid was removed by filtration. The filtrate was concentrated under reduced pressure, then the residue was dissolved in water and extracted with dichloromethane. Finally, the product was purified by ion-exchange column chromatography (Amberlite IR-120B, previously activated with 1 M NaOH, eluent; H₂O)[36] and concentrated under reduced pressure to give β-3′-sialyllactosyl azide (193 mg, 0.293

mmol, quant.).

^{1}H NMR (300 MHz, D$_2$O, δ): 4.7 (1H, H-1, included in DOH), 4.45 (d, 1H, H-1′, $J_{1′,2′}$ = 7.8 Hz), 4.05−3.46 (m, 18H, sugar-H), 3.22 (t, 1H, H-2), 2.67 (dd, 1H, H-3″$_{eq}$), 1.94 (s, 3H, CH$_3$), 1.71 (t, 1H, H-3″$_{ax}$); ^{13}C NMR (75 MHz, D$_2$O, δ): 175.0 (C=O of NHAc), 173.9 (COOH), 102.6 (C-1′), 99.8 (C-2″), 90.0 (C-1), 77.6, 76.7, 75.5, 75.2, 74.3, 72.9, 72.5, 71.8, 69.3, 68.3, 68.1, and 67.5 (sugar-C), 62.6 (C-9″), 61.0 (C-6), 59.8 (C-6′), 51.7 (C-5″), 39.8 (C-3″), 22.0 (CH$_3$).

Synthesis of 3′SALacAAm. *N*-Propargyl acrylamide (17 mg, 0.153 mmol), CuSO$_4$ (3.4 mg, 0.0137 mmol), AscNa (5.5 mg, 0.0274 mmol), and TBTA (7.7 mg, 0.0145 mmol) were added to a 50 % DMF aqueous solution (6 mL) of 3′SALac-N$_3$ (90 mg, 0.137 mmol), and the resulting mixture was stirred overnight at room temperature. After concentration of the reaction mixture under reduced pressure, the product was purified by silica gel column chromatography (CH$_3$CN/H$_2$O = 5/1) and then stirring with metal scavenger (SiliaMetS® Imidazole, 59 mg, 5 equiv. for Cu) overnight at room temperature. After removing of metal scavenger by filtration, the filtrate was concentrated under reduced pressure and freeze-dried to give 3′SALacAAm (69 mg, 0.0899 mmol, 66 %).

^{1}H NMR (300 MHz, D$_2$O, δ): 8.06 (s, 1H, triazole), 6.25−6.09 (m, 2H, vinyl), 5.71−5.66 (m, 2H, vinyl and H-1), 4.49 (s, 2H, N-CH$_2$), 4.49 (d, 1H, H-1′, $J_{1′,2′}$ = 7.5 Hz), 4.06-3.48 (m, 19H, sugar-H), 2.67 (dd, 1H, H-3″$_{eq}$), 1.94 (s, 3H, CH$_3$), 1.71 (t, 1H, H-3″$_{ax}$); ^{13}C NMR (75 MHz, D$_2$O, δ): 175.0 and 173.9 (C=O of SA), 168.6 (C=O of AAm), 145.0 (triazole), 129.6 (vinyl), 127.8 (vinyl), 123.0 (triazole), 102.6 (C-1′), 99.8 (C-2″), 87.3 (C-1), 77.7, 77.2, 75.5, 75.2, 74.5, 72.9, 72.0, 71.8, 69.4, 68.4, 68.1, and 67.5 (sugar-C), 62.6 (C-9″), 61.1 (C-6′), 59.7 (C-6), 51.7 (C-5″), 39.7 (C-3″), 34.5 (N-CH$_2$), 22.0 (CH$_3$); ESI-MS: [M−H]$^-$, M = C$_{29}$H$_{45}$N$_5$O$_{19}$, calcd. 766.2630, found 766.2611.

Synthesis of glycopolymers. RAFT polymerization reactions were carried out at 1.0 M in total monomer concentration. AAm, the glycomonomer, V-70, and BTSE were dissolved in 250 μL DMSO in a glass tube. The resulting solution was degassed by several freeze-thaw cycles, then the glass tube was sealed under vacuum and heated at 35 °C for 24 h. The products were purified by dialysis (Spectra/Por7 MWCO 3500) against deionized water and freeze-dried to give glycopolymers.

Assay of glycopolymers. HI assays were conducted using 96-well microtiter plates in PBS as described previously.[37] QCM binding assays were conducted on the gold-coated QCM sensor in PBS using thiol-terminated glycopolymers which were prepared by reducing with sodium borohydride in water as described previously.[23] These assays were conducted multiple times to confirm the repeatability.

ACKNOWLEDGMENTS

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