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 acting as receptors for the host cell. The hemagglutinin (HA) and neuraminidase (NA) proteins are the most important proteinaceous spikes of influenza virus. Although these two proteins are highly conserved, different strains of influenza virus have different antigenic properties, which make it difficult to develop an effective vaccine. In order to develop an effective vaccine, it is necessary to understand the molecular basis of these antigenic properties. In this paper, we report the synthesis of a glycopolymer bearing α2,3-linked sialyloligosaccharides as a multivalent glycoligand against avian and human influenza viruses.

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

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, tri[1-(benzyl-1H-tetrazol-4-yl)methyl]amine; 6´SA-Lac, 6´-sialyllactose; Lac, lactose; AAm, acrylamide; V-70, 2,2´-azo-bis(4-methoxy-2,4-dimethylvaleronitrile); BTSE, 2-(benzylsulfanylthiocarbonylsulfanyl) ethanol; BSA, bovine serum albumin.

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merization was conducted at lower temperature using V-70 group-free process, starting from the corresponding free moiety is easily cleaved at higher temperatures. Polyacrylamide carrying triazole-linked 3'-SALac was successfully obtained starting from free 3'-SALac with- out using protecting-groups. The 1H NMR spectra of the glycopolymers having 3'-SALac, 6'-SALac, and Lac (P3'-SALac, P6'-SALac, and PLac) were obtained in good yields after dialysis. All glycomonomers provided the desired glycopolymers with low dispersity (Mw/Mn < 1.22). The saccharide unit ratio in the polymer products was slightly lower (around 7%) than the glycomonomer ratio in the feed (10%). The 1H NMR spectra of the glycopolymers P3'-SALac 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 acetamido 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 glycopolymers against avian and human influenza viruses using the hemagglutination inhibition (HI) assay and the quartz crystal microbalance (QCM) method. A synthetic procedure for glycopolymers from free 3'-sialyllactose (3'SALac) via direct anomic azidation, subsequent CuAAC, followed by RAFT polymerization, is shown in Fig. 1. The β-glycosyl azide was directly synthesized from free saccharide using 2-chloro-1,3-dimethylimidazolinium chloride (DMC), sodium azide, and N,N-diisopropylethylamine (DIPEA) in water at 0 °C. The resulting β-glycosyl azide was reacted in aqueous DMF at room temperature with N-propargyl acrylamide in the presence of a catalytic amount of copper(II) sulfate pentahydrate (CuSO4), L-asparagine sodium salt (AscNa), and tris[1-(benzyl-1H-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 without using protecting-groups. The 1H NMR spectra of the glycopolymers having 3'SALac showed signals due to triazole, 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 lactose (Lac) were synthesized using 2-chloro-1,3-dimethylimino-sulfonyl ethanol (BTSE) as an initiator and a chain transfer agent, respectively (Table 1). RAFT polymeriza- tion was conducted at lower temperature using V-70 because sialyllinkages are generally unstable and the SA moiety is easily cleaved at higher temperatures. Polyacrylamides bearing 3'SALac, 6'SALac, and Lac (P3'SALac, P6'SALac, and PLac) were obtained in good yields after dialysis. All glycomonomers provided the desired glycopolymers with low dispersity (Mw/Mn < 1.22). The saccharide unit ratio in the product polymers was slightly lower (around 7%) than the glycomonomer ratio in the feed (10%). The 1H NMR spectra of the glycopolymers P3'SALac 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 acetamido 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 glycopolymers against avian and human influenza viruses using 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 required to obtain a positive result against both the avian and human influenza viruses was 6.3 × 10−4 g/mL. No activity was observed against the avian influenza virus P6'SALac and PLac, which lacked the α2,3-linked sialylgalacto moiety. Fetuin is a blood protein containing oligosaccharides having α2,3- and α2,6-linked sialylgalacto residues at the nonreducing ends of the saccharide chains.25,26,27 The minimum concentration of fetuin required to obtain a positive HI result against the avian influenza virus was 6.3 × 10−4 g/mL. P6'SALac and fetuin had activity against the human influenza virus, and the minimum concentration required to obtain a positive result was 2.5 × 10−4 and 7.8 × 10−3 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-sialygalacto moiety is a ligand for avian influenza virus. When

**Table 1. Synthesis of glycopolymers by RAFT polymerization.**

| Glycopolymer | Glycomonomer | Molar ratio of glycomonomer to AAm | Conv. (%) | Yield (%) | Mw(Mn) (g mol⁻¹) | Mw(Ma) (g mol⁻¹) | Mw/De | Saccharide unit ratio in polymer (%) |
|--------------|--------------|-----------------------------------|-----------|-----------|-----------------|-----------------|------|-----------------------------------|
| P3'SALac     | 3'SALacAAm   | 1/9                               | 88        | 63        | 24400           | 18100           | 1.22 | 7.0                               |
| 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 1H NMR. *c* Isolated yield by dialysis. *d* Determined by GPC.  

![Fig. 1. Protecting-group-free synthesis of glyco-monomers and polymers from free saccharides.](image-url)
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.

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/cm² 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 (Kₐ) were on the order of 10⁷ M⁻¹ (avian, 1.0 × 10⁷ M⁻¹; human, 3.1 × 10⁷ M⁻¹). No decrease in frequency was observed when BSA was added to the glycopolymer-immobi-

Fig. 2. ¹H NMR spectra of (a) the glycomonomer 3’SALacAAm, and (b) the glycopolymer P3’SALac, in D₂O.

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.
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$^{-1}$.

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 HAs 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.

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 sialyoligosaccharides amplified saccharide-protein interactions due to the multivalent forms of saccharides. This finding suggests that glycopolymers bearing sialyoligosaccharides 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. A-Propargyl acrylamide was synthesized using acryloyl chloride and propargylamine in the presence of triethylamine according to the literature. A chain transfer agent, BTSE, was synthesized using 2-mercaptopentanol, carbon disulfide, and benzyl bromide according to the literature. Avian influenza virus strain, A/duck/Hong Kong/313/4/1978 (H5N3), and its HA were propagated and purified as described previously. Human influenza virus strain, A/Memphis/1/1971 (H3N2), was propagated and purified as described previously. 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-N, 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 by ion-exchange column chromatography (Amberlite IR-120B), previously activated with 1 M NaOH, eluent; H$_2$O, and concentrated under reduced pressure to give β-3´-sialyllactosyl azide (193 mg, 0.293
Synthesis of glycopolymers.

Reduced pressure and freeze-dried to give 3´SALacAAm (69 mmol, 0.0899 mmol, 66 %).

Synthesis of 3´SALac-AAm. N-Propargyl acrylamide (17 mg, 0.153 mmol), CuSO$_4$(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$_2$ (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. AAm, the glycomonomer, V-70, and BTSE were dissolved in 250 μL DMSO in a glass tube. The resulting solution was stirred overnight at room temperature. After removal 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 %).

1H 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), 1.94 (s, 3H, CH$_3$). $^{13}$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, 72.4, 72.9, 72.5, 71.8, 69.3, 68.3, 68.1, and 67.5 (sugar-C), 62.6 (C-6), 59.8 (C-6´), 51.7 (C-5´), 39.8 (C-3´), 22.0 (CH$_3$).

Synthesis of glycopolymers. HI assays were conducted using 96-well microtiter plates in PBS as described previously. 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. These assays were conducted multiple times to confirm the repeatability.

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