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NOTE

Synthesis of well-controlled glycopolymers bearing oligosaccharides and their interactions with influenza viruses

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INTRODUCTION

Saccharides have a variety of different roles in living systems, such as providing energy sources and molecular recognition systems. Saccharides primarily exist on cell surfaces and facilitate the transfer of biological information between cells through the formation of interactions with specific proteins. The mechanism of this communication process is dependent on the molecular recognition ability of the saccharides, which can also have important roles in diseases, including the recognition of infectious pathogens, such as the influenza virus.[1] Pathogens recognize their host cells through the formation of specific sugar–protein interactions, where the sugar recognition proteins (lectins) on the surfaces of pathogens interact with the saccharide units on the cell surfaces. Based on the importance of these interaction mechanisms, considerable research efforts have been focused on the biological application of saccharides for the development of specific targeting systems.

In living systems, the sugar–protein interactions are typically enhanced by the cluster effects (multivalent effect) of sugar-accumulated compounds.[2] By mimicking this mechanism, glycopolymers that carry pendant saccharides in their structure can exhibit strong multivalent interactions.[3–5] However, the molecular recognition properties of oligosaccharides are typically more critical than those of the corresponding monosaccharides. For example, human influenza viruses can recognize the saccharide structure of Neu5Ac(α2-6)Gal (where Neu5Ac = *N*-glycolylneuraminic acid and Gal = galactose). However, avian influenza viruses recognize a slightly different structure of Neu5Ac(α2-3)Gal.[1] Sialyllactoses possess these structures and have consequently been used in a wide range of applications for the influenza virus. The slight differences in the structures of oligosaccharides can play an important role in host cell recognition. Therefore, glycopolymers bearing oligosaccharides are more important for the development of novel *in vivo* applications, such as polymer nanomedicines, than those bearing monosaccharides.

The *in vivo* application of synthetic polymers requires definition of the polymer structure and properties (for example, molecular weight and polydispersity index (PDI)). The precise polymerization of the vinyl derivatives of oligosaccharides is difficult and results in glycopolymers with broad PDIs because of the steric hindrance and multifunctionality of the saccharides. In addition, the protecting group chemistry required to prepare these materials in a controlled manner can be tedious. To overcome the synthetic challenges of monomers bearing oligosaccharides, the Haddleton group used a 'post-click' chemistry method, which was proposed by the Hawker group,[6] and reported the well-controlled synthesis of glycopolymers using living radical polymerization and click chemistry techniques.[7–9] These methods have enabled the synthesis of glycopolymers without the troublesome multistep reactions involving saccharide derivatives.

Herein, we report the synthesis of a series of glycopolymers bearing oligosaccharides using a 'post-click' chemistry strategy with an acrylamide (AAm) backbone. In addition, we evaluated their molecular recognition properties (Figure 1). This is the first report describing the synthesis of glycopolymers bearing sialyllactoses with narrow PDIs (<1.4). AAm was adopted as the polymer backbone because a previous report indicated that immobilization of linear polymers including AAm onto the surface of a material can inhibit nonspecific adsorption of proteins because of the hydrophilic layer on these surfaces that was formed by the linear polymers.[10] In addition, the steric effects of the saccharides on the Huisgen reaction, which is a typical 'click' chemistry reaction, would be reduced because the side chain of AAm is much smaller in size than those of other monomers, such as polyethylene glycol derivatives, that inhibit unspecific protein adsorption.

Our group has previously reported that glycopolymers can be immobilized onto various interfaces such as the surface of a device, by reaction of a terminal thiol group of a polymer derived from reversible addition-fragmentation chain transfer (RAFT) polymerization.[11] In this study, we functionalized an interface through the immobilization

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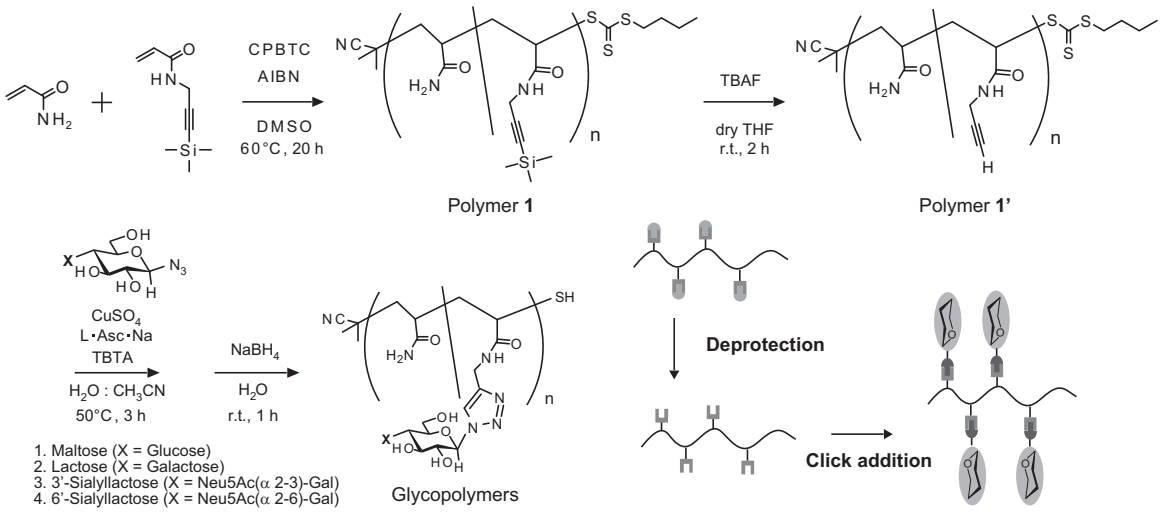

Figure 1 Synthesis procedure for well-controlled glycopolymers using 'post-click' chemistry. A full color version of this figure is available at *Polymer Journal* online.

of our newly synthesized glycopolymers and evaluated the interaction between the polymer surface and lectins using a quartz crystal microbalance (QCM) measurement.

Finally, we selected human and avian influenza viruses as practical targets. These viruses bind with sialyllactoses in their infection mechanism. 6′-Sialyllactose and 3′-sialyllactose can be found on the surfaces of human throat cells and avian colon cells, respectively. These saccharides can be used as potential ligands for binding human and avian influenza viruses.[12,13] Therefore, we synthesized several glycopolymers bearing sialyllactoses and evaluated the interactions between these glycopolymers and the influenza viruses. The interactions formed between two different types of influenza viruses and poly (AAm-*r*-6′-SALac) and poly(AAm-*r*-3′-SALac) were evaluated using a hemagglutination inhibition (HI) assay. The interactions between AAm polymers or dendrimers bearing sialic acids and influenza virus have been evaluated by Whitesides and Baker.[14–16] However, the glycopolymers structure must be determined for novel *in vivo* application, such as for polymer nano-medicine. This study is the first step in evaluating the potential of the glycopolymer.

EXPERIMENTAL PROCEDURE

Synthesis of polymer backbones

AAm and trimethylsilyl propargyl acrylamide (TMS PrAAm), which has an alkyne moiety, were copolymerized by RAFT polymerization in dimethylsulfoxide. The ratio of [Monomer]:[RAFT reagent]:[Initiator] was 100:1:0.4. 2-Cyanopropan-2-yl butyl trithiocarbonate, which was synthesized according to a previously reported method,[17] and 2,2′-azobis isobutyronitrile were used as the RAFT reagent and the initiator, respectively. The monomer ratio of [AAm]:[TMS PrAAm] was varied from 9:1 to 0:10, and the polymerization properties of this mixture were confirmed experimentally. Poly(AAm-*r*-PrAAm) (polymer **1′**) was obtained by removal of the TMS-protecting groups from poly(AAm-*r*-TMS PrAAm) (polymer **1**). The reaction proceeded with tetrabutylammonium fluoride in dry THF.

Synthesis of glycopolymers

Several saccharides including two types of sialyllactoses were subjected to a one-step azidation reaction to afford the corresponding azide derivatives of the oligosaccharides without the need for a protection step according to the protocol reported by Tanaka *et al.*[18] Then, the resulting azide saccharides were added to polymer **1′** using an optimized Huisgen reaction. The solvent was water, and a combination of copper sulfate and sodium ascorbate was used in the current study as a source of Cu(I) for the click chemistry reactions. Polymer **1′**, azide saccharides, copper sulfate and sodium ascorbate were dissolved in the solvent. The resulting solution was stirred for 3 h at a variety of different temperatures under an atmosphere of N_2.

Preparation of glycopolymer-immobilized surface and QCM measurement

The terminals of the glycopolymer were reduced to thiol with $NaBH_4$. The reduced glycopolymers were subsequently immobilized onto the SiO_2-coated QCM cell surfaces using our previously reported method.[11] Concanavalin A (Con A), peanut agglutinin, wheat germ agglutinin and sambucus sieboldiana lectin were used as corresponding lectins for maltose, lactose, 3′-sialyllactose and 6′-sialyllactose, respectively, in the glycopolymers. Bovine serum albumin (BSA) was used as a nonspecific protein. Each protein was successively injected into the glycopolymer-immobilized cells with phosphate-buffered saline buffer, and the delta frequencies were measured.

HI assay

The synthesized glycopolymers, polyAAm, 6′-SALac AAm and fetuin were used. The sample solution was prepared in the different concentrations of sialic acid in a 96-well plate. The influenza virus solution (4 HAU) and red blood cell suspension were successively injected into each well. The plate was incubated, and HI was observed. Red blood cells were added, and the aggregation was observed.

RESULTS AND DISCUSSION

Polymerization properties

The percentage conversion values of the different poly(AAm-*r*-TMS PrAAm) polymers were calculated for the formation of the controlled polymer of poly(AAm-*r*-TMS PrAAm), which yielded the highest conversion (Table 1, No. 1). When the molar ratio of [TMS PrAAm] was greater than 20%, the percentage conversion values were less than 40%. Furthermore, an increase in the initial molar ratio of [TMS PrAAm] resulted in a decrease in the conversion. The highest percentage conversion achieved in these polymerization reactions was 60%. Disappointingly, the RAFT and free radical processes failed to provide access to polyTMS PrAAm (Table 1, No. 6 and 7, respectively). Therefore, this result suggested that TMS PrAAm was an inappropriate substrate for radical polymerization reactions. The radical copolymerization of TMS PrAAm was also reported by the Hawker group. However, the polymerization properties were not

Table 1 Polymerization properties of poly(AAm-*r*-TMS PrAAm)

Run no.	*Molar ratio of AAm/TMS PrAAm*	*RAFT reagent*[a]	*Conv.*[b] *(%)*	*Yield (%)*	M_n[c]	M_w/M_n[c]	*Alkyne ratio*[b] *(%)*
1	9:1	+	62	58	5 000	1.26	8
2	8:2	+	32	27	ND	ND	18
3	7:3	+	22	19	ND	ND	26
4	6:4	+	20	6	ND	ND	ND
5	5:5	+	13	0	ND	ND	ND
6	0:10	+	0	0	ND	ND	ND
7	0:10	–	0	0	ND	ND	ND

Abbreviations: AAm, acrylamide; Conv, conversion; ND, not determined; RAFT, reversible addition-fragmentation chain transfer; TMS PrAAm, trimethylsilyl propargyl acrylamide.
[a]2-Cyanopropan-2-yl butyl trithiocarbonate, 10 mM.
[b]Determined by ^{1}H NMR before deprotection.
[c]Determined by gel permeation chromatography analysis calibrated for pullulan.

Table 2 Conditions for the Huisgen reaction

Sugar (eq)	*TBTA (eq)*	*Solvent*	*Time (h)*	*Temperture (°C)*	*Alkyne conv.*[a]*(%)*	*Sugar units*[b] *(%)*	M_n[c]	M_w/M_n[c]
Mal-N_3 (1.5)	–	H_2O	3	30	$\leqslant 70$	$\leqslant 5.2$		
Mal-N_3 (3)	–	H_2O	3	30	$\geqslant 70$	$\geqslant 5.6$		
	–	H_2O	3	50	$\geqslant 80$	$\geqslant 6.4$		
	–	H_2O:CH_3CN = 9:1	3	50	$\geqslant 90$	$\geqslant 7.2$		
	0.4	H_2O:CH_3CN = 9:1	3	50	$\geqslant 95$	$\geqslant 7.8$	8000	1.21
Lac-N_3 (3)	0.4	H_2O:CH_3CN = 9:1	3	50	$\geqslant 95$	$\geqslant 7.6$	7000	1.27
3′-SALac-N_3 (3)	0.4	H_2O:CH_3CN = 9:1	3	50	$\geqslant 95$	$\geqslant 7.6$	10 000	1.32
6′-SALac-N_3 (3)	0.4	H_2O:CH_3CN = 9:1	3	50	$\geqslant 80$	$\geqslant 6$		
	0.4	H_2O:CH_3CN = 9:1	6	60	$\geqslant 90$	$\geqslant 7.2$	10 000	1.34

Abbreviations: eq, equivalents; TBTA, tris(benzyltriazolylmethyl)amine.
[a]Calculated from ^{1}H NMR. The alkyne conversion = $[\int_{triazole}/[\int_{main\ chain}$, where $[\int_{triazole}$ and $[\int_{main\ chain}$ correspond to integral values of triazole and main chain in the each glycpolymers, respectively. The integral values are shown in Supplementary Figures 19-22.
[b]The % means sugar units in a polymer.
[c]Calculated from GPC analysis calibrated for pullulan standard.

sufficiently investigated.[6] The low reactivity of TMS PrAAm may have been due to π-electron resonance between the vinyl and acetylenyl groups through a propargyl methylene group, which would have a significant impact on the stability of the radical during the polymerization process. This hypothesis is under investigation.

The completion of deprotection was confirmed by proton nuclear magnetic resonance (^{1}H NMR; Supplementary Figures 1-5). The alkyne ratio in polymer **1** was determined to be 8%. The PDI of polymer **1** was determined to be 1.26 by gel permeation chromatography (GPC) analysis using phosphate-buffered saline buffer as the solvent (Supplementary Figure 2). Polymer **1** was employed in the next step because these results indicated that it was the best defined among all of the polymers prepared at this stage.

Saccharide addition by Huisgen reaction

The sugar ratio in the glycopolymer bearing maltose was determined based on its ^{1}H NMR spectrum (Table 2). The alkyne conversion was calculated by dividing the sugar ratio by the alkyne ratio. When an excess of maltose (1.5 equivalents) relative to the alkyne was used with a reaction temperature of 30 °C, the alkyne conversion was determined to be 70%. An increase in the amount of maltose to 3 equivalents along with an increased in the temperature to 50 °C led to an increase in the yield to 80%. The addition of tris(benzyltriazolylmethyl)amine, which is a ligand for Cu(I), was used to increase the yield. The addition of CH_3CN was required to allow for the dissolution of tris (benzyltriazolylmethyl)amine. However, several reports have demonstrated that CH_3CN can form stable complexes with Cu(I) ions.[19] This change in the solvent system and the addition of tris (benzyltriazolylmethyl)amine led to an increase in the reaction yield to greater than 95%, and the resulting polymer had a sugar unit content of 8% (Supplementary Figures 1-19 and Table 2). Therefore, these conditions were defined as the optimum conditions for the Huisgen reactions of polymer **1′** with the azide saccharides. The other azide saccharides were subsequently reacted with polymer **1′** under the optimized conditions. The results revealed that lactose azide and 3′-sialyllactose azide reacted with polymer **1′** to afford the desired products in greater than 95% reaction yield. However, the reaction yield of 6′-sialyllactose azide with polymer **1′** resulted in a lower yield that was less than 90%. This result was most likely due to its large structure, which would lead to steric hindrance. This yield can be increased by increasing the time and temperature of the reaction.

The molecular weight and PDI properties of the resulting glycopolymers were calculated by GPC analysis using phosphate-buffered saline buffer as a solvent (Table 2 and Supplementary Figure 2). The M_n values of the trisaccharide polymers were higher than those of the disaccharide polymers. All of the PDI values were less than 1.4. These results indicate that the addition of saccharides to the polymer backbone via the Huisgen reaction had no discernible effect on the polydispersity characteristics of the resulting glycopolymers. The residual copper levels in the glycopolymers were measured by atomic absorption spectroscopy. The results of this analysis indicated that the glycopolymers prepared in the current study contained less than 50 p.p.m. of copper in $1\,g\,l^{-1}$ of polymer solution. This value was sufficiently low for biological application of these materials (Supplementary Table 1).

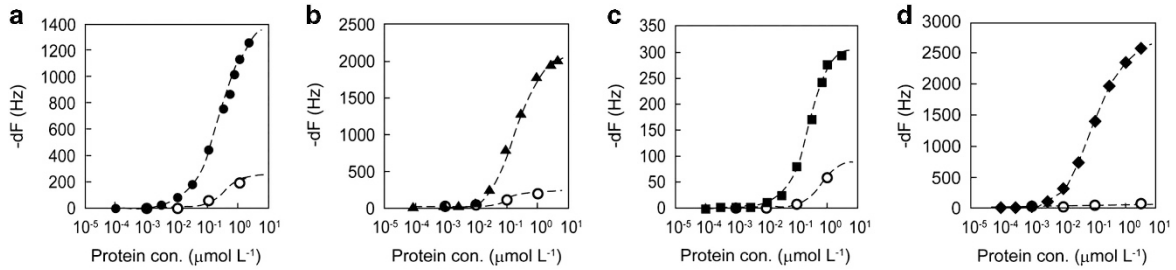

Figure 2 Protein adsorption measured by quartz crystal microbalance: (**a**) poly(AAm-*r*-Mal) surface, (**b**) poly(AAm-*r*-Lac) surface, (**c**) poly(AAm-*r*-3′-SALac) surface and (**d**) poly(AAm-*r*-6′-SALac) surface. The solid circle (●), solid triangle (▲), solid square (■), solid diamond (♦) and blank circle (○) represent Concanavalin A, peanut agglutinin, wheat germ agglutinin, sambucus sieboldiana lectin and bovine serum albumin, respectively.

Ability of the glycopolymer to recognize lectins

The existence of a terminal trithiocarbonate moiety in the poly(AAm-*r*-PrAAm) polymers was confirmed by ultraviolet–visible spectroscopy measurements (Supplementary Figure 3), and an absorbance peak that corresponded to the trithiocarbonate group was observed at nearly 310 nm. This peak disappeared after the reduction of the glycopolymers with $NaBH_4$, which confirmed that the terminal trithiocarbonate moiety in these polymers was completely reduced to a thiol under these conditions. The immobilization of the glycopolymers onto the SiO_2-coated QCM cell surface was confirmed by X-ray photoelectron spectroscopy measurements at each step (Supplementary Table 2 and Supplementary Figure 4). The residual maleimide moieties would be cleaved by visible light irradiation after cell preparation.

The protein adsorptions for each glycopolymer surface were evaluated using a QCM measurement (Figure 2 and Supplementary Figure 5). When the protein concentration was 1 μmol l^{-1}, the delta frequencies of Con A and BSA on the poly(AAm-*r*-Mal)-immobilized surface were 1400 and 200 Hz, respectively (Figure 2). In this case, the ratio of Con A to BSA was determined to be ~7. In the same manner, the ratio of lectin to BSA was determined to be ~9, ~5 and ~33 for poly(AAm-*r*-Lac), poly(AAm-*r*-3′-SALac) and poly(AAm-*r*-6′-SALac), respectively. The K_d values of the lectins were 2.4×10^{-7}, 1.7×10^{-7}, 2.9×10^{-7} and 8.9×10^{-8} M, respectively, and their order was less than 10^{-7}. Although the molecular weights of the target proteins and their delta frequencies varied, the delta frequency ratios of the lectins to BSA were high. The results indicated that the immobilized glycopolymers exhibited molecular recognition behavior toward the lectins. Furthermore, the K_d values of the immobilized glycopolymers were smaller than those of the monosaccharide for lectins.[20] This difference in the results indicated that the saccharides included in the polymer structure exhibited a multivalent effect. The delta frequencies for the adsorption of BSA were less than 300 Hz across the entire surface of the cell. Based on the results from previous reports in the literature,[10] grafted acrylamide-polymers can prevent the adsorption of proteins. These results suggest that immobilization of the well-controlled glycopolymers containing acrylamide onto QCM cells inhibit the nonspecific adsorption of BSA.

Ability of glycopolymer to recognize influenza viruses

We compared the performance of our glycopolymers with that of poly (AAm-*r*-Mal), poly(AAm-*r*-Lac) and polyAAm, which do not contain a saccharide. 6′-SALac AAm, which is a monomer that is expected to exhibit monovalent interactions with hemagglutinin, was also evaluated as a negative control. Fetuin, which is a natural protein composed of 6'-sialyllactose and 3′-sialyllactose units, was used as a positive control.

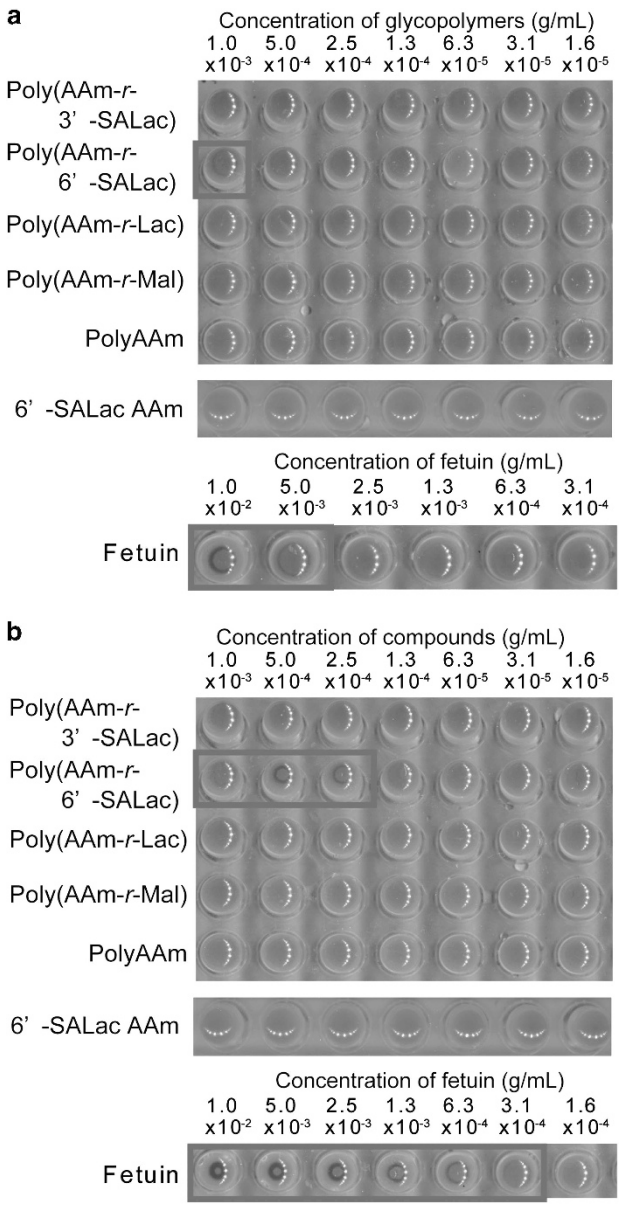

Figure 3 Hemagglutinin inhibition (HI) assays of the glycopolymers and fetuin against the influenza virus. (**a**) Avian influenza virus A/duck/Hong Kong/313/4/1978 (H5N3) and (**b**) human influenza virus A/Memphis/1/1971 (H3N2). The red squares show the minimum concentration required for HI activity. A full color version of this figure is available at *Polymer Journal* online.

As a result, only poly(AAm-*r*-6′-SALac) and fetuin inhibited the aggregation of red blood cells against both types of influenza virus. However, the other polymers and the 6′-sialyllactose monomer did not exhibit any effect (Figure 3 and Supplementary Table 3). This result suggested that poly(AAm-*r*-6′-SALac) exhibited a multivalent effect. The lowest concentrations of poly(AAm-*r*-6′-SALac) required to exhibit interactions with the avian and human influenza viruses were 1.0×10^{-3} and $2.5\times10^{-4}\,g\,ml^{-1}$, respectively. The corresponding values for fetuin were 5.0×10^{-3} and $3.1\times10^{-4}\,g\,ml^{-1}$, respectively. The lowest concentration of poly (AAm-*r*-6′-SALac) required for the human virus was lower than that of the avian virus. This result indicated that poly(AAm-*r*-6′-SALac) can be used to recognize a specific type of virus. The failure of poly(AAm-*r*-3′-SALac) to inhibit the influenza viruses was attributed to the inability of 3′-sialyllactose to form a meaningful interaction with the viruses. These results suggest that it is necessary to enhance the density of saccharides on the glycopolymers to allow for the formation of stronger interactions with the viruses through a multivalent effect.

CONCLUSION

In this study, well-controlled glycopolymers bearing oligosaccharides including sialyllactoses were synthesized using 'post-click' chemistry. The synthesized glycopolymers recognized the corresponding lectins, and poly(AAm-*r*-6′-SALac) exhibited interactions with influenza viruses. The results of this study represent a valuable contribution to the development of glycopolymers that are capable of interacting with influenza viruses as well as the development of *in vivo* applications, such as nano-medicines for pathogens.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Polymer Journal website (http://www.nature.com/pj)