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Protecting-Group-Free Synthesis of Glycopolymers Bearing Sialyloligosaccharide and Their High Binding with the Influenza Virus

Tomonari Tanaka,*[,†] Hideki Ishitani,[†] Yoshiko Miura,[‡] Kenta Oishi,[§] Tadanobu Takahashi,[§] Takashi Suzuki,[§] Shin-ichiro Shoda,[⊥] and Yoshiharu Kimura[†]

[†]Department of Biobased Materials Science, Graduate School of Science and Technology, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan

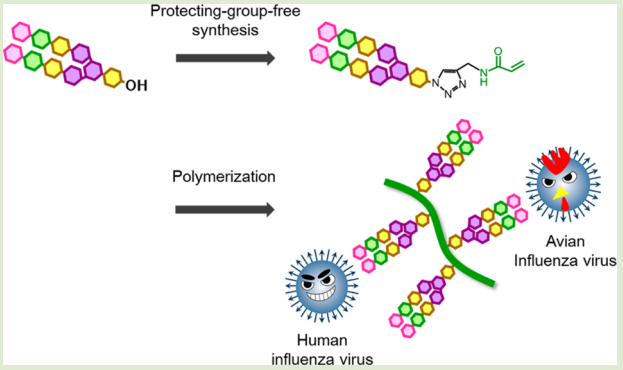
[‡]Department of Chemical Engineering, Graduate School of Engineering, Kyusyu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

[§]Department of Biochemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

[⊥]Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Aoba, Sendai, Miyagi 980-8579, Japan

Supporting Information

ABSTRACT: Glycopolymers having pendant triazole-linked sialyloligosaccharides were successfully synthesized from free saccharides without any protection of the hydroxy and carboxy groups on the saccharides. The glycomonomers were synthesized by the direct azidation of free saccharides using 2-chloro-1,3-dimethylimidazolinium chloride as a condensing agent followed by copper(I)-catalyzed azide−alkyne cycloaddition. The resultant glycomonomers were copolymerized with acrylamide by a reversible addition−fragmentation chain transfer technique. Each of the glycopolymers were obtained and then immobilized on a gold-coated sensor of quartz crystal microbalance to analyze their binding behavior with the lectin. The glycopolymers strongly bound with the corresponding lectin without nonspecific adsorption in aqueous solution. In addition, the glycopolymer bearing a complex-type sialyl N-linked oligosaccharide was found to strongly bind with both human and avian influenza A viruses. The strong binding, observed using the hemagglutination inhibition assay, was attributed to the glycocluster effect of the glycopolymer and the biantennary structure of the N-linked oligosaccharide.



Saccharides 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. Although saccharide−protein interactions are generally weak, these interactions are amplified by multivalent forms of saccharides called the "glycocluster effect"[1,2] to cause various biological processes. Sialic acid (SA) is a family of acidic monosaccharides consisting of a nine-carbon backbone that often terminates the oligosaccharide structure of cell surface glycoconjugates such as glycoproteins and glycolipids. Until now, many synthetic glycoclusters such as glycopolymers,[3−5] glycodendrimers,[6,7] and glyconanoparticles[8−12] have been reported to amplify the saccharide signals of natural ligands such as glycopeptides[13] and glycoproteins[14] existing on the cell surface. In particular, glycopolymers, which are synthetic polymers having pendant saccharides, have been receiving much attention in many fields, e.g., polymer chemistry, materials science, and biomedicine. However, the synthesis of glycopolymers is laborious and requires multistep processes including the protection and deprotection of hydroxy groups on the saccharide moieties. One typical route to the glycopolymers consists of the synthesis of glycomonomers with a polymerizable group at the anomeric position of the saccharide.[15,16] Another route involves the attaching of glycosyl derivatives to the polymer main chain. Click chemistry such as copper(I)-catalyzed azide−alkyne cycloaddition[17,18] is a powerful tool in these syntheses of glycomonomers and -polymers, although appropriate saccharide derivatives containing functional groups such as azide and acetylene must be synthesized by multistep processes. In contrast, the protecting-group-free synthesis of glycomonomers is simple and versatile in that glycomonomers can be synthesized via gluconolactone and glycosylamine as intermidiates.[19−24] For example, Kobayashi et

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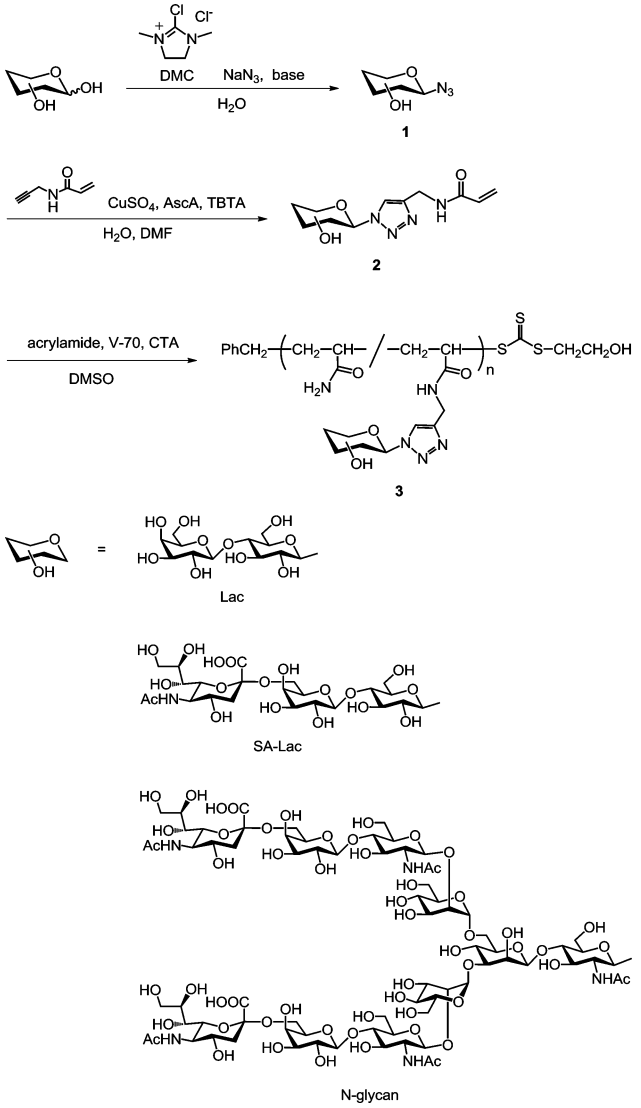
al. synthesized polystyrene bearing sialyllactose via glycosylamine, which was unstable and had to be handled carefully. Chemo-enzymatic methods were also used for the preparation of glycopolymers bearing sialyllactose.[25−27] This approach, however, is complicated because sialyltransferase must be used to attach sialyl moieties to the glycopolymer side chain lactose residues, being difficult to be applied to more complicated oligosaccharides such as N-linked oligosaccharides having SA. Therefore, a simpler and more efficient synthetic method that can be applied to various oligosaccharides is required for the preparation of glycopolymers from free saccharides.

We recently developed a one-step synthetic protocol for β-glycosyl azides from free saccharides by using a dehydrative condensing agent, 2-chloro-1,3-dimethylimidazolinium chloride (DMC).[28] This method, called "Shoda activation",[29] does not require the protection of a hydroxy group and allows the reaction in water. In this letter, we report the protecting-group-free synthesis of glycomonomers consisting of sialyloligosaccharide from free saccharides via the direct synthesis of β-glycosyl azide and click chemistry. The resultant monomers are then polymerized by the reversible addition−fragmentation chain transfer (RAFT) polymerization to obtain the glycopolymers. In addition, we demonstrate the detection of protein−saccharide interactions immobilized the glycopolymers on a gold-coated quartz crystal microbalance (QCM) sensor. The interaction with influenza viruses is also evaluated by the hemagglutination inhibition (HI) test.

A typical synthetic procedure of glycopolymers from free saccharides, lactose (Lac), 6′-sialyllactose (SA-Lac), and complex-type sialyl N-linked oligosaccharide (*N*-glycan), was as follows (Scheme 1). β-Glycosyl azides **1** were directly synthesized from free saccharides using DMC, sodium azide (NaN_3), and a base agent in water and reacted with *N*-propargyl acrylamide in the presence of a catalytic amount of copper(II) sulfate pentahydrate ($CuSO_4$), L-ascorbic acid sodium salt (AscA), and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)-methyl]amine (TBTA)[30] as a powerful stabilizing ligand for copper(I) in aqueous DMF. The resulting products **2**, acrylamide (AAm) derivatives having triazole-linked oligosaccharide residues, were obtained in good yields and purified by silica gel column chromatography (Table 1). The ^{1}H NMR spectra of **2** showed signals at 8.1, around 6.2, and 5.7 ppm, which were assigned to the triazole, vinyl, and anomeric protons, respectively. The ^{13}C NMR spectra showed signals at 87 ppm, due to the anomeric carbon of **2**, as well as the signals due to the carbonyl carbons of SA and *N*-acetylglucosamine residues around 174 ppm. The glycomonomers **2** were subjected to RAFT copolymerization with AAm to obtain glycopolymers **3**. The RAFT polymerization[31,32] was performed in DMSO at 35 °C using 2,2′-azobis(4-methoxy-2,4-dimethyl-valeronitrile) (V-70) and 2-(benzylsulfanylthiocarbonyl-sulfanyl)ethanol as the initiator and chain transfer agent (CTA), respectively (Table 2). The conversions of the polymerization reaction were over 70%. The glycopolymers **3** bearing oligosaccharides were isolated in good yields after dialysis. Lac-containing monomer (Lac-AAm) provided **3** with low dispersity, whereas SA-Lac-containing monomer (SA-Lac-AAm) and *N*-glycan-containing monomer (*N*-glycan-AAm) provided **3** with wide dispersities. It was therefore deduced that steric hindrance of the oligosaccharide residues increased the polydispersities of the glycopolymers. The saccharide unit ratio in the product polymers was slightly lower (around 7%) than the glycomonomer ratio (10%) in the feed. The separate RAFT

Scheme 1. Synthesis of Glycomonomers 2 and Glycopolymers 3 from Free Saccharides



polymerizations of AAm and triazole-linked glycomonomer **2** under the same conditions gave 79 and 24% conversions, respectively (data were not shown in Table 2), indicating the lower reactivity of **2** than AAm. The ^{1}H NMR spectra of the glycopolymer **3** showed the triazole, anomeric proton, and polymer main-chain signals at 8.1, 5.7, and 2.3−1.3 ppm, respectively, as well as the signals of the 3-position proton and methyl group of the acetamide group on SA at 2.6 and 2.0 ppm, respectively.

Next, we investigated the interaction between the glycopolymers and proteins. We first demonstrated detection of the interaction using the QCM method.[33] The thiol-terminated glycopolymers were easily prepared by reducing the trithiocarbonate terminal group with sodium borohydride, immobilized at a concentration of 150−200 ng/cm^2 on a gold-coated QCM sensor via Au−S bond formation in aqueous solution, and subjected to the binding test with proteins in PBS. When peanut agglutinin from *Arachis hypogaea* (PNA) that recognizes the β-galactoside (Gal) residue was added to the QCM sensor immobilized with Lac-bearing polyacrylamide (Lac-PAAm), the frequency (ΔF) significantly decreased, indicating an increase in mass due to the saccharide-bound lectin (Figure 1a). The

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Table 1. Synthesis of Glycomonomers 2 via 1

	1st step			2nd step	
free saccharide	DMC (equiv)	base (equiv)	yield (%)[a]	acetylene/$CuSO_4$/AscA (equiv)	yield (%)[b]
Lac	3	DIPEA[c] (9)	quant.	1.0/0.1/0.2	94
SA-Lac	3	DIPEA (9)	92	1.1/0.1/0.2	66
N-glycan	20	2,6-lutidine (40)	64	1.2/0.1/0.2	70

[a]Isolated yield of β-glycosyl azide **1**. [b]Isolated yield of glycomonomer **2**. [c]*N*,*N*-Diisopropylethylamine.

Table 2. Synthesis of Glycopolymers 3 by RAFT Polymerization of 2[a]

glycomonomer **2**	molar ratio of **2**/AAm	conv. (%)[b]	yield (%)[c]	M_n[b]	M_w/M_n[d]	saccharide ratio in polymer (%)[b]
Lac-AAm	1/9	71	65	23 000	1.16	7.2
SA-Lac-AAm	1/9	72	72	23 000	1.81	7.7
N-glycan-AAm	1/9	87	87	33 000	2.66	7.3

[a]At a molar ratio of total monomer/CTA/V-70 = 750/5/1 in DMSO at 35 °C for 24 h. [b]Determined by ^{1}H NMR. [c]Isolated yield after dialysis. [d]Determined by GPC.

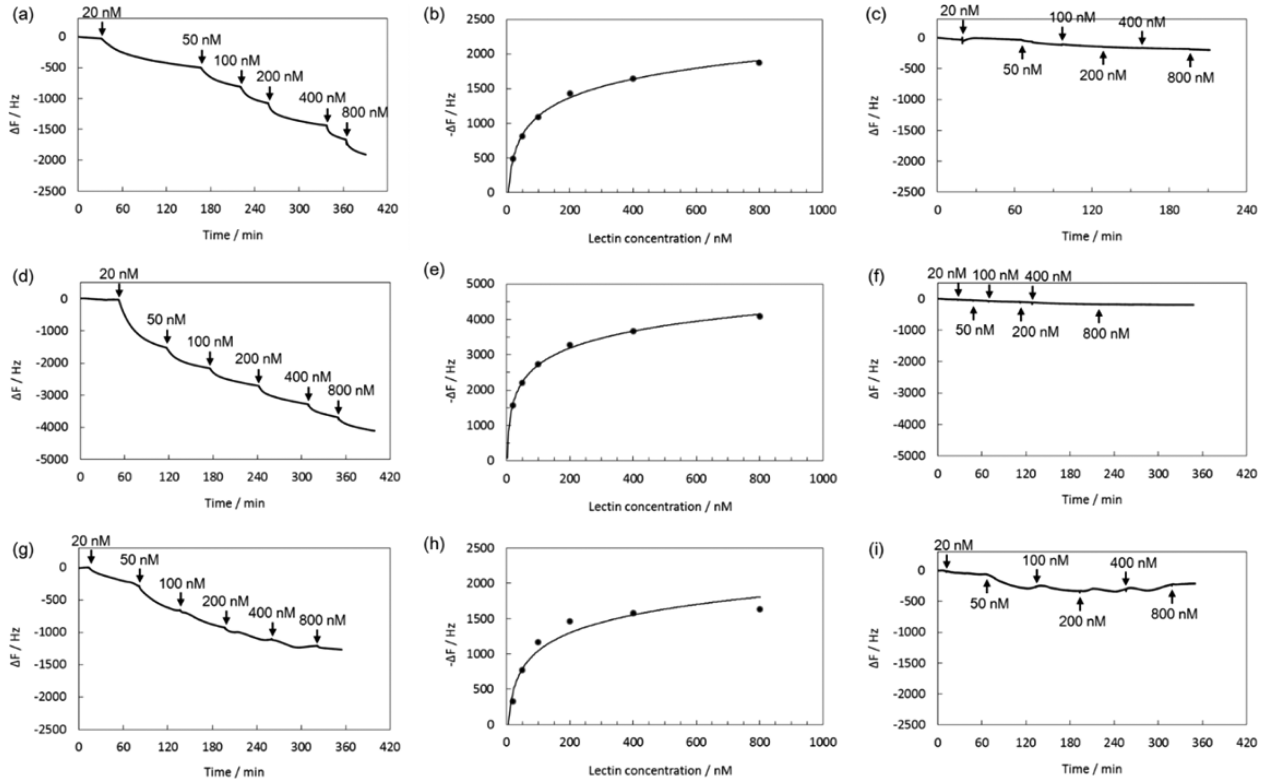

Figure 1. QCM analyses of the saccharide−protein interaction. Time courses of the frequency shift of (a) Lac-PAAm/PNA, (c) Lac-PAAm/BSA, (d) SA-Lac-PAAm/SSA, (f) SA-Lac-PAAm/BSA, (g) *N*-glycan-PAAm/SSA, and (i) *N*-glycan-PAAm/BSA. Equilibrium frequency shifts of (b) Lac-PAAm/PNA, (e) SA-Lac-PAAm/SSA, and (h) *N*-glycan-PAAm/SSA.

frequency decreased gradually as the amount of PNA added increased from 20 to 800 nM. On the other hand, when bovine serum albumin (BSA) was added to the QCM sensor immobilized with Lac-PAAm, no decrease in frequency was observed (Figure 1c). The association constant (K_a) for the lectin−saccharide interaction was estimated using a Langmuir-type isotherm based on the equilibrium frequency shift. The obtained K_a value was 1.28×10^7 M^{-1} (Figure 1b), supporting the strong binding of PNA by Lac-PAAm without nonspecific adsorption. Similarly, SA-Lac-bearing polyacrylamide (SA-Lac-PAAm) and *N*-glycan-bearing polyacrylamide (*N*-glycan-PAAm) immobilized on a QCM sensor chip were shown to bind *Sambucus sieboldiana* agglutinin (SSA), which is known specifically to recognize the α2−6 sialylgalactose (SAα2−6Gal) residue (Figure 1d and g). The K_a values for SA-Lac-PAAm/SSA and *N*-glycan-PAAm/SSA were 1.67×10^7 and 1.73×10^7 M^{-1}, respectively. It was previously reported that the K_a value for the binding of lectin and free saccharide is in the order of 10^3 M^{-1}.[34] The especially higher K_a values for glycopolymers are attributed to the glycocluster effect where the lectin−saccharide interactions are amplified by the multivalency of the saccharides on the glycopolymers.

Various substances carrying sialyloligosaccharides are known to inhibit hemagglutination by binding to viral hemagglutinin.[35,36] Here, the binding of the influenza virus by the glycopolymers was demonstrated by the HI assay. Figure 2a shows the results of HI assays for the glycopolymers by using human influenza virus A/Memphis/1/1971 (H3N2), which

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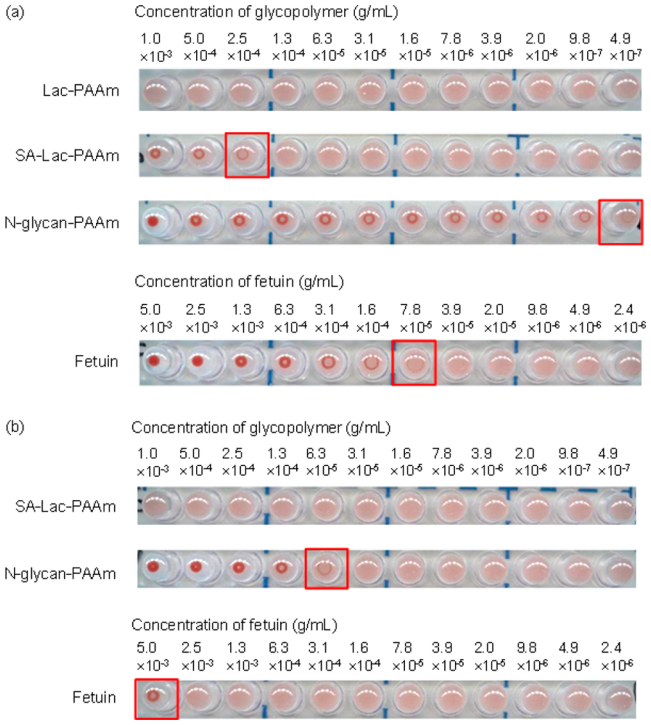

Figure 2. HI assays of the glycopolymers and fetuin against the influenza virus. (a) Human influenza virus A/Memphis/1/1971 (H3N2) and (b) avian influenza virus A/duck/Hong Kong/313/4/1978 (H5N3). The red squares show the minimum concentration required for HI activity.

binds to the SAα2−6Gal residue. The minimum concentrations of SA-Lac-PAAm and *N*-glycan-PAAm required to obtain a positive result were 2.5×10^{-4} and 4.9×10^{-7} g/mL, respectively. No activity was observed with Lac-PAAm that lacks a SA residue. Fetuin is a blood protein containing sialyloligosaccharides having SAα2−6Gal and α2−3 sialylgalactose (SAα2−3Gal) residues at the nonreducing ends of oligosaccharides;[37−41] a minimum of 7.8×10^{-5} g/mL of fetuin was required to obtain a positive HI result. Table 3 summarizes

Table 3. HI Activities of the Glycopolymers and Fetuin against Influenza Viruses[a]

	human influenza virus[b]	avian influenza virus[c]
Lac-PAAm	ND[d]	ND
SA-Lac-PAAm	2.5×10^{-4}	ND
N-glycan-PAAm	4.9×10^{-7}	6.3×10^{-5}
fetuin	7.8×10^{-5}	5.0×10^{-3}

[a]The minimum concentration required for HI activity (g/mL). [b]A/Memphis/1/1971 (H3N2). [c]A/duck/Hong Kong/313/4/1978 (H5N3). [d]Not detected (no activity).

the HI titers of the glycopolymers tested against two influenza viruses. It is evident that *N*-glycan-PAAm bound the human influenza virus 510 and 160 times more strongly than SA-Lac-PAAm and fetuin, respectively, suggesting that the biantennary structure of *N*-glycan with SA residues at the nonreducing ends increases the interaction between *N*-glycan-PAAm and the human influenza virus. Interestingly, *N*-glycan-PAAm also exhibited HI activity against avian influenza virus A/duck/Hong Kong/313/4/1978 (H5N3), which mainly binds to the SAα2−3Gal residue (Figure 2b). The minimum concentration of *N*-glycan-PAAm against the avian influenza virus was 6.3×10^{-5} g/mL, 80 times stronger than that of fetuin. No activity was observed with SA-Lac-PAAm against the avian influenza virus. Therefore, *N*-glycan-PAAm bound much more strongly with both human and avian influenza viruses than SA-Lac-PAAm and fetuin, suggesting that the glycocluster effect of the glycopolymer and the biantennary structure of N-glycan amplified the interaction between the glycopolymer and influenza virus.

In conclusion, we succeeded in synthesizing glycopolymers from free sialyloligosaccharides without any protection of the saccharide hydroxy and carboxy groups by using the direct azidation and click chemistry methods, followed by RAFT copolymerization. The present simple and efficient synthetic method for glycopolymers from free saccharides can be applied to various oligosaccharides with higher molecular weight and containing SA moieties such as N-linked and O-linked oligosaccharides typically found on a cell surface. Strong interactions between glycopolymers immobilized on a sensor chip and their corresponding lectins were confirmed by the QCM analysis. The glycopolymers were strongly and specifically recognized by their corresponding lectins, with K_a values in the order of 10^7 M^{-1}. These results indicated that the synthesized glycopolymers amplified the saccharide−protein interactions due to the glycocluster effect. In addition, *N*-glycan-PAAm bound both human and avian influenza viruses more strongly than SA-Lac-PAAm and fetuin. Our findings suggest that glycopolymers bearing sialyloligosaccharides will contribute to the development of various biomaterials for tissue engineering and biosensors for the detection of viruses and toxins.

■ ASSOCIATED CONTENT

Supporting Information

Experiment details and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: t-tanaka@kit.ac.jp. Tel.: +81 75 724 7802.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Lee, Y. C.; Lee, R. T. *Acc. Chem. Res.* **1995**, *28*, 321−327.
(2) Mammen, M.; Choi, S. K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2755−2794.
(3) Miura, Y. *J. Polym. Sci., Part A: Polym. Chem.* **2007**, *45*, 5031−5036.
(4) Le Droumaguet, B.; Nicolas, J. *Polym. Chem.* **2010**, *1*, 563−598.
(5) Miura, Y. *Polym. J.* **2012**, *44*, 679−689.
(6) Chabre, Y. M.; Roy, R. *Curr. Top. Med. Chem.* **2008**, *8*, 1237−1285.
(7) Tanaka, K.; Siwu, E. R. O.; Minami, K.; Hasegawa, K.; Nozaki, S.; Kanayama, Y.; Koyama, K.; Chen, W. C.; Paulson, J. C.; Watanabe, Y.; Fukase, K. *Angew. Chem., Int. Ed.* **2010**, *49*, 8195−8200.

(8) de la Fuente, J. M.; Barrientos, A. G.; Rojas, T. C.; Rojo, J.; Canada, J.; Fernandez, A.; Penades, S. *Angew. Chem., Int. Ed.* **2001**, *40*, 2258−2261.
(9) de la Fuente, J. M.; Penades, S. *Tetrahedron Asymmetry* **2005**, *16*, 387−391.
(10) Spain, S. G.; Albertin, L.; Cameron, N. R. *Chem. Commun.* **2006**, 4198−4200.
(11) Housni, A.; Cai, H.; Liu, S.; Pun, S. H.; Narain, R. *Langmuir* **2007**, *23*, 5056−5061.
(12) van Kasteren, S. I.; Campbell, S. J.; Serres, S.; Anthony, D. C.; Sibson, N. R.; Davis, B. G. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 18−23.
(13) Glunz, P. W.; Hintermann, S.; Williams, L. J.; Schwarz, J. B.; Kuduk, S. D.; Kudryashov, V.; Lloyd, K. O.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2000**, *122*, 7273−7279.
(14) Yamamoto, N.; Tanabe, Y.; Okamoto, R.; Dawson, P. E.; Kajihara, Y. *J. Am. Chem. Soc.* **2008**, *130*, 501−510.
(15) Roy, R.; Tropper, F. D.; Romanowska, A. *Bioconjugate Chem.* **1992**, *3*, 256−261.
(16) Fraser, C.; Grubbs, R. H. *Macromolecules* **1995**, *28*, 7248−7255.
(17) Kolb, H.; Finn, M.; Sharpless, K. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004−2021.
(18) Binder, W.; Sachsenhofer, R. *Macromol. Rapid Commun.* **2007**, *28*, 15−54.
(19) Kallin, E.; Lönn, H.; Norberg, T.; Elofsson, M. *J. Carbohydr. Chem.* **1989**, *8*, 597−611.
(20) Tsuchida, A.; Kobayashi, K.; Matsubara, N.; Muramatsu, T.; Suzuki, T.; Suzuki, Y. *Glycoconjugate J.* **1998**, *15*, 1047−1054.
(21) Kobayashi, K.; Sumitomo, H.; Ina, Y. *Polym. J.* **1985**, *17*, 567−575.
(22) Narain, R.; Armes, S. P. *Chem. Commun.* **2002**, 2776−2777.
(23) Narain, R.; Armes, S. P. *Macromolecules* **2003**, *36*, 4675−4678.
(24) Narain, R.; Armes, S. P. *Biomacromolecules* **2003**, *4*, 1746−1758.
(25) Ogata, M.; Murata, T.; Murakami, K.; Suzuki, T.; Hidari, K. I. P. J.; Suzuki, Y.; Usui, T. *Bioorg. Med. Chem.* **2007**, *15*, 1383−1393.
(26) Ogata, M.; Hidari, K. I. P. J.; Murata, T.; Shimada, S.; Kozaki, W.; Park, E. Y.; Suzuki, T.; Usui, T. *Bioconjugate Chem.* **2009**, *20*, 538−549.
(27) Narla, S. N.; Sun, X.-L. *Biomacromolecules* **2012**, *13*, 1675−1682.
(28) Tanaka, T.; Nagai, H.; Noguchi, M.; Kobayashi, A.; Shoda, S. *Chem. Commun.* **2009**, 3378−3379.
(29) Novoa, A.; Barluenga, S.; Serba, C.; Winssinger, N. *Chem. Commun.* **2013**, *49*, 7608−7610.
(30) Chan, T. R.; Hilgraf, R.; Sharpless, K. B.; Fokin, V. V. *Org. Lett.* **2004**, *6*, 2853−2855.
(31) Moad, G.; Rizzardo, E.; Thang, S. H. *Aust. J. Chem.* **2005**, *58*, 379−410.
(32) Lowe, A. B.; Sumerlin, B. S.; McCormick, C. L. *Polymer* **2003**, *44*, 6761−6765.
(33) Ebara, Y.; Okahata, Y. *J. Am. Chem. Soc.* **1994**, *116*, 11209−11212.
(34) Miura, Y.; Ikeda, T.; Kobayashi, K. *Biomacromolecules* **2003**, *4*, 410−415.
(35) Umemura, M.; Itoh, M.; Makimura, Y.; Yamazaki, K.; Umekawa, M.; Masui, A.; Matahira, Y.; Shibata, M.; Ashida, H.; Yamamoto, K. *J. Med. Chem.* **2008**, *51*, 4496−4503.
(36) Oka, H.; Onaga, T.; Koyama, T.; Guo, C.-T.; Suzuki, Y.; Esumi, Y.; Hatano, K.; Terunuma, D.; Matsuoka, K. *Bioorg. Med. Chem.* **2009**, *17*, 5465−5475.
(37) Spiro, R. G. *J. Biol. Chem.* **1960**, *235*, 2860−2869.
(38) Baenziger, J. U.; Fiete, D. *J. Biol. Chem.* **1979**, *254*, 789−795.
(39) Edge, A. S.; Spiro, R. G. *J. Biol. Chem.* **1987**, *262*, 16135−16141.
(40) Green, E. D.; Adelt, G.; Baenziger, J. U.; Wilson, S.; Van Halbeek, H. *J. Biol. Chem.* **1988**, *263*, 18253−18268.
(41) Townsend, R. R.; Hardy, M. R.; Cumming, D. A.; Carver, J. P.; Bendiak, B. *Anal. Biochem.* **1989**, *182*, 1−8.