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ORIGINAL ARTICLE

Polymeric water-soluble activated esters: synthesis of polymer backbones with pendant *N*-hydoxysulfosuccinimide esters for post-polymerization modification in water

Sotaro Tsuji[1] · Yuji Aso[1] · Hitomi Ohara[1] · Tomonari Tanaka[1]

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Abstract

Novel polymer backbones with water-soluble activated ester pendant groups were synthesized by a reversible addition-fragmentation chain transfer (RAFT) polymerization technique using an acrylamide derivative bearing an *N*-hydroxysulfosuccinimide (sulfoNHS) ester as a monomer substrate. The monomer bearing a sulfoNHS ester was newly synthesized by a dehydrative condensation reaction of *N*-hydroxysulfosuccinimide sodium salt with 6-acrylamidohexanoic acid. The molecular weight and polydispersity indexes of the resulting polymers were well controlled by the RAFT polymerization mechanism. To synthesize glycopolymers, the substitution of the sulfoNHS esters on the polymer side chain with an amine-containing saccharide derivative, *p*-aminophenyl *β*-D-galactopyranoside (*p*AP-Gal), was performed in water. The resulting glycopolymer bearing *p*AP-Gals exhibited a strong interaction with the corresponding lectin peanut agglutinin in aqueous solution, because the saccharide moieties are multivalent.

Introduction

Polymer backbones with reactive pendant groups, such as activated esters, anhydrides, isocyanates, oxazolines, epoxides, azides, alkynes, and thiols, are useful for synthesizing various functional polymers by post-polymerization modification approaches [1–3]. The substitution of activated esters, such as *N*-hydroxysuccinimide (NHS) esters [4] and pentafluorophenyl (PFP) esters [5], with amine-containing compounds via a substitution reaction is a well-known strategy for modifying polymers with dyes and biomolecules, such as fluorescent labels, saccharides, peptides, and proteins (Fig. 1). Because these activated esters are hydrophobic, the substitution of activated esters with amine-containing compounds is often limited in organic solvents and cosolvents.

Glycopolymers consisting of synthetic polymers with pendant saccharides have received much attention for their potential use as functional polymer materials [6–11]. The numerous glycopolymer synthesis methods reported to date can be classified into two categories. The first category is the polymerization of glycomonomers that are generally synthesized from free saccharides via multiple steps; at least two steps in this method occur without the protection of hydroxyl groups on the saccharides [12–18]. The second category is the post-polymerization modification of a polymer side chain by saccharide derivatives. Polymer backbones bearing activated esters, such as NHS [19–25] and PFP [26–28], have been widely used to synthesize functional polymers via post-polymerization modification approaches. Because these monomers and polymers bearing activated esters are hydrophobic and water-insoluble, it is difficult to perform the substitution reaction on the activated ester groups in water. Little research has been reported regarding the use of monomers with water-soluble activated esters for synthesizing functional polymers. Recently, water-soluble activated ester compounds, such as *N*-hydroxysulfosuccinimide (sulfoNHS) ester [29] and 4-sulfotetrafluorophenyl ester [30], were reported and used for protein labeling and functional polymer synthesis. Hawker et al. reported the synthesis of polymer backbones bearing water-soluble activated esters using an *N*-

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✉ Tomonari Tanaka
t-tanaka@kit.ac.jp

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

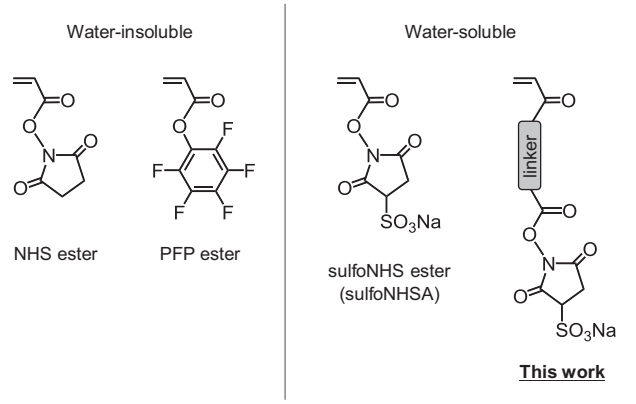

Fig. 1 Chemical structures of the monomers bearing an activated ester

hydroxysulfosuccimidyl acrylate (sulfoNHSA) monomer, in which a sulfoNHS ester group was directly linked to an acrylate group (Fig. 1) [31]. The acrylate derivative monomer bearing a sulfoNHS ester was subjected to rapid visible light-mediated reversible addition-fragmentation chain transfer (RAFT) polymerization. The resulting polymers bearing sulfoNHS esters were handled in situ and conjugated with amine-containing compounds in water, because the sulfoNHSA monomer and its polymer are unstable and degrade rapidly in water; the half-life of sulfoNHSA is ~60 min. Therefore, polymer backbones with water-soluble activated ester pendant groups that are stable in water must be developed to synthesize functional polymers by the post-polymerization modification with hydrophilic compounds, including biomolecules, such as saccharide derivatives. In this study, we report the development of a novel monomer compound bearing a water-soluble activated ester and the controlled polymerization of this monomer by a RAFT technique. The monomer is composed of a vinyl group and a sulfoNHS group, which are linked by an alkyl chain linker (Fig. 1). Moreover, the resulting polymer bearing water-soluble activated esters was used to synthesize glycopolymers in water by the post-polymerization modification with an amine-containing saccharide derivative.

Experimental

Materials

N-Hydroxysulfosuccinimide sodium salt and 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 6-Acrylamidohexanoic acid and *N*,*N*′-dicyclohexylcarbodiimide (DCC) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Nacalai Tesque Inc. (Kyoto, Japan), respectively. The chain transfer agent 2-(benzylsulfanylthiocarbonylsulfanyl)ethanol (BTSE) was synthesized using 2-mercaptoethanol, carbon disulfide, and benzyl bromide, according to a previously published method [32]. *p*-Aminophenyl-*β*-D-galactopyranoside (*p*AP-Gal) was synthesized from 4-nitrophenyl-*β*-D-galactopyranoside by reduction under hydrogen using Pd/C [33]. Fluorescein isothiocyanate (FITC)-labeled peanut agglutinin from *Arachis hypogaea* (FITC-PNA) and FITC-labeled bovine serum albumin (FITC-BSA) were purchased from Sigma-Aldrich Co., LLC (St. Louis, USA). All other reagents and solvents were commercially available and used without further purification.

Measurements

Bruker BioSpin AV-300 and AV-600 spectrometers were used to record the NMR spectra. A Bruker Daltonics micrOTOF Q-III spectrometer was used to record the ESI mass spectra. A PerkinElmer GX FT-IR spectrometer with an attenuated total reflection attachment was used to record the IR spectra. A system consisting of a JASCO PU-2089 pump, a CO-2065 column oven, and an RI-2031 refractive index detector was used to conduct the gel permeation chromatography (GPC) measurements. A Shodex OHpak SB-804 HQ (8.0 × 300 mm) column was used, and 20 mM phosphate buffer (pH 7.0) was used as the eluent and introduced at a flow rate of 0.5 mL min^{-1} at 30 °C. Pullulan samples were used as the standards. The fluorescence intensity was recorded using a JASCO FP-6500 spectrometer.

Synthesis of a monomer bearing a sulfoNHS ester

N-Hydroxysulfosuccinimide sodium salt (0.204 g, 0.94 mmol) and 6-acrylamidohexanoic acid (0.175 g, 0.94 mmol) were dissolved in dry DMF (6 mL) in a two-necked flask under a nitrogen atmosphere. DCC (0.573 g, 4.69 mmol) dissolved in dry DMF (2 mL) was added dropwise to the two-necked flask placed in an ice bath; then, the mixture was stirred at room temperature for 20 h. The reaction progress was confirmed by TLC (MeOH/$CHCl_3$ = 3/5 v/v). The solution was cooled in an ice bath and then filtered through a glass filter. The filtrate was reprecipitated with diethyl ether and dried under a reduced pressure to produce *N*-sulfosuccinyl-6-hexyloylacrylamide sodium salt (0.321 g, 0.835 mmol, 88.9%).

^{1}H NMR (600 MHz, DMSO-d_6, δ (ppm)): 8.06 (s, 1H, NH), 6.20 (dd, J = 10.2 and 7.2 Hz, 1H, vinyl), 6.06 (d, J = 16.8 Hz, 1H, vinyl), 5.56 (d, J = 9.6 Hz, 1H, vinyl), 3.96 (br, 1H, CH-SO_3Na), 3.2 (m, 3H, NH-C<u>H$_2$</u> and CH_2 of sulfoNHS), 2.80 (d, J = 6.9 Hz, 1H, CH_2 of sulfoNHS), 2.66 (s, 2H, CH_2-COO), 1.64 (quin, J = 7.2 Hz, 2H, NH-CH_2-C<u>H$_2$</u>-), 1.46 (quin, J = 7.2 Hz, 2H, C<u>H$_2$</u>-CH_2-COO), 1.37 (quin, J = 7.2 Hz, 2H, NH-CH_2-CH_2-C<u>H$_2$</u>-). ^{13}C NMR (151 MHz, DMSO-d_6, δ (ppm)): 168.8 (2C, C=O of

sulfoNHS and ester), 165.4 (C=O of sulfoNHS), 164.4 (C=O of amide), 131.9 (vinyl), 124.7 (vinyl), 56.3 (CH of sulfoNHS), 38.2 (NH-CH_2), 30.9 (CH_2 of sulfoNHS), 30.1 ($\underline{CH_2}$-COO), 28.5 ($\underline{CH_2}$-CH_2-COO), 25.5 (NH-CH_2-$\underline{CH_2}$-), 23.9 (NH-$\underline{CH_2}$-CH_2-). ESI-MS: $[M-Na]^-$, M = $C_{13}H_{17}N_2NaO_8S$, calculated 361.071, observed 361.074.

Synthesis of polymers bearing sulfoNHS esters

RAFT polymerization of a monomer bearing a sulfoNHS ester was performed with a monomer concentration of 1.0 M. The monomer, BTSE, and V-70 were dissolved in DMSO (0.39 mL) in a glass tube. The resulting solution was degassed via three freeze-thaw cycles; then, the glass tube was sealed under a vacuum and stirred at 35 °C. The products were purified by reprecipitation in a mixed solvent (MeOH/EtOH = 1/3 v/v) and dried under a reduced pressure to produce the polymers bearing sulfoNHS esters.

^{1}H NMR (300 MHz, DMSO-d_6, δ (ppm)): 7.8–7.0 (br, NH), 4.01(br, CH of sulfoNHS), 3.3–2.8 (m, NH-$\underline{CH_2}$- and CH_2 of sulfoNHS), 2.7–2.6 (br, CH_2-COO), 1.7–1.2 (m, (-$\underline{CH_2}$-$\underline{CH}$-)$_n$ and NH-CH_2-($\underline{CH_2}$)$_3$-CH_2-).

Synthesis of glycopolymers

A mixture of the polymer bearing sulfoNHS esters **P2** (10 mg) and *p*AP-Gal in water (0.2 mL) was stirred at 37 °C for 24 h. The product was purified by dialysis (Spectra/Por 7 MWCO 3500) and freeze-dried to produce the glycopolymers.

^{1}H NMR (300 MHz, D_2O, δ (ppm)): 7.3–6.7 (Ph), 3.9–3.4 (Gal), 3.2–2.7 (br, NH-$\underline{CH_2}$- and CH_2-COO), 2.3–1.8 ((-CH_2-$\underline{CH}$-)$_n$), 1.6–0.9 ((-$\underline{CH_2}$-CH-)$_n$) and NH-CH_2-($\underline{CH_2}$)$_3$-CH_2-).

Lectin binding assay

The FITC-labeled protein (FITC-PNA or FITC-BSA; 8 μM) and glycopolymer **P7** (1 mg) were mixed in PBS (pH 7.4) (1.015 mL), and the mixture was maintained in the dark at room temperature for 8 h. After centrifugation, the fluorescence intensity of the supernatant was measured by a fluorescence spectrophotometer (λ_{ex} = 495 nm and λ_{em} = 505 nm). The association constant (K_a) for the saccharide −lectin interaction was estimated using the Steck−Wallack equation [34, 35].

Results and discussion

Synthesis of polymers bearing sulfoNHS esters

The procedure used to synthesize the polymers bearing water-soluble activated esters is shown in Scheme 1. First, a monomer bearing a water-soluble activated ester was synthesized by a dehydrative condensation reaction of *N*-hydroxysulfosuccinimide sodium salt with 6-acrylamidohexanoic acid using a dehydrative condensing agent, DCC. The ^{1}H NMR spectrum of the monomer shows signals attributed to vinyl and sulfosuccinimidyl protons at 6.2–5.6 and 4.0–2.7 ppm, respectively (Fig. 2a). The monomer bearing a sulfoNHS ester was subjected to RAFT polymerization in DMSO at 35 °C to obtain polymers bearing sulfoNHS esters; the RAFT polymerization was performed using a trithiocarbonate derivative, BTSE, which is a useful CTA agent for the RAFT polymerization of acrylamide derivatives. The proportion of the remaining activated ester bonds in the monomer was 95% after maintaining at 35 °C for 24 h in DMSO. In contrast, 81% of the ester bonds in the monomer remained after maintaining at 65 °C for 24 h in DMSO. This result indicates that the monomer bearing a sulfoNHS ester is more stable at 35 °C than it is at 65 °C. Therefore, the RAFT polymerization was performed at 35 °C by using the azo initiator V-70, which has a low decomposition temperature, to synthesize the polymers bearing sulfoNHS esters. Table 1 summarizes the results of synthesizing the polymers bearing sulfoNHS esters. By performing the RAFT polymerization at different molar ratios of monomer (M) and chain transfer agent BTSE (M/BTSE = 50/1 and 150/1), two polymer backbones with different molecular weights, **P1** and **P2**, were obtained. For the polymerization conducted at a molar ratio of M/BTSE = 50/1, the plots show that $\ln[M]_0/[M]$ increased linearly with the reaction time (Fig. 3a). Additionally, the molecular weight of the produced polymers exhibited an increase proportional with the increasing conversion (Fig. 3b). The polydispersity index, $Đ$, remained low until the conversion approached its maximum value. When the polymerization reaction was performed without BTSE, the $Đ$ value of produced polymer **P3** exceeded that of the product of the reaction with BTSE. The GPC chromatograms obtained for polymers **P1** and **P2**, which were synthesized with BTSE, were monomodal (Fig. 4). These results indicate that the controlled synthesis of polymers bearing water-soluble activated esters was achieved by a RAFT polymerization technique using a monomer bearing a sulfoNHS ester. The produced polymers were successfully purified by reprecipitation with a mixed solvent (MeOH/EtOH = 1/3 v/v). The ^{1}H NMR spectrum of the polymers bearing sulfoNHS esters displayed signals arising from the polymer backbone and sulfosuccinimide protons at 1.7–1.1 and 4.0 ppm, respectively (Fig. 2b). The stability of the sulfoNHS ester group in the monomer and polymer was investigated in water at 25 °C by ^{1}H NMR (Fig. S2). The hydrolysis of the sulfoNHS ester on the polymer side chain was slower than that on the monomer. After 24 h, approximately 70% of the sulfoNHS ester groups remained on the polymer side chain in water. In contrast, the proportion of remaining sulfoNHS ester groups on the monomer was 37%

Scheme 1 Synthesis of the monomer, polymers bearing sulfoNHS esters, and glycopolymers

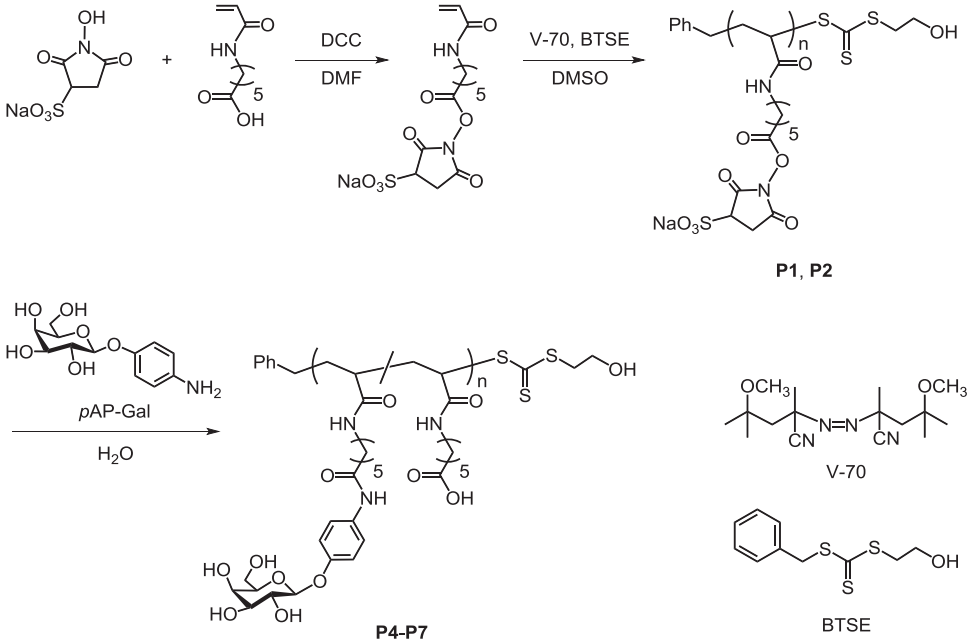

Fig. 2 ^{1}H NMR spectra of **a** the monomer and **b** polymer bearing sulfoNHS esters in DMSO-d_6

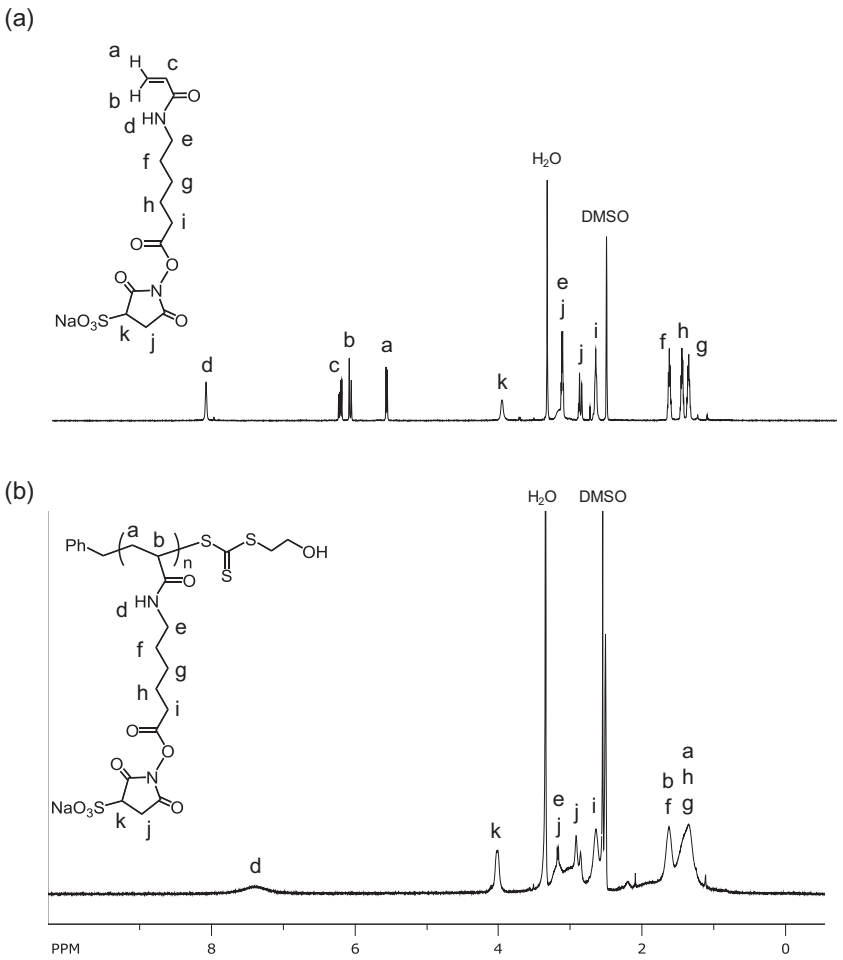

Table 1 Synthesis of polymers bearing sulfoNHS esters

Polymer	M/BTSE/V-70[a]	Time (h)	Conv. (%)[b]	Yield (%)[c]	M_n (g mol^{-1})[d]	M_n (g mol^{-1})[e]	DP[e]	$Đ$[d]
P1	50/1/0.5	8	89	40	36,400	18,000	47	1.34
P2	150/1/0.5	24	95	63	62,000	56,000	146	1.52
P3	150/0/0.5	8	61	33	135,000	–	–	2.39

[a]Feeding molar ratio of monomer (M), BTSE, and V-70

[b]Determined by ^{1}H NMR in DMSO-d_6

[c]Isolated yield

[d]Determined by GPC

[e]Determined by ^{1}H NMR in D_2O

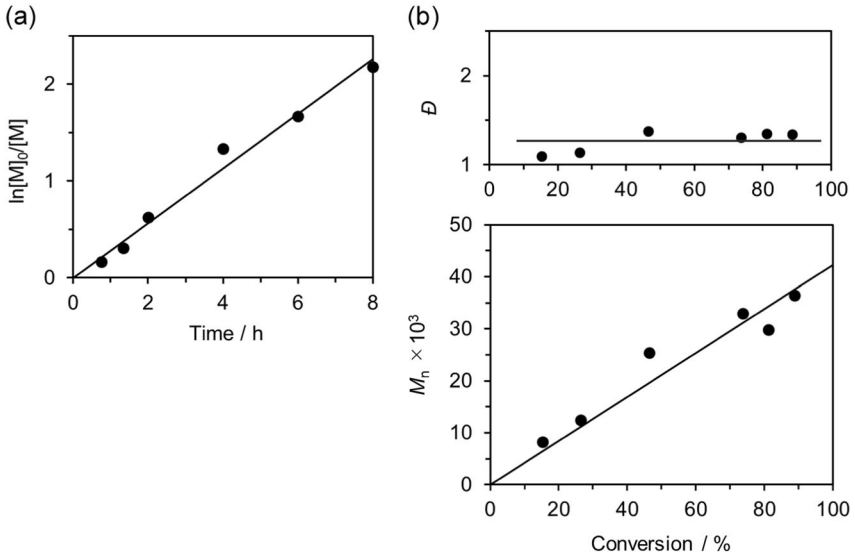

Fig. 3 **a** Kinetic plots of ln[M]$_0$/[M] vs. time and **b** plots of M_n and $Đ$ vs. conversion (M/BTSE/V-70 = 50/1/0.5)

after 24 h in water. This behavior agrees with previous reports; for example, Sakurada reported that the hydrolysis of a methyl ester bond in a polymer side chain is slower than that of the methyl esters in a monomer-like compound because of the steric hindrance of the polymer chain [36]. These results indicate that this polymer backbone bearing sulfoNHS esters can be modified with amine-containing derivatives in water via post-polymerization modification, because the polymer is water-soluble and the degradation of the sulfoNHS esters in the polymer is relatively slow.

Synthesis of glycopolymers

Post-polymerization modification was performed on the polymer bearing sulfoNHS esters **P2** to obtain glycopolymers in water (Scheme 1). By conducting nucleophilic substitution, where the amino group of *p*AP-Gal replaced the activated ester of the polymer side chain, glycopolymers bearing saccharide moieties were obtained. The resulting glycopolymers were purified by dialysis in water after the substitution reaction. The synthesized glycopolymer clearly exhibited ^{1}H NMR proton signals attributed to phenyl galactoside moieties at 7.3–6.7 and 3.9–3.4 ppm (Fig. 5). The IR spectrum of the glycopolymer clearly shows the increase in the absorption signal at approximately 3300 cm^{-1}, which is attributed to the hydroxyl and carboxy groups present after the substitution reaction (Fig. S4). The signal corresponding to the carbonyl groups, attributed to succinimide at 1737 cm^{-1}, disappeared after the reaction. In contrast, the signals attributed to the amide linkage at 1643 and 1545 cm^{-1} increased after the reaction. These analytical results support the successful substitution of the sulfoNHS esters with *p*AP-Gal on the polymer side chain. Table 2 summarizes the glycopolymer synthesis results. Changing the feeding amount of *p*AP-Gal with respect to the sulfoNHS ester groups present in the polymer side chain led to the production of glycopolymers having different degrees of substitution (DS) with galactoside moieties. The DS value reached approximately 25% as the feeding amount of *p*AP-Gal increased from 0.25 to 1.0 equivalents of the sulfoNHS ester groups on the polymer. Since the introduction of saccharide moieties results in steric hindrances, an upper limit of the DS is observed at approximately 25%, which is difficult to surpass. When the DS of the glycopolymers by *p*AP-Gal was low, the obtained glycopolymers **P4** and **P5** were partially water-insoluble. As the DS

increased, the glycopolymers **P6** and **P7** were water-soluble. Glycopolymers with a low DS by *p*AP-Gal were obtained by the hydrolysis of activated ester groups on the polymer side chain, suggesting that the water solubility of the glycopolymers with a high content of carboxy groups on the polymer side chain was reduced in neutral aqueous conditions. Therefore, a lectin binding assay was performed using **P7** with the highest DS by *p*AP-Gal under neutral aqueous conditions.

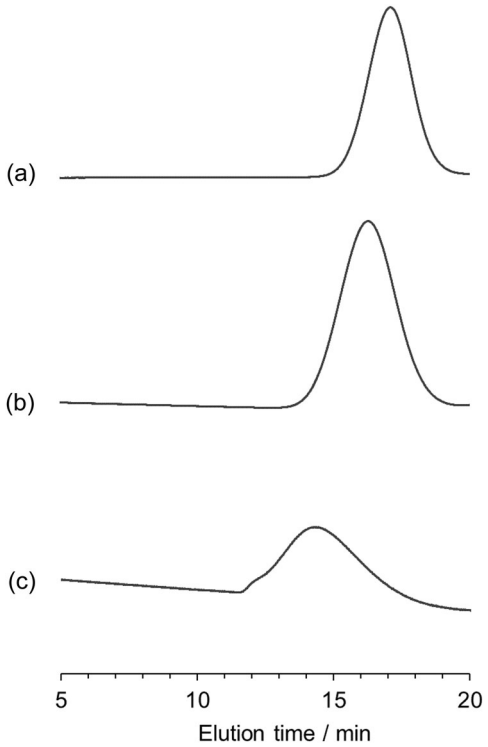

Fig. 4 GPC chromatograms of polymers bearing sulfoNHS esters: **a P1**, **b P2**, and **c P3**

Lectin binding assay

The binding of the glycopolymer with lectin was investigated using FITC-labeled lectin in PBS. The addition of glycopolymer **P7** to a FITC-PNA buffer solution caused a decrease in the fluorescence intensity (ΔF), as the lectin and glycopolymer aggregated and precipitated (Fig. 6). In contrast, no change in the fluorescence intensity was observed when FITC-BSA was added as a control protein. The association constant (K_a) of the saccharide−lectin interaction was estimated to be $1.3 \times 10^5\ M^{-1}$; the K_a of the saccharide−lectin is higher than that of the free saccharide, which was on the order of $10^3\ M^{-1}$ [37], due to the multivalency of the saccharide moieties on the polymer backbone, which is known as the glycocluster effect [38, 39]. The K_a value of the interaction of the glycopolymers with lectin in aqueous solution is generally reported on the order of $10^4-10^5\ M^{-1}$ [37]. The glycopolymer synthesized by postpolymerization modification using a novel polymer backbone bearing sulfoNHS esters has a lectin binding affinity on the order of $10^5\ M^{-1}$, which is similar to the lectin binding affinity of previously reported glycopolymers.

Conclusions

We synthesized novel polymer backbones with water-soluble activated ester pendant groups by the RAFT

Fig. 5 ^{1}H NMR spectrum of the glycopolymer in D_2O

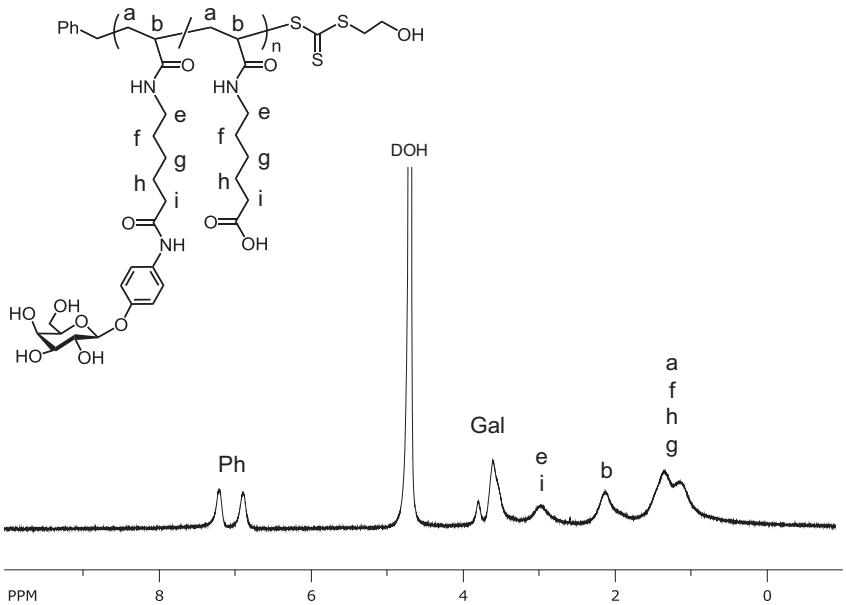

Table 2 Synthesis of glycopolymers using **P2**

Glycopolymer	*p*AP-Gal (equiv.)[a]	DS by *p*AP-Gal (%)[b]	Yield (%)[c]	M_n (g mol^{-1})[d]	$Đ$[d]
P4	0.25	7.7	46	—[e]	—[e]
P5	0.50	16.0	49	—[e]	—[e]
P6	1.0	25.0	46	20,500	1.59
P7	2.0	25.8	35	33,000	1.91

[a]Feeding amount of *p*AP-Gal with respect to the activated ester groups

[b]Degree of substitution by *p*AP-Gal, determined by ^{1}H NMR in D_2O

[c]Isolated yield

[d]Determined by GPC

[e]The sample is partially water-insoluble

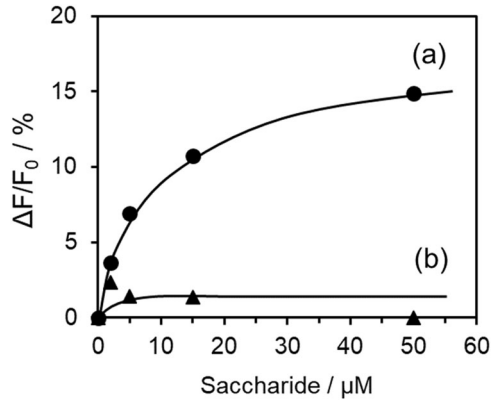

Fig. 6 Quenching of the fluorescence intensity in a lectin binding assay using **P7**: **a** FITC-PNA and **b** FITC-BSA

polymerization of a monomer bearing a sulfoNHS ester. The monomer was newly synthesized by the dehydrative condensation reaction of *N*-hydroxysulfosuccinimide sodium salt with 6-acrylamidohexanoic acid using a dehydrative condensing agent, DCC. The molecular weight and polydispersity indexes of the resulting polymers were well controlled by the RAFT polymerization mechanism. The stability of the sulfoNHS ester groups on the polymer synthesized in this study in water is higher than that of a previously reported acrylate monomer directly bearing sulfoNHS esters and the corresponding acrylate polymer in water [31]. These polymers bearing sulfoNHS esters can be isolated and reacted with an amine-containing saccharide derivative to produce glycopolymers in water. The resulting glycopolymers bearing saccharides exhibited a strong interaction with lectin in aqueous solution, because the saccharide moieties are multivalent and have higher values of K_a. This polymer backbone bearing water-soluble activated esters can be used to synthesize various functional polymers by post-polymerization modification in water.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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