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ORIGINAL ARTICLE

Reversible temperature-responsive and lectin-recognizing glycosylated block copolymers synthesized by RAFT polymerization

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Abstract

Reversible temperature-responsive and lectin-recognizing glycosylated block copolymers composed of polyacrylamide (PAAm) and poly(*N*-isopropylacrylamide) (PNIPAm) were synthesized by consecutive RAFT polymerization reactions. PAAm bearing maltose moieties was synthesized by RAFT polymerization with a trithiocarbonate derivative as a chain transfer agent for chain extension and with *N*-isopropylacrylamide to obtain block copolymers. The resulting glycosylated block copolymers were responsive to temperature at approximately 33 °C (lower critical solution temperature; LCST) and formed aggregates 100 nm in diameter in aqueous media above the LCST. The aggregates specifically interacted with lectin in aqueous media above the LCST, forming conjugates. When the temperature was decreased below the LCST, the conjugate dissociated into the aqueous medium. The conjugates of block copolymer and lectin were reversible in response to changes in temperature.

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Introduction

Glycopolymers are synthetic polymers with pendant saccharides and have received much attention in diverse fields, such as materials science and medicinal chemistry [1–6]. Although the binding affinity of saccharides with receptors, including lectins and viruses, is generally weak, the affinity is amplified by multivalent forms of saccharides in various biological processes. Such multivalent saccharides give rise to the "glycocluster effect" [7, 8] and allow the generation of artificial glycoclusters such as glycodendrimers [9–11] and glyconanoparticles [12–16].

Functional double-hydrophilic block copolymers containing at least one functional unit, such as a temperature or pH-responsive segment, have recently attracted significant interest for their potential applications in drug delivery systems and gene delivery vectors [17–20]. Temperature-responsive double-hydrophilic block copolymers are among the most important candidate biomaterials for these applications because they can reversibly form micelles and then dissociate in response to a change in temperature [21–23]. When the temperature is above the lower critical solution temperature (LCST) of the temperature-responsive copolymer [24], the copolymer forms aggregates in aqueous media, and when the temperature is below the LCST, the aggregates dissociate. Poly(*N*-isopropyl acrylamide) (PNIPAm) is one of the best-known temperature-responsive polymers, and its LCST is approximately 32 °C. In these previous reports, the glycomonomers for synthesizing glycosylated block copolymers were prepared by appropriate methods for each saccharide. For example, an amino group at the 2-position of glucosamine and a primary hydroxy group at the 6-position of glucose were used to obtain the glycomonomers. Therefore, a simpler and common synthetic method applicable to various saccharides is desirable for the preparation of glycopolymers from free saccharides.

We recently developed a protecting-group-free synthetic protocol to obtain glycopolymers composed of polyacrylamide (PAAm) from free saccharides via direct

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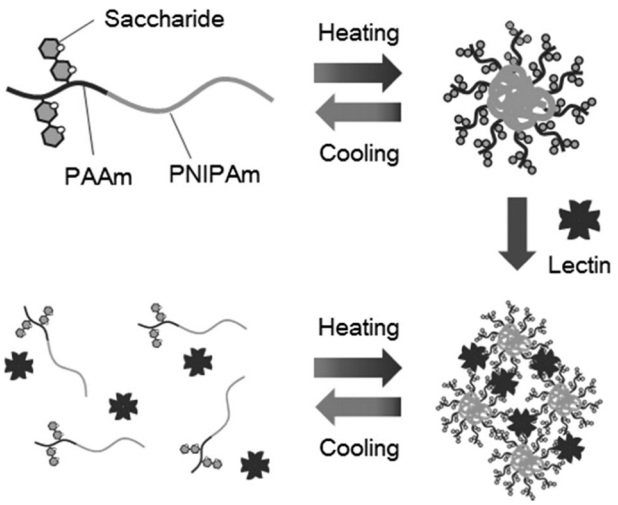


Fig. 1 Reversible temperature-dependent aggregation/dissociation behavior of block copolymer containing saccharide-bearing PNIPAm and PAAm

anomeric azidation with 2-chloro-1,3-dimethylimidazolinium chloride [25] (called "Shoda activation") [26], a subsequent copper-catalyzed azide-alkyne cycloaddition reaction (CuAAC) [27], and finally RAFT polymerization [28, 29]. This simple synthetic method for glycopolymers can be applied to various high-molecular-weight oligosaccharides and ones containing sialic acid moieties, such as sialyllactose and the complex-type biantennary *N*-linked oligosaccharides typically found on a cell surface [30–32]. We postulated that a double-hydrophilic glycosylated block copolymer with a polymeric backbone composed of PAAm and PNIPAm could form glycoclusters due to the temperature-dependent aggregation of PNIPAm. When a lectin is added to an aqueous solution of glycosylated block copolymer above the LCST, the aggregate interacts with the lectin and then precipitates because of the multivalent saccharide moieties on its surface. In addition, this conjugated lectin-copolymer binding complex can be dissociated by cooling below the LCST (Fig. 1). Based on this scheme, we here report the synthesis of reversible temperature-responsive and lectin-recognizing glycosylated block copolymers composed of PAAm and PNIPAm. PAAm bearing maltose (Mal) moieties was generated by protecting-group-free glycomonomer synthesis and RAFT polymerization. Chain extension by the RAFT process with the resulting glycopolymer and *N*-isopropyl acrylamide (NIPAm) as a macro chain transfer agent (CTA) and a monomer substrate, respectively, provided glycosylated block copolymers composed of PAAm and PNIPAm. We evaluated the temperature responsiveness and lectin binding properties of these block copolymers in aqueous media.

Experimental Procedures

Materials

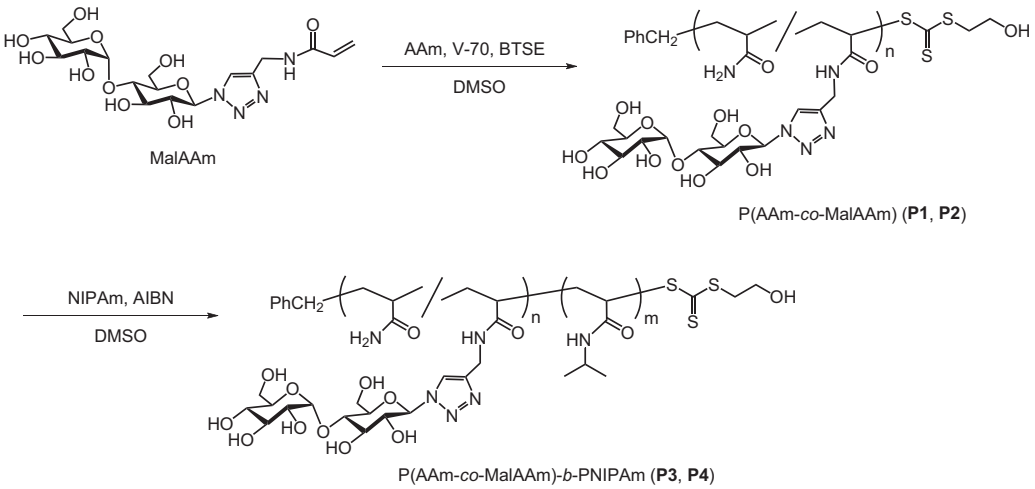
Mal and AAm were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). AAm was used after recrystallization from chloroform/methanol = 10/3. NIPAm was purchased from Wako Pure Chemical Industries (Osaka, Japan) and used after recrystallization from *n*-hexane. A Mal-bearing acrylamide derivative (MalAAm) was synthesized from β-maltosyl azide and *N*-propargyl acrylamide by the CuAAC reaction [16]. *N*-propargyl acrylamide was synthesized from acryloyl chloride and propargylamine in the presence of triethylamine by a literature method [33]. 2,2′-Azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) and α,α′-azobisisobutyronitrile (AIBN) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and Nacalai Tesque, Inc. (Kyoto, Japan), respectively. CTA 2-(benzylsulfanylthiocarbonylsulfanyl) ethanol (BTSE) was synthesized from 2-mercaptoethanol, carbon disulfide, and benzyl bromide according to a previously published method [34]. All other reagents were commercially available and used without further purification. Concanavalin A (Con A) from *Canavalia ensiformis* and bovine serum albumin (BSA) were purchased from J-Oil Mills, Inc. (Tokyo, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively.

Measurements

NMR spectra were recorded with a Bruker BioSpin AV-300 spectrometer. GPC measurements were performed with 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. Phosphate buffer (20 mM; pH 7.0) was used as the eluent at a flow rate of 0.5 mL min^{-1} at 30 °C for the analysis of P(AAm-*co*-MalAAm)s. Phosphate buffer (20 mM; pH 7.0) containing 20% dimethylformamide was used as the eluent for the analysis of P(AAm-*co*-MalAAm)-*b*-PNIPAms. Pullulan samples were used as standards. Transmittance and UV-Vis absorption were recorded with a JASCO V-550 spectrometer. Dynamic light scattering (DLS) analyses were conducted at 667 nm with an Otsuka Electronics ELSZ-1000 at 25 and 40 °C. The samples for DLS were prepared at 0.5 wt% in aqueous media.

Synthesis of P(AAm-*co*-MalAAm)

RAFT copolymerization reactions were carried out at a concentration of 1.0 M total monomer. MalAAm, AAm, V-70, and BTSE were dissolved in 1.0 mL DMSO in a glass



Scheme 1 Synthesis of P(AAm-*co*-MalAAm)-*b*-PNIPAm

tube. The feed molar ratio of AAm/MalAAm was 9/1. The resulting solution was degassed by three freeze-thaw cycles, and then the glass tube was sealed under vacuum and stirred at 35 °C for 24 h. The products were purified by dialysis (Spectra/Por 7 MWCO 3500) against deionized water and freeze-dried to give P(AAm-*co*-MalAAm) glycopolymers.

^{1}H NMR (300 MHz, D_2O, δ): 8.09 (triazole), 7.3–7.1 (Ph), 5.70 (H1), 5.40 (H1′), 4.41 (N–CH_2), 4.1–3.3 (Mal and S-CH_2–CH_2–), 2.3–2.0 ((–CH_2–C<u>H</u>–)$_n$), 1.8–1.4 ((–C<u>H</u>$_2$–CH–)$_n$).

Synthesis of P(AAm-*co*-MalAAm)-*b*-PNIPAm

Chain extension by the RAFT process was carried out at a monomer concentration of 1.0 M. P(AAm-*co*-MalAAm), NIPAm, and AIBN were dissolved in 0.25 mL DMSO in a glass tube. The resulting solution was degassed by three freeze-thaw cycles, and then the glass tube was sealed under vacuum and stirred at 65 °C for 24 h. The products were purified by dialysis (Spectra/Por 7 MWCO 3500) against deionized water and freeze-dried to give P(AAm-*co*-MalAAm)-*b*-PNIPAm block copolymers.

^{1}H NMR (300 MHz, D_2O, δ): 8.08 (triazole), 7.3–7.1 (Ph), 5.70 (H1), 5.40 (H1′), 4.40 (N-CH_2), 4.0–3.4 (Mal and S–CH_2–CH_2–), 3.80 (CH of PNIPAm) 2.3–1.9 ((–CH_2–C<u>H</u>–)$_n$), 1.8–1.3 ((–C<u>H</u>$_2$–CH–)$_n$), 1.06 (CH_3 of PNIPAm).

LCST analysis

A 1.0 wt% polymer aqueous solution was filtered through a membrane filter (0.45 μm), and the transmittance of the sample solution was measured in a quartz cell at 500 nm during heating or cooling at a rate of 0.5 °C min^{-1}.

Lectin binding test

A 0.5 wt% PBS polymer solution was filtered through a membrane filter (0.45 μm) and kept at 40 °C, and then Con A or BSA (final conc.: 2.0 μM) was added. After mixing and then standing for 2 h at 40 °C, the transmittance of the sample solution was measured in a quartz cell at 500 nm. To estimate the concentration of copolymer in the aqueous medium, the UV absorption at 214 nm of the sample solution was measured at 25 and 40 °C.

Results and discussion

Synthesis of glycosylated block copolymers

A synthetic procedure for the glycosylated block copolymer P(AAm-*co*-MalAAm)-*b*-PNIPAm, composed of PAAm bearing Mal moieties and PNIPAm, is shown in Scheme 1. The AAm derivative bearing the triazole-linked Mal moiety (MalAAm), synthesized from β-maltosyl azide and *N*-propargyl acrylamide by CuAAC, was subjected to RAFT copolymerization with AAm in dimethyl sulfoxide (DMSO) to obtain the P(AAm-*co*-MalAAm) glycopolymer by using the trithiocarbonate derivative BTSE as a CTA. Table 1 summarizes the P(AAm-*co*-MalAAm) synthesis results. Performing RAFT polymerization at different ratios of monomer and CTA (50/1 and 150/1) provided two desired glycopolymers, **P1** and **P2**, with different degrees of polymerization (DPs) and low dispersity. The ratio of the MalAAm unit in the product polymers agreed well (8–9%) with the feed ratio of MalAAm (10%).

The glycosylated block copolymers P(AAm-*co*-MalAAm)-*b*-PNIPAm were synthesized in DMSO with P

Table 1 Synthesis of P(AAm-*co*-MalAAm)

Polymer	M/CTA/I[a]	Conv. (%)[b]	Yield (%)[c]	M_n (g mol^{-1})[b]	M_w/M_n[d]	MalAAm ratio (%)[b,e]
P1	50/1/0.2	71	57	4,300	1.12	8.9
P2	150/1/0.2	69	47	12,400	1.14	7.9

[a] Molar ratio of total monomer (M: AAm and MalAAm), CTA, and initiator (I)

[b] Determined by ^{1}H NMR

[c] Isolated yield

[d] Determined by GPC

[e] MalAAm unit ratio in polymer calculated from the triazole proton and methine proton of the PAAm backbone

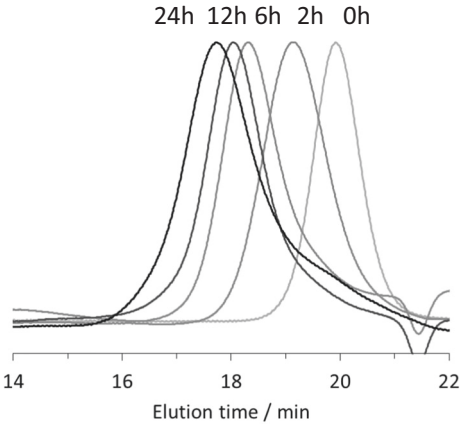


Fig. 2 GPC traces of the chain extension products with **P1** as the macro CTA

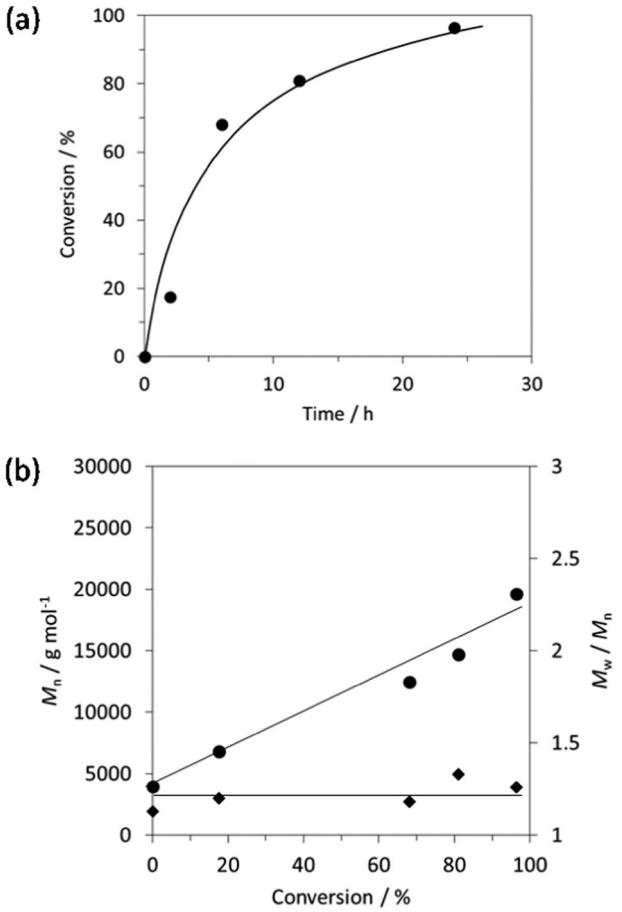


Fig. 3 Time-conversion curve (**a**) and product properties (**b**, ●: M_n, ◆: M_w/M_n) of chain extension with **P1** as the macro CTA

(AAm-*co*-MalAAm)s (**P1** and **P2**) and NIPAm as the macro CTA and a monomer substrate, respectively. When the chain extension by the RAFT process was performed with **P1** as the macro CTA, the GPC traces gradually shifted with time (2–24 h) towards the higher molecular weight region (Fig. 2), and the conversion and molecular weight increased (Fig. 3). The values of M_w/M_n remained low until the conversion approached its maximum value. The chain extension by the RAFT process to synthesize the block copolymers resulted in a clear increase in the ^{1}H NMR proton signals attributed to PNIPAm (Fig. 4). The proton signals of the polymer backbone and isopropyl groups of PNIPAm occurred at 2.3–1.3, 3.8, and 1.1 ppm, respectively. The Mal moiety signals occurred at 5.7, 5.4, and 4.0–3.4 ppm and the triazole and phenyl proton signals at 8.1 and 7.2, respectively. Table 2 summarizes the synthesis results for the block copolymers with **P1** or **P2** as the macro CTA. In both cases, the desired Mal-bearing block copolymers **P3** and **P4**, which contain a short and a long PAAm segment, respectively, were obtained as monomodal distribution polymers with low dispersity, as shown by GPC analysis.

Aggregation behavior of the block copolymers

The thermal properties of P(AAm-*co*-MalAAm)-*b*-PNIPAm were investigated. Figure 5 shows the transmittance of P(AAm-*co*-MalAAm)-*b*-PNIPAm and PNIPAm homopolymer in water. The block copolymer reacted for 2 h had short PNIPAm segments; its transmittance decreased slightly to 70% upon heating to 50 °C. In contrast, after >6 h reaction, the transmittance of the block copolymer was approximately 0%, similar to that of PNIPAm homopolymer. An increase in the DP of PNIPAm resulted in a shift of the LCST to the lower temperature region. This result indicated that PNIPAm required a higher DP than PAAm to exhibit a clear temperature response. Figure 6 shows the transmittance of **P3** and **P4** in water and in PBS. When each polymer solution was gradually heated from 20 to 50 °C in water, the transmittance of the **P3** solution significantly decreased to approximately 33 °C due to dehydration of the PNIPAm segment (Fig. 6a), whereas the decrease in transmittance of the **P4** solution was less pronounced (Fig. 6b). The dehydration of the PNIPAm segment in **P4** was less than that of **P3** because of the longer

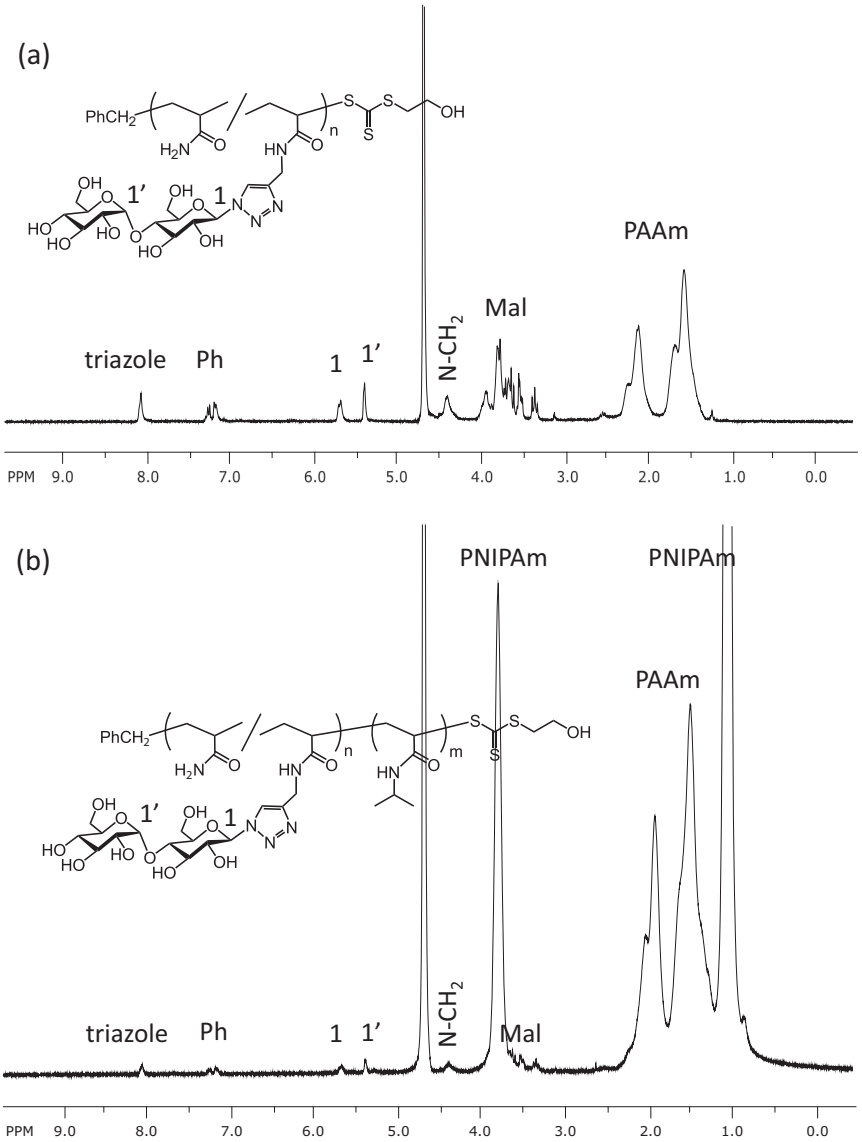


Fig. 4 ^{1}H NMR spectra of **P1** (**a**) and **P3** (**b**) in D_2O

Table 2 Synthesis of P(AAm-*co*-MalAAm)-*b*-PNIPAm

Polymer	M/CTA/I[a]	Macro CTA	Conv. (%)[b]	Yield (%)[c]	M_n (g mol^{-1})[b]	M_w/M_n[d]
P3	300/1/0.5	**P1**	96	84	46,600	1.30
P4	300/1/0.5	**P2**	49	46	35,600	1.33

[a] Molar ratio of monomer (M: NIPAm), macro CTA, and initiator (I)

[b] Determined by ^{1}H NMR

[c] Isolated yield

[d] Determined by GPC

hydrophilic PAAm segment in **P4**. The aggregation caused by the hydrophobicity of the block copolymer containing PNIPAm above the LCST could be controlled by the ratio of the PAAm/PNIPAm segments. When each polymer solution was cooled from 50 to 20 °C, the transmittance of the **P3** solution essentially returned to that before heating. The transmittance behaviors of both **P3** and **P4** in PBS were similar to those in water (Figs. 6c, d). Figure 7 shows the DLS analyses of **P3** in water and PBS. In both water and PBS, **P3** formed aggregates approximately 100 nm in diameter at 40 °C, which is above the LCST. In contrast, there was no aggregation at 25 °C, since the diameter of **P3** was below 10 nm. These results indicated that **P3**, which contains a short Mal-bearing PAAm segment and a long PNIPAm segment, clearly and reversibly responded to temperature and formed aggregates approximately 100 nm in diameter above the LCST in aqueous media.

Lectin binding test

The binding properties of P(AAm-*co*-MalAAm)-*b*-PNIPAm with lectin were investigated in PBS. Figure 8 shows

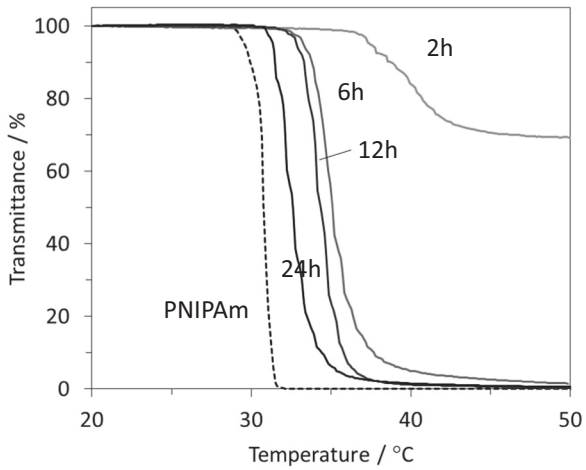


Fig. 5 Transmittance of the chain extension product P(AAm-*co*-MalAAm)-*b*-PNIPAm in water with **P1** as the macro CTA. Dashed line: PNIPAm homopolymer

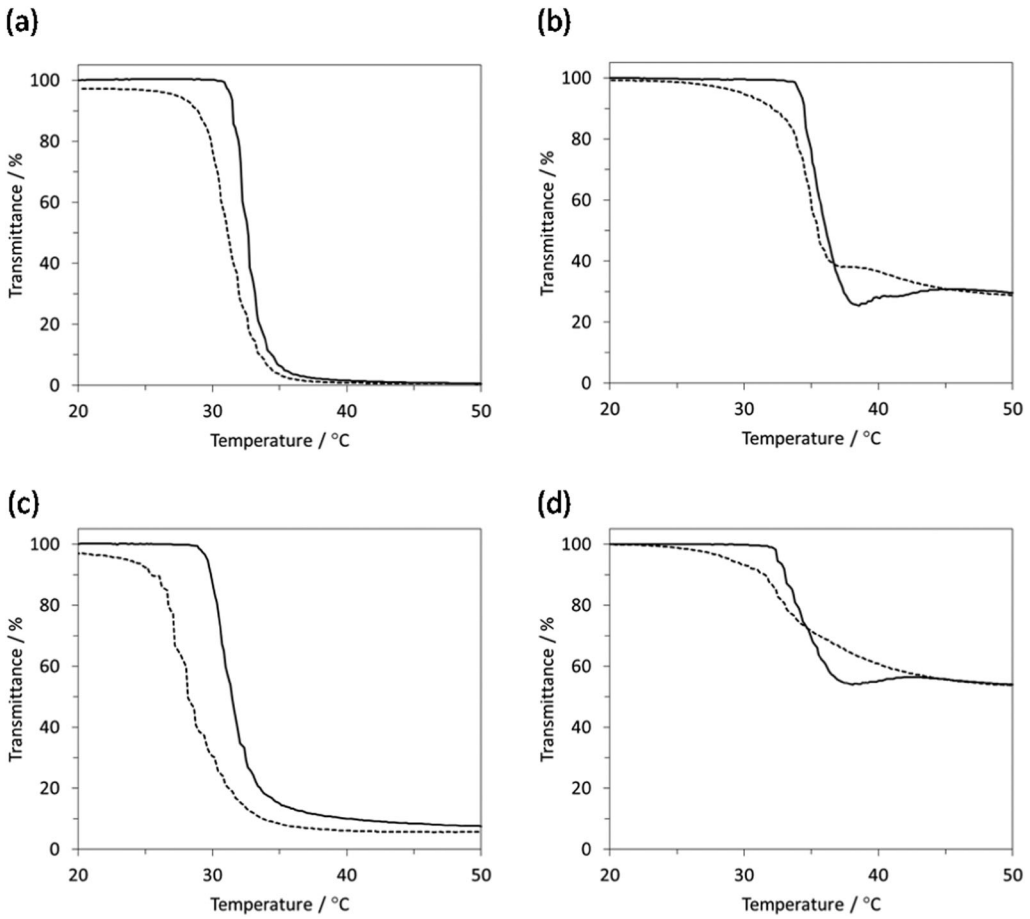


Fig. 6 Transmittance of P(AAm-*co*-MalAAm)-*b*-PNIPAm. **a P3** in water, **b P4** in water, **c P3** in PBS, **d P4** in PBS. Solid line: 20→50 °C. Dashed line: 50→20 °C

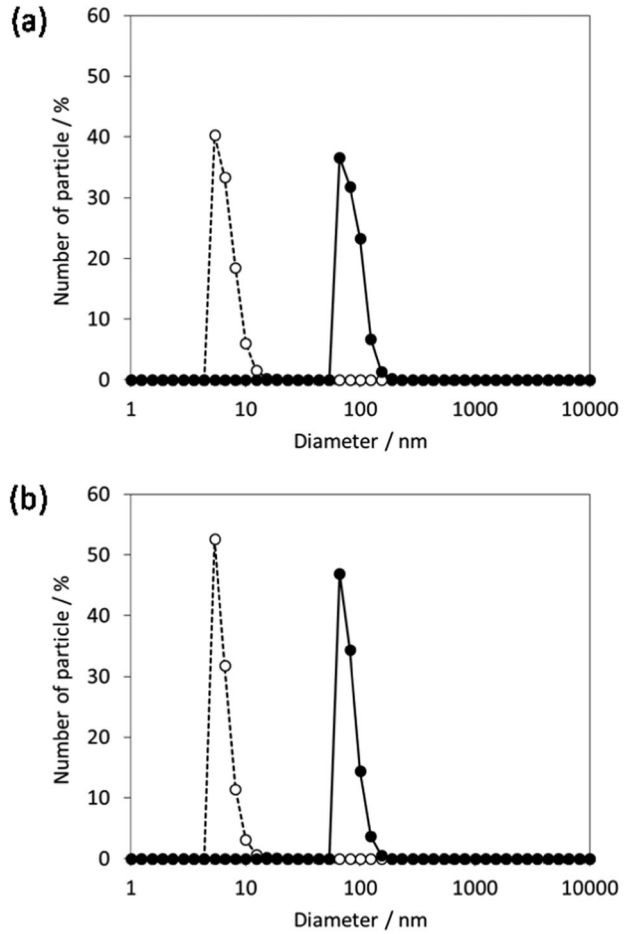


Fig. 7 DLS analysis of **P3** in water (**a**) and PBS (**b**). ○: 25 °C, ●: 40 °C

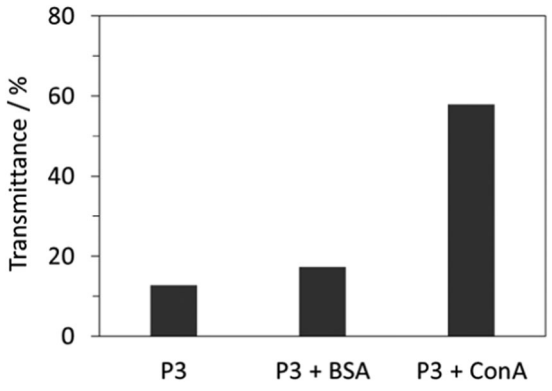


Fig. 8 Transmittance of **P3** solution at 40 °C

the transmittance of the **P3** solution at 40 °C, above the LCST. After the addition of Con A to the **P3** solution at 40 °C, the transmittance increased due to **P3** aggregation and subsequent precipitation of the **P3**/Con A conjugate. In contrast, no increase in transmittance was observed when BSA was added to the **P3** solution. We investigated the reversibility of the **P3**/Con A conjugate. Figure 9a shows

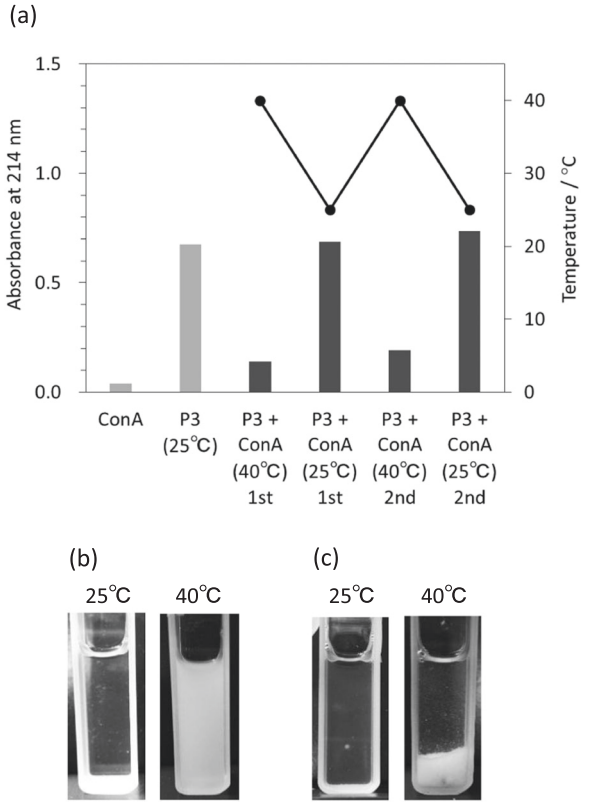


Fig. 9 **a** Absorbance of **P3** solution upon a change in temperature after the addition of Con A. Bar graph: absorbance, Line graph: temperature. **b**, **c** Photos of **P3** solution before (**b**) and after (**c**) the addition of Con A

the absorbance at 214 nm of the **P3** solution, providing an estimate of the concentration of **P3** dissolved into the aqueous medium. The addition of Con A to the **P3** solution at 40 °C resulted in a decrease in absorbance, indicating that the concentration of **P3** in the solution decreased due to precipitation of the polymer aggregate/lectin conjugate (Fig. 9a, 40 °C 1st and Fig. 9c, 40 °C). When the temperature was decreased from 40 to 25 °C, the absorbance increased due to dissociation of the conjugate (Fig. 9a, 25 °C 1st and Fig. 9c, 25 °C). Repetition of this heating and cooling cycle resulted in a corresponding cycling in the absorbance (Fig. 9a, 40 °C 2nd and 25 °C 2nd), indicating that the **P3** block copolymer aggregate interacted with Con A above the LCST (40 °C) due to the multivalent forms of Mal moieties displayed upon aggregation. In contrast, the binding affinity of **P3** to Con A below the LCST (25 °C) in aqueous media was weak because of the absence of polymer/lectin precipitation. The lack of aggregation of **P3** caused weak interaction with lectin, suggesting that the dissolved block copolymer hardly bound with lectin because **P3** has a short PAAm segment and a low ratio (8.9%) of MalAAm units. P(AAm-*co*-MalAAm)-*b*-PNIPAm including short PAAm segments and long PNIPAm segments is a dual-responsive glycosylated block

copolymer, responsive to both lectin and temperature. Our findings suggest that the aggregation and dissociation behavior of glycosylated block copolymers in response to changes in temperature makes them promising functional materials for protein purification, drug delivery systems, and gene delivery vectors.

Conclusions

We synthesized temperature-responsive and lectin-recognizing glycosylated block copolymers composed of PAAm bearing Mal moieties and PNIPAm by a RAFT polymerization technique. The resulting glycosylated block copolymers had an LCST of approximately 33 °C and formed aggregates 100 nm in diameter in aqueous media. These aggregates interacted strongly with the lectin Con A and formed precipitates above the LCST. When the temperature was decreased below the LCST, the copolymer/lectin conjugates dissociated and dissolved into the aqueous medium. The aggregation and dissociation were reversible, indicating that the interaction between the copolymer aggregate and the lectin was strong despite the weak binding affinity of the copolymer chain. This finding suggests that glycosylated double-hydrophilic block copolymers containing a PNIPAm segment will contribute to the development of various temperature-responsive and lectin-recognizing biomaterials for use in, for example, protein purification, drug delivery systems, and gene delivery vectors.

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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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