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NOTE

Synthesis of terminal-functionalized thermoresponsive diblock copolymers using biodegradable macro-RAFT agents

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INTRODUCTION

Amphiphilic diblock copolymers form a variety of multi-molecular assemblies (for example, polymeric micelles and polymer vesicles) in aqueous phases and are being explored for biomedical applications, including biomaterials and drug delivery.[1] Currently, the introduction of various functional moieties (for example, biorelated molecules for increasing active interactions with target cells or fluorescent units for visualization) to hydrophilic block termini is being used to provide additional unique functions to polymeric assemblies.[2–4] In addition, stimuli-responsive polymers applied to single or both blocks of diblock copolymers provide the controlled structural and property changes of polymer assemblies in response to physical and chemical signals (for example, heat, light and pH).[5–7]

In recent years, well-defined polymers have been efficiently synthesized, using the recently developed living radical polymerizations, such as the reversible addition-fragmentation chain transfer radical (RAFT) polymerization[8] and atom-transfer radical polymerization.[9] Living radical polymerizations produce polymers with controlled molecular weights that are applicable for a wide range of monomers under facile conditions. Among several living radical polymerizations, the RAFT polymerization possesses an attractive advantage for functionalizing polymer termini in biomedical applications.[10] Dithiobenzoate groups at the ω-position of RAFT-mediated polymers are sensitive to nucleophiles and are converted to thiol groups, which can be utilized for introducing biorelated molecules and other functional moieties via thiol chemistry. In addition, functional RAFT agents allow polymers to be functionalized at the α-positions. By utilizing these unique features of RAFT polymerization, our laboratory has synthesized diblock copolymers comprised of thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm)-based blocks and biodegradable blocks by combining the RAFT and ring opening polymerization (ROP) techniques.[2,11,12] During our synthetic process for block copolymers, the α-hydroxyl, ω-dithiobenzoate poly(*N*-isopropylacrylamide-*co*-*N*,*N*-dimethylacrylamide) (P(IPAAm-*co*-DMAAm)) block was prepared using hydroxylated RAFT agents. DMAAm was copolymerized with IPAAm for regulating the lower critical solution temperature of the thermoresponsive block around a physiological temperature for use in biomedical applications. Subsequently, the fabrication of biodegradable blocks, which was initiated from the α-hydroxyl groups of the thermoresponsive blocks, was achieved by the ROP of D,L-lactide. Intelligent polymeric micelles comprised of block copolymers were investigated for use in biomedical applications involving thermally modulated drug release and intracellular micellar uptake. However, this synthetic approach includes some problems associated with preparing dithiobenzoate-terminated block copolymers with various molecular weights. The deactivation of the terminal dithiobenzoate groups is caused by thermal decomposition during the ROP process using tin (II) 2-ethylhexanoate with heating at 120 °C.[12,13] In addition, polyester blocks with short chain lengths are often obtained because of the low solubility of α-hydroxylated PIPAAm derivatives, which are used as the macro-initiators in reactive solvents (for example, toluene and xylene). To solve these issues, the present study focused on the acid-based cationic ROP (CROP)[14] in conjunction with RAFT polymerization. The advantages of CROP include a fast polymerization rate at a low temperature (for example, 0 °C), free heavy metal catalysts and a controlled polymer molecular weight. Herein, the CROP-mediated synthesis of poly(ε-caprolactone) (PCL) as a macro-RAFT agent was first performed using a hydroxyl-dithiobenzoate compound. Subsequently, the thermoresponsive PIPAAm-based block was propagated using PCL with a terminal dithiobenzoate moiety, and the polymerization kinetics were

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compared with that of a low molecular weight RAFT agent. Furthermore, the terminal dithiobenzoate groups were converted to thiol groups, which were biotinylated via thiol-maleimide chemistry for preparing bioactive-terminated block copolymers comprised of thermoresponsive, biodegradable blocks.

MATERIALS AND METHODS

Materials

Dichloromethane (Wako Pure Chemicals, Osaka, Japan) and ε-caprolactone (CL; Wako Pure Chemicals) were dried over calcium hydride and distilled under reduced pressure. IPAAm, which was provided by Kojin (Tokyo, Japan), was recrystallized twice from *n*-hexane. DMAAm and *N*,*N*-dimethylformamide (DMF) were obtained from Wako Pure Chemicals and distilled under reduced pressure. Triethylamine, 2,2′-azobis[2-methyl-*N*-(hydroxyethyl)]propionamide (VA-086), 1.0 mol l^{-1} hydrochloric acid in diethyl ether, *n*-hexane and diethyl ether were purchased from Wako Pure Chemicals, and used as received. A RAFT agent, 2-[*N*-(2-hydroxyethyl)carbamoyl]prop-2-yl dithiobenzoate (HECPD), was prepared using previously reported methods with a slight modification.[15] The water used in this study was purified using a Milli-Q Synthesis A10 system (Millipore, Billerica, MA, USA) unless otherwise specified.

Synthesis of the biodegradable macro-RAFT agents

HECPD was dried under reduced pressure for 2 h to remove moisture. CL, dehydrated dichloromethane and hydrochloric acid in diethyl ether were added, and the polymerization proceeded at 0 °C for 4 h under a dry nitrogen atmosphere. The reaction was then stopped by the addition of triethylamine. Polymers were purified by repeated precipitation into excess hexane, followed by drying *in vacuo*. The number-average molecular weight (M_n) was estimated from the ultraviolet absorbance of the terminal dithiobenzoate groups at 512 nm (ε: 109.5 l mol^{-1} cm in DMF). The composition of the monomer was determined using a ^{1}H nuclear magnetic resonance (NMR) spectrometer (400 MHz, Varian Inc., Lake Forest, CA, USA) with chloroform-*d* (Sigma-Aldrich, St Louis, MO, USA). The polydispersity indexes (PDIs) of the polymers were determined using a gel permeation chromatography (GPC) instrument (SC-8020; Tosoh, Tokyo, Japan) with two-connected columns (TSKgel-G3000H HR and TSKgel-G4000H HR columns; Tosoh) that were calibrated with polystyrene standards. All GPC measurements were performed at a flow rate of 1.0 ml min^{-1} at 45 °C using DMF containing 10 mmol l^{-1} LiCl (Wako Pure Chemicals) as an eluent, unless otherwise noted.

Synthesis of the amphiphilic diblock copolymers

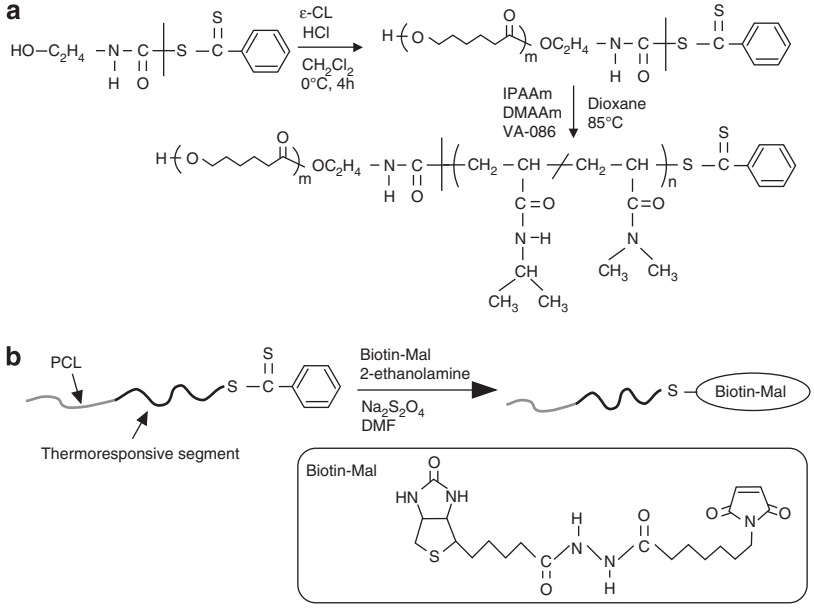
IPAAm and DMAAm were copolymerized using a macro-RAFT agent. IPAAm (2.93 mol l^{-1}), DMAAm (1.57 mol l^{-1}), PCL as the macro-RAFT agent (M_n: 2800, 15.0 mmol l^{-1}), VA-086 (3 mmol l^{-1}) and 1,3,5-trioxane (one fifteenth mol equivalent to the total monomers; Sigma-Aldrich) as an internal reference for ^{1}H NMR measurements were dissolved in DMF. The solution was degassed under reduced pressure by three freeze–pump–thaw cycles, and polymerization was performed at 85 °C for 18 h. The polymers were purified by repeated precipitation in excess diethyl ether, followed by thoroughly drying under vacuum. The composition of the diblock copolymer was determined from the ^{1}H NMR spectrum in DMSO-d_6 (Sigma-Aldrich) from PCL of methylene ($-OCH_2-$, 4.0 p.p.m.), IPAAm of methine (3.9 p.p.m.) and DMAAm of methyl (2.9 p.p.m.). The M_n and PDI were calculated from the GPC profile that was calibrated with poly(ethylene glycol) (PEG) standards.

Kinetic analysis of RAFT polymerization

IPAAm and DMAAm were copolymerized using HECPD or a macro-RAFT agent. IPAAm (2.93 mol l^{-1}), DMAAm (1.57 mol l^{-1}), macro-RAFT agent (M_n: 2800, 15.0 mmol l^{-1}), VA-086 (3 mmol l^{-1}) and 1,3,5-trioxane (one fifteenth mol equivalent to the total monomers) were dissolved in DMF. The solutions were degassed under reduced pressure by three freeze–pump–thaw cycles; the samples were then divided to vials (1 ml) and polymerization was performed at 85 °C under a nitrogen atmosphere. After polymerizing for specific periods of time, the polymerization was stopped by cooling and stirring under air. The conversion was determined by the ^{1}H NMR signals observed from the vinylic protons (IPAAm: 5.5 p.p.m. and DMAAm: 5.6 p.p.m.) and 1,3,5-trioxane (5.1 p.p.m.) in DMSO-d_6. The M_n and PDI were determined using GPC, which was calibrated using PEG standards.

Aminolysis and terminal biotinylation of block copolymers

Block copolymers (64 mg) and *N*-biotinoyl-*N*′-(6-maleimidohexanoyl)hydrazide (Biotin-Mal; Sigma-Aldrich) (22.6 mg, 10 mol equivalents to the polymer terminal dithiobenzoate groups) were dissolved in 6 ml DMF that was deoxidized by nitrogen gas bubbling; sodium hydrosulfite (0.1 mg) was then added to the reaction solution. Then, 2-hydroxyethylamine (10 mol equivalents to the dithiobenzoate groups) in deoxidized DMF (1 ml) was added dropwise to the polymer solutions; the reaction then proceeded at room temperature for 20 h in a nitrogen atmosphere (Scheme 1(b)). After the reaction, the polymer solutions were dialyzed using a dialysis membrane (MWCO 1000, Spectra/Por 6; Spectrum Medical Industries, Los Angeles, CA, USA) against a water/



Scheme 1 (**a**) Synthetic scheme of the macro-RAFT agents and subsequent diblock copolymers. (**b**) Terminal aminolysis and biotinylation of the block copolymer terminus.

methanol/DMF (1/1/1) mixture solvent for 3 days, followed by dialysis against water for 2 days. The polymers were recovered by freeze-drying. The composition of the diblock copolymers was calculated from the ^{1}H NMR spectrum in DMF-d_6 (Sigma-Aldrich). The M_n and PDI were determined using GPC, which was calibrated using PEG standards.

Preparation and characterization of the polymeric micelles

Diblock copolymers (20 mg) were dissolved in 4 ml of *N,N*-dimethylacetoamide (Wako Pure Chemical) and then dialyzed against water using a dialysis membrane (MWCO 1000, Spectra/Por 6) for 16 h. The hydrodynamic diameter of the polymeric micelles in Dulbecco's phosphate saline without calcium chloride and magnesium chloride (Sigma-Aldrich) was determined by dynamic light scattering using a Nano-ZS instrument (Malvern Instruments, Worcestershire, UK) equipped with a He-Ne laser (633 nm) at a scattering angle of 173°. The optical transmittance of the polymeric micelles (5.0 mg ml^{-1}) in Dulbecco's phosphate saline without calcium chloride and magnesium chloride at various temperatures was measured at 600 nm with a UV–vis spectrophotometer (V-530; JASCO, Tokyo, Japan) equipped with a sample cell thermostat (EHC-477S; JASCO). The heating rate was 0.1 °C min^{-1}.

RESULTS AND DISCUSSION

Biodegradability is one of the interesting properties regarding the *in vivo* use of biomaterials.[16] The biodegradable properties of polymers are promising for more effective and safer clinical applications, especially in applications for drug carrier systems, because of the acceleration of renal excretion from living body systems by decomposing the polymeric drug carriers. For this purpose, PCL with a controlled molecular weight for use as biodegradable macro-RAFT agents was prepared by CROP using a hydroxylated RAFT agent (HECPD) as an initiator and HCl as a catalyst (Scheme 1). A series of 4-h polymerization procedures resulted in relatively high yields of PCL (the initial feed ratios of CL to initiator ([CL]/[initiator]; 10, 66%; 20, 91%; 30, 99%), and the obtained polymers were easily purified by a repeated precipitation process in *n*-hexane. The biodegradable macro-RAFT agents were characterized using ^{1}H NMR, ultraviolet spectrometry and GPC. The ^{1}H NMR spectrum of the macro-RAFT agent (in $CDCl_3$) is shown in Figure 1a, and the peaks derived from the terminal dithiobenzoate groups and the PCL backbones were easily assigned. PCL dissolved in DMF showed an n-π^* absorption band at 514 nm, which corresponded to terminal dithiobenzoate groups. Therefore, the M_n of PCL was estimated by an end-group analysis using the molar extinction coefficient of terminal dithiobenzoate. From the characterization of PCL, their molecular weights could be regulated by arranging the initial feed ratios of [CL]/[initiator] and were found to be comparable to the theoretical values. Furthermore, the macro-RAFT agents demonstrated unimodal GPC profiles with low PDIs (less than 1.10). Therefore, under the CROP process, the polymerization of CL was confirmed to initiate from HECPD and proceed effectively without decomposing the PCL backbones and dithiobenzoate-based RAFT agents by the addition of acid as the catalyst.

Diblock copolymers were synthesized from the RAFT-mediated random copolymerization of IPAAm and DMAAm using the biodegradable macro-RAFT agents. The terminal activity of the RAFT-mediated dithiobenzoate groups was confirmed by the ^{1}H NMR spectra in $CDCl_3$. The peaks derived from the dithiobenzoate groups were clearly assigned at 7.3–7.9 p.p.m. The monomer compositions of IPAAm, DMAAm and CL of the block copolymers were also calculated from the relative proton intensities of methine (IPAAm, 3.9 p.p.m.), methyl (DMAAm, 3.4 p.p.m.) and methylene (CL, 4.0 p.p.m.) in DMSO-d_6, respectively. For investigating the controlled radical process, the polymerization kinetics with macro-RAFT agents

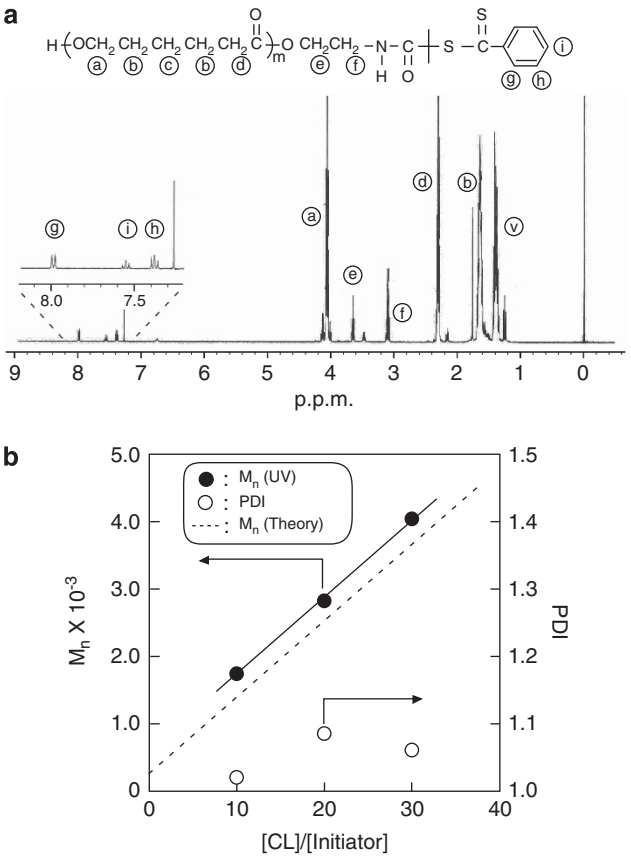


Figure 1 (**a**) ^{1}H NMR spectrum of PCL (M_n: 4000) in $CDCl_3$. (**b**) M_n and PDI against the initial feed molar ratio of CL to initiator. [CL] and [Initiator] stand for the initial concentration of CL and initiator, respectively. The dashed line represents the theoretical line.

were compared with that of HECPD (Figure 2). The monomer conversions were estimated from the monomer consumptions of vinylic protons corresponding to IPAAm (5.5 p.p.m.) and DMAAm (5.6 p.p.m.) by comparison with the peak of 1,3,5-trioxane (5.1 p.p.m.) as the internal reference. The relationships between the polymerization time and conversion, as well as the first-order kinetic plots of the RAFT copolymerization were investigated using the PCL-based macro-RAFT agent compared with HECPD (low-molecular RAFT agent; Figures 2a and b). The copolymerization of IPAAm and DMAAm in the presence of HECPD or the macro-RAFT agent was observed with a slight initial retardation (within 30 min), which commonly appeared in the RAFT polymerization process using dithiobenzoate-RAFT agents.[17] During the initial stage of polymerization, within 4 h, the monomer consumption rate for the polymerization using the macro-RAFT agent was comparable to that of HECPD. However, the polymerization rate in the presence of the macro-RAFT agent became slower than that of HECPD during the later stage. This decelerated polymerization rate was speculated to be caused by the increase in the viscosity of reaction solution, and thus, the monomer diffusion in the solution was reduced. The first-order kinetic plots were relatively linear at a conversion of less than 40%, indicating that the monomers and their concentrations of active species constantly remained. From the GPC results, the polymerization was well regulated at a conversion less than 50% (PDI < 1.1), whereas the PDIs increased with increasing monomer conversion. Figures 2c and d shows the relationship between the M_n determined by GPC calibrated with PEG standards and the monomer

conversion. The M_n determined by GPC calibrated with PEG standards showed relatively comparable values to those determined from ^{1}H NMR analysis and the end-group using the molar extinction coefficient of terminal dithiobenzoate.[2] The theoretical M_n ($M_{n(theo)}$) was calculated from the following equation:

$$M_{n(theo)} = \frac{[M]_0}{[HECPD]}(M_{IPAAm}\chi_{IPAAm} + M_{DMAAm}\chi_{DMAAm}) + M_{HECPD} \quad (1)$$

where $[M]_0$ and [HECPD] are the concentrations of the initial monomers and HECPD, respectively. M_{IPAAm}, M_{DMAAm} and M_{HECPD} are the molecular weights for individual compounds, and χ_{IPAAm} and χ_{DMAAm} are monomer conversions for each monomer. M_n linearly increased with increasing monomer conversion and was consistent with the theoretical line, indicating that controlled behavior was maintained during the copolymerization. In addition, the monomer reactivity ratios of IPAAm (r_1) and DMAAm (r_2) determined using the Mayo–Lewis methods[18] were 0.67 and 0.72, respectively (Table 1). Therefore, the consumption of DMAAm was greater than that of IPAAm in the copolymerization during the RAFT polymerization.

The terminal conversion of the obtained block copolymer was demonstrated by aminolysis and the subsequent thiol-maleimide coupling reaction (Scheme 1 (B)). The polymer termini were substituted with the biotin-related maleimide derivative for installing biomolecules using biotin–avidin chemistry. Biotin is known to strongly bind to avidin with an extremely high affinity (dissociation constant (K_d): 10^{-15} M). Therefore, biomolecules can be installed on the polymer termini using the essentially irreversible avidin–biotin interaction.[19] Terminal conversion was confirmed from the ^{1}H NMR and GPC analyses. The progression of the terminal aminolysis was confirmed by the disappearance of ^{1}H NMR signals corresponding to the dithiobenzoate moieties (7.3–8.0 p.p.m.). The methine and amine proton signals derived from the Biotin-Mal were assigned to be 4.1 and 6.40–6.45 p.p.m., respectively. The terminal conversion was estimated from the relative proton intensities of methine (IPAAm, 3.9 p.p.m.) with methine (Biotin-Mal, 4.1 p.p.m.) and the conversion efficiency was determined to be greater than 90% (Figure 3). From the GPC analysis, the biotin-Mal-terminated block copolymers (M_n: 11 400, PDI: 1.16) showed comparable GPC profiles to the pre-reacted block copolymers (M_n: 10 600, PDI: 1.17). From these results,

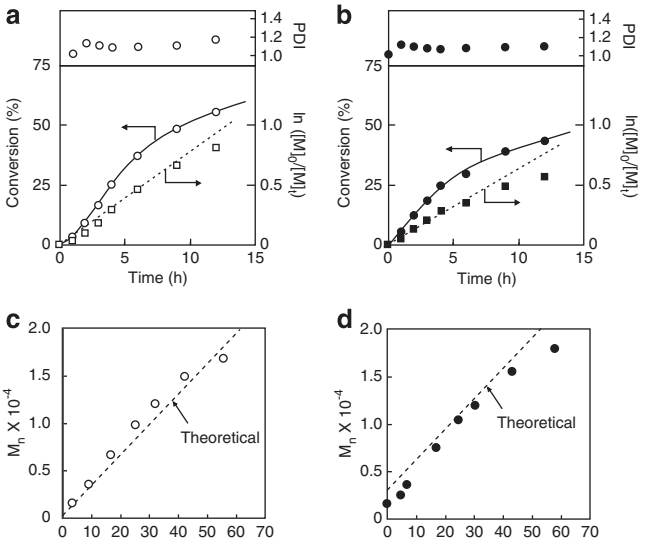


Figure 2 Time-conversion, first-order kinetic plots and polydispersity indexes after copolymerization of IPAAm and DMAAm in the presence of (**a**) HECPD and (**b**) macro-RAFT agents ($M_{n(PCL)}$: 2800). $[M]_0$ and $[M]_t$ are the total monomer concentrations of polymerization time at 0 h and a specific time, respectively. Dependence of M_n on monomer conversion in the copolymerization of IPAAm and DMAAm using (**c**) HECPD and (**d**) macro-RAFT agent ($M_{n(PCL)}$: 2800). M_n was determined using GPC calibrated with PEG standards. The dashed lines represent the theoretical lines.

Table 1 Monomer compositions of IPAAm and DMAAm in feed solution and the obtained copolymers

Feed composition (mol%)		*Copolymer composition (mol%)*		
IPAAm	*DMAAm*	*IPAAm*	*DMAAm*	*Total monomer conversion (%)*
67.7	32.3	64.1	35.9	4.9
68.6	31.4	65.0	35.0	7.0
69.5	30.5	65.7	34.3	18.6

Abbreviations: DMAAm, *N,N*-dimethylacrylamide ; IPAAm, *N*-isopropylacrylamide.

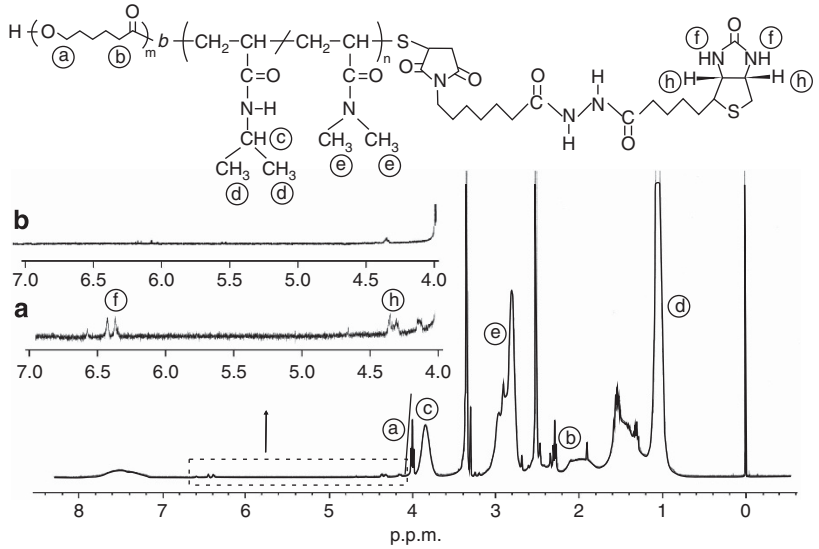


Figure 3 ^{1}H NMR spectra of the biotinylated block copolymers possessing terminal groups of (**a**) dithiobenzoate and (**b**) biotin in DMF-d_6.

the terminal conversion was speculated to proceed effectively without significant side reactions. The multi-assemblies of the obtained diblock copolymers resulted in the formation of polymeric micelles that possessed thermoresponsive coronas and biodegradable cores in aqueous media. The properties of the polymeric micelles were investigated using dynamic light scattering measurements and turbidity changes at various temperatures. From the dynamic light scattering study, the micelles showed a monodisperse distribution with a weight-averaged diameter of 17.3 nm at 25 °C. The micellar solution was highly transparent at a temperature below the lower critical solution temperature of the corona-forming PIPAAm derivatives and abruptly clouded upon heating to a temperature of 43.5 °C. The micellar lower critical solution temperature was successfully adjusted to that of body temperature (37 °C) by introducing the DMAAm comonomer into the PIPAAm main chain. In addition, the micelles contained the biodegradable PCL-based cores. PCL was able to be decomposed by hydrolysis in response to environmental pH and enzymes in an *in vivo* situation.[12,20] Therefore, the degradation rate of the polymeric micelles can be controlled for releasing incorporated drugs with an effective excretion of residual polymers from living systems.

CONCLUSIONS

In summary, diblock copolymers with controlled molecular weights that possessed thermoresponsive and biodegradable blocks were successfully synthesized by the combination of RAFT polymerization and CROP. CROP is available for various cycloesters and cyclocarbonates, and allows well-defined biodegradable macro-RAFT agents to be fabricated using hydroxylated dithiobenzoate compounds as initiators. By using the biodegradable macro-RAFT agent, the propagation of the thermoresponsive block was achieved while preserving the terminal dithiobenzoate functionality. In addition, terminal dithiobenzoate groups were able to be effectively converted to thiol groups by aminolysis, which was utilized for introducing various bioactive compounds at the terminal of the diblock copolymers. This is a promising method for preparing block copolymer-based multifunctional biomaterials.

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