Facile Fabrication of Reversible Core Cross-Linked Micelles Possessing Thermosensitive Swellability

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ABSTRACT: Poly(ethylene oxide)-*b*-poly(*N*-isopropylacrylamide-*co*-*N*-acryloxysuccinimide), PEO-*b*-P(NIPAM-*co*-NAS), was synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization in dioxane at 70 °C employing a poly(ethylene oxide) (PEO)-based macroRAFT agent. The obtained double hydrophilic block copolymer molecularly dissolves in aqueous solution at room temperature. Above the lower critical solution temperature (LCST) of P(NIPAM-*co*-NAS) block, it self-assembles into micelles consisting of thermoresponsive P(NIPAM-*co*-NAS) cores and well-solvated PEO coronas. Cross-linking of the P(NIPAM-*co*-NAS) cores was facilely achieved via the reaction of NAS residues with cystamine at elevated temperatures in aqueous media, forming structurally permanent core cross-linked micelles. Most importantly, the disulfide bonds within the cross-linker can be conveniently cleaved in the presence of dithiothreitol and re-formed again upon addition of cystamine as a thiol/disulfide exchange promoter, leading to the reversible core cross-linking of micelles. The P(NIPAM-*co*-NAS) cores of the obtained core cross-linked (CCL) micelles exhibit tunable swelling/deswelling behavior below and above the critical phase transition temperature. Dynamic laser light scattering, optical transmittance, ^{1}H NMR, and transmission electron microscopy were used in combination to investigate the thermoresponsive micellization of PEO-*b*-P(NIPAM-*co*-NAS) and the subsequent reversible core cross-linking.

Introduction

Double hydrophilic block copolymers (DHBCs) can play an important role in diverse fields such as drug delivery, interface mediators, biosensing, soft actuators/valves, and catalysis.[1−6] Under a proper combination of external stimuli such as pH, temperature, and ionic strengths, one of the blocks of DHBCs can be rendered insoluble, while the other block still remains solvated; these copolymers then supramolecularly self-assemble in aqueous solution into colloidal aggregates with various morphologies such as micelles or vesicles, starting from the molecularly soluble state.[7−20]

Past studies of DHBCs containing pH- or thermoresponsive blocks have focused on the characterization of equilibrium structures of the self-assembled aggregates. Recent developments include investigations concerning the kinetic aspect, that is, the process of formation, disintegration, and structural inversion of DHBC micelles.[21−23] It has been established that the pH- or thermoresponsive unimer-to-micelle transition typically completes within seconds, whereas for the reverse process, micelle disintegration upon dilution or pH alterations occurs even faster, the time scale of which is typically on the order of several tens of milliseconds. Thus, in practical applications of DHBCs, the stability of the formed micelles will be an important issue, considering that the stimuli-responsive self-assembly process is fully reversible in most cases.

To account for the stability issue of block copolymer micelles, two strategies, that is, core cross-linking (CCL)[24−34] and shell cross-linking (SCL),[17,35−41] have been developed, leading to structurally stable nanostructures. The CCL method was originally reported by Liu et al.,[30−32] in which 2-cinnamoylethyl methacrylate residues were selectively incorporated into the core-forming block. For example, polystyrene-*b*-poly(2-cinnamoylethyl methacrylate) (PS-*b*-PCEMA) diblock copolymer formed PCEMA-core starlike or crewcut micelles in THF/cyclohexane mixtures. Upon UV irradiation, photo cross-linking of the micelle cores can be successfully achieved.[32] Kataoka et al.[29] synthesized poly(ethylene oxide)-*b*-poly(lactic acid) (PEO-*b*-PLA) possessing a methacryloyl group at the PLA chain end. In aqueous solution, this diblock copolymer self-assembles into PLA-core micelles, and the polymerization within the micelle cores leads to the facile preparation of CCL micelles. For block copolymers in a common organic solvent, selective chemical reaction of one of the blocks with a difunctional reagent also leads to highly efficient preparation of CCL micelles.[33,42,43] Chen et al.[44,45] utilized the sol−gel chemistry to fix the micellar and vesicular morphologies assembled from poly(3-(trimethoxysilyl)propyl methacrylate)-containing amphiphilic block copolymers. Just recently, Wooley reported the preparation of CCL micelles of polystyrene-*b*-poly(acrylic acid) (PS-*b*-PAA). The hydrophobic PS block contains alkynyl residues; the "click" core cross-linking was then achieved upon addition of dendrimers of different generations, the periphery of which was functionalized with azide groups.[34] In most of the above cases, a cosolvent approach has been typically employed to actuate the self-assembly process; moreover, the cross-linked core was water-insoluble and did not exhibit any stimuli-responsiveness.

The SCL approach was originally reported by Wooley et al.[46,47] for polystyrene-*b*-poly(4-vinyl pyridine). Armes et al.[48,49] further applied this strategy to the field of DHBC micelles. Typically, a stimuli-responsive triblock copolymer was em-

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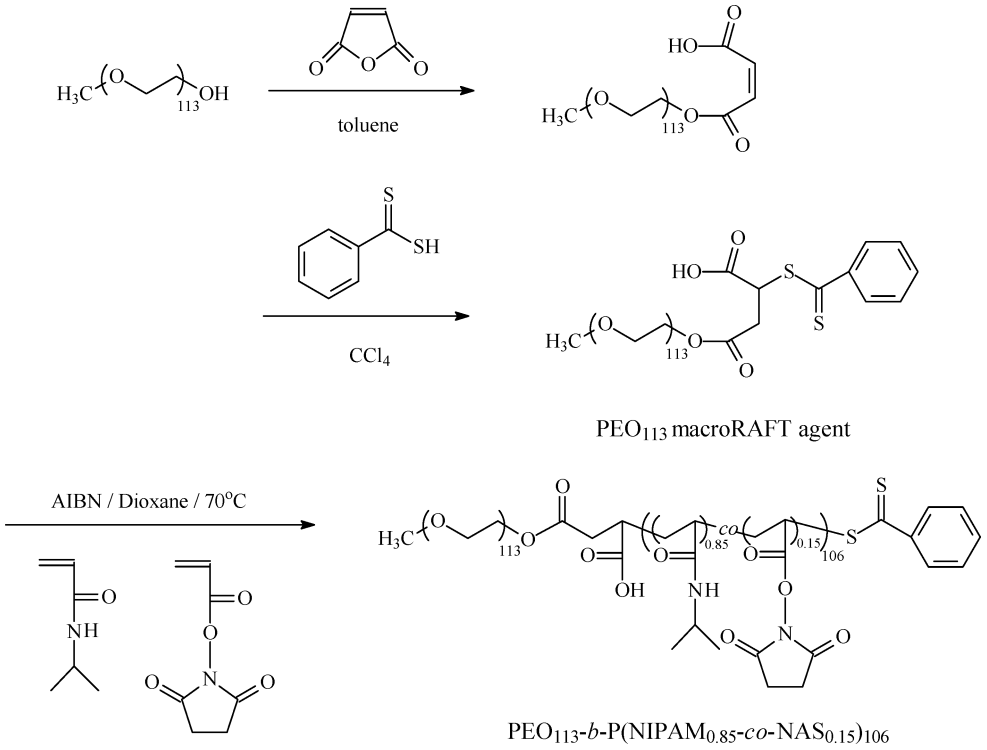
Scheme 1. Reaction Scheme for the Preparation of PEO_{113}-*b*-P($NIPAM_{0.85}$-*co*-$NAS_{0.15}$)$_{106}$ Diblock Copolymer



ployed, and shell cross-linking can be conducted at relatively high concentrations due to steric stabilization of the well-solvated outer corona and consequently the avoidance of intermicellar cross-linking. A more recent stimulating work concerning shell cross-linking of DHBC micelles was reported by McCormick and co-workers.[50,51] They synthesized a novel triblock copolymer, poly(ethylene oxide)-*b*-poly(*N*-dimethylacrylamide-*co*-*N*-acryloxysuccinimide)-*b*-poly(*N*-isopropylacrylamide) (PEO-*b*-P(DMA-*co*-NAS)-*b*-PNIPAM). As the middle block contains reactive NAS residues, the formed PNIPAM-core micelles at elevated temperatures can be facilely shell cross-linked upon addition of cystamine, a disulfide-containing difunctional primary amine. Most importantly, reversible shell cross-linking can be achieved due to the presence of disulfide bonds within the cross-linker, the formation and cleavage of which can be fully controlled.[50]

Recently, Pitt et al.[33] successfully prepared CCL micelles in aqueous media utilizing the Ce^{4+}-initiated copolymerization of NIPAM and a disulfide-containing difunctional monomer, *N*,*N*-bis(acryloyl)cystamine, employing PEO monomethyl ether as the initiator. Model hydrophobic drugs can then be loaded into the lightly cross-linked PNIPAM core, and the controlled release of them can be facilely achieved upon addition of excess β-mercaptoethanol, which can disintegrate the cross-linking sites within nanoparticle cores. It should be noted the obtained nanoparticles generally possess a relatively broad distribution of particle sizes. Moreover, due to different rates of free-radical polymerization between mono- and difunctional monomers, the cross-linking densities may vary considerably from the inner to the outer part of nanoparticle cores, which will probably lead to nonuniform release of guest molecules.

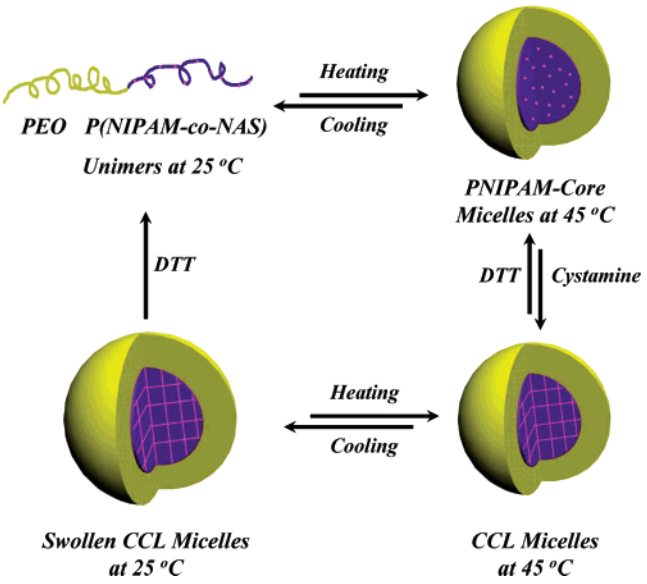
Following the promising reversible cross-linking chemistry as demonstrated by McCormick et al.[50,51] and Pitt et al.,[33] we further fabricated reversible CCL micelles starting from a NAS-containing double hydrophilic diblock copolymer. As compared to triblock copolymers, the synthesis of diblock copolymers by "living" free radical polymerization techniques, for example, atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer (RAFT) polymerization, should be more straightforward and well-controlled. Moreover, the core of the resulting CCL micelles can be designed to possess stimuli-responsiveness, and this provides additionally tunable parameters for the controlled release of encapsulated guest molecules.

Herein, we synthesized poly(ethylene oxide)-*b*-poly(*N*-isopropylacrylamide-*co*-*N*-acryloxysuccinimide), PEO-*b*-P(NIPAM-*co*-NAS), via RAFT polymerization starting from a PEO-based chain transfer agent (Scheme 1). The obtained double hydrophilic diblock copolymer molecularly dissolves in aqueous solution at room temperature and supramolecularly self-assembles into P(NIPAM-*co*-NAS)-core micelles at elevated temperatures due to the well-known LCST phase behavior of PNIPAM block.[52−54] As the micelle cores contain reactive NAS residues, CCL micelles can be facilely fabricated upon reaction with a difunctional primary amine, cystamine (Scheme 2). We successfully demonstrated that the core cross-linking could be fully reversible, via the controllable formation and cleavage of disulfide bonds within the cross-linker. The cross-linked P(NIPAM-*co*-NAS) cores of the prepared CCL micelles also exhibit thermosensitive swelling/deswelling behavior. As far as we know, this represents the first report of reversible CCL micelles with thermoresponsive cores starting from a double hydrophilic block copolymer.

Experimental Section

Materials. *N*-Isopropylacrylamide (NIPAM) (97%, Tokyo Kasei Kagyo Co.) was purified by recrystallization from a mixture of benzene and *n*-hexane (1/3, v/v). Monohydroxy-capped poly(ethylene oxide) (PEO_{113}−OH, $M_n = 5000$, $M_w/M_n = 1.06$, mean degree of polymerization, DP, is 113) was purchased from Aldrich and used as received. 2,2′-Azobisisobutyronitrile (AIBN, 98%, Fluka) was recrystallized from 95% ethanol. Maleic anhydride (MAh) was sublimed at 50 °C at reduced pressure and then stored at −20 °C. *N*-Hydroxysuccinimide (NHS, 97%, Aldrich) was recrystallized from toluene prior to use. *N*-Acryloxysuccinimide

Scheme 2. Schematic Illustration of the Fabrication of Reversible CCL Micelles from PEO_{113}-*b*-P($NIPAM_{0.85}$-*co*-$NAS_{0.15}$)$_{106}$ Diblock Copolymer



(NAS)[55] and dithiobenzoic acid (DTBA)[56] were prepared according to literature procedures. All other chemicals were purchased from Shanghai Chemical Reagent Co. and used as received.

Synthesis of PEO MacroRAFT Agent. Esterification of PEO with MAh. PEO_{113}−OH (16.0 g, 3.2 mmol) was dissolved in 30 mL of dry toluene, MAh (1.96 g, 20.0 mmol) was then added, and the reaction mixture was stirred at 60 °C overnight. After toluene was removed, the residues were dissolved in CH_2Cl_2 and precipitated into an excess of *n*-hexane to remove unreacted MAh. This purification cycle was repeated three times. The obtained PEO−MAh was dried in a vacuum oven at 45 °C overnight with a yield of ∼85%. ^{1}H NMR ($CDCl_3$, δ): 6.2−6.4 (2H, −C*H*═C*H*COOH), 4.3 (2H, −CH_2OOCH═CHCOOH), 3.6 (450H, OCH_2CH_2O).

Synthesis of PEO MacroRAFT Agent. The addition reaction of DTBA with PEO−MAh leads to the facile preparation of PEO-based macroRAFT agent. PEO−MAh (5.1 g, 1.0 mmol) and DTBA (3.1 g, 20.0 mmol) were charged into a glass ampule containing 20 mL of CCl_4. The mixture was degassed through three freeze−thaw cycles. The ampule was then flame sealed under vacuum and kept in an oil bath preheated at 65 °C for 24 h. After being cooled to room temperature, the reaction mixture was precipitated into diethyl ether three times to remove the unreacted DTBA. The obtained PEO macroRAFT agent was dried in a vacuum oven at room temperature for 24 h with a yield of ∼92%.

Synthesis of PEO-*b*-P(NIPAM-*co*-NAS) Diblock Copolymer. PEO-*b*-P(NIPAM-*co*-NAS) was prepared by the statistical RAFT copolymerization of NIPAM and NAS employing PEO macroRAFT agent. In a typical run, PEO macroRAFT agent (0.26 g, 0.05 mmol), NIPAM (1.13 g, 10 mmol), NAS (0.19 g, 1.1 mmol), and AIBN (2.0 mg, 0.01 mmol) were charged into a glass ampule containing 4 mL of dioxane. The mixture was degassed through three freeze−thaw cycles. The ampule was then flame sealed under vacuum and kept in an oil bath preheated at 70 °C to conduct the polymerization. After 24 h, the ampule was quenched into liquid nitrogen to stop the polymerization. The reaction mixture was diluted with THF and precipitated into an excess of cold diethyl ether. This purification cycle was repeated twice. After being dried in a vacuum oven overnight at room temperature, slightly pink solids were obtained with a yield of ∼59%. The molecular weight and molecular weight distribution of PEO-*b*-P(NIPAM-*co*-NAS) diblock copolymer were determined by GPC using THF as eluent: M_n = 23 500, M_w/M_n = 1.12. The overall DP of the P(NIPAM-*co*-NAS) block and its NAS content were determined to be 106 and 15 mol %, respectively, by ^{1}H NMR analysis in $CDCl_3$. The obtained diblock copolymer was denoted PEO_{113}-*b*-P($NIPAM_{0.85}$-*co*-$NAS_{0.15}$)$_{106}$.

Thermoresponsive Micellization and Preparation of CCL Micelles. PEO_{113}-*b*-P($NIPAM_{0.85}$-*co*-$NAS_{0.15}$)$_{106}$ was dissolved in water at a concentration of 15.0 g/L (25 °C). The aqueous solution (30 mL) was then heated to 40 °C. A bluish tinge characteristic of colloidal aggregates appeared immediately upon heating, indicating the formation of micelles. After equilibration for 10 min at 40 °C, 6.1 mL of an aqueous solution of cystamine (5.0 g/L, pH 9.0) preheated to 40 °C was injected. The cystamine/NAS molar ratio was kept constant at 1:2. The mixture was stirred at 40 °C for 5 h. After the mixture was cooled to 25 °C, the bluish tinge characteristic of micelles persisted, suggesting successful core cross-linking. The aqueous solution of CCL micelles was further diluted for subsequent transmittance, LLS, and TEM studies.

Reversible Disintegration and Re-formation of CCL Micelles. The disintegration of CCL micelles was facilitated with the addition of DTT, which can readily cleave disulfide bonds of the cross-linker. DTT (0.28 g) was added to 3.0 mL of an aqueous solution of the above-prepared CCL micelles in a 5 mL vial; after it was degassed with nitrogen bubbling for 3 min, the vial was sealed with a rubber septum and placed in a water bath at 40 °C for 8 h to cleave the CCL micelles.

After cleavage of CCL micelles with DTT, the solution was dialyzed against deionized water at room temperature for 3 days to remove DTT and other byproducts. Upon reheating to 40 °C, micellization occurred as evidenced by the appearance of a bluish tinge. Cystamine (30 mg) was added, and the solution mixture was maintained at 40 °C for 6 h to conduct the core cross-linking.

Characterization. Nuclear Magnetic Resonance (NMR) Spectroscopy. All ^{1}H NMR spectra were performed in D_2O or $CDCl_3$ on a Bruker AV300 NMR spectrometer (resonance frequency of 300 MHz for ^{1}H) operating in the Fourier transform mode.

Gel Permeation Chromatography (GPC). Molecular weights and molecular weight distributions were determined by gel permeation chromatography (GPC) equipped with a Waters 1515 pump and a Waters 2414 differential refractive index detector (set at 30 °C). It used a series of three linear Styragel columns HT2, HT4, and HT5 at an oven temperature of 45 °C. The eluent was THF at a flow rate of 1.0 mL/min. A series of low polydispersity PEO standards were employed for the GPC calibration.

Laser Light Scattering (LLS). A commercial spectrometer (ALV/DLS/SLS-5022F) equipped with a multi-tau digital time correlator (ALV5000) and a cylindrical 22 mW UNIPHASE He−Ne laser (λ_0 = 632 nm) as the light source was employed for dynamic LLS measurements. Scattered light was collected at a fixed angle of 90° for duration of ∼10 min. Distribution averages and particle size distributions were computed using cumulants analysis and CONTIN routines. All data were averaged over three measurements.

Transmittance Measurements. The transmittance of the aqueous solution was acquired on a Unico UV/vis 2802PCS spectrophotometer and measured at a wavelength of 500 nm using a thermostatically controlled couvette.

Transmission Electron Microscopy (TEM). TEM observations were conducted on a Philips CM 120 electron microscope at an acceleration voltage of 100 kV. Samples for TEM observations were prepared by placing 10 μL of a micellar solution at a concentration of 0.1 g/L on copper grids coated with thin films of Formvar and carbon successively. No staining was required.

Results and Discussion

Syntheses of PEO MacroRAFT Agent and PEO-*b*-P(NIPAM-*co*-NAS). The general approach employed for the preparation of PEO macroRAFT agent and PEO-*b*-P(NIPAM-*co*-NAS) diblock copolymer was shown in Scheme 1. The target block copolymer was synthesized by statistical RAFT copolymerization of NIPAM and NAS monomers using PEO-based chain transfer agent.

PEO-based macroRAFT agent was synthesized via the esterification of the terminal hydroxyl group of PEO_{113}−OH with MAh, followed by the addition reaction of DTBA with

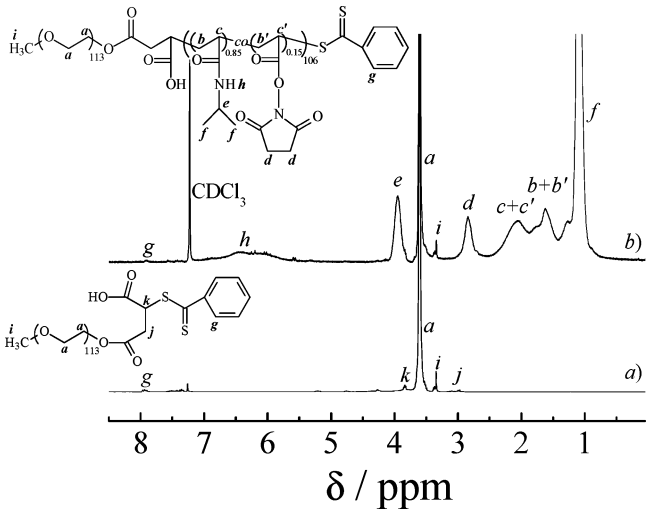


Figure 1. ^{1}H NMR spectra of (a) PEO macroRAFT agent and (b) PEO_{113}-*b*-$P(NIPAM_{0.85}$-*co*-$NAS_{0.15})_{106}$ diblock copolymer in $CDCl_3$.

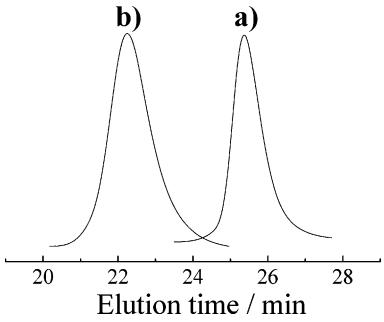
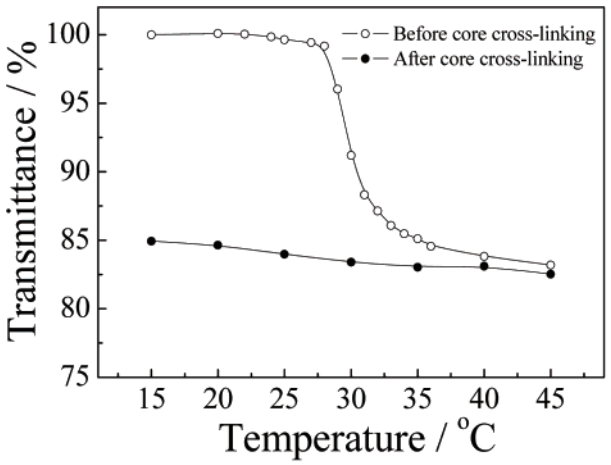


Figure 2. GPC traces of (a) PEO macroRAFT agent and (b) PEO_{113}-*b*-$P(NIPAM_{0.85}$-*co*-$NAS_{0.15})_{106}$ diblock copolymer.

PEO−MAh (Scheme 1).[57] Figure 1a shows the ^{1}H NMR spectrum of PEO macroRAFT agent in $CDCl_3$. The signal at $\delta = 3.6$ ppm (*a*) is ascribed to methylene protons of PEO. Signals at $\delta = 7.9$ (*g*, 2H), 3.8 (*k*), and 3.0 ppm (*j*) are attributed to aromatic protons of dithiobenzoate, methine proton, and methylene protons neighboring to the thiocarbonyl thio moiety. The integral ratio of peaks *g* to *k* is determined to be ~2:1, indicating a quantitative end group transformation. GPC analysis of PEO macroRAFT agent in THF reveals a monomodal peak with an M_n of 5200 and a polydispersity, M_w/M_n, of 1.10 (Figure 2).

The RAFT polymerization of NIPAM monomer has been well-documented.[58,59] For the NAS monomer, although its RAFT homopolymerization is relatively less controlled,[60−62] it can be copolymerized with other (meth)acrylate or acrylamido monomers via the RAFT method in a controlled manner.[51,63,64] Thus, the statistical RAFT copolymerization of NIPAM and NAS using dithiobenzoate-terminated PEO as the chain transfer agent should lead to a well-defined PEO-*b*-P(NIPAM-*co*-NAS) diblock copolymer.

The ^{1}H NMR spectrum of the obtained diblock copolymer in $CDCl_3$ is shown in Figure 1b. Besides signals characteristic of the PEO block, the ^{1}H NMR spectrum also reveals the presence of characteristic signals of NIPAM residues at $\delta = 5.8-7.0$ (*h*), 4.0 (*e*), and 1.1 ppm (*f*), and NAS residues at $\delta = 2.8$ (*d*) ppm. Most importantly, GPC traces in Figure 2 clearly show that the elution peak shifts to higher molecular weight after the RAFT copolymerization of NIPAM and NAS. The diblock copolymer elution peak is relatively symmetric and shows no discernible tailing at the lower molecular weight side, confirming a complete consumption of macroRAFT agent. The



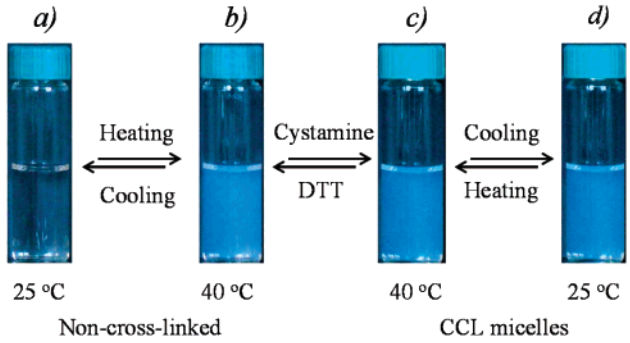


Figure 3. Temperature-dependent optical transmittance obtained for 1.0 wt % aqueous solution of PEO_{113}-*b*-$P(NIPAM_{0.85}$-*co*-$NAS_{0.15})_{106}$ diblock copolymer micelles before and after core cross-linking. Also shown are digital photographs of aqueous solutions of PEO_{113}-*b*-$P(NIPAM_{0.85}$-*co*-$NAS_{0.15})_{106}$ diblock copolymer: (a) non-cross-linked, 25 °C; (b) non-cross-linked, 40 °C; (c) CCL micelles, 40 °C; and (d) CCL micelles, 25 °C.

NAS content and the overall DP of P(NIPAM-*co*-NAS) block are determined to be 15 mol % and 106, respectively, by ^{1}H NMR. Thus, the obtained diblock copolymer is denoted as PEO_{113}-*b*-$P(NIPAM_{0.85}$-*co*-$NAS_{0.15})_{106}$. Its molecular weight and molecular weight distribution are characterized by GPC analysis in THF: $M_n = 23\,500$, $M_w/M_n = 1.12$.

Thermoresponsive Micellization. PEO has been extensively used as the hydrophilic block due to its excellent biocompatibility and nontoxicity. PNIPAM homopolymer dissolves in cold and dilute aqueous solution but gets insoluble above ~32 °C due to its well-known LCST phase behavior. Several research groups reported the thermoresponsive micellization behavior of PEO-*b*-PNIPAM, forming PNIPAM-core micelles stabilized by PEO chains in the corona.[65−69] To achieve the feasibility of reversible core cross-linking, NAS residues need to be incorporated into the PNIPAM block; however, the NAS monomer and its homopolymer are basically hydrophobic. Based on previous reports of the water-solubility of PEO-*b*-P(DMA-*co*-NAS)[51] and poly(glycerol monomethacrylate) partially esterified with cinnamoyl chloride,[70] this dilemma can be solved by the incorporation of relatively low content of NAS into the PNIPAM block. Preliminary experiments indicated that P(DMA-*co*-NAS) remained water-soluble if the NAS content was ≤25%. In the current case, PEO_{113}-*b*-$P(NIPAM_{0.85}$-*co*-$NAS_{0.15})_{106}$ was synthesized and used for subsequent studies of thermoresponsive micellization and reversible core cross-linking.

PEO_{113}-*b*-$P(NIPAM_{0.85}$-*co*-$NAS_{0.15})_{106}$ is directly soluble in water at 25 °C. Upon heating to 40 °C, a bluish tinge characteristic of micellar solutions appears (Figure 3). The

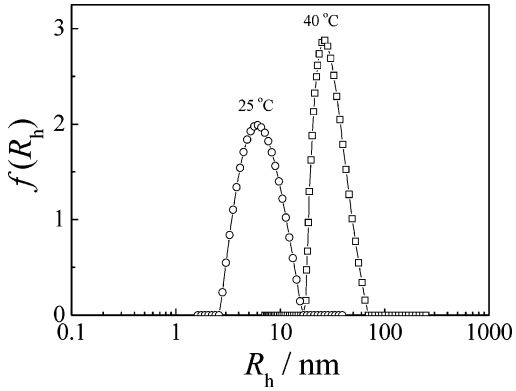


Figure 4. Hydrodynamic radius distributions, $f(R_h)$, obtained for 0.3 g/L aqueous solution of PEO_{113}-*b*-P($NIPAM_{0.85}$-*co*-$NAS_{0.15}$)$_{106}$ at 25 °C (○) and 40 °C (□).

temperature-dependent transmittance of a 1.0 wt % aqueous solution of PEO_{113}-*b*-P($NIPAM_{0.85}$-*co*-$NAS_{0.15}$)$_{106}$ is shown in Figure 3. The transmittance starts to decrease considerably at 28 °C, indicating that the P(NIPAM-*co*-NAS) block is water-insoluble due to its thermal phase transition. Above 40 °C, the transmittance stabilizes out, suggesting the complete thermo-induced micellization. Based on chemical intuition, the formed micelle should possess a core consisting of P(NIPAM-*co*-NAS) and a PEO corona (Scheme 2). As compared to the LCST of PNIPAM homopolymer with similar molecular weight (∼32 °C), the critical micellization temperature (CMT) (∼28 °C) observed for the PEO_{113}-*b*-P($NIPAM_{0.85}$-*co*-$NAS_{0.15}$)$_{106}$ block copolymer is slightly lower. This is quite reasonable because hydrophobic NAS residues are incorporated into the PNIPAM block. It is well-known that the LCST of PNIPAM homopolymer can be finely tuned when they are random or block copolymerized with hydrophilic or hydrophobic monomers, which will then accordingly increase or decrease its LCST.[71,72]

Dynamic LLS was employed to characterize the thermoresponsive micellization behavior. Figure 4 shows typical hydrodynamic radius distributions, $f(R_h)$, of the aqueous solution of PEO_{113}-*b*-P($NIPAM_{0.85}$-*co*-$NAS_{0.15}$)$_{106}$ at two different temperatures. At 25 °C, the diblock copolymer molecularly dissolves, with an average hydrodynamic radius, $\langle R_h \rangle$, of ∼5−6 nm, confirming that the diblock copolymer molecularly dissolves. Above the CMT, micellization of the diblock copolymer occurs. Dynamic LLS at 40 °C reveals relatively narrow size distributions, with an intensity-average hydrodynamic radius, $\langle R_h \rangle$, of 26 nm and a polydispersity (μ_2/Γ^2) of 0.11. The shift to larger $\langle R_h \rangle$ and the dramatic increase of scattered light intensity at elevated temperatures clearly indicate the formation of core−shell nanoparticles (Scheme 2).

The thermoresponsive micellization of PEO_{113}-*b*-P($NIPAM_{0.85}$-*co*-$NAS_{0.15}$)$_{106}$ was further investigated by ^{1}H NMR spectra. At 25 °C, the copolymer is fully solvated, and characteristic signals of both blocks are visible. The signals at $\delta = 1.1$, 2.8, and 3.6 ppm are characteristic of NIPAM, NAS residues, and the PEO block, respectively (see peak assignments in Figure 5A). At 40 °C, the ^{1}H NMR spectrum clearly tells us that the relative intensity of signal characteristic of PEO at 3.6 ppm remains unchanged, while characteristic signals of NIPAM and NAS residues at 1.1 and 2.8 ppm are invisible (Figure 5B). This indicates that the P(NIPAM-*co*-NAS) block becomes insoluble at 40 °C, leading to the formation of P(NIPAM-*co*-NAS)-core micelles stabilized by the well-solvated PEO coronas.

From Figure 5A, we can also discern a small resonance peak at $\delta = 2.7$ ppm, which should be ascribed to methylene protons of the hydrolysis product of NAS residues, that is, NHS. The

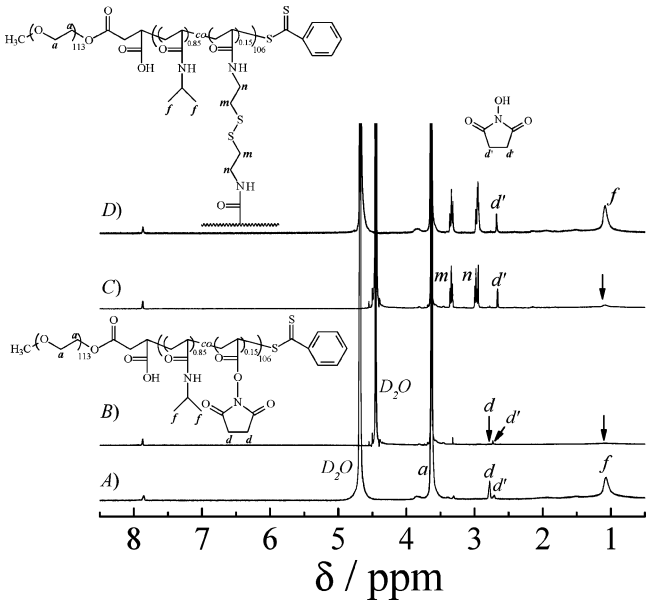


Figure 5. ^{1}H NMR spectra of PEO_{113}-*b*-P($NIPAM_{0.85}$-*co*-$NAS_{0.15}$)$_{106}$ in D_2O: (A) unimers at 25 °C; (B) PNIPAM-core micelles at 40 °C; (C) CCL micelles at 40 °C; and (D) CCL micelles at 25 °C.

presence of NHS residues was further confirmed by the ^{1}H NMR results shown in Figure 5B. Although characteristic signals of NAS residues in the P(NIPAM-*co*-NAS) block at 2.8 ppm completely disappeared at 40 °C, the signal due to methylene protons of NHS was still evident. From the time evolution of the integral ratios of characteristic resonance peaks of NAS residues to that of NHS, preliminary experiments revealed that the hydrolysis rate is ∼0.7% at 25 °C for the first 12 h. This indicates that the active ester of NAS residues is not stable in aqueous solution, even in neutral water.[64] However, primary amines should react with the active ester at a much faster rate than the hydrolysis reaction. In subsequent preparation of CCL micelles, freshly prepared block copolymer solutions were employed; moreover, 10 min after the solution was heated to 40 °C, cystamine was added to actuate the cross-linking reactions.

CCL Micelles. Various cross-linking approaches, such as photoinduced cross-linking, free-radical polymerization within the core, sol−gel chemistry, and click chemistry, have been utilized to fabricate CCL micelles.[24−34,39,45] In the current case, vinyl monomer containing an active ester, NAS, is incorporated into the thermoresponsive PNIPAM block. Because the NAS residues are highly reactive toward primary amines and they possess relatively low susceptibility to hydrolysis,[64] McCormick et al.[50,51] successfully prepared SCL micelles starting from a NAS-containing triblock copolymer in the presence of a difunctional primary amine. Using cystamine as the cross-linker, they further demonstrated that reversible SCL micelles could be facilely prepared.[50]

In the current study, we employ cystamine as a cross-linker to fabricate CCL micelles from PEO_{113}-*b*-P($NIPAM_{0.85}$-*co*-$NAS_{0.15}$)$_{106}$. At 40 °C, this diblock copolymer forms P(NIPAM-*co*-NAS)-core micelles stabilized by PEO coronas. Five hours after the addition of cystamine, we can apparently tell that the core cross-linking is successful, as revealed by the fact that the bluish tinge persists after the micellar solution is cooled back to 25 °C (Figure 3, bottom). The temperature-dependent transmittance of the aqueous solution of CCL micelles is shown in Figure 3. As compared to that of non-cross-linked micelles, which exhibit a large decrease of transmittance above the CMT

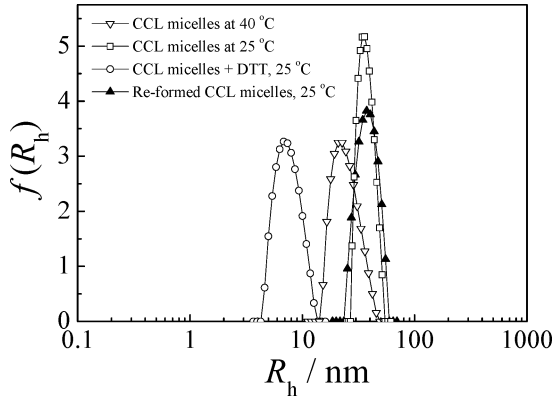


Figure 6. Hydrodynamic radius distributions, $f(R_h)$, obtained for 0.3 g/L aqueous solution of PEO_{113}-*b*-P(NIPAM$_{0.85}$-*co*-NAS$_{0.15}$)$_{106}$ micelles after core cross-linking, cleavage, and re-cross-linking.

of ~28 °C, the transmittance of the aqueous solution of CCL micelles exhibits negligible changes with increasing temperature, indicating that the P(NIPAM-*co*-NAS) core is successfully cross-linked. Above the thermal phase transition temperature of the cross-linked P(NIPAM-*co*-NAS) cores of CCL micelles, inter-micellar aggregation is excluded due to steric shielding and stabilization of the well-solvated PEO coronas (Scheme 2).

^{1}H NMR spectroscopy was further employed to study the extent of core cross-linking and the thermo-induced swelling/deswelling of the cross-linked core. We can clearly see from Figure 5C and D that after core cross-linking, the resonance peaks characteristic of NAS residues at 2.8 ppm completely disappeared. Further studies revealed that the cross-linking reaction is actually completed after only ~2 h at 40 °C, indicating the high reactivity of the active ester toward difunctional primary amine, cystamine. The peak at 2.7 ppm should be ascribed to the aminolysis product, that is, NHS, while resonance peaks at $\delta = 3.0$ and 3.3 ppm were ascribed to the methylene protons of cystamine.

For the CCL micelles at 40 °C, signal characteristic of NIPAM residues at 1.1 ppm was invisible, suggesting that the cross-linked P(NIPAM-*co*-NAS) core was in the fully desolvated state. Upon cooling back to 25 °C, the characteristic PNIPAM signal (*f*) reappeared, indicating that the PNIPAM segments within the cross-linked core became solvated again. Further ^{1}H NMR studies of the obtained CCL micelles under a cycling of temperature between 25 and 40 °C revealed that the swelling/deswelling of the cross-linked P(NIPAM-*co*-NAS) core upon temperature decrease/increase was fully reversible.

The above arguments were further corroborated by the subsequent LLS studies. Typical hydrodynamic radius distributions, $f(R_h)$, of the CCL micelles at 25 and 40 °C were shown in Figure 6. Both distributions were relatively monodisperse, with μ_2/Γ^2 values being 0.07 and 0.06 for CCL micelles at 25 and 40 °C, respectively. Most importantly, the intensity-average hydrodynamic, $\langle R_h \rangle$, of the former is much larger than the latter, being 35 and 24 nm, respectively. This suggests that upon heating from 25 to 40 °C, the hydrodynamic volume of the CCL micelles shrinks ~3 times. The shrinking of CCL micelles upon heating should be due to the thermal phase transition of cross-linked P(NIPAM-*co*-NAS) cores, which gets desolvated above the critical temperature. The CCL micelles exhibited a $\langle R_h \rangle$ of ~24 nm at 40 °C, which was slightly smaller than that of the P(NIPAM-*co*-NAS)-core micelles before core cross-linking (26 nm). This was reasonable considering that the micelle core may contract a little upon cross-linking.

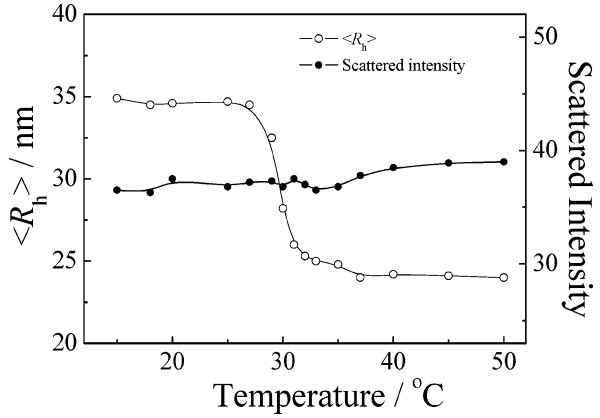


Figure 7. Temperature dependence of the average hydrodynamic radius, $\langle R_h \rangle$, and the scattered light intensity obtained for 0.3 g/L aqueous solution of CCL micelles prepared from PEO_{113}-*b*-P(NIPAM$_{0.85}$-*co*-NAS$_{0.15}$)$_{106}$ diblock copolymer.

Figure 7 illustrated the temperature dependence of dynamic LLS results of the obtained CCL micelles. In the temperature range of 15−50 °C, the scattered light intensity exhibited no appreciable changes. This clearly confirmed that the core cross-linking was successful. Otherwise, the micelles will dissociate into unimers upon cooling, leading to a large reduction of scattered intensity. On the other hand, in the temperature range of 27−32 °C, $\langle R_h \rangle$ exhibited the most dramatic decrease. It then stabilized out above 35 °C, remaining constant at ~24 nm. Further LLS studies revealed that the size variation with temperature was fully reversible. This should be due to the thermosensitive swelling/deswelling of the cross-linked core, exhibiting a critical phase transition temperature of ~27 °C. Below this critical temperature, the cross-linked P(NIPAM-*co*-NAS) core was getting solvated, and the infusion of solvents led to swelling of the cross-linked P(NIPAM-*co*-NAS) core. This was in excellent agreement with previous ^{1}H NMR results (Figure 5). A schematic illustration of the thermoresponsive micellization, core cross-linking, and the subsequent thermosensitive swelling/deswelling of the CCL micelle core was shown in Scheme 2.

Reversible Disintegration and Re-formation of CCL Micelles. The disulfide bonds within the cross-linker, cystamine, can also be readily cleaved using a well-known exchange reaction with dithiol compounds such as DTT.[73,74] At 40 °C, excess DTT was employed to disintegrate the obtained CCL micelles. After the mixture was stirred for 10 h, the initially bluish dispersion at 40 °C became apparently clear upon cooling to 25 °C, indicating the successful disintegration of cross-linked cores (Figure 3, bottom). Dynamic LLS studies further confirmed that the CCL micelles could be completely de-cross-linked (Figure 6). The average hydrodynamic radius, $\langle R_h \rangle$, obtained for the aqueous solution of CCL micelles after treatment with DTT was determined to be ~6 nm at 25 °C, which is comparable to that of the unimer state at 25 °C before cross-linking (Figure 4).

After the cleavage of CCL micelles with DTT, the disulfide bonds of cystamine were converted to thiol groups, while the primary amine groups reacted with the active ester of NAS residues to form stable amide bonds. Upon heating to 40 °C, the bluish tinge characteristic of colloidal dispersion can be observed again, indicating the thermoresponsive unimer-to-micelle transition. Most interestingly, after thoroughly removing DTT through dialysis, the core cross-linking can be facilely achieved again upon addition of excess cystamine, leading to the re-formation of disulfide bonds between thiol groups

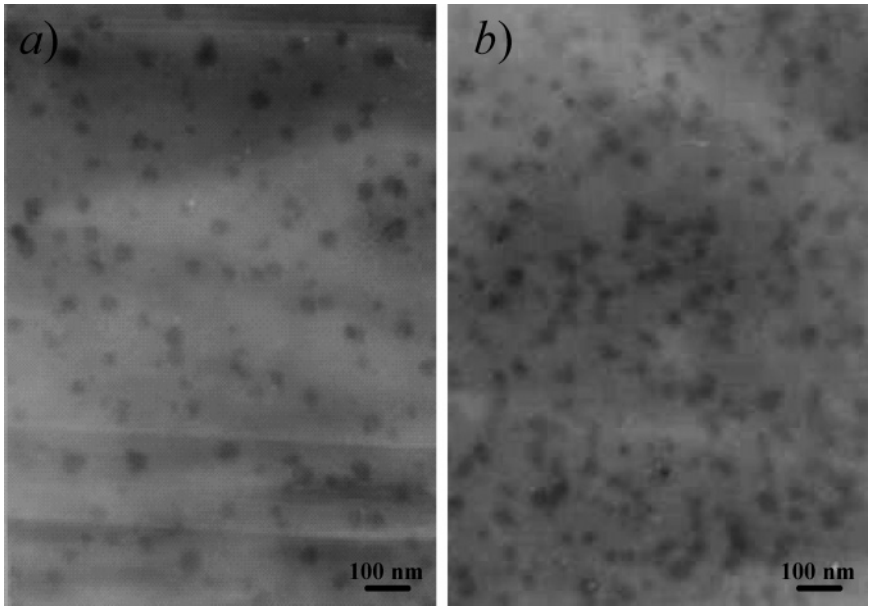


Figure 8. Typical transmission electron microscopy (TEM) images obtained for (a) the initially prepared CCL micelles and (b) the re-formed CCL micelles in aqueous solution at 25 °C.

attached to PNIPAM chain segments.[50] The successful re-cross-linking was apparently revealed by the fact that the characteristic bluish tinge persisted upon cooling back to 25 °C (Figure 3, bottom), exhibiting an intensity-average hydrodynamic radius of ~37 nm (Figure 6), which is comparable to that obtained for the initially prepared CCL micelles at 25 °C.

Visual inspection of the appearance of copolymer solution at 25 °C revealed that core cross-linking and de-cross-linking can be reversibly cycled. The fully reversible processes of core cross-linking and the subsequent de-cross-linking were also shown in Scheme 2.

The fabrication of CCL micelles and their re-formation after degrading the cross-linking sites were further confirmed by TEM observations. Figure 8 showed typical TEM images of CCL micelles (Figure 8a) and the re-formed CCL micelles (Figure 8b) at 25 °C. Both of them revealed the presence of spherical and narrowly distributed nanoparticles. The particle sizes determined by TEM were in the range of 30−40 nm in diameter, which were smaller than that obtained by dynamic LLS for the CCL micelles at 25 °C (~70 nm in diameter). For block copolymer micelles, it has been well-established that diameters determined by TEM are typically much smaller than those by dynamic LLS because the former reflects conformations in the dry state, while those determined by dynamic LLS reflect the dimension of both the swollen P(NIPAM-*co*-NAS) core and the stretched PEO shell. The general agreement between sizes measured by dynamic LLS and TEM suggests that the spherical morphologies observed by TEM are present in solution. The sizes of re-formed CCL micelles were comparable to those of the originally prepared CLL micelles, suggesting cross-linking, de-cross-linking, and re-cross-linking of PEO-*b*-P(NIPAM-*co*-NAS) micelles were fully reversible.

Conclusion

We successfully demonstrated that reversible CCL micelles can be facilely fabricated from a novel double hydrophilic block copolymer, PEO-*b*-P(NIPAM-*co*-NAS). This diblock copolymer is molecularly soluble in aqueous solution at room temperature, but forms P(NIPAM-*co*-NAS)-core micelles stabilized with soluble PEO coronas at elevated temperatures. As the micelle core contains reactive NAS residues, core cross-linking can be conveniently achieved upon addition of a disulfide-containing difunctional cross-linker, that is, cystamine. The obtained CCL micelles can be conveniently de-cross-linked by DTT, and the re-formation of CCL micelles is realized by the addition of cystamine after removing previously added DTT via dialysis. Moreover, the cross-linked PNIPAM cores of CCL micelles exhibit thermo-tunable swelling/deswelling behavior. The intriguing properties of fully reversible CCL micelles may find potential applications in drug delivery, sensors, and diagnosis.

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