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Thermo-responsive shell cross-linked PMMA-*b*-P(NIPAAm-*co*-NAS) micelles for drug delivery

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ABSTRACT

Thermo-responsive amphiphilic poly(methyl methacrylate)-*b*-poly(*N*-isopropylacrylamide-*co*-*N*-acryloxysuccinimide) (PMMA-*b*-P(NIPAAm-*co*-NAS)) block copolymer was synthesized by successive RAFT polymerizations. The uncross-linked micelles were facilely prepared by directly dissolving the block copolymer in an aqueous medium, and the shell cross-linked (SCL) micelles were further fabricated by the addition of ethylenediamine as a di-functional cross-linker into the micellar solution. Optical absorption measurements showed that the LCST of uncross-linked and cross-linked micelles was 31.0 °C and 40.8 °C, respectively. Transmission electron microscopy (TEM) showed that both uncross-linked and cross-linked micelles exhibited well-defined spherical shape in aqueous phase at room temperature, while the SCL micelles were able to retain the spherical shape with relatively smaller dimension even at 40 °C due to the cross-linked structure. *In vitro* drug release study demonstrated a slower and more sustained drug release behavior from the SCL micelles at high temperature as compared with the release profile of uncross-linked micelles, indicating the great potential of SCL micelles developed herein as novel smart carriers for controlled drug release.

1. Introduction

The self-assembly of amphiphilic block copolymers into core-shell micelle is a subject of much concern for their potential applications in biomedicine as nano-sized carriers for drug and gene delivery (Wang et al., 2005b, 2007; Wu et al., 2009; Ten Cate and Borner, 2007; Tian et al., 2006). Reversible addition–fragmentation chain transfer radical (RAFT) polymerization, as one of the controlled living radical polymerizations (CLRP), is a powerful synthetic technique for the preparation of amphiphilic block copolymers with well-defined structure, pre-determined composition, and narrow-distributed molecular weight. It gives access to a wide range of various monomers, such as acrylic acid (AA), hydroxyethyl methacrylate (HEMA), and *N*-isopropylacrylamide (NIPAAm), which can insert into the chain transfer agent (CTA) or macro-CTA (de Lambert et al., 2005, 2007).

During the past decade, the fabrication of intelligent particles capable of changing its structure according to the alteration of environmental signals (e.g., temperature and pH) has attracted increasing attention (Rapoport et al., 2002; Ramesh Babu et al., 2006; Mundargi et al., 2010; Reddy et al., 2008). The most extensively investigated temperature-sensitive polymer, poly(*N*-isopropylacrylamide) (PNIPAAm), exhibits a reversible thermo-responsive phase transition at around 33 °C in aqueous medium, termed as the lower critical solution temperature (LCST). It undergoes a transition from hydrophilic to hydrophobic state in aqueous solution when the temperature is increased from below to above its LCST (Zhang and Zhuo, 2001; Shelke et al., 2008). Our previous study demonstrated that the drug release from PNIPAAm-*b*-poly(methyl methacrylate) (PNIPAAm-*b*-PMMA) micelles was accelerated dramatically at the high temperature above the LCST due to the deformation of micellar structure (Wei et al., 2006b).

Although polymeric micelles exhibit kinetic stability (Yokoyama et al., 1996), the inevitable dissociation of micellar structure into individual polymer chains under dilution by gastric, blood, or other body fluids in the biological environment after administration hampers their practical applications (Jiang et al., 2007; Huo et al., 2006). To address such crucial issue, the strategies of covalent stabilization via cross-linking of the micellar core or shell were explored in order to enhance the structural stability and integrity of the supramolecular architecture (Murthy et al., 2001; Ding and Liu, 1998). For example, Bontha and co-workers reported the construction of core cross-linked (CCL) micelles based on poly(ethylene oxide)-*b*-poly(methacrylic acid) (PEO-*b*-PMA) using divalent metal cations (Ca^{2+}) as the cross-linker, and further investigated the release behavior of cisplatin from the CCL micelles (Bontha et al., 2006).

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Hu et al. (2006) used glutaraldehyde (GA) to cross-link stearic acid grafted chitosan oligosaccharide (CSO-*g*-SA), and studied the release of paclitaxel from the resultant shell cross-linked (SCL) micelles. Sairam et al. (2006) investigated the encapsulation efficiency and drug release behaviors of cross-linked poly(acrylamide) particles prepared by dispersion polymerization.

In this study, we synthesized amphiphilic diblock copolymer PMMA-*b*-poly(NIPAAm-*co*-*N*-acryloxysuccinimide) (PMMA-*b*-P(NIPAAm-*co*-NAS)) by successive RAFT polymerization techniques and further constructed SCL micelles using ethylenediamine as a di-functional cross-linker. Reactive NAS units were introduced into the polymer structure as the cross-linking sites by RAFT copolymerization given that NAS could offer an optimized accessibility for compound containing amino group (Li et al., 2006; Hakwere and Perrier, 2009; Zhang et al., 2007). PMMA was chosen as the hydrophobic building block for its good biocompatibility (Cheng et al., 2009; Wei et al., 2008a; Hayashi et al., 2005; de Brabander et al., 2003). In comparison to the uncross-linked micelle, the resultant SCL micelle exhibited enhanced stability at the high temperature as well as in the organic solvent. Moreover, *in vitro* drug release study demonstrated that the resultant SCL micelle could achieve slower and steadier drug release at 45 °C, indicating its potential as intelligent carrier for sustained drug release.

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (NIPAAm) was purchased from Acros and used as received. Methyl methacrylate (MMA), carbon disulfide (CS_2), 1,4-dioxane, tetrahydrofuran (THF), chloroform and triethylamine (TEA) were purchased from Shanghai Chemical Reagent Company and used after distillation. *N*-hydroxysuccinimide (NHS) was purchased from Shanghai Chemical Reagent Company. *N*-acryloxysuccinimide (NAS) and 2-(2-Carboxyethylsulfanylthiocarbonylsulfanyl)propionic acid (CPA) were prepared according to the reported methods (Li et al., 2006; Wang et al., 2005a; Chang et al., 2009). *N*, *N*′-azobisisobutyronitrile (AIBN) was purchased from Shanghai Chemical Reagent Company and used after recrystallization with 95% ethanol. Dialysis tube (molecular weight cut-off: 8000–12,000 g/mol) and pore-sized syringe filter (0.45 μm) were purchased from Shanghai Chemical Reagent Company. All other reagents and solvents were used as received.

2.2. Synthesis of N-acryloxysuccinimide (NAS)

NAS monomer was synthesized according to reference (Li et al., 2006). Briefly, NHS (4.25 g) and TEA (4.10 g) were dissolved in 80 mL of distilled chloroform in a flask preserved in an ice–salt bath. Acryloyl chloride (3.34 g) in 20 mL of distilled chloroform was added dropwise under stirring. After 3 h, the reaction mixture was rinsed with distilled water and saturated salt solution three times respectively, then dried over anhydrous magnesium sulfate overnight. After removal of chloroform by rotary evaporation, NAS was harvested by recrystallization in ethyl acetate/n-hexane (volume ratio, 1:1) as colorless crystalline. ^{1}H NMR ($CDCl_3$, 300 MH_z): δ (ppm from TMS) 2.9 (s, –C***H***$_2$–C***H***$_2$–), 6.2 (d, –CH=C***H***$_2$), 6.4 (t, –C***H***=CH_2), 6.7 (d, –CH=C***H***$_2$).

2.3. Synthesis of 2-(2-carboxyethylsulfanylthiocarbonylsulfanyl)propionic acid (CPA)

3-mercaptopropionic acid (10.6 g, 0.1 mol), distilled water (100 mL), and 50 wt.% NaOH solution (16.0 g, 0.2 mol) were added to a 250 mL round-bottomed flask equipped with a magnetic stir bar. The mixture was stirred for 30 min prior to the addition of carbon disulfide (6.0 mL, 0.1 mol) dropwise. The resulting yellow solution was stirred overnight at room temperature. Thereafter 2-bromopropionic acid (15.3 g, 0.1 mol) was added dropwise to the yellow solution, and the mixture was further stirred overnight at room temperature. Then the reaction mixture was acidified by the addition of concentrated hydrochloric acid, and the resulting precipitate was extracted with acetic ether. Finally, the concentrated yellow acetic ether solution was poured into petroleum to precipitate the product. The product was separated by filtration and further purified twice by redissolving/reprecipitating with acetic ether/petroleum, and dried under vacuum overnight. ^{1}H NMR (d_6-DMSO) δ (ppm): 1.53 (d, –C***H***$_3$–CH–), 2.70 (t, –C***H***$_2$–COOH), 3.56 (t, –S–C***H***$_2$–), 4.68 (quar, –S–C***H***–), 12.8 (s, –COO***H***).

2.4. Synthesis of PMMA (macro-CTA) by RAFT polymerization

MMA (776 mg, 7.76 mmol), CPA(25.4 mg, 0.1 mmol), and AIBN (1.64 mg, 0.01 mmol) were dissolved in 15 mL of freshly distilled THF in a 50 mL round-bottom flask with a magnetic stir bar. The flask was purged with dry N_2 for 30 min prior to being immersed in a preheated oil bath at 70 °C. After reaction for 22 h, the reaction mixtures were poured into a large excess of n-hexane to precipitate the resulting PMMA. The product was collected by filtration and further purified twice by redissolution/reprecipitation with THF/n-hexane, and finally dried under vacuum overnight.

2.5. Synthesis of PMMA-b-P(NIPAAm-co-NAS) by RAFT polymerization

In a 50 mL round-bottom flask charged with a magnetic stir bar, NIPAAm (1.14 g, 10 mmol), NAS (84.5 mg, 0.5 mmol), macro-CTA PMMA (150 mg, 0.025 mmol), and AIBN (0.8 mg, 0.005 mmol) were dissolved in 8 mL of freshly distilled 1,4-dioxane. The flask was purged with dry N_2 for 30 min prior to being immersed in a preheated oil bath at 70 °C. After reaction for 22 h, the reaction mixtures were poured into a large excess of diethyl ether to precipitate the resulting PMMA-*b*-P(NIPAAm-*co*-NAS). The product was collected by filtration and purified twice by redissolution/reprecipitation with THF/diethyl ether, and finally dried under vacuum overnight.

2.6. ^{1}H NMR characterization

^{1}H NMR spectra were recorded on a Varian Unity 300 MHz spectrometer using $CDCl_3$ as a solvent. The testing temperature was fixed at 20 °C without specific clarification.

2.7. SEC–MALLS measurements

The size-exclusion chromatography and multi-angle laser light scattering (SEC–MALLS) analysis were used to determine the molecular weight of the as-prepared polymers. A dual-detector system, consisting of a MALLS device (DAWN EOS, Wyatt Technology) and an interferometric refractometer (Optilab DSP, Wyatt Technology) were used. The columns used were styragel HR1 and HR4. The concentration of the polymer was kept constant at 10 mg/mL and THF (chromatographic grade) was used as the eluent at a flow rate of 0.3 mL/min. The MALLS detector was operated at a laser wavelength of 690 nm. The dn/dc of PMMA and PMMA-*b*-P(NIPAAm-*co*-NAS) were 0.185 and 0.077, respectively.

2.8. Micelle formation

The uncross-linked micelle was prepared by directly dissolving 20 mg of PMMA-*b*-P(NIPAAm-*co*-NAS) copolymer in 40 mL of

distilled water at 20 °C. The SCL micelle was fabricated by adding 0.5 mg of ethylenediamine into the above micelle solution for cross-linking. After reaction for 24 h, the mixture solution was transferred into a dialysis tube and dialyzed against distilled water for 24 h to remove any unreacted ethylenediamine and byproduct NHS resulting from the aminolysis of NAS.

2.9. Transmission electron microscopy (TEM) observation

The sample was prepared by dipping a carbon film coated copper grid into the uncross-linked and cross-linked micelle aqueous solutions (500 mg/L). After the deposition of particles, the residue of sample solution was blotted away with a strip of filter paper. The samples were further stained with 0.2% phosphatetungstic acid aqueous solution, and dried in air prior to TEM observation on a JEM-100CX II instrument operating at an acceleration voltage of 80 kV. For DMF suspension, the sample was prepared in the same way. The specimens at high temperature were prepared according to our previous study (Chang et al., 2009). Briefly, a drop of micelle suspension preheated at 40 °C using a water bath was placed onto a carbon film coated copper grid, which was then placed in a baking oven thermostated at 40 °C for quick drying.

2.10. Size distribution measurements

Zetasizer Nano ZS (Malvern Instruments) was used to determine the average size and size distribution of uncross-linked and cross-linked micelles at different temperatures. The micelle solution (800 mg/L) was passed through a 0.45 μm pore-sized syringe filter prior to measurements.

2.11. LCST

The optical absorbance of uncross-linked and cross-linked micelle solutions (800 mg/L) at various temperatures was measured at 500 nm using a Lambda Bio40 UV–Vis spectrometer (Perkin-Elmer). Sample cells were thermostated in a circulator bath at different temperatures from 23 to 49 °C prior to the measurements, and the heating rate was set at 0.1 °C min^{-1}. The LCST was defined as the temperature producing a half increase of the total increase in optical absorbance.

2.12. Drug loading and in vitro drug release

Lyophilized PMMA-*b*-P(NIPAAm-*co*-NAS) copolymer (25 mg) and prednisone acetate (5 mg) were dissolved in 2 mL of DMF, which was then added into 50 mL of distilled water under stirring. The mixture solution was placed into a dialysis tube and dialyzed against 1 L of distilled water for 24 h to obtain the drug-loaded uncross-linked micelles. The distilled water was renewed every 8 h to remove the unloaded free drug and DMF, and then the solution in the dialysis tube was freeze-dried. Thereafter, 20 mg of drug-loaded uncross-linked micelle was re-dispersed in 10 mL of distilled water. The solution was equipartitioned into two parts, and each was placed into a dialysis tube followed by immersing the tube into the PBS (pH = 7.4, I = 0.1) at 20 °C (labeled as A-1) and 45 °C (labeled as A-2), respectively.

Drug-loaded SCL micelles were prepared in the same way as that of drug-loaded uncross-linked micelles mentioned above, except for the addition of ethylenediamine (0.5 mg) as the cross-linker into the micelle solution and further stirring for 24 h prior to dialysis. The two samples for drug release study of SCL micelles at 20 °C and 45 °C were labeled as B-1 and B-2, respectively.

The volume of PBS for drug release study was 10 mL, which was withdrawn periodically and held constant by adding 10 mL of fresh medium after each sampling. The total amount of prednisone acetate loaded in the micelles was calculated as the summation of cumulative amount of drug released from the micelles and the amount of drug remained in the micelles after release. The amount of prednisone acetate released from micelles was measured using UV absorbance at 242 nm (Wei et al., 2007; Zou et al., 2007; Li et al., 2007). To determine the amount of drug retained in micelle, the micelle suspension after drug release was freeze-dried, and then dissolved in DMF and analyzed by UV absorbance at 270 nm (Chang et al., 2009). The entrapment efficiency (EE) and drug loading (DL) capacity were calculated based on the following formulas,

$$EE = \frac{\text{mass of drug loaded in micelles}}{\text{mass of drug fed initially}} \times 100\%,$$

$$DL = \frac{\text{mass of drug loaded in micelles}}{\text{mass of micelles}} \times 100\%.$$

Table 1
Molecular weight of PMMA and PMMA-*b*-P(NIPAAm-*co*-NAS) determined by SEC–MALLS.

Polymer	M_n bar[a]	M_w bar[b]	PDI (M_w bar/M_n bar)
PMMA	3,300	6,000	1.82
PMMA-*b*-P(NIPAAm-*co*-NAS)	32,800	48,500	1.48

[a] M_n bar = $\sum N_i M_i / \sum N_i$
[b] M_w bar = $\sum W_i M_i / \sum W_i = \sum N_i M_i^2 / \sum N_i M_i$

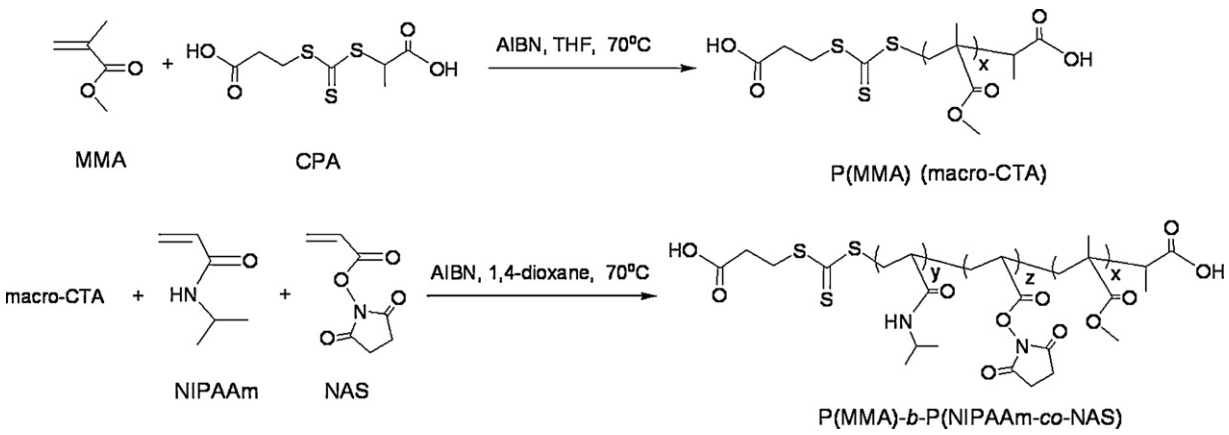
3. Results and discussion

3.1. Synthesis of PMMA-b-P(NIPAAm-co-NAS) copolymer

The order of constructing the blocks of a block copolymer can be very important in the RAFT polymerization. The primary rule that should follow is the propagating radical for the first synthesized block must be a good homolytic leaving group with respect to that of the second block, thus in the synthesis of a methacrylates–styrene or methacrylates–acrylamide diblock copolymer, the methacrylate block should be prepared first. The styrene or acrylamide propagating radicals are very poor leaving groups as compared with methacrylates propagating radicals. Herein, PMMA-*b*-P(NIPAAm-*co*-NAS) diblock copolymer was synthesized by a two-step procedure, i.e., the synthesis of PMMA by RAFT polymerization using CPA as a CTA, followed by the synthesis of PMMA-*b*-P(NIPAAm-*co*-NAS) diblock copolymer via RAFT polymerization using PMMA as a macro-CTA. The detailed synthesis route is illustrated in Scheme 1.

^{1}H NMR was utilized to characterize the structure of the as-prepared copolymers. As shown in Fig. 1A, the characteristic peaks of MMA units (1.0–2.0 ppm (a + b, –C***H***$_2$C(C***H***$_3$)–, 3.6 ppm (c, –COOC***H***$_3$)) are readily visible in the ^{1}H NMR spectrum of PMMA using $CDCl_3$ as a solvent. In comparison to Fig. 1A, all the characteristic peaks of NIPAAm (5.9–7.0 (d, –N***H***–), 4.0 ppm (e, –C***H***$(CH_3)_2$), 1.0 ppm (f, –CH(C***H***$_3)_2$)), and NAS (2.9 ppm (g –C***H***$_2$–C***H***$_2$–)) units appear in the ^{1}H NMR spectrum of purified PMMA-*b*-P(NIPAAm-*co*-NAS) (Fig. 1B), confirming the successful synthesis of the target diblock copolymer. The molar ratio of NIPAAm and NAS units in P(NIPAAm-*co*-NAS) block was calculated to be 15:1 based on the integral ratio of resonance signals at 4.0 ppm (e) and 2.9 ppm (g).

SEC–MALLS analysis was used to determine the molecular weight and distribution of the copolymers. It can be seen from Table 1 that M_w bar of PMMA-*b*-P(NIPAAm-*co*-NAS) (48,500) is larger than that of PMMA (6000). The SEC chromatograms in Fig. 2 demonstrate that both synthesized polymers are unimodal. Most importantly, in comparison to the SEC trace obtained for the PMMA precursor (Fig. 2A), there is a clear shift to the higher molecular



Scheme 1. Synthesis of PMMA-*b*-P(NIPAAm-*co*-NAS) diblock copolymer.

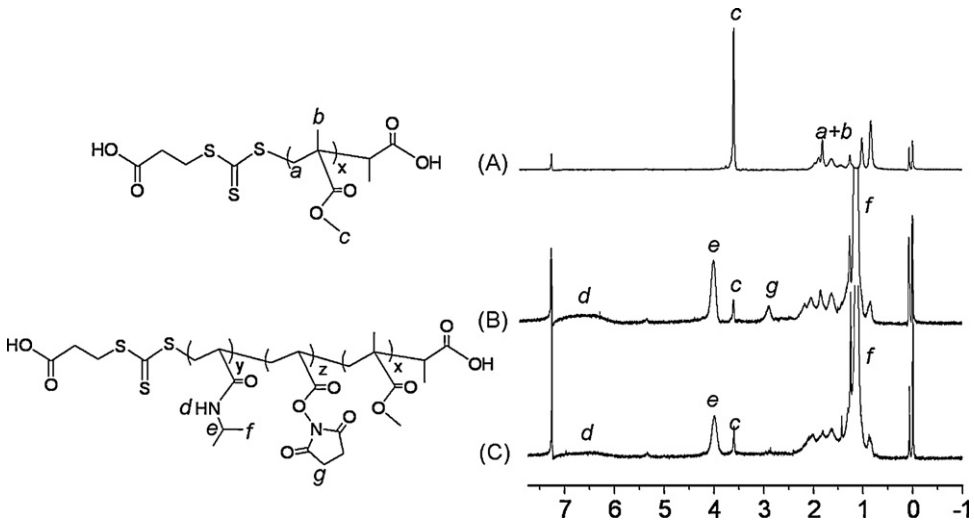


Fig. 1. ^{1}H NMR spectra of (A) PMMA, (B) PMMA-*b*-P(NIPAAm-*co*-NAS), and (C) lyophilized SCL micelles in $CDCl_3$.

weight for the PMMA-*b*-P(NIPAAm-*co*-NAS) (Fig. 2B) diblock copolymer, indicating a high reactivity of PMMA as a macro-CTA and a successful preparation of the target PMMA-*b*-P(NIPAAm-*co*-NAS) diblock copolymers by consecutive RAFT techniques.

The degree of polymerization (DP) of MMA units was determined to be 60, based on the molecular weight of PMMA block (6000). Thereafter, the DP of NIPAAm and NAS units was calculated to be 330 and 22 respectively, based on the molar ratio (15:1) obtained from ^{1}H NMR analysis and the molecular weight of P(NIPAAm-*co*-NAS) block (48,500). Thus the diblock copolymer was labeled as $PMMA_{60}$-*b*-P($NIPAAm_{330}$-*co*-NAS_{22}). It should be pointed out that the actual composition of the copolymer can be calculated based on either the integrity ratio in the NMR spectra or the absolute molecular weight determined by SEC–MALLS. Both methods were used in our research, however, the results obtained from molecular weight were closer to the molar feed ratio of the copolymer, therefore we, herein, only reported the calculation of the real composition using the data from molecular weight. The same method has been used in our recent publication (Chang et al., 2011) as well.

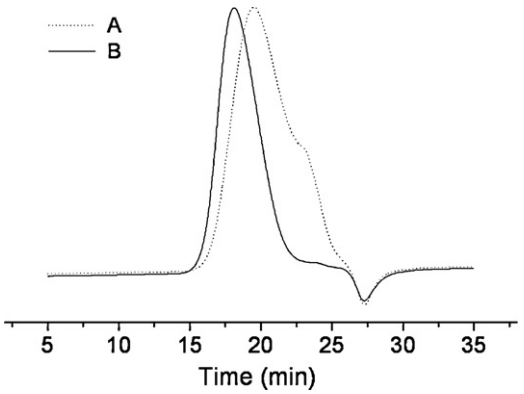


Fig. 2. SEC traces of (A) PMMA and (B) PMMA-*b*-P (NIPAAm-*co*-NAS).

3.2. SCL micelle formation

In this study, SCL micelles were designed and prepared to enhance the micellar stability and increase the drug loading capacity as well as achieve the sustained drug release. Uncross-linked micelles were fabricated simultaneously to make a control. The schematic illustration of SCL micelle formation and proposed thermo-induced drug release behavior from SCL micelle are presented in Fig. 3.

^{1}H NMR spectrum of lyophilized SCL micelle in $CDCl_3$ (Fig. 1C) was employed to confirm the occurrence of cross-linking reaction in the micellar shell. Upon comparison of part B and C in Fig. 1, we observe the significant attenuation of characteristic signal (g) of NAS units, which is feasibly attributed to the transformation of

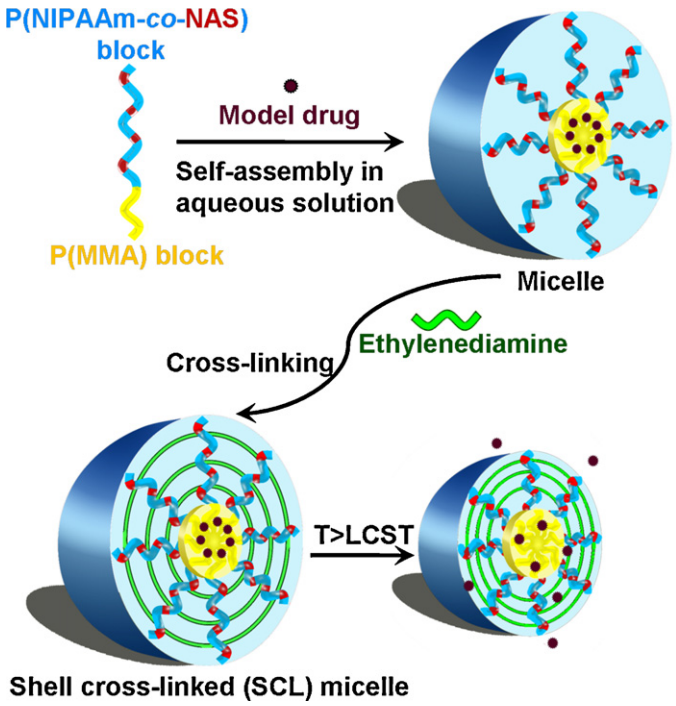


Fig. 3. Schematic illustration of the formation of SCL micelles and temperature controlled drug release from SCL micelles in aqueous solution.

activated ester group in NAS unit to the amide linkage after cross-linking by ethylenediamine.

The lyophilized SCL micelles were further dissolved in organic solvent to prove sufficient cross-linking. Fig. 4A displays the TEM image obtained for the SCL micelles in DMF, in which the particles exhibit well-defined spherical shape with diameter ranging from 90 to 140 nm. The results convincingly show that the cross-linked structure of the micelle remains intact after transfer into organic solvent, in which the assembly would be destroyed. It turns out that cross-linking of micellar shell is effective and the micellar structure is locked by cross-linking of the hydrophilic shell layer. Dynamic light scattering (DLS) reveals that the average size of SCL micelles in DMF is 255 nm (Fig. 4B). The difference between the sizes measured by DLS and visualized by TEM is due to the fact that the dimension determined by the DLS is based on the swollen micelles in solution, while the size observed by TEM is for the dried particles. Similar

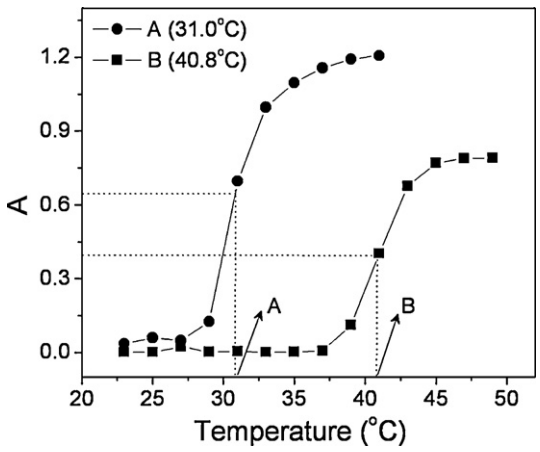


Fig. 5. Thermo-sensitive behavior of aqueous solution of (A) uncross-linked micelles and (B) SCL micelles.

results were also reported in our previous studies (Chang et al., 2009; Wei et al., 2008b).

3.3. Thermo-responsive properties of uncross-linked and cross-linked micelles

The optical absorbance of uncross-linked micelle and SCL micelle solution as a function of temperature was examined to confirm their thermo-responsive properties. As presented in Fig. 5, the uncross-linked PMMA-*b*-P(NIPAAm-*co*-NAS) micelle undergoes a change in the thermoresponsive P(NIPAAm-*co*-NAS) shell at a temperature around 31 °C. Generally, in the case of a random copolymer of NIPAAm with hydrophobic comonomer, the LCST shifts to a lower temperature because incorporation of the hydrophobic comonomer favors chain aggregation, resulting in a decrease of the LCST (Polozova and Winnik, 1999; Koga et al., 2001). Since NAS is hydrophobic, the P(NIPAAm-*co*-NAS) copolymer exhibits a lower LCST than that (33 °C) of the PNIPAAm homopolymer. Additionally, it can be seen from Fig. 5 that the response rate of the LCST behavior for PMMA-*b*-P(NIPAAm-*co*-NAS) micelle remains fast, indicating that the introduction of an appropriate amount of NAS comonomer into the PNIPAAm chain as the cross-linking sites results in P(NIPAAm-*co*-NAS) copolymer with unaltered response rate, albeit a small decrease in the LCST value with respect to the PNIPAAm homopolymer.

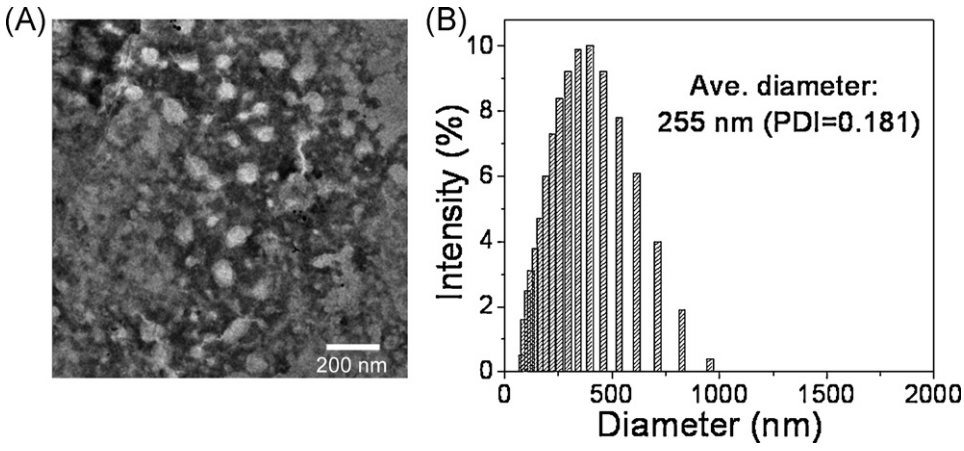


Fig. 4. TEM micropicture (A) and size distribution (B) of SCL micelles in DMF.

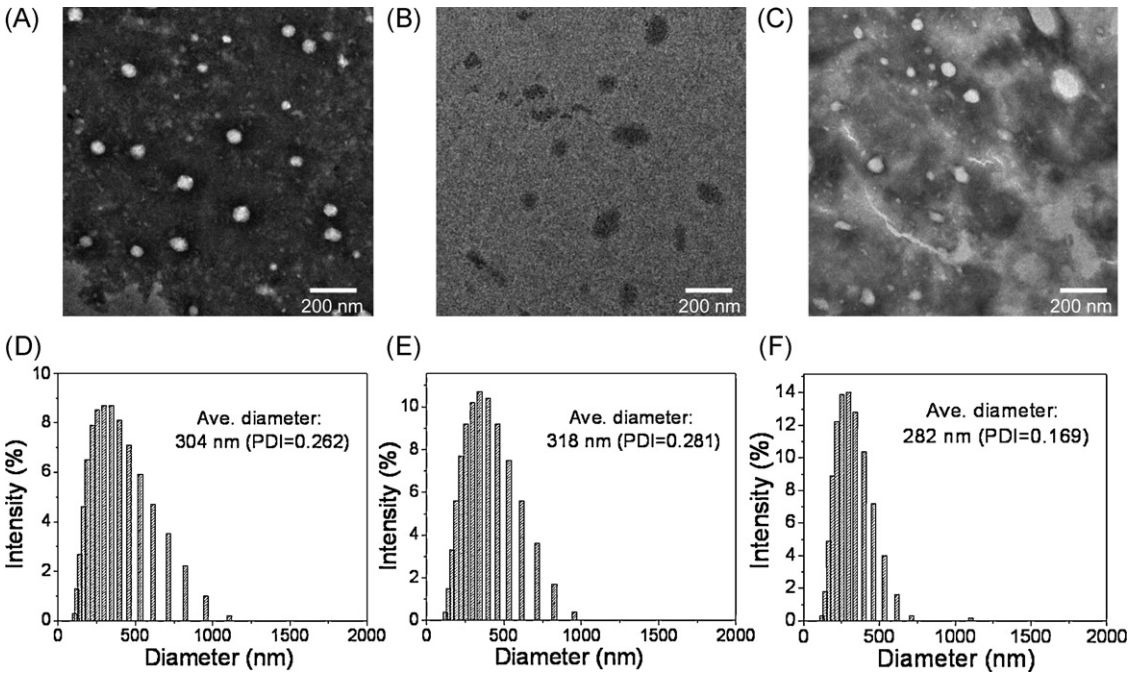


Fig. 6. TEM micropictures and size distributions of (A and D) uncross-linked micelles at 20 °C, SCL micelles at (B and E) 20 °C and (C and F) 40 °C.

However, a significantly increased LCST at 40.8 °C is recorded for the SCL micelles, which feasibly results from the following two reasons, 1) due to the incorporation of ethylenediamine as the cross-linker into the micellar structure, the neighboring thermosensitive P(NIPAAm-*co*-NAS) chains in the shell are diluted and interrupted; as a result, the temperature sensitivity of the shell is a little weakened, namely, from Fig. 5 it is apparent that the temperature response rate of the SCL micelle becomes slightly slower as compared with that of the uncross-linked micelle, and the relatively retarded response rate accounts for an elevated LCST for the SCL micelle; 2) the hydrophobic activated ester group of NAS converts to the hydrophilic amide linkage after the formation of SCL micelles, leading to the increase of LCST as well.

Variation of morphology and dimension at different temperatures were investigated to demonstrate the temperature sensitivity of uncross-linked and cross-linked micelles. As presented in Fig. 6, both uncross-linked and cross-linked micelles are well-dispersed with regularly spherical shape at 20 °C, and their diameter is estimated to be 80–130 nm and 110–180 nm, indicating that cross-linking of micellar shell has no obvious effect on the morphology of particles. Instead of deformation and aggregation, the shrinkage of SCL micelles takes place at 40 °C, leading to the observation of smaller nanoparticles with 70–130 nm in diameter (Fig. 6). Although the SCL micelles tend to form aggregates via hydrophobic interactions at the elevated temperature, the existence of cross-linked structure in micellar shell prevents such tendency and stabilizes the micelles, as a result, SCL micelles can maintain the spherical shape even at the high temperature. The average particle size observed by TEM is in close agreement with the results determined by particle-size analyser as depicted in Fig. 6.

The average size of uncross-linked and cross-linked micelles in aqueous media was further measured as a function of temperature by DLS. As shown in Fig. 7, the average size of uncross-linked micelles decreases obviously from 25 to 37 °C, suggesting the shrinkage of micellar shell resulting from the collapse of P(NIPAAm-*co*-NAS) chains. In the case of SCL micelles, slower and steadier decrease of size is observed over the whole recorded temperature range, confirming the apparent stability of cross-linked structure even at high temperatures. The change of micelle diameter against temperature alteration is coincident with the TEM observation presented in Fig. 6.

3.4. In vitro drug loading and drug release

Prednisone acetate (molecular weight: 400.47, melting point: 235–242 °C) is an anti-inflammatory drug with rather low solubility in aqueous phase. It can be easily dissolved in many organic solvents, such as DMF and $CHCl_3$ (Wei et al., 2006a). In order to improve its solubility in aqueous medium and to enhance its therapeutic efficiency, prednisone acetate was chosen as a model drug to be encapsulated into the hydrophobic cores of the micelles in this study. The drug-loaded uncross-linked and cross-linked micelles were both prepared by a classical dialysis technique to investigate the difference of their drug release characteristics. The EE and DL were calculated to be 30.7% and 5.9% for uncross-linked micelles, and 27.3% and 5.5% for SCL micelles, respectively.

As shown in Fig. 8, the drug release profiles of uncross-linked and cross-linked micelles are close to each other at 20 °C,

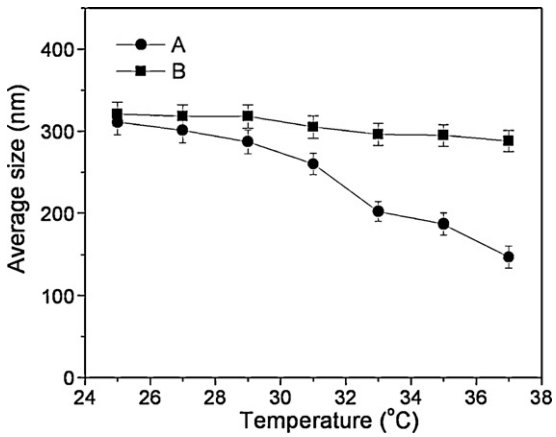


Fig. 7. The average sizes of (A) uncross-linked and (B) SCL micelles against temperature change from 25 to 37 °C.

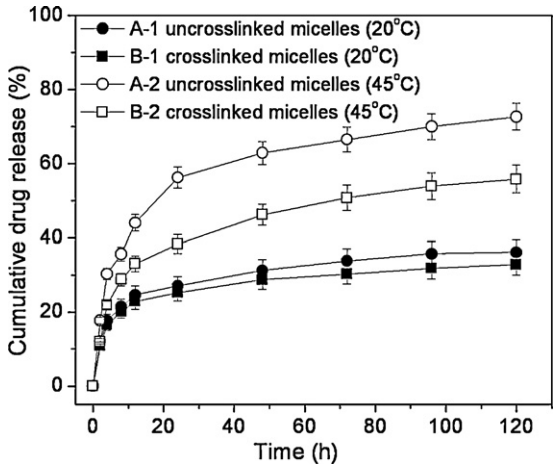


Fig. 8. Cumulative drug release from uncross-linked and cross-linked micelles: uncross-linked micelles at (A-1) 20 °C and (A-2) 45 °C, SCL micelles at (B-1) 20 °C and (B-2) 45 °C.

i.e., the cumulative drug release is 36.1% and 32.9% within 120 h, respectively, indicating that cross-linking affects little the release behaviors at low temperature. However, when temperature increases to 45 °C, uncross-linked micelles exhibit faster drug release than SCL micelles, which seems plausibly attributed to the deformation and aggregation of uncross-linked micelles at high temperature. Due to the preservation of micelle structure, a slower and more sustained drug release behavior is recorded for the SCL micelles at 45 °C, which can maintain the drug concentration at a more stable level, resulting in the reduction of dosing frequency and improvement of patients' compliance (Kostewicz et al., 1996; Kim et al., 2005).

4. Conclusion

Thermo-responsive diblock copolymer PMMA-*b*-P(NIPAAm-*co*-NAS) was synthesized by successive RAFT polymerization and SCL micelles were further fabricated using ethylenediamine as a di-functional cross-linker. Due to the cross-linked structure, the resultant SCL micelles were able to maintain well-defined spherical shape in organic solvent as well as at high temperature. Most importantly, the SCL micelles exhibited more sustained drug release behavior than uncross-linked micelles at high temperature. Based on the results, the SCL micelles developed herein will have great potential as novel smart carriers for controlled drug release.

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