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Fabrication of star-shaped, thermo-sensitive poly(*N*-isopropylacrylamide)–cholic acid–poly(ε-caprolactone) copolymers and their self-assembled micelles as drug carriers

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ABSTRACT

Novel star-shaped copolymers, comprised of a thermo-sensitive poly(*N*-isopropylacrylamide) (PNIPAAm) segment and three hydrophobic poly(ε-caprolactone) (PCL) arms were fabricated. The copolymers were prepared by stannous octoate ($Sn(Oct)_2$) catalyzed ring-opening polymerization of ε-caprolactone (CL) using cholic acid functionalized PNIPAAm as the macroinitiator. The lower critical solution temperatures (LCST) of the copolymer solutions are attractively close to the nominal physiologic temperature at around 37 °C. The *in vitro* cytotoxicity test indicated no apparent cytotoxicity. The amphiphilic star-shaped copolymers were capable of self-assembling into spherical micelles in water at room temperature, and they possessed low critical micelle concentrations (CMCs) of 3 ~ 8 mg/L in aqueous solution determined by fluorescence spectroscopy using pyrene as a probe. Transmission electron microscopy (TEM) measurement showed that the micelles exhibited a spherical shape with a size range of 30 ~ 75 nm in diameter. In addition, the anticancer drug, methotrexate (MTX) can be loaded effectively in the polymeric micelles and its release was temperature-stimulated, which suggests that these materials have good potential as "intelligent" drug carriers.

1. Introduction

Clinical applications of many therapeutic anticancer drugs are hampered due to their low water solubility and high toxicities [1]. To overcome these obstacles, considerable attentions have been devoted to the construction of drug-loaded polymeric nanoparticle systems, which are known to allow solubilizing drugs for intravascular delivery, crossing biological barriers, sustained drug release and passive target effect to tumor tissues [2,3]. Among these systems, the micelle usually prepared from biocompatible amphiphilic block copolymers in aqueous medium seems more promising for potential application [4–10]. The research in these fields is further propelled by the interest in the "intelligent" polymeric micelles to respond reversibly to external stimuli, such as temperature, pH and ionic strength [11–19].

Relative to linear polymers, the characteristic properties of star-shaped polymers derived from their unique shape have been observed in solution or in bulk. It has been shown that star-shaped polymers exhibit a smaller hydrodynamic radius and lower solution viscosity when compared to linear polymers of the same molecular weight and composition. The most significant point for star polymer-based unimolecular micelles is its marked improvement of micelle stability relative to linear polymers [20,21]. Note that compared with lots of published data concerning the block linear copolymers and their micelles, the study of star-shaped copolymer, especially star-shaped environmental sensitive copolymers is relatively limited and more work is in great need to understand the particular characteristics involving self-assembly, drug release behavior as well as related application.

In this study, thermo-sensitive nano-sized micelles were attempted to prepare by self-assembly of the PNIPAAm-based amphiphilic polymers. As known, PNIPAAm shows a reversible thermo-sensitive soluble-to-insoluble phase transition in aqueous medium at around 32 °C, and of particular importance it (molecular weight <50,000) exhibits very low toxicity due to the excretion through glomerular filtration [22,23]. Herein PCL served as the hydrophobic segments of the micelles and cholic acid, one of the main bile acids, was selected as the joint to combine PNIPAAm and PCL segments in terms of their excellent biodegradability and biocompatibility [24]. Moreover, cholic acid (CA)-tailed PNIPAAm with three hydroxyl terminal groups afforded multi-functional macroinitiator for CL ring-opening polymerization to obtain three-armed star-shaped copolymers (PNIPAAm–CA–$(PCL)_3$). The rigid polycyclic steroidal structure and well-spaced array of three hydroxyl groups may endow the copolymer with well-defined

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geometrical architectures [25,26], thus possibly resulting in other particular properties. The lower critical solution temperature (LCST) of the micelles may be tuned by modulating the ratio of PCL chain to PNIPAAm chain to meet practical demands, for instance at nominal physiologic temperature of around 37 °C. The self-assembling behavior of the PNIPAAm–CA–$(PCL)_3$ was investigated and the resulted micelles were further studied as the drug carriers to examine their thermo-sensitive properties in controlled release.

2. Experimental

2.1. Materials

N-Isopropylacrylamide (NIPAAm), 2-amino ethanethiol hydrochloride (AET·HCl) and cholic acid (CA) were purchased from ACROS and used as received. ε-Caprolactone (CL) was purchased from Sigma and was dried over CaH_2 for 2 days, and then distilled under reduced pressure prior to use. Stannous octoate ($Sn(Oct)_2$), toluene, *N*,*N*′-dimethylformamide (DMF) and dimethyl sulphoxide (DMSO) were obtained from Shanghai Chemical Reagent Co., China and used after distillation. *N*,*N*′-Azobisisobutyronitrile (AIBN) provided by Shanghai Chemical Reagent Co. was used after recrystallization with 95% ethanol. Methotrexate (MTX) was kindly provided by SuRi Biochem Co. Ltd., Suzhou, China. All other reagents and solvents were used without further purification.

For *in vitro* cytotoxicity test, NIH 3T3 mouse fibroblasts were obtained from China Center for Typical Culture Collection. Dulbecco's Modified Eagle's Medium (DMEM) and RPMI1640 were obtained from GIBCO Invitrogen Corporation. 3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was from Amresco and dimethylsulfoxide (DMSO) was from Sigma.

2.2. Preparation of the amino-terminated PNIPAAm

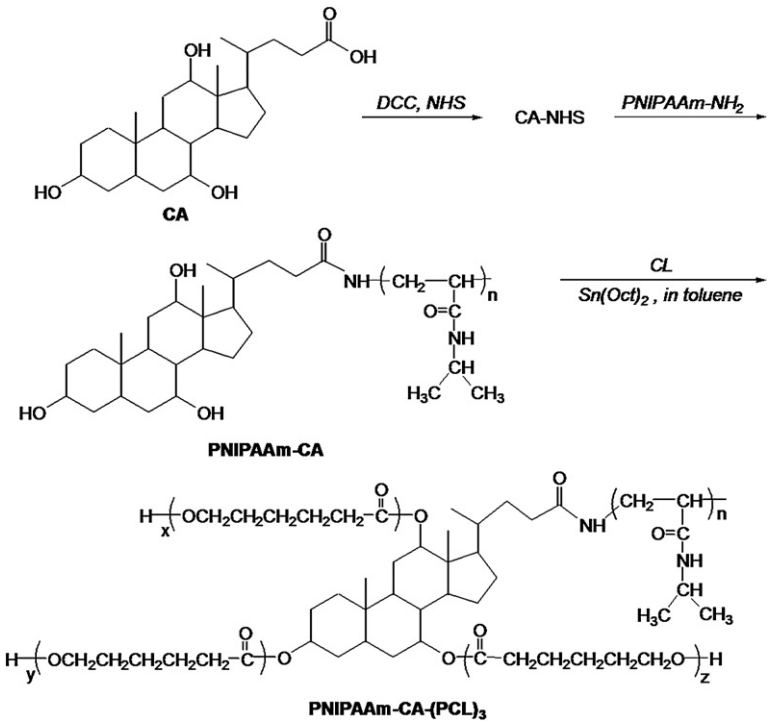
Amino-terminated PNIPAAm (PNIPAAm-NH_2) was prepared by radical polymerization in the presence of AIBN as the initiator. To introduce a terminal amino group, AET·HCl was employed as the chain transfer agent. NIPAAm (2.74 g, 0.024 mol), AET (0.02 g, 1.76×10^{-4} mol) and AIBN (2.3 mg, 1.83×10^{-6} mol) were dissolved in 27.5 ml DMF. The mixed solution was degassed by bubbling nitrogen for 30 min. Then the reaction solution was refluxed at 70 °C for 24 h under nitrogen atmosphere. After the reaction, the solution was precipitated into 400 ml chilled dry diethyl ether. The obtained polymer was purified by further precipitation in diethyl ether twice and finally dried in vacuum.

2.3. Preparation of macroinitiator PNIPAAm–CA

The carboxylic acid group of the cholic acid (CA) (1.0 g, 2.58×10^{-3} mol) was activated by *N*-hydroxysuccinimide (NHS) (0.46 g, 3.40×10^{-3} mol) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (0.5 g, 2.42×10^{-3} mol) as the catalyst and condensing agent, respectively. The reaction was carried out in DMSO at room temperature for 24 h under nitrogen atmosphere. After filtrating, the isolated NHS-activated CA was coupled with the amine group of PNIPAAm-NH_2 (1.7 g) at room temperature under nitrogen atmosphere for 48 h. After the reaction, the solution was placed in a dialysis membrane (MWCO = 8000) and dialyzed at room temperature for 48 h against DMSO (500 ml) which was changed every 24 h to remove the excess CA. Then the solution was dialyzed against water at room temperature for 48 h to remove DMSO. During the dialysis, the water was renewed every 3 h. Finally the solution was lyophilized to obtain the macromolecular initiator PNIPAAm–CA as white powder.

2.4. Preparation of PNIPAAm–CA–$(PCL)_3$

The star-shaped copolymers of PNIPAAm–CA–$(PCL)_3$ were synthesized by $Sn(Oct)_2$-catalyzed ring-opening polymerization of CL at different monomer/initiator feed ratios. PNIPAAm–CA with three hydroxyl terminal groups actually served as the macroinitiator in the polymerization. The typical polymerization procedures are as follows (Scheme 1): a predetermined amount of PNIPAAm–CA and



Scheme 1. Scheduler illustration of the synthesis of star-shaped PNIPAAm–CA–$(PCL)_3$ copolymer.

Table 1
Preparation of the PNIPAAm–CA–$(PCL)_3$ copolymers using PNIPAAm–CA as macroinitiator[a]

Sample	PNIPAAm–CA (g)	CL	DP of CL	M_n	M_w/M_n
Polymer-a	0.4	0.4 g (3.51 mmol)	38	12,500[b]/14,700[c]	1.28[b]/1.76[c]
Polymer-b	0.3	0.45 g (3.95 mmol)	51	16,400[c]	1.79[c]

[a] Both the reactions were conducted at 100 °C for 24 h and $Sn(OCt)_2$ concentration was fixed at 0.1 mol% to CL. M_n of macroinitiator PNIPAAm–CA is 8100 determined by GPC with $d = 1.26$ and DP (degree of polymerization) = 72.
[b] Determined by GPC.
[c] Determined by SEC–MALLS.

toluene (25 ml) were placed in a flask, and moisture was removed by azeotropic drying through evaporation of about 15 mL toluene at 140 °C. After cooling, a mixture solution of a certain amount of CL and catalyst $Sn(Oct)_2$ in toluene was placed into the flask. The concentration of catalyst $Sn(Oct)_2$ was fixed at 0.1 mol% to CL monomer. The solution was degassed followed by bubbling nitrogen for 30 min and then the reaction mixture was refluxed at 100 °C for 24 h under nitrogen atmosphere. After cooled to the room temperature, the solution was poured into 400 ml diethyl ether and this precipitation process was repeated twice. The isolated product was dissolved in THF (15 mL) and this solution was further poured into cold, dried methanol (400 mL) to remove the insoluble by-product of PCL. The obtained solution was rotated under reduced pressure to get rid of extra solvents. The final products were dried in vacuum and yielded as yellow powder. Here as shown in Table 1, two products of PNIPAAm–CA–$(PCL)_3$ with different PCL lengths were labeled as polymer-a and polymer-b.

2.5. Micelle formation

We employed dialysis method to fabricate the micelles. Briefly, each PNIPAAm–CA–$(PCL)_3$ polymer (10 mg) was dissolved in 4 ml THF. The solution was put into a dialysis tube and subjected to dialysis against 1000 ml distilled water, which was renewed every 3 h, then lyophilized before further examinations.

2.6. GPC measurements and SEC-MALLS measurements

The gel permeation chromatographic (GPC) system was used to determine the number-average molecular weights (M_n) of PNIPAAm–CA and polymer-a. The GPC system was equipped with Waters 2690D separations module, Waters 2410 refractive index detector. THF was introduced as the eluent at a flow rate of 0.3 ml/min. Waters millennium module software was used to calculate molecular weight on the basis of a universal calibration curve generated by polystyrene standard with narrow molecular weight distribution.

The molecular weights of polymer-a and polymer-b were determined by the size-exclusion chromatography and multi-angle laser light scattering (SEC–MALLS) analysis, a dual detector system, consists of a MALLS device (DAWN EOS, Wyatt Technology) and an interferometric refractometer (Optilab DSP, Wyatt Technology). Each copolymer concentration was 10 mg/ml and THF served as the eluent at a flow rate of 0.3 ml/min. The MALLS detector was operated at a laser wavelength of 690.0 nm.

2.7. FT-IR measurements

To study the core–shell structure of the micelles, FT-IR spectra of PNIPAAm–CA–$(PCL)_3$ and freeze-dried micelles were recorded on a Perkin–Elmer-2 spectrometer. The freeze-dried micelles were prepared by dialysis method, and then lyophilized prior to measurements. The samples of freeze-dried micelles were pressed into potassium bromide (KBr) pellets. Additionally, other samples for FT-IR measurements were prepared by dissolving them in 1.5 wt% chloroform solution on the surface of a silicon wafer, and the solvent was completely evaporated before measurements.

2.8. ^1H NMR characterization

^1H NMR spectra were recorded on a Mercury VX-300 spectrometer at 300 Hz using $CDCl_3$ or D_2O as the solvent.

2.9. Transmission electron microscopy (TEM)

The morphology of the self-assembled micelles was performed on a JEM-100CX II transmission electron microscopy operating at an acceleration voltage of 80 kV. TEM sample was prepared by dipping a copper grid with Formvar film into the freshly prepared micelles solution. A few minutes after the deposition, the aqueous solution was blotted away with a strip of filter paper and stained with phosphotungstic acid aqueous solution, then dried in the air.

2.10. Micelles size and their distributions in aqueous solution

The mean particle size in aqueous solution at various temperatures and size distribution of self-assembled micelles were determined by Nano-ZS ZEN3600. Each sample was diluted to the appropriate concentration with distilled water. The polymeric solutions (mg/L) were passed through a 0.45 μm pore size filter before measurement.

2.11. Optical absorption measurements

Optical absorbance of PNIPAAm–CA–$(PCL)_3$ aqueous solution (250 mg/L) at various temperatures was measured at 542 nm with a Lambda Bio40 UV–vis spectrometer (Perkin–Elmer). Sample cell was thermostated in a refrigerated circulator baths at different temperatures from 26 to 42 °C prior to measurements. The heating rate was 0.1 °C/min. The LCST of the polymer solution was defined as the temperature producing a half increase of the total increase in optical absorbance.

2.12. Fluorescence measurements

Fluorescence spectra were recorded on a RF-5301PC spectrofluorophotometer (Shimadzu). Pyrene was used as a hydrophobic fluorescent probe to determine the CMC. Aliquots of pyrene solutions (6×10^{-6} M in acetone, 1 ml) were added to containers, and acetone was allowed to evaporate at room temperature. Ten-milliliter aqueous copolymer solutions at different concentrations were then added to the containers containing the pyrene residue. It should be noted that all the aqueous sample solutions contained excess pyrene residue at the same concentration of 6×10^{-7} M. The solutions were kept at room temperature for 24 h to reach the solubilization equilibrium of pyrene in the aqueous phase. Excitation was carried out at 340 nm, and emission spectra were recorded ranging from 360 to 460 nm. The excitation and emission bandwidths were 10 nm and 5 nm, respectively. From the pyrene emission spectra, the peak height-intensity ratio (I_3/I_1) of the third peak (I_3 at 391 nm) to the first peak (I_1 at 371 nm) was plotted against the logarithm of the copolymer concentrations. A CMC value was determined from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration.

2.13. Cytotoxicity

For each well in a 96-well plate, 200 μL of NIH 3T3 mouse fibroblasts in DMEM, with a concentration of 1.25×10^5 cells/mL, was

added. The number of NIH 3T3 mouse fibroblasts in each well was 2.5×10^3. After incubation for 24 h in incubator (37 °C, 5% CO_2), the culture medium was changed to 200 μL of DMEM containing polymer-a with particular concentrations and the mixture was further incubated for 48 h. Then, DMEM with polymers was replaced by fresh DMEM and 20 μL of MTT solution (5 mg/mL) was added to the fibroblasts. After incubation for 4 h, 200 μL of DMSO was added and shaken at room temperature. The optical density (OD) was measured at 570 nm with a Microplate Reader Model550 (BIO-RAD, USA). The viable rate was calculated by the following equation:

$$\text{Viable rate} = (\text{OD}_{\text{treated}}/\text{OD}_{\text{control}}) \times 100\%$$

where $\text{OD}_{\text{control}}$ was obtained in the absence of polymer-a and $\text{OD}_{\text{treated}}$ was obtained in the presence of polymer-a.

2.14. Drug loading

PNIPAAm–CA–$(PCL)_3$ (8 mg) and MTX (2 mg) were dissolved in 2 mL DMF, then the solution was put into a dialysis tube and subjected to dialysis against 1000 ml distilled water. The whole dialysis process lasted for 24 h and the water was renewed every 2 h during the initial 12 h to remove the unloaded drug. To determine the entrapment efficiency (EE), the drug-loaded micelle solution was lyophilized, and then dissolved in DMF and analyzed by UV absorbance at 303 nm, using a standard calibration curve experimentally obtained with MTX/DMF solutions. The EE was calculated based on the following formula:

$$\text{EE (wt\%)} = (\text{mass of drug in micelles}/\text{mass of drug fed initially}) \times 100\%$$

2.15. In vitro *drug release*

After dialysis, the dialysis tube was directly immersed into 400 mL distilled water thermostated at 26 or 42 °C. Aliquots of 3 mL were withdrawn from the solution periodically. The volume of solution was held constant by adding 3 mL distilled water after each sampling. The amount of MTX released from micelles was determined according to the UV absorbance intensity at 303 nm.

3. Results and discussion

3.1. Synthesis of PNIPAAm–CA–$(PCL)_3$ and self-assembled micelles

GPC, SEC-MALLS, HNMR and IR measurements were utilized to determine the structure of the resulting copolymers and micelles. It demonstrated in our previous study that the initiation occurred for all the three hydroxyl groups when cholic acid was employed to initiate the ring-opening polymerization of CL [24–27]. In this contribution, cholic acid-tailed PNIPAAm served as the macroinitiator for the ring-opening polymerization of CL. Thus three-armed star-shaped copolymers comprised of a hydrophilic PNIPAAm segment as well as three hydrophobic PCL arms were actually afforded. By changing the feed ratio of monomer to macroinitiator, two copolymers of polymer-a and polymer-b with different lengths of PCL chain were easily obtained (Table 1). For both the copolymers, only one single symmetric peak was observed in the GPC or SEC–MALLS curves as shown in Fig. 1.

As exhibited in the FT-IR spectrum (Fig. 2(a-1, b-1)), the absorbance of carbonyl amide groups in polymer occurs at 1648 cm^{-1} and the bending frequency of the amide N–H appears at 1545 cm^{-1}. In addition, a peak exists simultaneously at 1735 cm^{-1} attributed to the stretching vibration of C═O in PCL, which confirms the

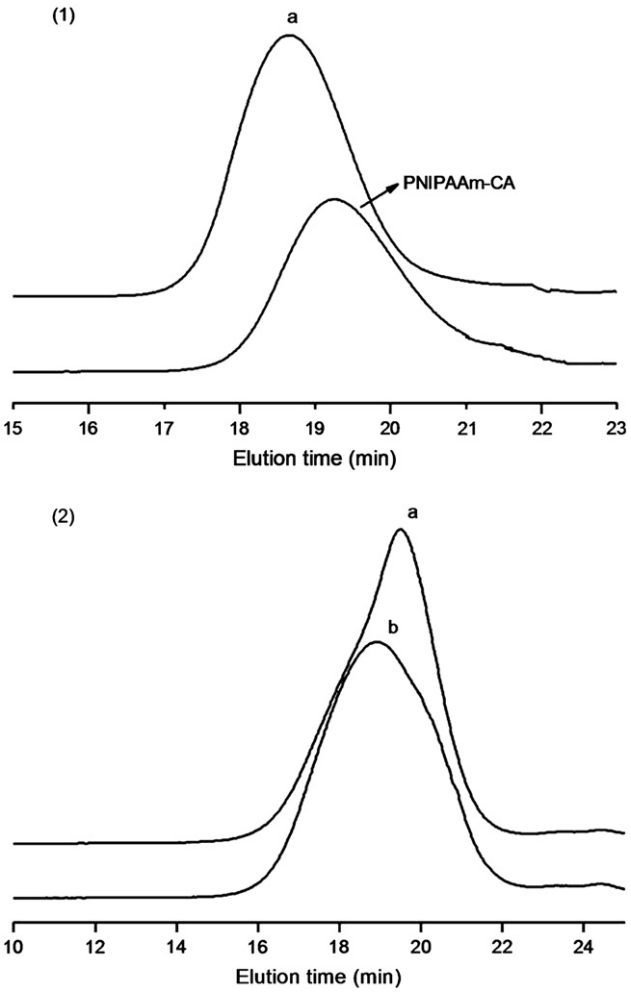


Fig. 1. (1) GPC traces of PNIPAAm–CA, $M_n = 8100$, $M_w/M_n = 1.26$ and (a) polymer-a, $M_n = 12{,}500$, $M_w/M_n = 1.28$ and (2) SEC traces of (a) polymer-a, $M_n = 14{,}700$, $M_w/M_n = 1.76$ (b) polymer-b, $M_n = 16{,}400$, $M_w/M_n = 1.79$.

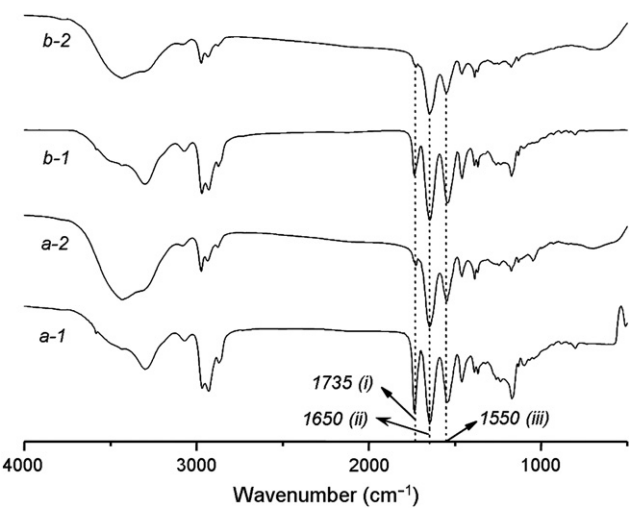


Fig. 2. FT-IR spectra of polymer-a (a-1), micelles derived from polymer-a (a-2), polymer-b (b-1) and micelles derived from polymer-b (b-2).

successful synthesis of the copolymers in combination with the GPC result of unimodal peak. By comparison of the IR spectra between the prepared copolymers and their corresponding micelles, it was found that the absorbance of C═O in the PCL segments of

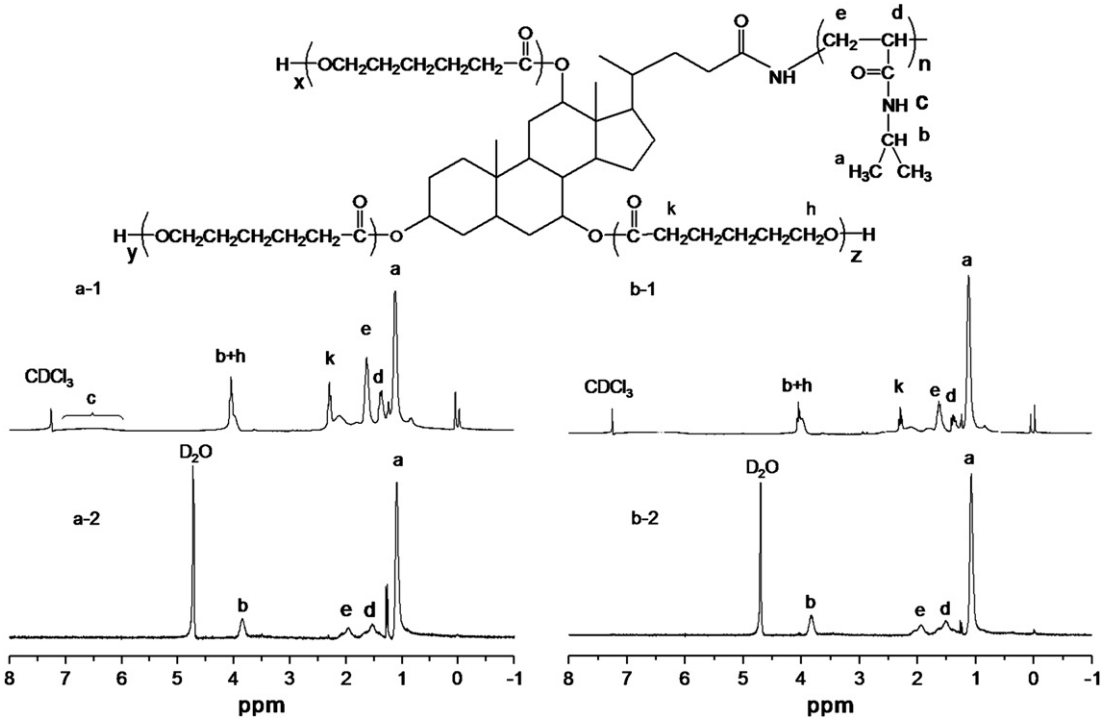
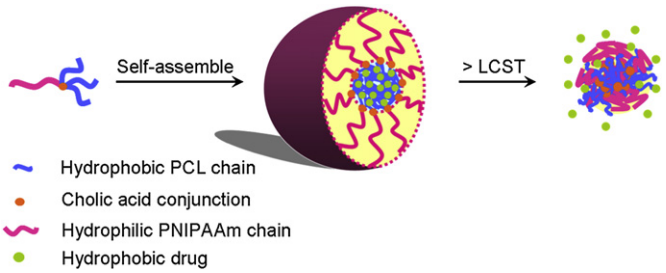


Fig. 3. ^{1}H NMR spectra of polymer-a in $CDCl_3$ (a-1), polymer-a in D_2O (a-2), polymer-b in $CDCl_3$ (b-1) and polymer-b in D_2O (b-2).

micelles (Fig. 2(a-2, b-2)) was remarkably weaker while the C═O peak attributed to PNIPAAm segments had the similar intensity regardless of the formation of micelle. The explanation should be ascribed to the complete isolation of hydrophobic inner cores and hydrophilic shells of the micelles, resulting in the inner cores comprised of PCL segments are shielded. Fig. 3 shows ^{1}H NMR spectra of the copolymers in $CDCl_3$ or in D_2O. For the copolymer spectra in $CDCl_3$ as shown in Fig. 3(a-1, b-1), a single peak as well as triple peaks at around δ 4.0 ppm was found, attributed to the characteristic signals of hydrogen of NC*H*$(CH_3)_2$ in the PNIPAAm and OC*H*$_2$ in the PCL. Besides, the signals at around δ 1.1 ppm assigned to methyl protons of isopropyl unit of PNIPAAm that to the methylene connected with the C═O in PCL at around δ 2.3 ppm also exists, which further proved the synthesis of copolymers. In contrast, complete loss of characteristic resonance originating from CL was found when using D_2O as NMR solvent, as shown in Fig. 3(a-2, b-2), due to the suppressed molecular motion of the aggregated hydrophobic PCL chains in the inner cores in the case of D_2O medium. Thus IR and ^{1}HNMR experiments demonstrated a stable core–shell micelle structure of PNIPAAm–CA–$(PCL)_3$ copolymers with a hydrophobic PCL inner core and a hydrophilic PNIPAAm outer shell as illustrated in Scheme 2.



Scheme 2. Schematic representation of PNIPAAm–CA–$(PCL)_3$ and the self-assembled thermo-sensitive core–shell micelles for temperature-stimulated drug release.

3.2. CMC of PNIPAAm–CA–$(PCL)_3$ micelles

The formation of micelles from PNIPAAm–CA–$(PCL)_3$ in aqueous medium was also verified by a fluorescence probe technique. Pyrene was used here as a hydrophobic fluorescent probe for its high sensitivity to the local polarity of the medium. When pyrene is located in a hydrophobic environment, it shows strong fluorescence. On the contrary, in a hydrophilic environment a weak fluorescent intensity and a shift of wavelength are observed in the emission spectra. Since the peak height-intensity ratio (I_3/I_1) of the third peak (I_3 at 391 nm) to the first peak (I_1 at 371 nm) can be used as a sensitive parameter to represent the polarity of the microenvironment, the I_3/I_1 values of each copolymer have been plotted as a function of copolymer concentration as shown in Fig. 4. It was found that the ratio was almost constant at relatively low concentration. After the concentration of the copolymer got to a critical value, ratio started to enhance dramatically with the increasing concentration, which indicated the creation of micelles as well as the simultaneous transfer of pyrene into the hydrophobic micellar core. Thus the transition concentration was defined as the critical micellar concentration (CMC) of 7.86 and 3.85 mg/L for polymer-a and polymer-b, respectively. The discrepancy of the CMC values is probably due to the relatively stronger hydrophobic interactions or decreased solvency resulting from the more hydrophobic core in the presence of longer PCL chains. Thus the CMC value exhibits a decreasing tendency with the increased length of PCL chain and can be tuned accordingly to a certain extent.

To understand the micellization behavior, HLB values were estimated based on determined composition of the copolymers according to the equation [28,29]:

$$\mathrm{HLB} = \frac{W_\mathrm{H}}{W_\mathrm{H} + W_\mathrm{L}} \times 20$$

Here, W_H and W_L correspond to the weight fraction of the hydrophilic segment (PNIPAAm) and hydrophobic segment (PCL), respectively. HLB values of the copolymers are 13.0 and 11.6 for

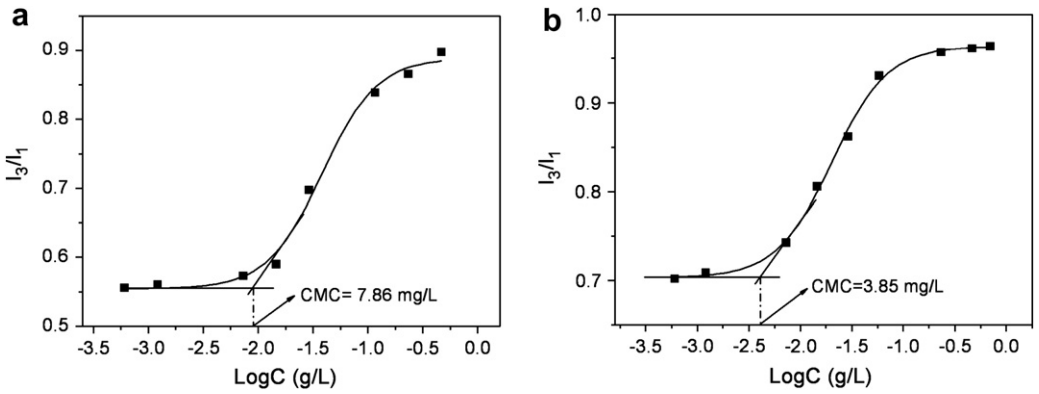


Fig. 4. I_3/I_1 plotted as a function of polymer concentrations of polymer-a (a) and polymer-b (b); [Pyrene] = 1.2×10^{-6} M.

polymer-a and polymer-b, respectively. The HLB values decrease as the length of PCL chain increases, which behaves with the similar variation tendency with the CMC values, and this partly sustains the conclusion concerning the CMC result.

3.3. Morphology of micelles

The morphology and size distribution of polymer micelles were investigated by TEM measurement and dynamic light scattering (DLS). It can be seen from TEM pictures (Fig. 5(a,b)) that the self-assembled micelles are well dispersed as individual nanoparticles with regularly spherical shape, which confirms that the micellization does take place. Furthermore, the diameters of the micelles are around 30–75 nm. The data are more likely to support the opinion that the micellization takes place as a result of molecular association, rather than aggregation of smaller micelles. The PDI of corresponding micelles, extremely low about 0.235 and 0.182 determined by DLS also reinforced this opinion although the micelles exhibited larger average diameter of 98 nm and 130 nm, respectively, determined by DLS (Fig. 5(c,d)). This difference in micelles size measured by TEM and DLS should be attributed to that the latter is the hydrodynamic diameter of micelles in water, whereas the former reveals the morphology size of the micelles in solid state. Similar difference in size as a result of different measuring techniques was also reported in our previous studies [22,30–32], as well as others [33–35].

3.4. Cytotoxicity of the PNIPAAm–CA–$(PCL)_3$

Biocompatibility is a great concern for biomaterials and in this study we performed preliminary evaluation on the cytotoxicity of obtained materials by the MTT assay. The results in Fig. 6 show that resulted copolymer did not exhibit apparent cytotoxicity although the cell viability decreases slightly with increasing polymer concentration.

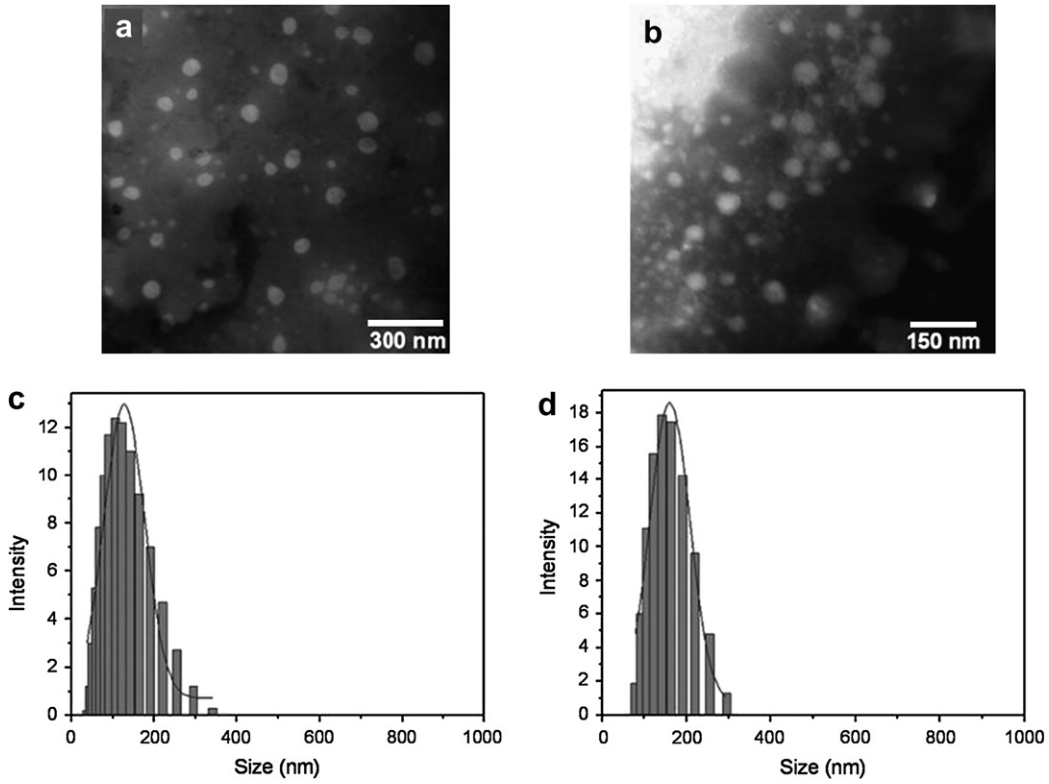


Fig. 5. TEM micropictures of the micelles of polymer-a (a), polymer-b (b) and size distribution detected by DLS of the micelles of polymer-a (c), polymer-b (d) in distilled water.

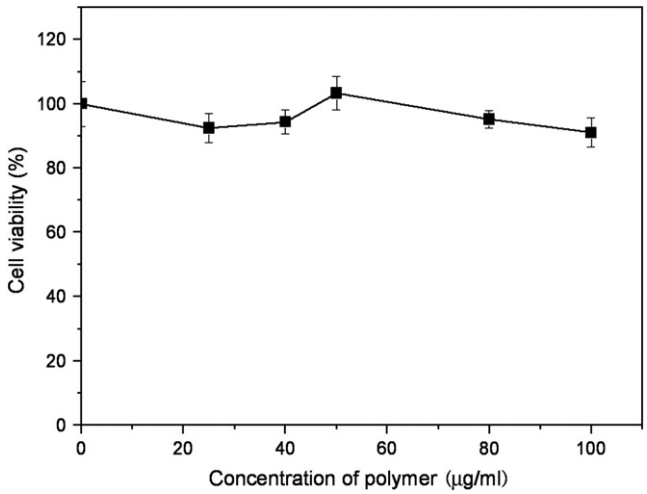


Fig. 6. In vitro cytotoxicity of the polymer-a with different concentrations.

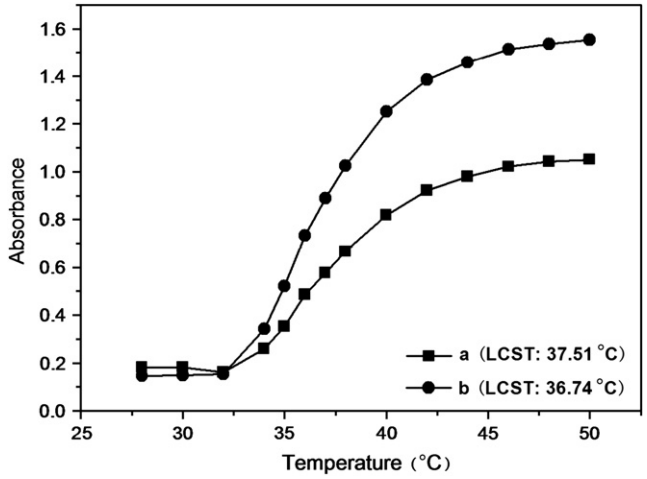


Fig. 7. Plots of the absorbance variation at 542 nm in UV spectroscopy upon temperature change for polymer-a (a) and polymer-b (b).

3.5. LCST determination

As known, PNIPAAm behaves a reversible thermo-sensitive soluble-to-insoluble phase transition in aqueous medium at around 32 °C. As shown in Fig. 7, the influence on the LCST after the introduction of three-arm PCL to PNIPAAm were investigated by monitoring the absorbance change of aqueous copolymers solutions at 542 nm as a function of temperature by UV spectroscopy. The LCSTs of the copolymers rose up relative to that of PNIPAAm itself. Note that the LCSTs of polymer-a and polymer-b are both close to the nominal physiologic temperature at 37.5 and 36.7 °C, respectively. Furthermore, the LCST of polymer-b is slightly lower than that of polymer-a. This small shift might be ascribed to the longer chain length of PCL segments which results in relatively stronger tendency to aggregate. Thus the LCST of the self-assembled micelles may be tuned by modulating the length ratio of PCL chain to PNIPAAm to meet practical demands.

3.6. Thermo-sensitive structural changes of micelles

In order to study the thermo-sensitive changes of the micelles, we further investigated the changes of cumulative average micelle diameter in aqueous solution as a function of temperature in the range from 25 to 49 °C through the LCST. As shown in Fig. 8, the hydrodynamic diameter of the polymeric micelles slowly decreased with the increasing temperature in the initial stage. The result is reasonable since with increasing temperature, the weaken PNIPAAm hydration led to the shrinking of the PNIPAAm chains and subsequent relatively tighter contact of the PCL chains. However, a dramatic increase in hydrodynamic diameter is further observed once the temperature increases through the LCST (36–37 °C). Obviously the aggregation of micelles started to happen and finally form the aggregate by strengthened hydrophobic interactions around the phase transition temperature, where the micellar outer shell (PNIPAAm segment) turns to be more hydrophobic and sticky resulting in the deformation of the core–shell micelle structure.

3.7. Drug loading and thermo-sensitive release

In terms of the unique thermo-sensitive properties of prepared copolymers, their potential application in controlled drug release was evaluated by investigating the drug release profile from the corresponding micelles. It is known that the hydrophobic drug could be physically loaded and stabilized in the hydrophobic micellar inner core by hydrophobic interactions. The anticancer drug, MTX-loaded micelles were prepared by the dialysis method. About 0.327 mg drug was loaded in 8 mg polymer micelles with an EE of around 16% when the initial feed drug is 2 mg, which indicated that MTX can be effectively loaded into the micelles.

The MTX release profiles from the thermo-sensitive micelles were performed in distilled water at two different temperatures of 26 or 42 °C, below or above the LCST. As illustrated in Fig. 9, only about 56% drug was released after 8 days at 26 °C for polymer-a, resulting from the slow drug leaching from the micelles for inevitable out-diffusion process. It indicated that the highly hydrated

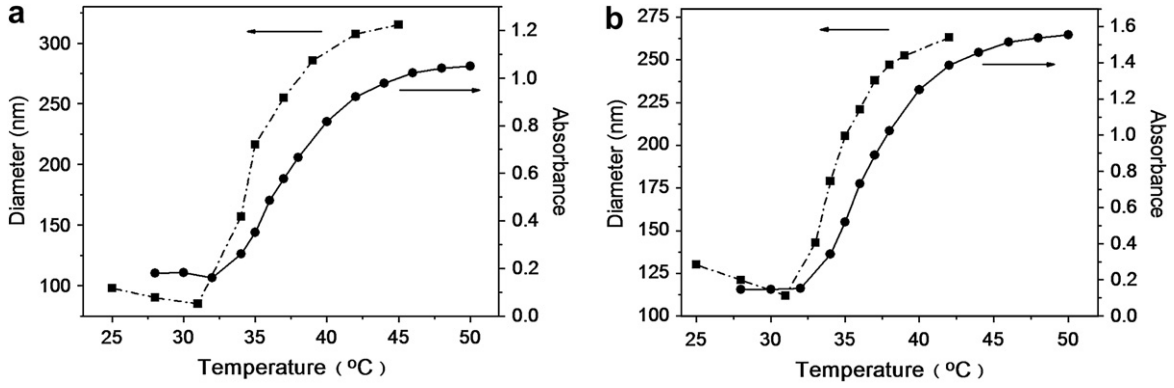


Fig. 8. Thermo-sensitive behaviors of the copolymer micelles upon temperature changes: real lines represented as absorbance variation at 542 nm in UV spectroscopy and broken lines represented as diameter changes as a function of temperature. Fixed angle = 90° for polymer-a (a) and polymer-b (b).

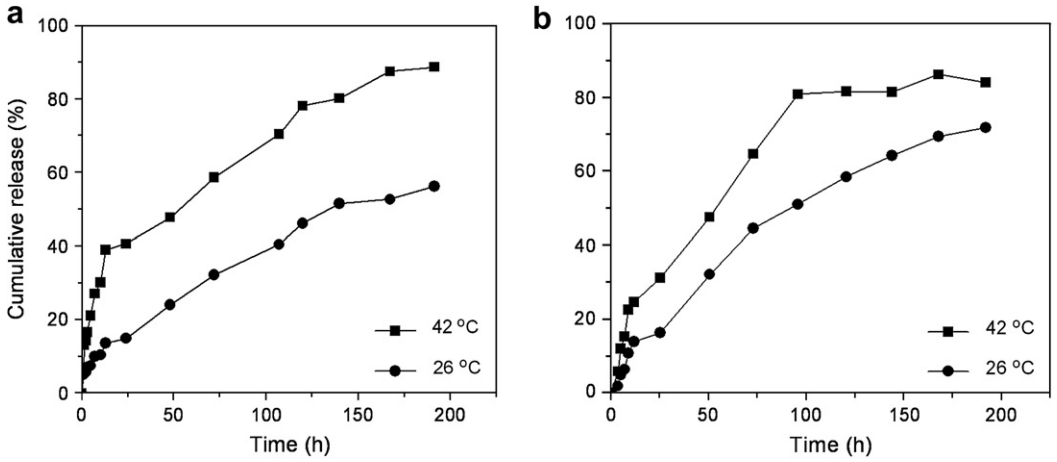


Fig. 9. Cumulative drug release profiles of the polymer-a (a) and polymer-b (b) at 26 °C or 42 °C.

PNIPAAm segment at the temperature below the LCST would stabilize the drug loaded in micellar cores. When the temperature rose up to 42 °C (above the LCST), an obvious acceleration of drug release occurred. For instance, 39% drug in the starting 13 h and 89% drug during 8 days for polymer-a was released at 42 °C as reflected in Fig. 9(a). Similar acceleration phenomenon was also found for polymer-b. The drastically different MTX release behavior indicated that the nano-sized micelles loaded with MTX would show a slow/fast thermo-sensitive switching behavior for drug release upon temperature change around LCST. Similar results were reported in our previous studies [36,37] as well as others [38]. Obviously the transformation of the PNIPAAm shell from hydrophilic to hydrophobic phase through the LCST should account for the complex thermo-sensitive release process. Generally speaking, this phase transition would provide a circumstance with enlarged hydrophobic volume, which may retard the drug molecules' diffusion into the aqueous solution. However, the phase transition of micelle also resulted in the shrinkage of PNIPAAm chains and the destruction of the steady micelle structure, which may lead to the expulsion of the loaded drug. Although the release mechanism is not explicit until now, it is more likely that the factor of structural changes of the micelles resulted from micelle deformation is dominating with respect to the drug release behavior, which was also reflected from thermo-sensitive size changes of micelles in Fig. 8.

4. Conclusions

Novel thermo-sensitive star-shaped copolymers PNIPAAm–CA–$(PCL)_3$ were designed and synthesized. The LCSTs of resulted copolymers in water are close to the nominal physiologic temperature of around 37 °C. These amphiphilic copolymers are able to self-assemble into nano-sized micelles in aqueous solution at temperature below the LCST. The copolymers exhibit no apparent cytotoxicity. Moreover, the micelles can effectively load hydrophobic MTX and show a fast/slow thermo-sensitive switching behavior for drug release due to the temperature-responsive phase transition of micelles, which endows them good potential as intelligent drug delivery systems.

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