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Synthesis of Stimuli Responsive Graft Triblock Polymers *via* Combination of Reversible Addition-Fragmentation Chain Transfer Polymerization and Ring Opening Polymerization

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A well-defined triblock copolymer, poly(ethylene glycol)-*b*-poly(2-hydroxyethyl methacrylate-*g*-lactide)-*b*-poly(N-isopropylacrylamide) [PEG-P(HEMA-PLA)-PNIPAM], containing a biodegradable PLA block and a thermo-sensitive PNIPAM block, was synthesized by a combination of ring opening polymerization (ROP) and reversible addition-fragmentation chain transfer polymerization (RAFT). The copolymer could self-assemble into core-shell micelles with PLA block as the core and PEG/PNIPAM blocks as the mixed shell at ambient temperature. With increasing temperature, the micelles converted into the core-shell-corona (CSC) structure because of the collapsed PNIPAM block. Dynamic light scattering shows that the hydrodynamic diameter (D_h) of the micelles is about 140 nm at low temperature (25 ºC) and 110 nm at high temperature (45 ºC).

Key Words: Stimuli-responsive polymer, Reversible addition-fragmentation chain transfer polymerization, Ring opening polymerization, Core-shell-corona structure, Graft copolymers.

INTRODUCTION

Polymeric micelles have attracted great attention as drug carrier and gene delivery in recent years, because they possess many advantages over others[1-5]. Since the advanced polymerization techniques such as controlled radical polymerization method were developed, more kinds of precise and tunable structures have been self-assembled by block copolymers. Especially, the stimuli-responsive polymeric micelles, which can respond to environmental stimuli such as pH, temperature and ionic strength, are well investigated systems for controlled drug release.

Temperature sensitivity is one of the interesting properties in stimuli-responsive polymers. Poly(N-isopropylacrylamide) (PNIPAM), an extensively investigated thermo-responsive polymer, possesses a lower critical solution temperature (LCST) of *ca.* 32 ºC in aqueous solution[6]. In addition, the LCST of PNIPAM-based polymers can be tuned *via* copolymerization with hydrophilic or hydrophobic polymers[7,8]. Many PNIPAM containing copolymers have been prepared and self-assemble into thermo-responsive micelles as drug carriers[9-12]. Wang *et al.*[11], synthesized triblock copolymers of PCL-PNIPAM-PCL and prepared micelles with a biodegradable PCL core and thermo-responsive PNIPAM corona for hydrophobic drug release.

However, thermo-sensitive micelles formed by self-assembly of diblock copolymers is limited due to the possibility of inter-micellar aggregation at pH 7.4 in human surroundings when the temperature is raised above the LCST of the polymer. The problem can be solved by using triblock copolymer and complex diblock copolymers containing another hydrophilic segment to stabilize the micelles at high temperature[13-15]. Triblock copolymer with a hydrophobic block and two hydrophilic blocks linked in sequence often self-assemble into core-shell-corona (CSC) micelles with a three-layered structure[16-18] and that with two outer hydrophilic blocks and one inner hydrophobic block could form more kinds of special morphologies to offer additional properties[19-22].

Herein, a well-defined graft triblock copolymer, poly-(ethylene glycol)-*b*-poly(2-hydroxyethyl methacrylate-*g*-lactide)-*b*-poly(N-isopropylacrylamide), [PEG-P(HEMA-PLA)-PNIPAM], was synthesized by a combination of ring opening polymerization (ROP) and reversible addition-fragmentation chain transfer polymerization (RAFT) (Fig. 1). The copolymer was self-assembled into the core-shell micelles with the biodegradable PLA as the core and PEG/PNIPAM as the mixed shell in aqueous solution at 25 ºC. When the temperature was raised up to the LCST of PNIPAM, the core-shell micelles converted into CSC micelles due to the collapse of PNIPAM block onto the PLA core and the soluble PEG chain could stretch out the PNIPAM shell to avoid aggregation.

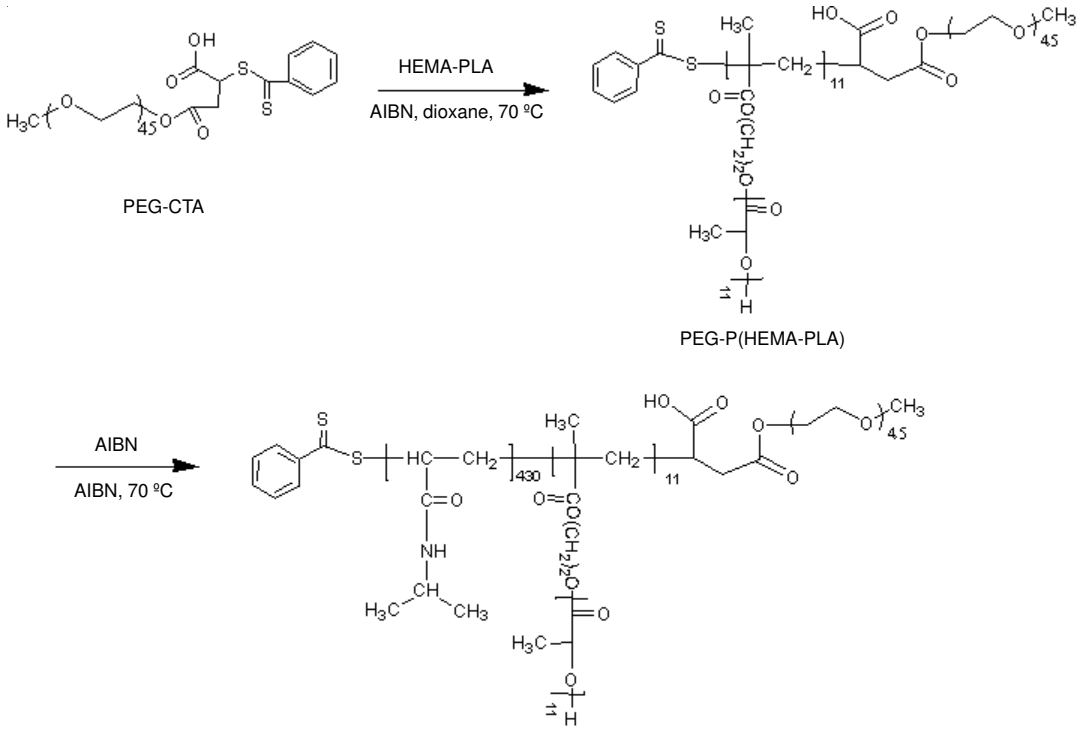

Fig. 1. Outline of synthesis of PEG-P(HEMA-PLA)-PNIPAM by ROP and RAFT polymerization

EXPERIMENTAL

D,L-Lactide (97 %, Alfa) was purified by recrystallization from ethyl acetate twice and dried in a vacuum prior to use. N-Isopropylacrylamide (NIPAM) (97 %, Aldrich) was purified by recrystallization from hexane and dried in a vacuum prior to use. Methoxy poly(ethylene glycol) (mPEG) (M_w = 2,000 and polydispersity index (PDI) = 1.05, Aldrich) was dried in a vacuum for 24 h prior to use. 2-Hydroxyethyl methacrylate (HEMA) (Alfa) was distilled under reduced pressure. 2,2'-Azobisisobutyronitrile (AIBN) was purified by recrystallization from ethanol twice and dried in a vacuum. Dithiobenzoic acid (DTBA) was prepared according to the previous literature[23]. Stannous octoate [$Sn(Oct)_2$] was used without further purification. The dialysis bag was purchased from Beijing Dingguo Biotech Co. (Molecular weight cut-off (MWCO): 7,000 Da and 14,000 Da). All other solvents were redistilled before use.

General procedure

Synthesis of HEMA-PLA macro-monomer: Macromonomer of 2-hydroxyethyl methacrylate-terminated D,L-lactide was synthesized by ROP, using HEMA as the initiator and $Sn(Oct)_2$ as the catalyst[24]. The details are as follows: a mixture of D,L-lactide (9.7 g, 67.4 mmol), HEMA (2.07 g, 15.9 mmol) and $Sn(Oct)_2$ (88.4 mg, 0.218 mmol) was added into a flask and then degassed by three freeze-vacuum-thaw cycles. The mixture was maintained at 130 ºC for 4 h under nitrogen atmosphere and dissolved in tetrahydrofuran and precipitated in cold water and dried in vacuum. ^{1}H NMR ($CDCl_3$, 400 MHz, TMS), 6.11-6.20 (1H, *cis*), 5.58-5.64 (1H, *trans*), 5.10-5.35 (1H, -CH). The molecular weight of HEMA-PLA was characterized by ^{1}H NMR and the number average molecular weight (M_n) was about 820 calculated by the integration ratio of 6.11-6.20 (1H, *cis*) and 5.10-5.35 (1H, -CH).

Synthesis of marco-PEG chain transfer agent: PEG-CTA was synthesized according to the literature with some modifications to the purification procedure[25]. Briefly, PEG-OH (10.0 g, 5.0 mmol) was dissolved in dry toluene (20 mL) and then maleic anhydride (MAh) (3.4 g, 34.5 mmol) was added. The reaction was stirred at 60 ºC for 48 h. After removal of the toluene, the mixture was dissolved in CH_2Cl_2 and precipitated into an excess of diethyl ether to remove the unreacted MAh. The PEG-MAh (4.0 g, 2.0 mmol) and DTBA (3.1 g, 20 mmol) were dissolved in CCl_4 (30 mL) in a flask and degassed through five freeze-vacuum-thaw cycles. The solution was maintained at 65 ºC for 24 h and precipitated into diethyl ether and dried in a vacuum.

Synthesis of PEG-P(HEMA-PLA): HEMA-PLA (2.01 g, 2.46 mmol), PEG-CTA (0.20 g, 0.10 mmol) and AIBN (6.1 mg, 0.037 mmol) were dissolved in 1,4-dioxane in a flask. The solution was degassed by three freeze-vacuum-thaw cycles. The polymerization was carried out at 70 ºC for 48 h. The solution was added dropwise into a large excess of diethyl ether and the precipitate was washed with methanol to remove the homopolymer. The precipitate was dried under a vacuum to a constant weight.

Synthesis of PEG-P(HEMA-PLA)-PNIPAM: PEG-P(HEMA-PLA) (0.495 g, 24.8 μmol), NIPAM (1.00 g, 8.85 mmol) and AIBN (1.0 mg, 6.1 μmol) were dissolved into 1,4-dioxane in a flask. The solution was degassed by three freeze-vacuum-thaw cycles. The polymerization was carried out at 70 ºC for 60 h and then cooled by iced water. The triblock copolymer was purified by dialysis against distilled water for 4 days (MWCO: 14,000) and recovered by freeze-drying.

Preparation of polymeric micelles: PEG-P(HEMA-PLA)-PNIPAM was first dissolved in DMF and stirred overnight to

form the original polymer/DMF solution with a concentration of 1.0 g/L at room temperature. Subsequently, deionized water was added slowly (*ca.* 10 µL every 10 s) into polymer/DMF solution with vigorous stirring until turbidity appeared. The solution was strongly stirred overnight to avoid the appearance of transient morphologies and eliminate the effect of the rate of stirring. After that, an excess of distilled water was rapidly added to the solution. The resultant solution was placed into a dialysis bag and dialyzed against water to remove DMF. Final concentration of the polymer was 0.1 g/L.

Detection method: ^{1}H NMR measurements were performed on a Varian UNITY-plus 400 M nuclear magnetic resonance spectrometer using $CDCl_3$ as solvent. The number-average molecular weight (M_n), weight-average molecular weight (M_w) and polydispersity (M_w/M_n) of the polymers were determined by gel permeation chromatograph (GPC) at 35 ºC with a Waters 1525 chromatograph equipped with a Waters 2414 refractive index detector. THF was used as the mobile phase at a flow rate of 1 mL/min. Polystyrene standards was employed for calibration. Steady-state fluorescence spectra were obtained on a Hitachi F-4600 spectrofluorometer (Japan). Dynamic light scattering (DLS) measurements were performed on a laser light scattering spectrometer (BI-200SM) equipped with a digital correlator (BI-9000AT) at 636 nm at given temperature. Transmission electron microscopy (TEM) measurements were conducted using a Philips T20ST electron microscopy at an acceleration voltage of 200 kV.

RESULTS AND DISCUSSION

Polymer synthesis: The polymerization of NIPAM and LA monomers generally undergoes two different polymerization mechanisms (free radical polymerization and ROP). RAFT and atom transfer radical polymerization (ATRP) are feasible for controlling the polymerization of NIPAM[26,27] and HEMA[28]. The RAFT technique could be more advantageous over ATRP for controlled drug release, because a metal catalyst is not required which might carry a risk in the drug release process, while certain RAFT agents have low level cytotoxicity[29]. The triblock copolymer containing PEG, PLA and PNIPAM is difficult to synthesize *via* one polymerization technique, especially the copolymer with two outer hydrophilic blocks and an inner long hydrophobic block. PEG-P(HEMA-PLA)-PNIPAM was synthesized by ROP and RAFT polymerization starting from PEG-based chain transfer agent (Fig. 1). The first step was to prepare PEG-CTA. At the same time, HEMA-PLA was synthesized by ROP, using HEMA as an initiator and $Sn(Oct)_2$ as a catalyst and the PLA length could be controlled by the ratio of D,L-lactide to HEMA (Fig. 1). Second, the graft copolymer PEG-P(HEMA-PLA) was synthesized by RAFT polymerization, which used PEG-CTA as a chain transfer agent, AIBN as the initiator and 1,4-dioxane as the solvent. The ratio of HEMA-PLA to PEG-CTA (mol:mol = 25:1) was precisely modulated to control the chain length of HEMA-PLA. Third, the PEG-P(HEMA-PLA) was taken as a macro-RAFT agent to complete the polymerization of NIPAM monomer with a ratio of 400:1:0.25 (mol/mol/mol) for feed monomer: macro-RAFT agent: initiator.

Fig. 2 shows a comparison of 1H NMR spectra of PEG-CTA, PEG-P(HEMA-PLA) diblock copolymer and PEG-P(HEMA-PLA)-PNIPAM triblock copolymer in $CDCl_3$. The degree of polymerization (DP) of PHEMA block was *ca.* 11 and it was calculated from the ^{1}H NMR, which was calculated by the integration ratio of c (4.3 ppm) and e (3.6-3.8 ppm), assigned to the pendent ethylene protons of P(HEMA-PLA) and the ethylene protons of the PEG backbone. The degree of polymerization of PNIPAM block was about 430, based on the integration ratio of b (4.0 ppm) and e (3.6-3.8 ppm) and assigned to the pendent methine protons of PNIPAM and the ethylene protons of PEG backbone. Besides, the degree of polymerization of PLA was *ca.* 11, calculated by the methine protons of PLA and alkene protons of HEMA. The triblock copolymer could be denoted as PEG_{45}-P(HEMA-$PLA_{11})_{11}$-$PNIPAM_{430}$, where the subscripts indicate the number of repeating units. The polydispersity indices (PDIs) of the diblock copolymer and triblock copolymer measured by gel permeation chromatograph (GPC) using THF as the eluent were 1.47 and 1.35, respectively (Fig. 3). Table-1 summarized the react conditions and the results for the block copolymers.

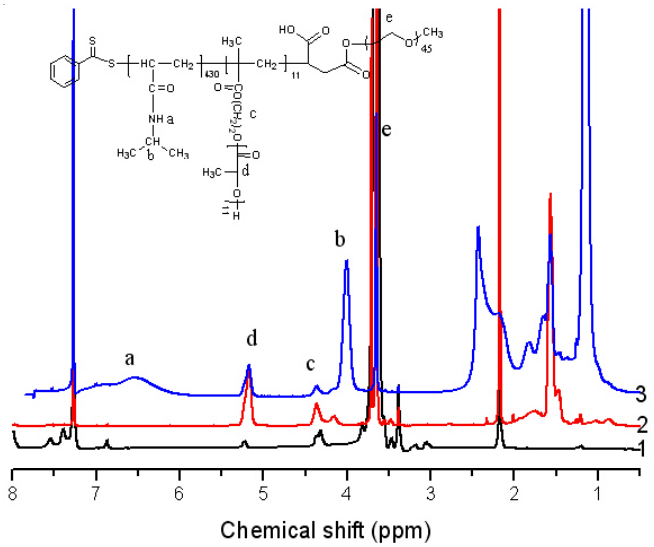

Fig. 2. ^{1}H NMR spectra of PEG-CTA (curve 1), PEG-P(HEMA-PLA) (curve 2) and PEG-P(HEMA-PLA)-PNIPAM (curve 3) taken in $CDCl_3$

Formation and characterizations of the micelles: It is well known that the amphiphilic block copolymers could form micelles though self-assembling. The triblock copolymer PEG-P(HEMA-PLA)-PNIPAM are composed of hydrophilic PEG as well as PNIPAM and hydrophobic PLA. Therefore, It can be self-assembled into micelles with a biodegradable PLA block as the core and PEG/PNIPAM block as the mixed shell at room temperature (lower than the LCST of PNIPAM).

The critical micelle concentration (CMC) was measured by a fluorescence technique using pyrene as a probe according to the previous literature[30]. The pyrene fluorescence intensity ratio (I_{336}/I_{334}) was plotted of against the logarithm of polymer concentration in Fig. 4. I_{336}/I_{334} is constant below a certain polymer concentration. Above this concentration, I_{336}/I_{334} increases with increasing log C. From this plot, the CMC of 1.84×10^{-2} g L^{-1} was obtained.

The size and size distribution of the micelles were measured by DLS. Fig. 5 shows the hydrodynamic diameter distributions $f(D_h)$ of the PEG-P(HEMA-PLA)-PNIPAM micelles at different

TABLE-1
REACTION CONDITIONS OF RAFT POLYMERIZATION AND CHARACTERISTICS OF POLYMERS

Sample	CTA/AIBN (mol/mol)	Temp. (°C)	Reaction time (h)	M_n[a]	M_n[b]	PDI[b]
PEG-P(HEMA-PLA)	3/1	70	48	10,200	11,300	1.47
PEG-P(HEMA-PLA)-PNIPAM	4/1	70	60	59,000	51,000	1.35

[a]Determined by ^{1}H NMR. [b]Determined by GPC.

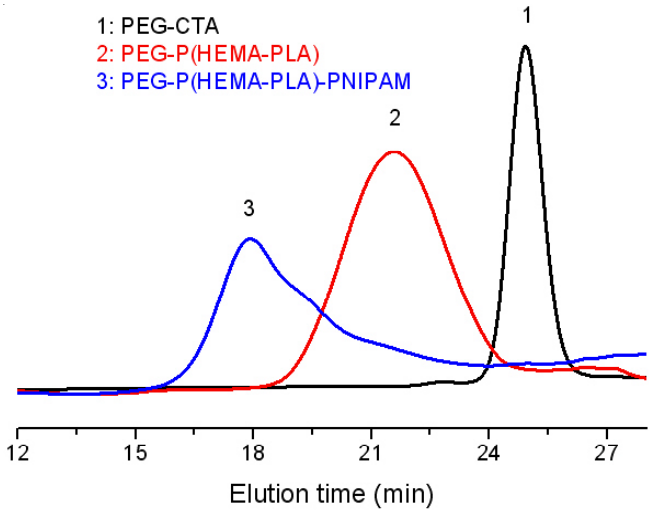

Fig. 3. GPC curves of PEG-CTA (1), PEG-P(HEMA-PLA) (2) and PEG-P(HEMA-PLA)-PNIPAM (3)

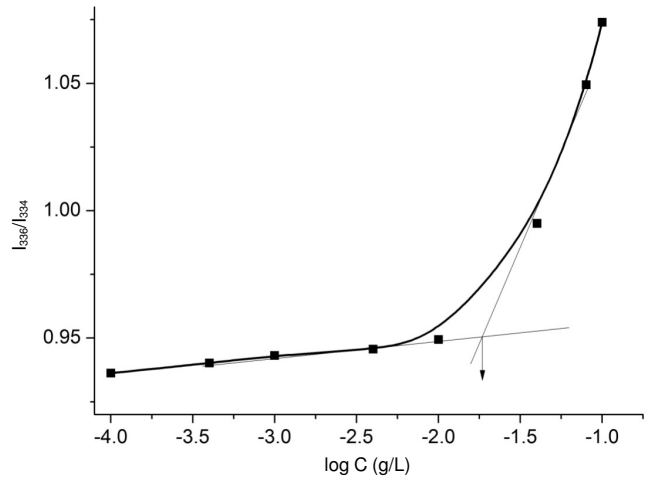

Fig. 4. Plot of I_{336}/I_{334} *versus* log C of polymeric micelles

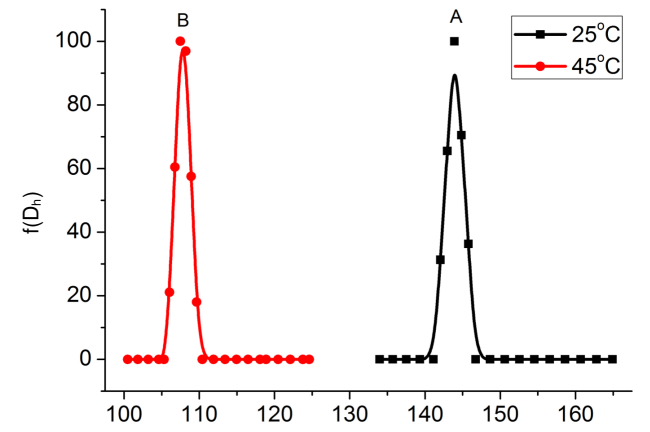

Fig. 5. Hydrodynamic diameter distributions ($f(D_h)$) of the polymeric micelles in aqueous solution at 25 °C (right) and 45 °C (left), where the polymer concentration is 0.1 g/L and the scattering angle is 90°

temperatures. Clearly, the hydrodynamic diameters (D_h) of the micelles show a narrow distribution. D_h of the micelles is 144 nm at 25 °C and 108 nm at 45 °C. With increasing temperature, the size of the micelles decreases, because PNIPAM possessing a longer block length (DP: 430) than PEG (DP: 45) collapses.

The micelle morphology and size could be studied by TEM. Fig. 6 shows the TEM images of the micelles prepared at different temperatures (25 and 45 °C). It can be seen that the micelles are generally spherical and their average diameters are around 100 nm, smaller than that measured by DLS. The reason for this can be ascribed to the different methods, *i.e.*, the DLS results show the hydrated state of the micelles in which the shell chains of the micelles remains swollen or stretched, while the TEM result is obtained from a dried state of the micelles in which the free hydrophilic chains collapse and the polymer chains are dehydrated.

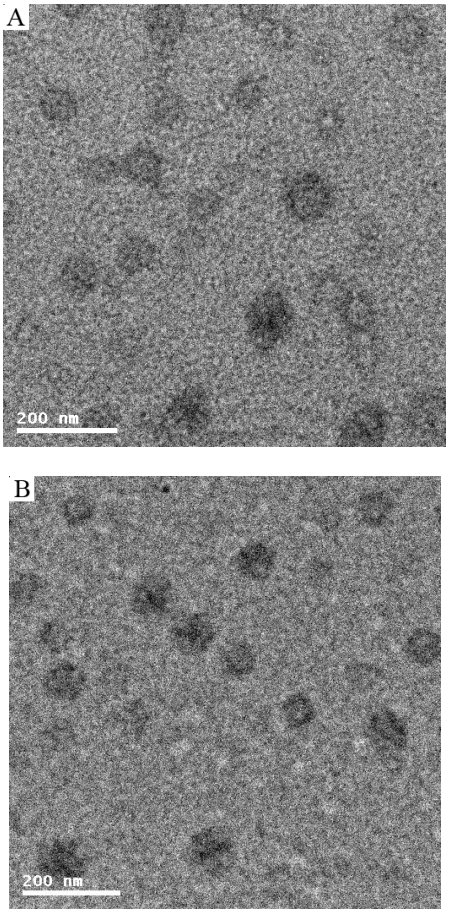

Fig. 6. Transmission electron microscope (TEM) images of the polymeric micelles at 25 °C (A) and 45 °C (B)

When the temperature increases, water progressively becomes a poor solvent for PNIPAM blocks and thus the stretched PNIPAM chain can collapse on the surface of the hydrophobic core. For the core-shell micelles of PLA-b-

PNIPAM, large aggregates can be formed when the temperature rises above the LCST of PNIPAM, because of the insolubility of both the PLA and PNIPAM blocks. However, the micelles of PEG-P(HEMA-PLA)-PNIPAM keep stable even at high temperature because of the hydrophilic PEG chain.

The thermo-responsive behaviour of the PEG-P(HEMA-PLA)-PNIPAM micelles was measured by DLS in aqueous solution at different temperatures (Fig. 7). When the temperature is below the LCST of PNIPAM, the micelles form core-shell structure with the P(HEMA-PLA) segment as the core and the PEG/PNIPAM segments as the mixed shell. DLS data shows that the micelles disperse well in water and the D_h value is *ca*. 140 nm. As the temperature increases, the D_h value decreases due to the collapse of PNIPAM. When the temperature is higher than 35 ºC, their diameters shrink to the D_h value of *ca*. 100 nm. When the temperature is above the LCST of PNIPAM, the stretched PNIPAM chain of the micelles collapses onto the biodegradable PLA core and the micelles can convert into the CSC structure with PLA as the core, the collapsed PNIPAM as the shell and the water-soluble PEG as the corona. It is worth noting that the diameter of the micelles almost becomes constant above 35 ºC, indicating that the PNIPAM block is fully collapsed and there is no aggregation of the micelles. The LCST of PNIPAM is *ca*. 33-34 ºC.

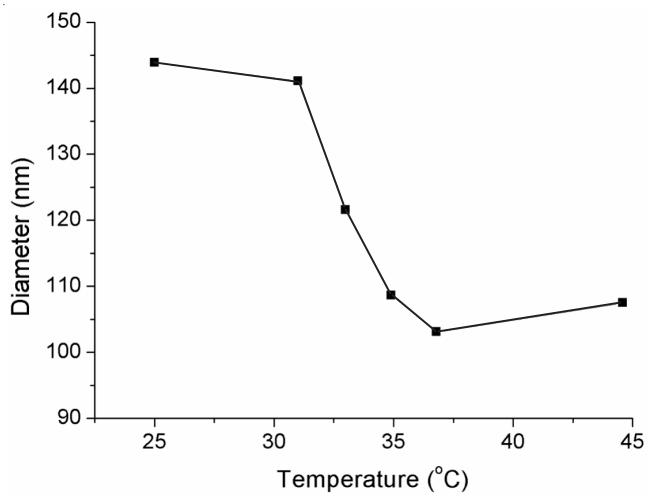

Fig. 7. Hydrodynamic diameter (D_h) of PEG-P(HEMA-PLA)-PNIPAM micelles in aqueous solution as a function of the temperature, where the polymer concentration is 0.1 g/L and the scattering angle of DLS is 90º

Conclusion

In summary, PEG-P(HEMA-PLA)-PNIPAM graft triblock copolymer containing a biodegradable PLA block, a thermo-responsive PNIPAM block and a hydrophilic PEG block was synthesized by a combination of ROP and RAFT. The copolymer could self-assemble to the core-shell micelles with PLA as the core and PEG/PNIPAM as the mixed shell at ambient temperature. Upon increasing temperature to LCST of PNIPAM, the micelles converted into the CSC structure because the PNIAPM block collapsed onto the PLA core. The hydrophilic PEG chain stretched outside from the core throughout the collapsed PNIPAM shell, which formed hydrophilic PEG channels on the PNIPAM shell.

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