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Synthesis and characterization of temperature-responsive copolymer of PELGA modified poly(*N*-isopropylacrylamide)

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Abstract

Novel amphiphilic PELGA modified temperature-responsive copolymer, [(poly(methoxyethylene glycol)-*co*-poly(lactic acid)-*co*-poly-(glycolic acid))acrylate-*co*-poly(*N*-isopropylacrylamide)-*co*-poly(*N*-hydroxymethylacrylamide)] (PELGAA-*co*-PNIPAAm-*co*-PNHMAAm) was synthesized by incorporating PELGA as the amphiphilic moiety into poly(*N*-isopropylamide) with various LA/GA ratios. Polymers obtained were characterized by FT-IR, GPC, ^{1}H-NMR and DSC. The lower critical solution temperature (LCST) of the copolymeric nanoparticles was 40 ± 0.6 °C, the critical aggregation concentration (CAC) was 18 mg L^{-1}, and reversible change in nanoparticle size related to temperature was fluctuated between 210 ± 10 and 109 ± 26 nm, while change in zeta potential of the nanoparticles was between -36 ± 6 and -26 ± 4 mV. The transmission electron microscopy (TEM) images of nanoparticles were also presented.

Keywords: Temperature-responsive; PELGA modified poly(*N*-isopropylamide); Nanoparticles

1. Introduction

Temperature-responsive polymeric nanoparticles have attracted much attention in recent years for their potential applications in targeted drug delivery, especially in delivering drug to tumor sites [1] and arthritis sites [2]. These 'intelligent' polymeric nanoparticles can undergo reversible structural transitions from a closed state to an open state with the help of external stimuli, giving on-off switches for modulated drug delivery [3,4]. It is well known that polymers that exhibit a lower critical solution temperature (LCST) transition are soluble in aqueous solutions below their LCST, but collapse and aggregate at temperatures above their LCST [5]. In this approach, the temperature-responsive polymer is systemically soluble when injected in vivo, but become insoluble and accumulated in a locally heated target site [1]. Thus the nanoparticles made of such material also have temperature-related properties, enabling thermal targeting of heated sites.

Among those polymers that can respond to external stimuli, poly(*N*-isopropylacrylamide) (PNIPAAm) hydrogel has been widely examined as an 'intelligent' drug delivery material due to its unique phase separation behavior upon external temperature changes [6,7]. Its phase transition occurs at about 32 °C and the reversibility of its transition allows repeated thermal 'switchings'. However, an obvious limitation of the normal PNIPAAm hydrogel is its poor mechanical property in a highly swollen state when used as a drug delivery device [8]. To overcome the deficiency of PNIPAAm, some hydrophobic modifications have been reported [9–13]. However, nanoparticles prepared by such materials are mainly used for hydrophobic drug loading [2,14,15]. There were few reports focusing on incorporating an amphiphilic moiety into PNIPAAm backbone and on the preparation of copolymeric nanoparticles as temperature-responsive carriers to load hydrophobic, amphiphilic, as well as hydrophilic drugs.

Synthetic copolymers such as [poly(methoxyethylene glycol)-*co*-poly(lactic acid)-*co*-poly(glycolic acid)] (PELGA) have been commonly introduced into medical materials, especially in the field of drug delivery systems because of their amphiphilicity and biodegradability [16,17]. Here, by

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incorporating PELGA as the amphiphilic moiety into PNIPAAm, a novel amphiphilic PELGA modified temperature-responsive copolymer, [(poly(methoxyethylene glycol)-*co*-poly(lactic acid)-*co*-poly-(glycolic acid)) acrylate-*co*-poly(*N*-isopropylacrylamide)-*co*-poly(*N*-hydroxylmethylacrylamide)] (PELGAA-*co*-PNIPAAm-*co*-PNHMAAm), was obtained. In this paper, we reported for the first time the synthesis and characterization of this novel copolymer. The structure of the copolymer was investigated via FT-IR, GPC, ^{1}H-NMR and DSC. Some properties of nanoparticles prepared from the copolymer such as LCST, critical aggregation concentration (CAC), reversible changes in size and zeta potential related to temperature and transmission electron microscopy (TEM) were also investigated.

2. Experimental part

2.1. Material

N-Isopropylacrylamide (NIPAAm) (Acros Organics) was recrystallized twice from hexane before use. Monomethoxypoly(ethylene glycol) (mPEG)(Av. Mol. Wt.:2000, SIGMA), Stannous octoate and acryloyl chloride (both from Aldrich), and Benzoyl peroxide (BPO) (Chengdu Kelong Chemicals, China) were used as received. D,L-lactide (DLLA) and glycolide (GA) were purchased from Shandong Medical Equipments Corp., China and were purified by recrystallization for four times. *N*-hydroxymethylacrylamide (NHMAAm) (obtained from Tianjing Shuangze Chemical Tech. Corp., China) was used after recrystallization from chloroform.

2.2. Synthesis of PELGA and PELGAA

PELGA was synthesized by ring opening copolymerisation [18]. Briefly, LA (4.0 g), GA (1.0 g), $mPEG_{2000}$ (0.56 g) and 0.05% (w/w) stannous octoate were mixed and maintained at 170 °C for 4 h under constant vacuum. The synthesized PELGA (LA/GA = 80/20) was dissolved in dichloromethane and precipitated twice in methanol. The resultant jelly precipitation was dried under vacuum and dissolved in anhydrous tetrahydrofuran. Acryloyl chloride was dripped into the above polymeric solution at 0 °C. After stirring for 4 h at room temperature, the PELGAA was recovered by precipitation thrice into anhydrous ether to remove the un-reacted acryloyl chloride and dried under vacuum. PELGAA (LA/GA = 70/30, 60/40) were prepared with the corresponding feeding ratio using the same method.

2.3. Synthesis of PELGAA-co-PNIPAAm-co-PNHMAAm

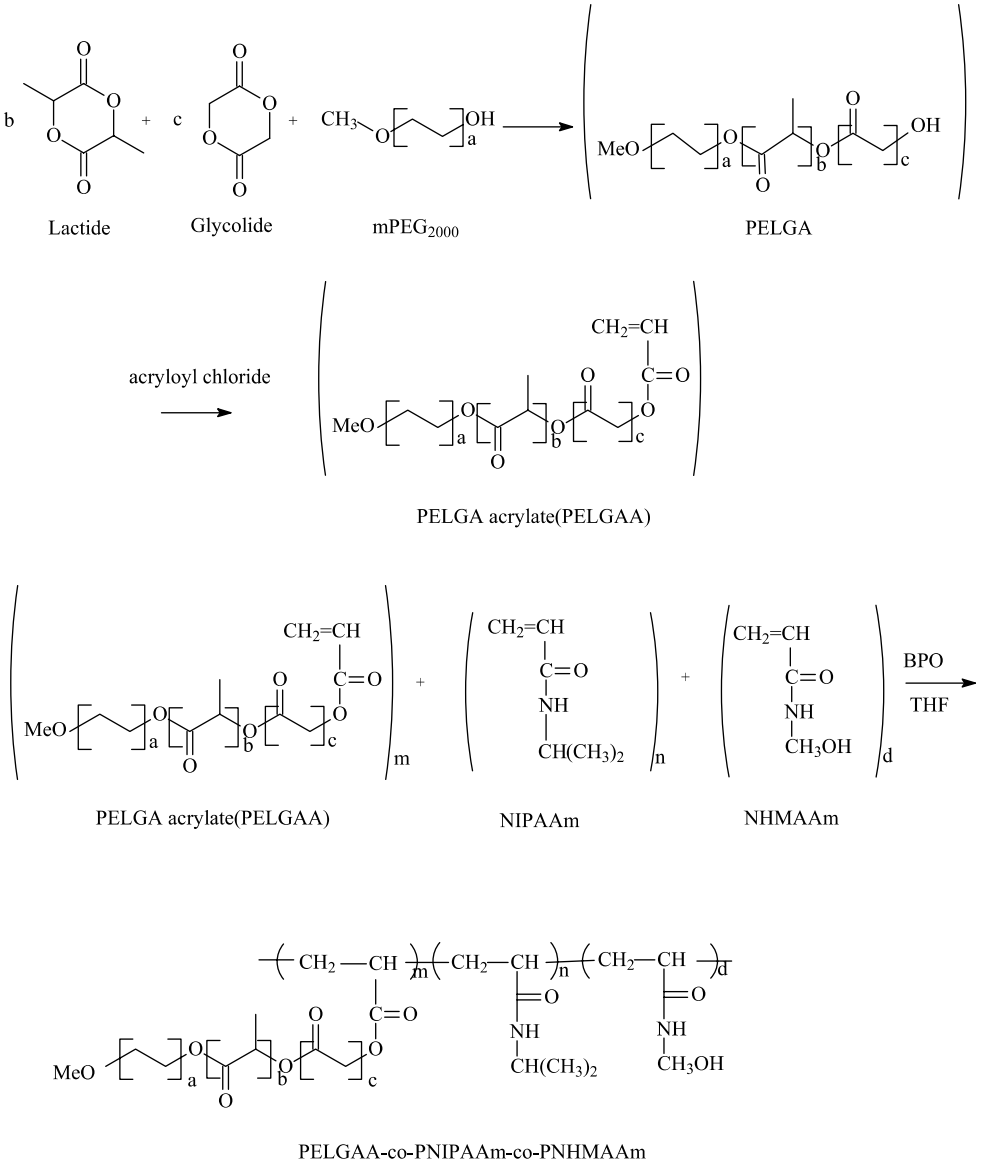
The synthesis route of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm is shown in Scheme 1. As an example, PELGAA (LA/GA = 80/20) (0.40 g), NIPAAm (1.60 g) and NHMAm (0.112 g) were dissolved in anhydrous tetrahydrofuran. After 30 min nitrogen bubbling, BPO (10.6 mg) was introduced as an initiator. The reaction mixture was kept stirring at 55 °C under nitrogen for 24 h. Then the reaction solution was dripped twice in an excessive amount of anhydrous ether to remove un-reacted monomers and the catalyst. The precipitates were dried under vacuum. The dried polymer was dissolved in tetrahydrofuran (THF) and dialyzed against distilled water using a dialysis membrane with 8000 molecular weight cut-off, the goal copolymer as white needles was obtained using lyophilization. The copolymers of other feeding ratio (LA/GA = 70/30 and 60/40) were also prepared with the same method. Poly(*N*-isopropylacrylamide-*co*-*N*-hydroxyl-methylacrylamide) (P(NIPAAm-*co*-NHMAAm)) copolymer was also synthesized as described previously and dialyzed using a dialysis membrane with 1000 molecular weight cut-off.

2.4. Preparation of polymeric nanoparticles

The copolymeric nanoparticles of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm formed spontaneously when the copolymer THF solution was added into water [19]. Briefly, the amphiphilic copolymers (50 mg) were dissolved in 1.5 ml of anhydrous tetrahydrofuran, and then the solution was added into water (4–5 ml) and sonicated for 10 min. The organic solvent was evaporated off and water was added until the polymer concentration reached 0.5 wt%.

2.5. Characterization

The ^{1}H-NMR spectra were recorded with an UNITY INOVA-400 NMR spectrometer using tetramethylsilane (TMS) as the internal reference (Varian, USA). FT-IR spectra of PELGAA-*co*-PNIPAAm-*co*-PNIHAAm and related copolymers were recorded on a Perkin–Elmer spectrum using the KBr pellet technique. The number-average and mass-average relative molecular masses were determined by gel permeation chromatography (GPC) (HP 1100) equipped with PLgel-800 columns and RI refraction index detector, using THF as eluent at flow rate of 1.0 ml min^{-1} at 30 °C against polystyrene standards. The difference scanning calorimetry (DSC) was performed on DSC6200 instrument (Seiko, Japan) with a heating rate of 10 °C min^{-1}. The LCSTs of amphiphilic copolymers were measured by optical methods. A UV–visible spectrophotometer (GBC cintra10e, Australia) equipped with a temperature controller was used to trace the phase transition of the copolymer by monitoring the transmittance at 500 nm. The heating rate was 0.5 °C min^{-1}. The hydrodynamic diameters and zeta potentials of the copolymeric nanoparticles were measured by dynamic laser scanning (DLS) with a Malvern Zetasizer nano-ZS90 at a wavelength of 633 nm and scattering angle of 90°. Fluorescence spectra were recorded on a spectrofluorometer (RF-5301PC, Shimadzu Corp., Japan). Pyrene was used as a hydrophobic fluorescent probe. These samples containing pyrene (6×10^{-7} mol l^{-1}) were kept at 20 °C for 24 h to equilibration before measurements. Excitation was carried out at 340 nm, and emission spectra were recorded from 350 to 600 nm. The ratio of I_{374}/I_{384} intensity (height) was monitored as a function of polymer concentration. Transmission electron microscopy (TEM) images were obtained using JEM-instrument (Hitachi H-600, Japan) at an



Scheme 1. Synthetic route of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm amphiphilic random copolymer.

acceleration voltage of 100 kV. All the concentrations of copolymers measured were 0.5 wt%.

3. Results and discussion

3.1. Polymer synthesis

The synthesis of well-defined random copolymers is critical for the preparation of the novel biomaterials. The aim of our study is to synthesize a novel copolymer that can not only respond to temperature, but also can be used as a carrier to deliver hydrophobic, amphiphilc and hydrophilic agents to target organs.

NHMAAm was used as a functional monomer as well as a component to adjust the LCST of the goal copolymers [2,14]. The weight ratios between LA and GA in copolymers were designed as 80/20, 70/30 and 60/40, respectively, to meet the requirements of different drugs with various hydrophilic/lipophilic coefficients. PELGAA-*co*-PNIPAAm-*co*-PNHMAAm was obtained as white needles with 58–64% yields after lyophilization. The synthesized PNIPAAm, P(NIPAAm-*co*-NHMAAm) and PELGAA in our laboratory could be easily dissolved in $CHCl_3$ and DMSO, while the goal copolymer of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm could not be dissolved in $CHCl_3$ at all, however, it could be dissolved in DMSO. The difference in solubility might be attributed to the conformational change of the polymers [8]. The loose clustering of P(NIPAAm-*co*-NHMAAm) and PELGAA units leads to the flexibility of polymers in $CHCl_3$, then the solubility in $CHCl_3$. After PELGAA-*co*-PNIPAAm-*co*-PNHMAAm is formed, the entanglement of segments chains restricts the sharp conformational change; its PNIPAAm bone chain attached with PELGAA could not stretch their linear chains into $CHCl_3$ as before, resulting in the insolubility of the copolymer in $CHCl_3$. The differences in physical properties among PELGAA-*co*-PNIPAAm-*co*-PNHMAAm, PNIPAAm and

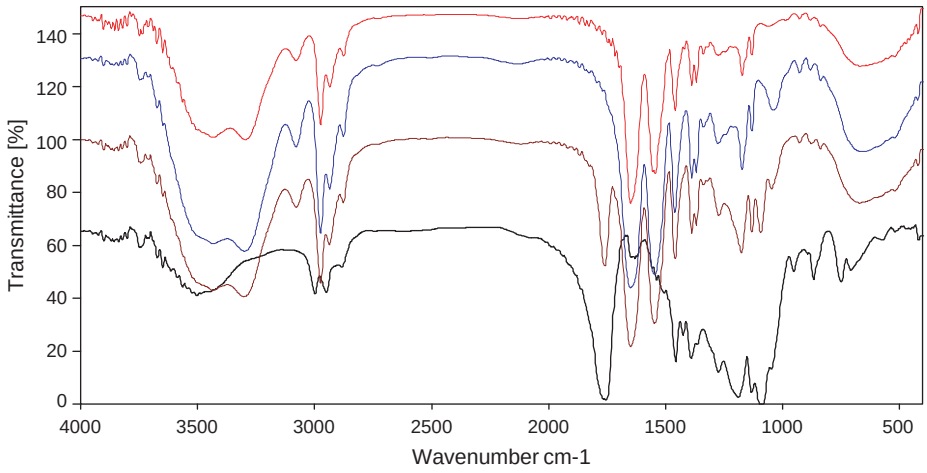


Fig. 1. IR spectra of copolymer of PNIPAAm, P(NIPAAm-*co*-NHMAAm), PELGAA-*co*-PNIPAAm-*co*-PNHMAAm PELGAA(LA/GA = 80:20) (from top to bottom).

P(NIPAAm-*co*-NHMAAm) confirmed the successful preparation of the goal copolymer to some extent.

IR spectra of the PNIPAAm, P(NIPAAm-*co*-NHMAAm) and PELGAA-*co*-PNIPAAm-*co*-PNHMAAm were shown in Fig. 1. In the spectrum of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm copolymer, in addition to the characteristic stretching vibrations of amide I and II (at 1649 and 1544 cm^{-1}) corresponding to PNIPAAm and P(NIPAAm-*co*-NHMAAm), peaks at 3507 and 1047 cm^{-1} were attributed to hydroxyl groups of NHMAAm, and peaks at 1757 cm^{-1} was assigned to the ester bond of PELGAA. All the bands of amides, ester and hydroxyl groups were found in the spectra of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm copolymer [20]. The peaks at 2988 cm^{-1} assigning to alkenes stretching vibration of carbon–hydrogen of PELGAA had disappeared in the spectra of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm because of polymerization, where the intensity of the peak at 952 cm^{-1} also decreased compared with that of PELGAA. The results testified the occurrence of copolymerization.

The ^{1}H-NMR of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm copolymer displayed two well-separated peaks at δ 3.8 and 4.5 ppm, which were assigned to NIPAAm (C*H*Me) and NHMAAm (CH_2OH), respectively. The integration ratio of these two peaks is approximately 12.2:1, which is equal to the feed ratios (12.5:1). Also, a singlet at δ 3.4 ppm was attributed to ethylene groups of PEG and the multibelts at δ 4.8 and 5.1 ppm were assigned to GA and LA. Besides that, there were characteristic peaks at δ 3.8 ppm (C*H*Me of NIPAAm) and 4.5 ppm (CH_2OH of NHMAAm). The entire spectrum above suggested the target copolymer had been synthesized.

The thermal properties for P(NIPAAm-*co*-NHMAAm) copolymers were determined by DSC. The differences of T_g and $T_{m,onset}$ of PNIPAAm, P(NIPAAm-*co*-NHMAAm), PELGAA-*co*-PNIPAAm-*co*-PNHMAAm also confirmed the successful synthesis of the goal copolymer. Along with the higher GA portions in the goal copolymer, T_g decreased from 35.7 to 34.5 °C while the $T_{m,onset}$ increased from 51.4 to 75.6 °C. The $\bar{M}_n$ of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm ranged from 13,300 to 14,700 g mol^{-1}. The small value of polydispersity indexes ($\bar{M}_w/\bar{M}_n$) (PDI) (with a range of 1.20–1.26) demonstrated that the copolymers had narrow molecular weight distribution. The yields, $\bar{M}_n$, the PDI, T_g, $T_{m,onset}$ and other properties of the copolymers were shown in Tables 1 and 2. Based on the ratio of $\bar{M}_n$ of PELGAA to that of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm, every random copolymer molecule contained approximately six NIPAAm and 0.5 NHMAAm units.

3.2. LCSTs of copolymeric nanoparticles

In order to adjust the phase transition temperature of PNIPAAm towards tumor temperature, a highly hydrophilic group, NHMAAm, was introduced in PNIPAAm to raise the LCST to a higher value for the reason of strengthening interactions between polymeric chains and water [21]. The LCSTs of the copolymeric nanoparticles were measured by turbidimetry. LCST was defined as the temperature where the transmittance was decreased about 50%. The copolymeric nanoparticles started to precipitate as the temperature increased near LCSTs, showing the onset of turbidity.

Fig. 2 showed the thermosensitivity and the appearance change of copolymeric nanoparticles related to temperature. The LCST values of copolymeric nanoparticles with various LA/GA ratios (80:20, 70:30, 60:40) were 40.6, 40.0 and 39.5 °C, respectively. A trend could be seen from the variation in LCSTs that the phase transition temperature enhanced with the increase of the proportion of LA in the copolymer. It is important to note that the PELGAA-*co*-PNIPAAm-*co*-PNHMAAm copolymeric nanoparticles exhibited a LCST (∼40 °C) greater than physiological body temperature (T_b ∼ 37 °C), but less than the temperature of a heated tumor or a heated inflammatory site (T_h ∼ 42 °C). As novel drug carriers, these copolymers can be employed for the controlled release of anti-tumor and anti-inflammatory drugs at the target sites by a local hyperthermia method [22]. Also, the nanoparticles exhibited a sharp complete phase transformation within a very narrow temperature range of 2.5 °C. This imply that the nanoparticles have smartly and rapidly swelling and

Table 1
The physical characterization of polymer series

Samples	Ratio of LA/GA	Yields (%)[a]	$\bar{M}_n$[b] (g mol^{-1})	($\bar{M}_w/\bar{M}_n$) (PDI)[b]	T_g (°C)[c]	$T_{m,onset}$ (°C)[d]	Grafted (%)[e]
PNIPAAm	–	72	1100	1.40	–	42.1	–
P(NIPAAm-*co*-NIHAAm)	–	66	850	1.39	–	54.9	–
PELGAA-1	80:20	62	14,000	1.26	28.2	56.2	–
PELGAA-2	70:30	68	12,600	1.48	23.3	55.1	–
PELGAA-3	60:40	60	12,000	1.50	–	–	–
PELGAA-*co*-PNIPAAm-*co*-PNHMAAm-1	80:20	59	14,700	1.26	35.7	51.4	5.0
PELGAA-*co*-PNIPAAm-*co*-PNHMAAm-2	70:30	64	13,300	1.26	34.5	65.9	5.5
PELGAA-*co*-PNIPAAm-*co*-PNHMAAm-3	60:40	58	14,100	1.20	–	75.6	17.5

[a] Yield was calculated according to equation: yield (%) = $W_p/(W_1+W_2)$, where W_p, W_1 and W_2 are the weight of the final copolymer, the feeding monomer of NIPAAm or/and, or/and NHMAAm PELGAA.
[b] Measured by GPC using THF as eluent (1.0 ml min^{-1}) against polystyrene standards.
[c] Measured by DSC. T_g, abbreviation of glass transition temperature.
[d] Measured by DSC. $T_{m,onset}$, the start point in the course of melting.
[e] Calculated according to the equation: grafted (%) = $(W_1-W_2)/W_1$, where W_1 and W_2 are $\bar{M}_n$ of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm and PELGAA.

contracting properties, and the loaded drug can be released from nanoparticles as soon as possible when outer temperature surpasses LCSTs.

3.3. The reversible changes in size and zeta potential of nanoparticles

It was known that amphiphilic polymers had the ability to form nanoparticles in aqueous media [23]. In the media of water, there are three main forces (hydrogen bonding among hydrophilic segments and water molecules, the hydrogen bonding among hydrophilic chains, and the hydrophobic–hydrophobic associations among hydrophobic segments) controlling the aggregation of amphiphilic polymers [24,25]. The hydrogen bonding among hydrophilic chains tends to draw them together, whereas that of water-hydrophilic chain serves as a repulsive force to keep the hydrophilic chains away from each other and therefore stabilize the nanoparticles. That's why the goal polymeric nanoparticles keep stable below LCST. When the temperature is over LCST, for temperature-responsive amphiphilic polymers, the self-association of the hydrophobic segments becomes stronger and accordingly reduces the water solubility of the polymer [26]. This property is very useful in the field of controlled drug delivery. The goal copolymer of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm was well soluble in several organic solvents (such as DMSO, THF and acetonitrile), while not soluble in water. In order to form nanoparticles in the aqueous media, direct adding copolymer THF solution into water was a simple and effective method. The hydrophilic chains (such as NIPAAm moiety) could stretch into water, and the amphiphilic PELGAA moiety could construct inner core automatically. Because of the temperature-responsive and hydrophilic outer shell, the copolymeric nanoparticles exhibited relatively larger diameter and were more stable when outer temperature was below LCST. And when the temperature was higher than LCST, the contract behavior of temperature-responsive nanoparticles could be observed. Fig. 3(a) and (b) showed the changes in nanoparticles outer diameter and zeta potential against temperature fluctuation. The size of three types of copolymeric nanoparticles changed with the fluctuation

Table 2
The physical characterization of polymeric nanoparticles

Samples	LCST (°C)[a,b]	Range of change in mean size (nm)[c]	Mean size change (nm)[c]	Range of change in mean zeta potential (mV)[c]	Mean zeta potential change (mV)[c]
PNIPAAm	33.5	214.1±14.0 to 53.8±1.1	160.2	−13.3±4.0 to −12.1±0.8	1.2
P(NIPAAm-*co*-NIHAAm)	39.8	274.0±25.2 to 108.2±2.0	165.8	−9.0±4.0 to −13.5±1.8	4.5
PELGAA-*co*-PNIPAAm-*co*-PNHMAAm-1	40.6	210.7±3.8 to 133.6±2.1	77.1	−40.4±3.7 to −28.4±1.3	12
PELGAA-*co*-PNIPAAm-*co*-PNHMAAm-2	40.0	198.0±2.3∼108.7±1.3	89.3	−35.8±1.1 to −22.0±1.4	13.8
PELGAA-*co*-PNIPAAm-*co*-PNHMAAm-3	39.5	219.7±3.2 to 82.3±1.0	137.4	−41.0±1.3 to −26.4±1.2	14.6

[a] LCST was determined spectroscopically at 500 nm in 0.5 wt% in water media.
[b] The heating rate was controlled as 0.5 °C min^{-1}.
[c] Measured by dynamic light scanning (DLS), temperature fluctuated between 25 and 50 °C.

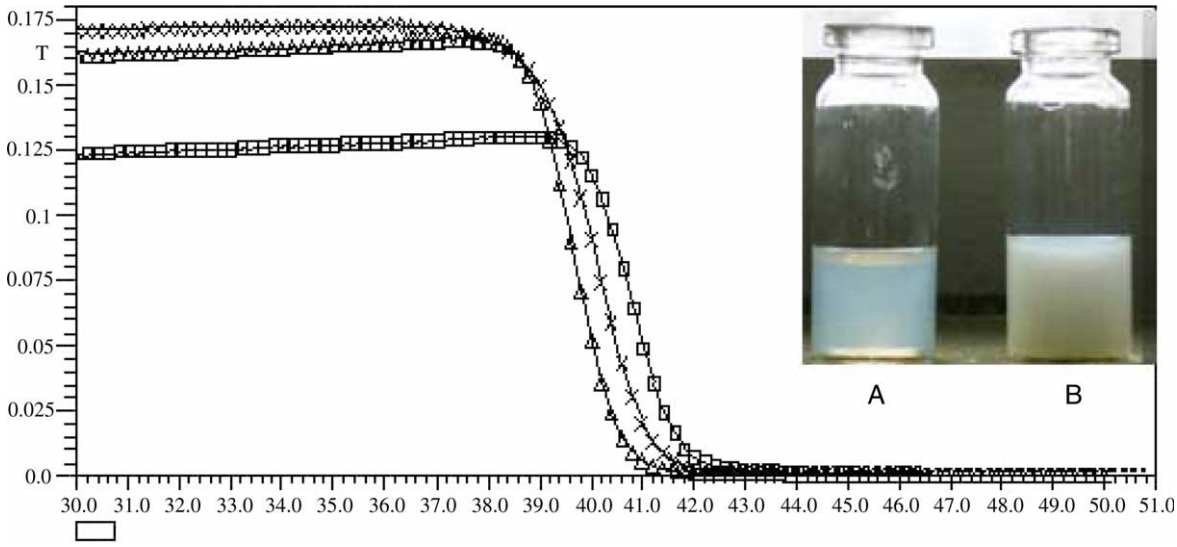


Fig. 2. The LCST curves of PELGAA-*co*-PNIPAAm-*co*-PNIHAAm copolymer nanoparticles (□, LA/GA = 80:20; ×, LA/GA = 70:30; △, LA/GA = 60:40), and the appearance change of the nanoparticles related to temperature (A, 30 °C; B, 50 °C).

of temperature and these procedures were repeatable and reversible with polydispersity index less than 0.3. Table 2 showed that at the temperature of 25 °C the mean size of copolymeric nanoparticles were 210.7 ± 3.8 nm (copolymer of LA/GA = 80:20), 198.0 ± 2.3 nm (LA/GA = 70:30) and 219.7 ± 3.2 nm (LA/GA = 60:40), respectively. When the temperature was raised to 50 °C, the nanoparticles diameters changed to 133.6 ± 2.1 nm (LA/GA = 80:20), 108.7 ± 1.3 nm (LA/GA = 70:30), and 82.3 ± 1.0 nm (LA/GA = 60:40) (n = 9), respectively. And the mean size of P(NIPAAm-*co*-NHMAAm) micelle could fluctuate between 274.0 ± 25.2 and 108.2 ± 2.0 nm (n = 9) [27]. The changes in mean size of the three types of novel copolymeric nanoparticles, and P(NIPAAm-*co*-NHMAAm) micelle were 77.1, 89.3, 137.4, and 165.8 nm, respectively. There were great differences in the changes of mean size among PNIPAAm, P(NIPAAm-*co*-NHMAAm), and PELGAA-*co*-PNIPAAm-*co*-PNHMAAm nanoparticles, which may be due to the different grafting efficiency of P(NIPAAm-*co*-NHMAAm) and the ratios of LA/GA in the copolymer of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm. Tables 1 and 2 showed that the copolymeric nanoparticles had larger range of mean size changes when the copolymer had more grafting efficiency, namely the more numbers of thermosensitive group P(NIPAAm-*co*-NHMAAm) in the copolymer. And the copolymer that had more LA in its core structure would have stronger interaction among relatively hydrophobic groups when outer temperature rose over LCST.

Both particle size and zeta potential are important physicochemical properties of particles because they determine the physical stability as well as the biopharmaceutical properties of the preparations [28]. In theory, more pronounced zeta potential values tend to stabilize particle suspensions. The electrostatic repulsion between particles with the same electrical charge prevents the aggregation of the spheres [29]. The stability of the disperse systems by non-ionic copolymers is mainly due to steric repulsion between dispersed polymer

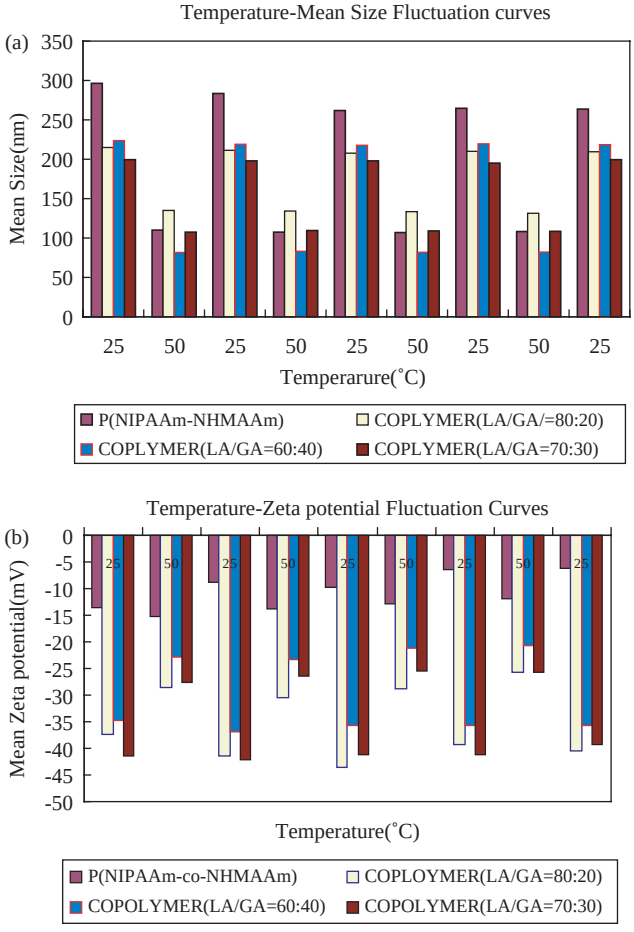


Fig. 3. (a) The temperature-hydrodynamic diameter fluctuation curves of the PELGAA-*co*-PNIPAAm-*co*-PNIHAAm series, P(NIPAAm-*co*-NHMAAm) nanoparticles (0.5 wt%). (b) The temperature-zeta potential fluctuation curves of the PELGAA-*co*-PNIPAAm-*co*-PNIHAAm series, P(NIPAAm-*co*-NHMAAm) nanoparticles (0.5 wt%).

covered particles. However, when nanoparticles bear surface charge, the adsorption of non-ionic polymers leads to changes in surface electrical properties [27,30]. The mean zeta potential results (Fig. 3(b)) showed that under the temperature of 25 °C the copolymeric nanoparticles with three LA/GA ratios and P(NIPAAm-*co*-NHMAAm) micelle exhibited a negative charge, the mean zeta potentials of copolymeric nanoparticles were -40.4 ± 3.7 mV (copolymer of LA/GA = 80:20), -35.8 ± 1.1 mV (LA/GA = 70:30) and -41.0 ± 1.3 mV (LA/GA = 60:40), respectively. As the temperature was increased to 50 °C, the value of zeta potential changed to -28.4 ± 1.3 mV (LA/GA = 80:20), -22.0 ± 1.4 mV (LA/GA = 70:30) and -26.4 ± 1.2 mV (LA/GA = 60:40) ($n=9$), respectively. And the mean zeta potential of P(NIPAAm-*co*-NHMAAm) micelle could fluctuate between -9.0 ± 4.0 mV and -13.5 ± 1.8 ($n=9$) mV. Table 2 revealed that the mean zeta potential changes of PNIPAAm and P(NIPAAm-*co*-NHMAAm) were 1.2 and 4.6 mV, respectively. While the mean zeta potential changes of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm (LA/GA = 80/20, 70/30 and 60/40) were altered from 12.0 to 14.6 mV. The differences in the range of changes in zeta potential maybe also explained by the grafting efficiency of P(NIPAAm-*co*-NHMAAm) and the ratios of LA/GA in the copolymer of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm. And this trend was consistent to that of mean size variation.

As a hydrophilic moiety, NIPAAm gave a strong interaction with outer water molecules. This experiment also demonstrated that the incorporation of PELGA into the system could elevate the negative zeta potential, possibly because the hydrophobic PELGA incorporated into P(NIPAAm-*co*-NHMAAm) could change the binding affinity of water in the aqueous solution to the surface of particles. Strategies for the development of new materials with improved biocompatibility have to be based on comprehensive studies of interfacial properties [31,32]. Positively charged surfaces of materials in contact with blood are suspicious to induce the formation of primary platelet clots [33]. It was found that the intact intima of the vascular system is negatively charged; surfaces with excellent blood compatibility may bear negatively charged groups [34]. We can conclude that the negative charge of the nanoparticles will be advantageous due to negative charge of human blood.

All these processes were reversible, and the results were summarized in Table 2. It indicated a unique feature that the shell of nanoparticles was widely spread in the space without aggregation below LCST, and the copolymeric nanoparticles could change their structure from a hydrated state below the LCST to a dehydrated state above the LCST. As we know, the interior temperature of tumor and inflammatory tissue is higher than that of normal tissues [35]. After intravenous administration, the copolymeric nanoparticles could 'burst' its entrapped drug at the targeting site.

3.4. Determination of critical aggregation concentration (CAC)

In the present study, fluorescence spectroscopy measurement was performed to investigate the formation of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm copolymeric nanoparticles. The CAC is an important parameter to evaluate the stability of amphiphilic polymeric nanoparticles. Above the CAC, amphiphilic polymeric molecules can self-assemble into an ordered structure, in which a relatively hydrophobic core is surrounded with a relatively hydrophilic shell. Amphiphilic polymers with low CAC are desirable with high stability during extreme dilution, as would be the case in the physiological environments after administration.

Fluorescence probing is a frequently used method to study the self-association of amphiphilic copolymers in water [36]. One of the most widely used probes is pyrene, whose fluorescence spectrum is sensitive to the change of polarity of the medium. At low polymer concentration, this ratio takes the value characteristic of pyrene in water, and at high concentration it takes the value of pyrene in a hydrophobic environment. The CAC is taken from the midpoint of the plot for I_{374}/I_{384} changes against polymer concentration. Our experiments were performed according to literature [14,37]. Pyrene dissolved in acetone (6.0×10^{-4} mol l^{-1}, 10 μl) was added to 10 ml of nanoparticles in aqueous media at concentrations ranging from 0.1 to 400 mg l^{-1}. These samples containing pyrene (6.0×10^{-7} mol l^{-1}) were kept for 24 h at room temperature to allow complete evaporation of acetone. Fig. 4 showed the CAC points of three ratios LA/GA nanoparticles of copolymers. The CAC of the three LA/GA ratios copolymeric nanoparticles were almost the same as 18 mg L^{-1}. These results suggested that the copolymeric nanoparticles were indeed formed in aqueous media and the CAC was very low.

From pyrene emission spectra, the intensity (peak height) ratios (I_1/I_3) of the first band (374 nm) to the third band (384 nm) were analyzed as a function of the polymer concentration [12,37]. It was demonstrated the micropolarity changes probed by pyrene at different temperatures for PNIPAAm and its hydrophobic modified PNIPAAm solutions [12]. The I_{374}/I_{384} value of pure PNIPAAm solution remained nearly unchanged at temperature below 30 °C. While for hydrophobically modified PNIPAAm showed a totally different I_{374}/I_{384} dependence upon temperature. The hydrophobic microdomains formed facilitate the solubilization of pyrene,

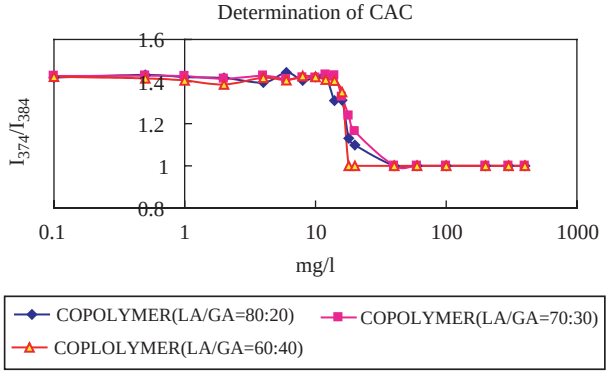


Fig. 4. Plots of relative fluorescence intensities ratio (I_{374}/I_{384}) of pyrene (6.0×10^{-7} mol l^{-1}) as a function of the PELGAA-*co*-PNIPAAm-*co*-PNIHAAm copolymeric nanoparticles concentration at 25 °C ($\lambda_{ex}=340$ nm).

generating a value of 1.4 at room temperature. This result was consistent with the value in our test. We also measured the values of I_{374}/I_{384} of pyrene in pure water. The water containing pyrene (6.0×10^{-7} mol l^{-1}) was measured at 25 °C, and the value of I_{374}/I_{384} was 1.41 too. This result was consistent with the I_{374}/I_{384} value obtained at very low polymer concentration, which takes the value characteristic of pyrene in water [38].

Although both hydrophilic and hydrophobic segment affect the CAC value, the hydrophobic segment plays a more important role [39]. The increase in the length or number of hydrophobic segment can cause a significant decrease in CAC value and increase in nanoparticles stability [2]. This might have resulted in the lower CAC value of the PELGAA-*co*-PNIPAAm-*co*-PNHMAAm copolymer. Therefore, it is anticipated that—in a normal physiological environment—after considerable dilution, the copolymeric nanoparticles are also stable, and it has enough capability to deliver the drug to the target tissue.

3.5. The transmission electron microscopy (TEM) images of nanoparticles

The size and morphology of the nanoparticles are important considerations when evaluating the possibility of their use as carriers for targeted drug delivery. Nanoparticles formation was frequently observed for the amphiphilic copolymers in aqueous solution [19]. A drop of the resultant copolymeric nanoparticles containing 0.01 (w/v) % of phosphotungstic acid was placed on a copper grid and air-dried at room temperature. The morphology of the nanoparticles was spherical (Fig. 5(a)–(c)). The diameter of the polymeric nanoparticles was found to be somewhat smaller than that measured from Malvern Zetasizer instrument. This may be due to the measuring principle of TEM instrument that assumes all the nanoparticles in the copper grip were orbicular in advance and all the measured nanoparticles in TEM were individuals.

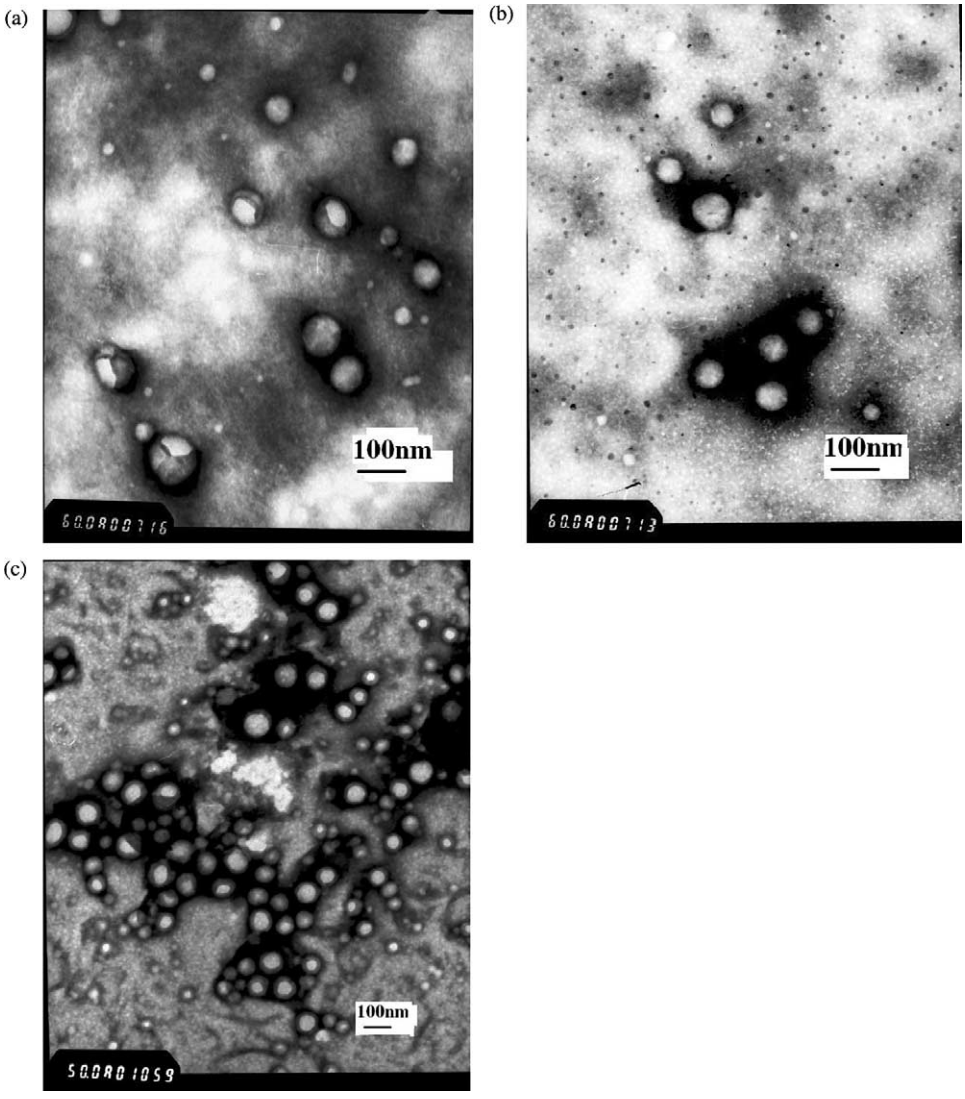


Fig. 5. TEM images of copolymer nanoparticles of PELGAA-*co*-PNIPAAm-*co*-PNIHAAm ((a), LA/GA = 80:20), ((b) LA/GA = 70:30) and ((c) LA/GA = 60:40) at an initial concentration of 0.5 wt% at magnification 60,000× (a) and (b) and 50,000× (c). Scale bar: 100 nm.

4. Conclusions

By incorporating of PELGA as an amphiphilic moiety into poly(*N*-isopropylamide), novel temperature-responsive random copolymer of PELGAA-*co*-PNIPAAm-*co*-PNHMAAm was successively synthesized. FT-IR, ^{1}H-NMR, GPC and DSC measurements confirmed the architecture of the copolymers. The $\bar{M}_n$ of the copolymer series ranged from 13,300 to 14,700 g mol^{-1} and the molecular weight distribution was narrow. The P(NIPAAm-*co*-NHMAAm) grafting ratios ranged from 5 to 17.5%. The novel copolymeric nanoparticles had appropriate LCST of 40 ± 0.6 °C higher than the normal body temperature. The hydrodynamic diameters and zeta potentials showed a reversible and repeatable procedure along with the temperature, fluctuating between 210 ± 10 and 109 ± 26 nm and between -36 ± 6 and -26 ± 4 mV, respectively. The CAC of the nanoparticles was considerable low, which was around 18 mg l^{-1}. The electronic micrographs clearly indicated formation of spherical nanoparticles. We expect that this novel temperature-responsive nanoparticle with amphiphilic block incorporated may display well prospects in targeted drug delivery systems for agents with various hydrophilic/lipophilic coefficients. Further studies addressing the drug encapsulation and releasing properties from the nanoparticles and copolymeric cytotoxicity testing are now underway.

Acknowledgements

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