

THERMOSENSITIVE POLYION COMPLEX MICELLES PREPARED BY SELF-ASSEMBLY OF TWO OPPOSITELY CHARGED DIBLOCK COPOLYMERS*

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Abstract Polyion complex (PIC) micelles were spontaneously formed in aqueous solutions through electrostatic interaction between two oppositely charged block copolymers, poly(*N*-isopropylacrylamide)-*b*-poly(L-glutamic acid) and poly(*N*-isopropylacrylamide)-*b*-poly(L-lysine). Their controlled synthesis was achieved via the ring opening polymerization of *N*-carboxyanhydrides (NCA), ϵ -benzyloxycarbonyl-L-lysine (Lys(Z)-NCA) or γ -benzyl-L-glutamate (BLG-NCA) with amino-terminated poly(*N*-isopropylacrylamide) macroinitiator and the subsequent deprotection reaction. The formation of PIC micelles was confirmed by dynamic light scattering and transmission electron microscopy. Turbidimetric characterization suggested that the formed PIC micelles had a concentration-dependent thermosensitivity and their phase transition behaviors could be easily adjusted either by the block length of copolymers or the concentration of micelles.

Keywords: Polyion complex; Micelles; Thermosensitivity; Poly(amino acid)s.

INTRODUCTION

Polyion complex (PIC) micelles were first reported by Kataoka *et al.*^[1] and Kabanov *et al.*^[2] in 1995, using a pair of oppositely charged PEG-polyelectrolyte block copolymers. Other than micelles of amphiphilic polymers formed by hydrophobic interaction^[3, 4], the driving force of PIC micelles is the electrostatic interaction. This micelles system reveals valuable advantages including a narrow size distribution on a mesoscopic scale, total water solubility and good biocompatibility. What's more, PIC micelles were promising vehicles for peptide or protein drugs sensitive to the environment, because they were all prepared under mild conditions in aqueous solutions without using harmful solvents which may cause denaturation of fragile protein drugs^[5, 6].

The functional properties of PIC micelles could be enhanced by combination with a responsivity to external chemical and physical stimuli such as pH, ionic strength, magnetism, light and temperature. Temperature-sensitive polymeric micelles represent one of the most important micelle systems. Poly(*N*-isopropylacrylamide) (PNIPAM) exhibits a reversible coil-to-globule transition at about 32°C (lower critical solution temperature, LCST) in aqueous media, below which it is well soluble in water and above which it becomes water-insoluble and precipitates out from its aqueous solution^[7]. This stimulus-responsive property is particularly useful for targeted micelles carriers for drugs. However, there are not many studies on PIC micelles bearing a thermosensitive shell layer^[8, 9].

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In this study, we report the preparation of novel PIC micelles based on the complexation of a pair of oppositely charged block ionomers with thermosensitive PNIPAM segments, poly(*N*-isopropylacrylamide)-*b*-poly(L-glutamic acid) (PNIPAM-*b*-PLG) and poly(*N*-isopropylacrylamide)-*b*-poly(L-lysine) (PNIPAM-*b*-PLL). The formed PIC micelles were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). Their thermosensitive behavior was explored by the turbidimetric method.

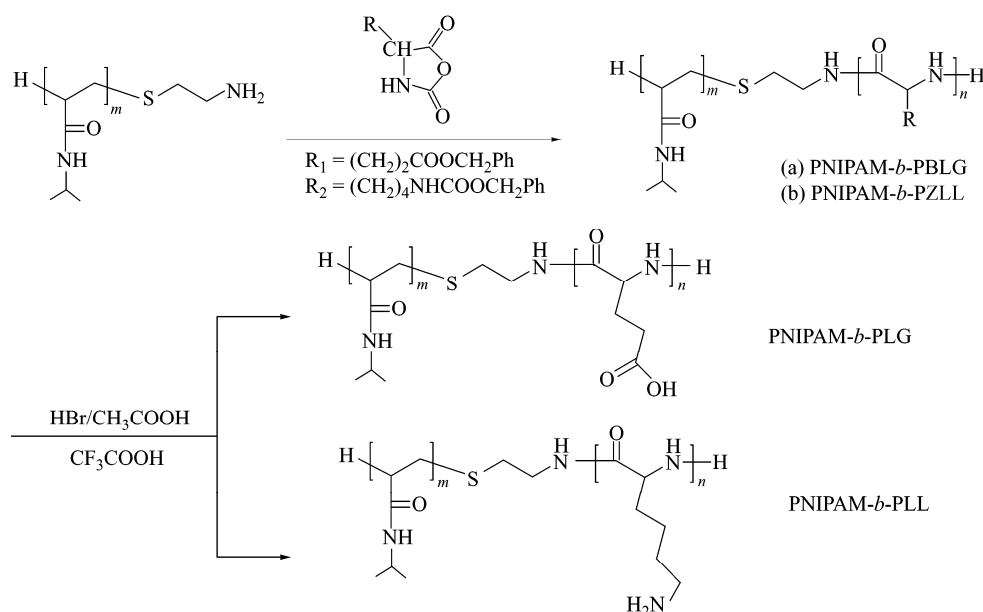
EXPERIMENTAL

Materials

N-isopropylacrylamide (NIPAM, 99%, Sigma) was recrystallized from hexane and dried under vacuum for 24 h prior to use. Azobisisobutyronitrile (AIBN, Beijing Chemical Co., China) was recrystallized thrice from methanol. ϵ -(Benzyloxycarbonyl)-L-lysine and trifluoroacetic acid were purchased from GL Biochem (Shanghai) Ltd., China. Lys(Z)-NCA and BLG-NCA were prepared according to the method reported by our previous work^[10, 11]. 33% HBr solution in acetic acid (HBr/AcOH) was purchased from Acros. Dialysis membrane (MWCO 7000 g/mol) was from Greenbird Company, Shanghai, China. Dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) were dried over CaH₂ and distilled prior to use. All other chemicals were of analytical grade and used as received.

Synthesis of PNIPAM-*b*-Poly(γ -benzyl-L-glutamate) (PNIPAM-*b*-PBLG)

Amine-terminated poly(*N*-isopropylacrylamide) (PNIPAM-NH₂) was synthesized by monomer polymerization of NIPAM using AIBN as the initiator and AET-HCl as the chain transfer reagent, respectively, following the procedure as reported by our previous work^[12]. The PNIPAM-*b*-PBLG diblock copolymers were synthesized by the ring opening polymerization (ROP) of BLG-NCA using PNIPAM-NH₂ as the macroinitiator (Scheme 1, a). Predetermined amount of PNIPAM-NH₂ and BLG-NCA were dissolved in anhydrous DMSO (1:15, *W/V*). The solution was stirring for 72 h at ambient temperature. Then the mixture was dialyzed against ethanol to remove unreacted PNIPAM homopolymers and DMSO. The remained product was redissolved in THF, followed by pouring slowly the polymer solution into excessive diethyl ether. The precipitated product was filtered and dried under vacuum at room temperature for 24 h.



Scheme 1 Synthesis of poly(*N*-isopropylacrylamide)-*b*-poly(amino acid)s

Synthesis of PNIPAM-*b*-Poly(ϵ -benzyloxycarbonyl-L-lysine) (PNIPAM-*b*-PZLL)

The PNIPAM-*b*-PZLL diblock copolymers were synthesized by the ROP of Lys(Z)-NCA using PNIPAM-NH₂ as the macroinitiator (Scheme 1, b). Predetermined amounts of PNIPAM-NH₂ and Lys(Z)-NCA were dissolved in anhydrous DMF (1:15, *W/V*) in a dried flask. The solution was stirred for 72 h at 40°C under argon atmosphere. Then DMF was evaporated under reduced pressures, and the resulting product was dissolved in 40 mL of chloroform and then precipitated into an excess of diethyl ether.

Synthesis of Poly(*N*-isopropylacrylamide)-*b*-Poly(amino acid)s

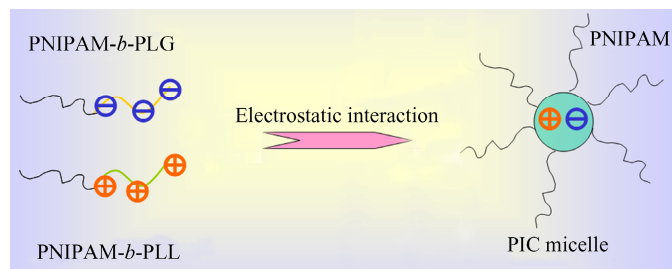
Acidolysis deprotection^[13] was used to remove the benzyl groups of PNIPAM-*b*-PBLG or the benzyl carbamate (Z) group of PNIPAM-*b*-PZLL (Scheme 1). In a typical procedure, 1 g PNIPAM-*b*-PZLL was dissolved in 10 mL trifluoroacetic acid, followed by addition of 4 equiv of 33% HBr/AcOH with respect to the benzyl carbamate (Z) group. The mixture was gently stirred at ambient temperature overnight, and then precipitated with diethyl ether. The resultant product was purified by washing with acetone and diethyl ether repeatedly to remove the residual HBr and then dried under vacuum. Subsequently, the product was dissolved in DMF (PNIPAM-*b*-PLG) or in double distilled water (PNIPAM-*b*-PLL) and dialyzed with a cellulose membrane for 3 days. After the dialysis solution was lyophilized, the final product was obtained.

Characterization of the Block Copolymers

The chemical structures of the copolymers were confirmed by ¹H-NMR spectra recorded on a Bruker AV 400 NMR spectrometer at room temperature. The weight-average molecular weight (*M_w*) was determined by gel permeation chromatography using a series of linear Tskgel Super columns (AW3000 and AW5000) and Waters 515 HPLC with an OPTILAB DSP Interferometric Refractometer (Wyatt Technology) as the detector. The mobile phase was *N,N*-dimethyl formamide (DMF) containing 0.01 mol/L LiBr at a flow rate of 1.0 mL/min.

Preparation of Polyion Complex Micelles

Given amounts of PNIPAM-*b*-PLL and PNIPAM-*b*-PLG were separately dissolved in a phosphate buffer solution (PBS, 10 mmol/L, pH 7.4). After filtration through a 0.45 μ m filter, the polyion complex micelles were prepared by mixing the two solutions in an equal unit ratio of L-lysine and L-glutamic acid residues in the block copolymers as shown in Scheme 2. The micelles solution was stirred for half an hour and then kept in refrigerator at 4°C.



Scheme 2 Formation of PIC micelles by a pair of oppositely charged block copolymers

Characterization of Polyion Complex Micelles

Dynamic laser scattering (DLS) measurements were performed on a Wyatt QELS instrument with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology). The scattering angle was fixed at 90°. All the samples were measured at 25°C without further purification.

Transmission electron microscopy (TEM) measurements were performed on a JEOL JEM-1011 electron microscope operating at an acceleration voltage of 100 kV. A drop of PIC micelles solution (concentration = 0.25 mg/mL) was deposited onto a copper grid for about 5 min and then was blotted up with a piece of filter paper. The sample was dried at room temperature before the measurement.

Turbidimetric measurements were carried out on a UV-Vis spectrometer (Shimadzu UV-2401PC) equipped with a temperature controller (Shimadzu S-1700) at a heating rate of 0.5 K/min. Cloud-point values of the PIC

micelles solution with different concentrations were determined as the temperature corresponding to a 10% decrease in the optical transmittance.

RESULTS AND DISCUSSION

Synthesis and Characterization of Poly(*N*-isopropylacrylamide)-*b*-Poly(amino acid)s

It has been well established that PNIPAM with molecular weight lower than 10 kDa can be excreted by glomerular filtration without long-term accumulation *in vivo*^[14]. So PNIPAM-NH₂ with number average molecular weight of 3.9 kDa (DP = 34) was synthesized and used as macroinitiator for NCA polymerization. The length of poly(amino acid)s (PAA) blocks can be easily adjusted by the feed ratio. After polymerization, the protective groups of amino acids were removed by acidolysis deprotectin.

The typical ¹H-NMR spectrum of poly(*N*-isopropylacrylamide)-*b*-poly(amino acid)s is shown in Fig. 1. The disappearance of signals of benzyl groups or Z groups indicated the complete removal of the protecting groups (¹H-NMR spectra of copolymers before deprotection are not shown). The compositions of the copolymers were also analyzed by ¹H-NMR. Taking PNIPAM-*b*-PLG as example, The DP of PLG block was calculated by comparing the integral value of the characteristic peak of PNIPAM at $\delta = 3.9$ with that of PLG at $\delta = 4.7$.

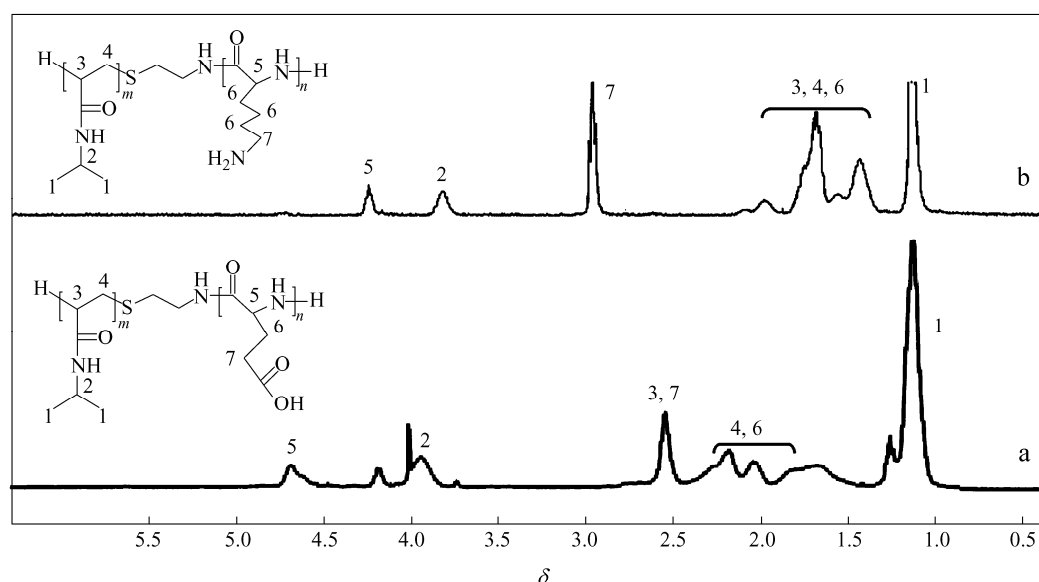


Fig. 1 ¹H-NMR spectra of PNIPAM-*b*-PLG (a) in d-TFA and PNIPAM-*b*-PLL (b) in D₂O

Herein, two pairs of oppositely charged copolymers with identical PNIPAM block but with different lengths of the PAA block were synthesized, and the results are listed in Table 1. All the copolymers before deprotection have relatively narrow polydispersity. It also should be noted that DP of poly(L-glutamic acid) and poly(L-lysine) were almost equal in each pair of copolymers (Table 1). This is important for PIC micelles preparation, since it has been proved that PIC micelles were formed by matched pairs with the same block lengths of polyanions and polycations under stoichiometric conditions^[15].

Table 1. Molecular weight and distribution of two pairs of poly(*N*-isopropylacrylamide)-*b*-poly(amino acid)s

Polymer	NCA:PNIPAM mol:mol (feed)	NCA:PNIPAM mol:mol (obtained)	M_n^a	M_n^b	M_w/M_n^b
PNIPAM- <i>b</i> -PBLG1	25:1	26:1	9.6×10^3	2.1×10^4	1.3
PNIPAM- <i>b</i> -PZLL1	25:1	25:1	9.9×10^3	1.7×10^4	1.6
PNIPAM- <i>b</i> -PBLG2	80:1	86:1	2.2×10^4	3.4×10^4	1.2
PNIPAM- <i>b</i> -PZLL2	80:1	79:1	2.3×10^4	2.7×10^4	1.2

^a Calculated by ¹H-NMR; ^b Obtained by SEC-MALLS

Formation of PIC Micelles

The polyion complex micelles were prepared by mixing 1.0 mg/mL PBS solutions of PNIPAM-*b*-PLG and PNIPAM-*b*-PLL under the stoichiometric condition that the mole ratio of L-lysine and L-glutamic acid residues in the block copolymers was equal. The formed complex micelles were named PIC25 and PIC80 micelles, with number representing the DP of poly(amino acid)s block. It was supposed that the formed PIC micelles had a neutral PAA core surround by thermosensitive PNIPAM as the shell layer^[9].

Hydrodynamic radius (R_h) distribution of the PIC micelles was studied by DLS technique. The average R_h calculated by DLS for PIC25 and PIC80 micelles are (37.8 ± 5.5) and (56.3 ± 20.5) nm, respectively. It seems that the longer PAA block leads to the bigger particle size. As can be seen in Fig. 2, the formed PIC micelles had a rather narrow size distribution, the same as reported for PIC micelles by Kataoka's group^[1].

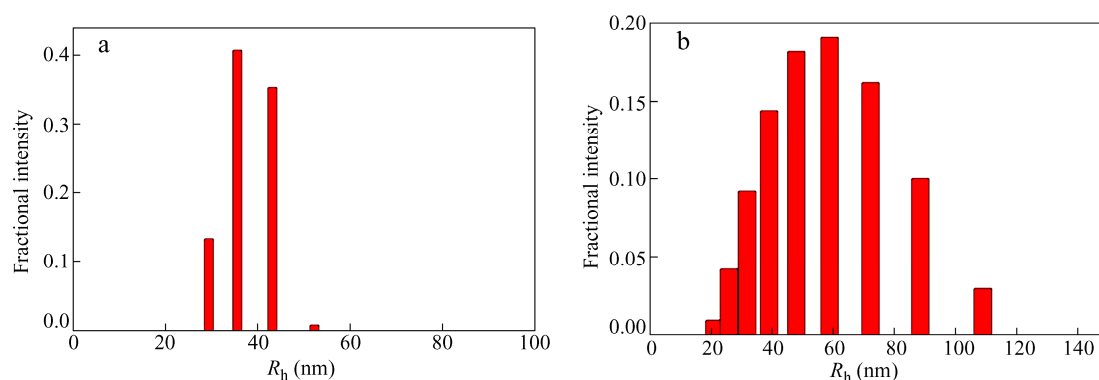


Fig. 2 Hydrodynamic radius distribution of the PIC25 (a) and PIC80 (b) micelles with concentration of 0.125 mg/mL formed in PBS at 25°C

Figure 3 shows the morphology of PIC25 micelles characterized by TEM. The micelles are spherical particles with a diameter of about 50 nm and with narrow size distribution, which is in coordination with the DLS tests. However, the particle diameters measured by TEM are a little smaller than the hydrodynamic diameters measured by DLS. This may be because of the volume shrinkage during sample drying^[16].

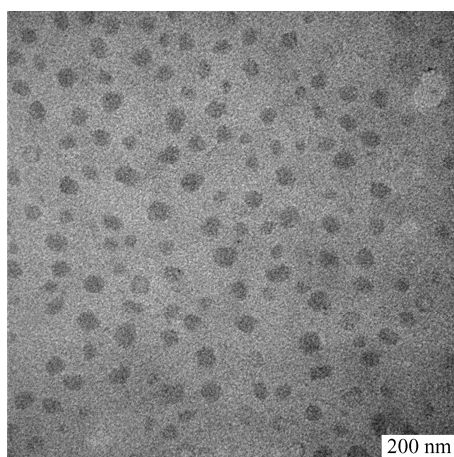


Fig. 3 TEM image of PIC25 micelles at room temperature with concentration of 0.25 mg/mL

Thermosensitive Behavior of PIC Micelles

In our previous report, PNIPAM-*b*-PLL and PNIPAM-*b*-PLG had their own LCST behavior in solution and responded to a narrow pH change. This work focused on the thermosensitivity of their complex micelles. And

the phase transition behaviors of charged copolymers were also studied separately as contrast.

Figure 4 shows the transmittance change with temperature for PIC25 micelles solutions at different concentrations. The transmittance of solution gradually decreased when the temperature was higher than 32°C, the LCST of PNIPAM shell. It was found that the cloud points of PIC micelles shifted to a lower temperature with increasing the micelles concentration in solution. For example, the LCST of micelles at 1.0 mg/mL was 34.3°C, lower than that at 0.25 mg/mL (36.1°C). And PIC micelles with a higher concentration showed a faster phase transition behavior, because the higher local micelles concentration led to a faster aggregation of PNIPAM shell in solution at temperatures above LCST. When the temperature decreased from 40°C to 25°C, the transmittance of solution was back to nearly 100 percent, indicating that the thermal-induced phase transition of PIC micelles was reversible.

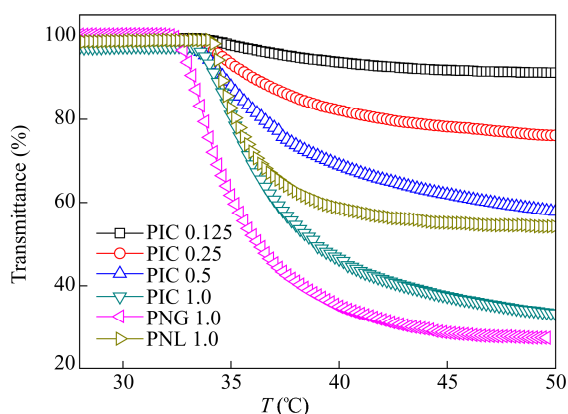


Fig. 4 The temperature-dependent phase-transition curves of PIC25 micelles solutions with different concentrations (PNL and PNG are short for PNIPAM-*b*-PLL and PNIPAM-*b*-PLG with DP 25 of PAA blocks, respectively.)

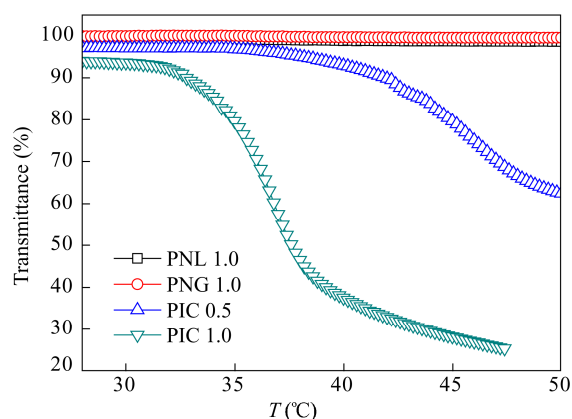


Fig. 5 The phase-transition curves of PIC80 micelles and their oppositely charged copolymers solution with concentration of 1.0 mg/mL (PNL and PNG are short for PNIPAM-*b*-PLL and PNIPAM-*b*-PLG with DP 80 of PAA blocks, respectively.)

From Fig. 4, it can also be seen that the phase transition behavior became slower as the PIC25 micelles concentration in solution decreased. So the thermosensitivity of PIC80 micelles was tested from 0.5 mg/mL to 1.0 mg/mL. A similar concentration-dependent thermosensitivity was also observed for PIC80 micelles (Fig. 5). As the concentration of micelles increased from 0.5 mg/mL to 1.0 mg/mL, the LCST of PIC80 micelles decreased significantly from 40°C to 34.5°C. However, in sharp contrast with the PIC80 micelles, there was no thermo phase transition behavior for PNIPAM-*b*-PLL or PNIPAM-*b*-PLG with DP 80 of PAA block. When the charged block of PNIPAM-*b*-PAA is long enough, the whole copolymer is so hydrophilic that it can not precipitate out from the solution even when PNIPAM has become insoluble at a temperature much higher than 32°C. The formation of PIC micelles was further proved by the totally different curves of transmittance versus temperature for the PNIPAM-*b*-PAA and their complex micelles.

The size distribution change of PIC25 micelles at a temperature above LCST was studied by DLS measurements. As can be seen in Fig. 6, PIC micelles have a rather narrow size distribution at 25°C with an average R_h of (72.5 ± 16.30) nm. However, when the temperature raised to 40°C (above LCST of PNIPAM), the size distribution became broader due to the formation of some large aggregates of PIC micelles. Meanwhile part of the micelles still existed in solution at 40°C and had a little smaller size than that at 25°C. The reason of this phenomenon needs further investigation.

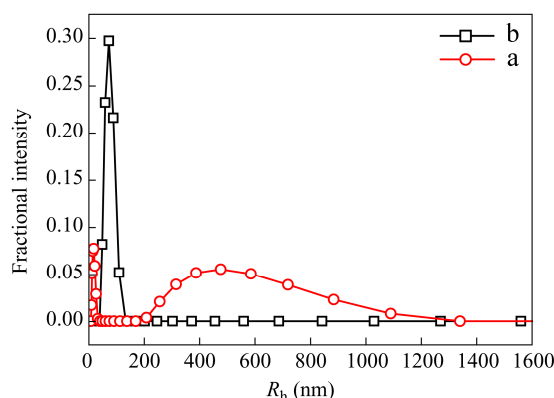


Fig. 6 Hydrodynamic radius distributions of the PIC25 micelles with concentration of 0.25 mg/mL in PBS solution at 40°C (a) and at 25°C (b)

CONCLUSIONS

Two pairs of PNIPAM-*b*-PLG and PNIPAM-*b*-PLL were synthesized with identical temperature-sensitive PNIPAM segment and different length of the poly(amino acid) block. Polyion complex micelles were spontaneous formed by mixing solutions of the oppositely charged block ionomers under stoichiometric conditions. It was confirmed that the PIC micelles were spherical particles with narrow size distribution. PIC25 and PIC80 micelles had concentration-dependent thermosensitivity. And the phase transition temperature could be easily adjusted by the block length of copolymers and the micelles concentration. The thermosensitive PIC micelles developed in this study have potential applications in biomedical areas especially in charged drug delivery systems.

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