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Synthesis and micellization behavior of stimuli-responsive polypeptide hybrid triblock copolymer

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Polypeptide hybrid triblock copolymer, poly(L-glutamic acid)-b-poly(propylene oxide)-b-poly (L-glutamic acid) (PLGA-b-PPO-b-PLGA), was synthesized by the ring-opening polymerization of benzyl-L-glutamic N-carboxyanhydride (BLG-NCA) using poly(propylene glycol) *bis*(2-aminopropyl ether) as initiator, followed by the subsequent deprotection step. The obtained double hydrophilic triblock copolymer exhibits "schizophrenic" micellization behavior in aqueous solution upon dually playing with solution pH and temperature. The multi-responsive micellization behavior of this polypeptide hybrid triblock copolymer has been thoroughly investigated by ^{1}H NMR, laser light scattering (LLS), temperature-dependent optical transmittance, and circular dichroism spectroscopy (CD).

poly(L-glutamic acid), poly(propylene glycol), coil, *α*-helix, micellization

Block copolymers can self-assemble into mesophases with a variety of morphologies, such as micelles, vesicles, tubules, and complex super-aggregates, depending on the relative block length, solvent composition, polymer concentration, external additives, and temperature[1–5]. Among them, polypeptide hybrid block copolymers consisting of synthetic polymer blocks and polypeptide segments represent a special type[6–8]. The presence of peptide segment endows block copolymers with intriguing supramolecular nanostructures through hierarchical self-assembly in bulk and solution, partially due to the formation of characteristic protein folding motif via inter- and intra-molecular interactions. Molecular structure of the polypeptide hybrid block can be tailor-made via N-carboxyanhydride (NCA) ring opening polymerization (ROP) technique[9–11].

A large amount of literature has documented the supramolecular self-assembly of peptide hybrid block copolymers in aqueous solution. They can be further categorized into two main types. The first one involves nanostructures with corona-forming poly-peptide segments, starting from amphiphilic copolymers consisting of hydrophobic synthetic block and hydrophilic blocks polypeptide block such as poly(L-glutamic acid)(PLGA) or poly(L-lysine) (PLL)[12–18]. The second type typically involves water-insoluble polypeptide sequence as one of the building blocks[19,20].

Our recent research interests partially involve double hydrophilic block copolymers (DHBCs)[21–23]. Subjected to physical or chemical transformations in aqueous solution, one of the blocks of DHBCs can be selectively rendered water insoluble, while the other block still remains hydrophilic to stabilize the self-assembled aggregates. Moreover, certain DHBCs exhibit "schizophrenic" micellization properties, forming two or more types of aggregates with "invertible" nanostructures upon judicious adjustment of external conditions.

PLGA and PLL can reversibly switch between *α*-helix, *β*-sheet, and random coil conformations when external conditions, such as pH, ionic strength, and temperature are properly tuned[24–27]. These conformational changes concomitantly result in changes of their water-solubility.

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Recently, Schlaad et al.[28] described the synthesis of poly(2-isopropyl-2-oxazoline)-b-poly(L-glutamate) (PiPrOx-b-PLGA), using amino-terminated PiPrOx as the initiator for the ROP of NCA. However, the macroinitiator contains tertiary amine residues, which are also capable of initiating NCA polymerization via a different mechanism from that by primary amines. This might result in structural uncertainties for the target hybrid polypeptide diblock copolymers.

Poly(propylene oxide) (PPO) has been well known as a thermoresponsive polymer, exhibiting a lower critical solution temperature (LCST) at ~20℃[29]. Herein, we synthesized a novel polypeptide hybrid DHBC, PLGA-b-PPO-b-PLGA, via the ROP of γ-benzyl-L-glutamate *N*-carboxyanhydride (BLG-NCA) using diamino-terminated PPO as the macroinitiator, followed by deprotection. The pH- and thermo-responsive "schizophrenic" micellization properties of PLGA-b-PPO-b-PLGA associated with coil-to-helix transitions were thoroughly investigated by ^{1}H NMR, fluorescence measurement, laser light scattering (LLS), optical transmittance, and circular dichroism (CD).

1 Experimental

1.1 Materials

Poly(propylene glycol) bis(2-amino- propyl ether) (NH_2-PPO_{33}-NH_2) (Aldrich) was used as received. γ-Benzyl-L-glutamate (BLG) was prepared according to literature procedures[30]. THF was dried by refluxing over sodium/benzophenone and distilled prior to use. CH_2Cl_2 was vacuum-distilled from CaH_2. All other chemicals were purchased from Shanghai Chemical Reagent Co.

1.2 Synthesis

The general synthetic routes for the preparation of polypeptide containing triblock copolymers, PBLG-b-PPO-b-PBLG and PLGA-b-PPO-b-PLGA, are shown in Figure 1.

NH_2-PPO-NH_2 (0.5 g, 0.25 mmol) was dissolved in ~30 mL anhydrous CH_2Cl_2. BLG-NCA (5.2 g, 20 mmol) was dissolved in 30 mL anhydrous CH_2Cl_2 in a separate flask. The BLG-NCA/CH_2Cl_2 mixture was then cannulated into the NH_2-PPO-NH_2/CH_2Cl_2 solution via a double-tipped stainless needle. The reaction mixture was allowed to stir for 3 d at room temperature under dry N_2 atmosphere. After partially removing the solvents, the reaction mixture was precipitated into an excess of anhydrous diethyl ether. This purification cycle was repeated for 3 times. The obtained white solids were dried in a vacuum oven overnight at room temperature.

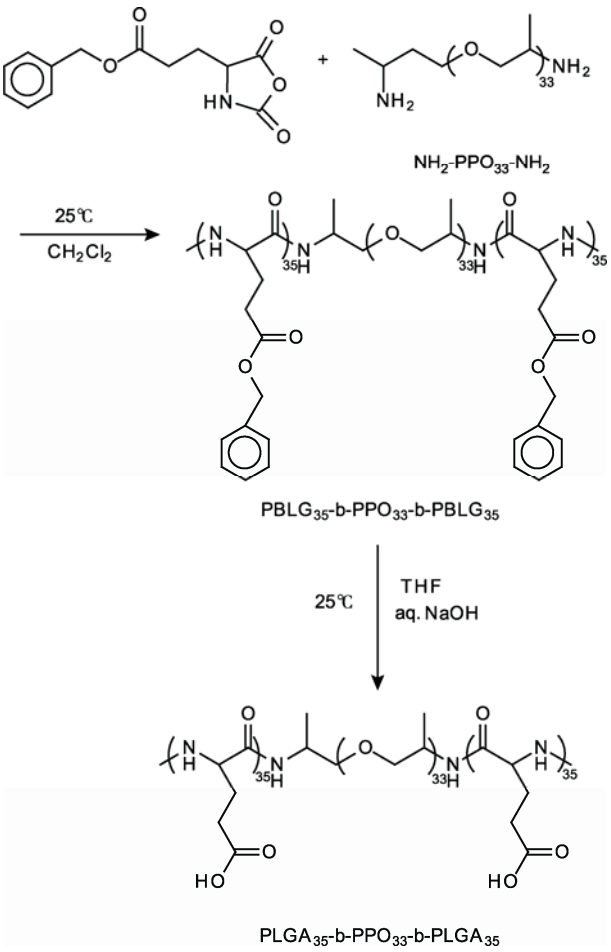

Figure 1 Reaction scheme for the preparation of $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$.

0.5 g PBLG-b-PPO-b-PBLG was dissolved in 40 mL THF. A solution of NaOH (0.6 mL, 25 wt%) was added. After 12 h stirring, the solvent was removed under vacuum and dissolved in acidic water, then dialyzed against water for 48 h. The final product, PLGA-b-PPO-b-PLGA, was obtained as white powder after being freeze-dried.

1.3 Characterization

Molecular weights and molecular weight distributions were determined by gel permeation chromatography (GPC) equipped with Waters 1515 pump and Waters 2414 differential refractive index detector. It used a series of three linear Styragel columns HT2, HT4, and HT5 at an oven temperature of 45℃. The eluent was DMF at a flow rate of 1.0 mL/min. All ^{1}H NMR spectra

were performed at 25℃ on a Bruker AV300 NMR spectrometer (resonance frequency of 300 MHz for 1H) operating with the Fourier transform mode. The optical transmittance of the solution was acquired on a Unico UV/vis 2802PCS spectrophotometer and measured at a wavelength of 600 nm using a thermostatically controlled couvette. Circular dichroism spectra were recorded on Jasco J-720 circular dichroism spectroscopy. A commercial spectrometer (ALV/DLS/ SLS-5022F) equipped with a multi-tau digital time correlator (ALV5000) and a cylindrical 22 mW UNIPHASE He-Ne laser (λ_0 = 632 nm) as the light source was employed for dynamic laser light scattering (LLS) measurements.

2 Results and discussion

2.1 Synthesis of PLGA-b-PPO-b-PLGA triblock copolymer

General synthetic routes for the preparation of polypeptide hybrid triblock copolymers, PBLG-b-PPO-b-PBLG and PLGA-b-PPO-b-PLGA, are shown in Figure 1. The target triblock copolymer, PBLG-b-PPO-b-PBLG, was synthesized via the ROP of BLG-NCA using NH_2-PPO-NH_2 as the macroinitiator, followed by deprotection. The GPC trace clearly shows that the elution peak shifts to higher molecular weight after the ROP of BLG-NCA (Figure 2), compared to that of NH_2-PPO-NH_2 precursor. The elution peak of triblock copolymer is relatively symmetric and shows almost no discernible tailing on the lower molecular weight side, confirming a complete consumption of NH_2-PPO-NH_2. The molecular weight and molecular weight distribution of PBLG-b-PPO-b-PBLG were characterized by GPC analysis in DMF: M_n = 18900, and M_w/M_n = 1.12. The relatively narrow polydispersity of the obtained triblock copolymer could be explained by the living character of the ROP of NCAs initiated by primary amine residues.

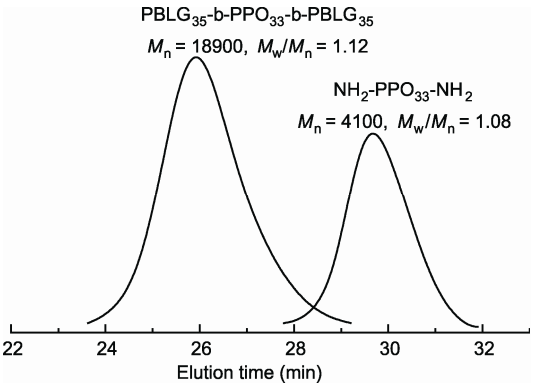

Figure 2 DMF GPC traces of NH_2-PPO-NH_2 and PBLG-b-PPO-b-PBLG ABA triblock copolymers.

Figure 3(A) shows ^{1}H NMR spectrum of PBLG-b-PPO-b-PBLG in $CDCl_3$. It can be clearly seen that all signals characteristic of PPO and PBLG blocks are visible. The signal at δ = 1.0 is ascribed to methyl protons (a) of PPO block, whereas signal at δ = 5.0 can be ascribed to methylene group of benzyl (e). Based on the integral ratio of peaks a and e, the degree of polymerization, DP, of PBLG block was calculated to be 70. Thus, the triblock copolymer was denoted as $PBLG_{35}$-b-PPO_{33}-b-$PBLG_{35}$. The obtained $PBLG_{35}$-b-PPO_{33}-b-$PBLG_{35}$ was then subjected to hydrolysis to deprotect benzyl groups under alkaline condition.

Figure 3(B) shows the ^{1}H NMR spectrum of the hydro lyzed product, $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$, in D_2O

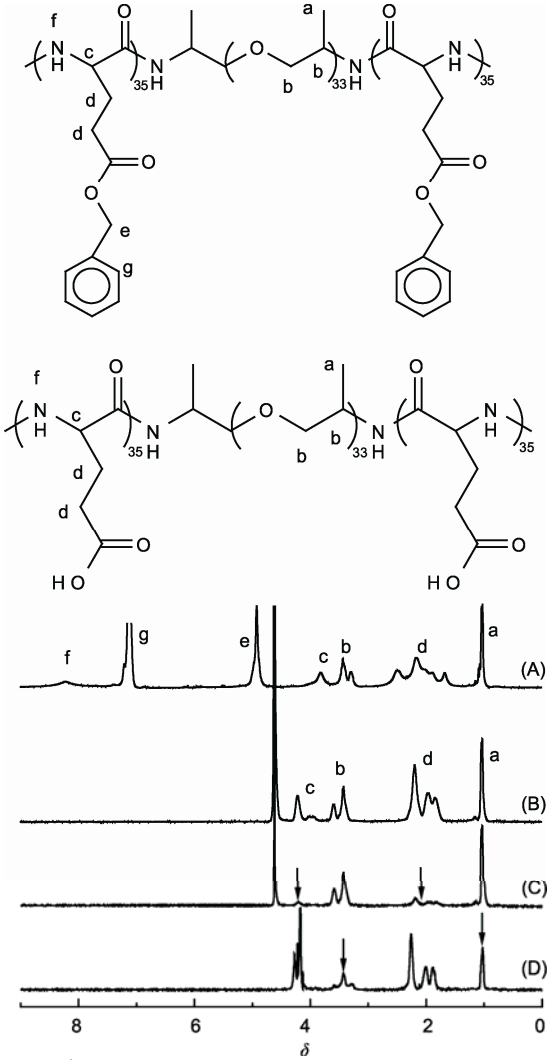

Figure 3 ^{1}H NMR spectra of (A) $PBLG_{35}$-b-PPO_{33}-b-$PBLG_{35}$ in $CDCl_3$ containing 10 % TFA (*V*/*V*); (B) $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ at pH 10; (C) pH 2 at 5℃, and (D) pH 10 at 45℃ in D_2O.

at pH 10. We can clearly see that signals characteristic of benzyl groups (peaks e and g) of PBLG block at δ 7.1 and 5.0 completely disappear, indicating the complete removal of benzyl groups. It should be noted that the DP of PLGA blocks was also determined to be ~70 based on comparison of the integration areas of peaks a and d. This indicates that both PPO and polyamide backbone of polypeptide blocks are unaffected during hydrolysis.

2.2 "Schizophrenic" micellization of PLGA-b-PPO-b-PLGA triblock copolymer

It is well known that PPO homopolymer dissolves in cold and dilute aqueous solution but gets insoluble at ~20℃ due to its lower critical solution temperature (LCST) phase behavior[29]. On the other hand, poly(L-glutamic acid) takes a random coil conformation in aqueous solution at alkaline pH due to ionization of side carboxyl groups, and an α-helix conformation in acidic media (the pK_a value of glutamic acid is 4.32). Interestingly, the pH-induced coil-to-helix transition is also accompanied with a considerable decrease of the water-solubility of PLGA block. Thus, $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ might exhibit thermo- and pH-responsive "schizophrenic" micellization behavior in aqueous solution, accompanied with coil-to-helix transitions.

^{1}H NMR was also used to investigate the micellization of triblock copolymer. Figure 3 shows the ^{1}H NMR spectra recorded for the $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ in D_2O at different solution conditions. At 5℃ and pH 10, both PPO and PLGA blocks were hydrophilic, thus the triblock copolymer dissolved molecularly in dilute aqueous solution and ^{1}H NMR signals due to both blocks were visible (Figure 3(B)). Upon addition of a small amount of DCl into the molecularly dissolved solution at 5℃, micellization occurred at pH 2 or lower (Figure 3(C)). Comparing Figure 3(B) and (C), it is clear that the signals due to the PLGA block at δ = 1.2, 3.1, 3.5, and 4.4 completely disappeared. This suggested that PLGA-core micelles were formed, with the still-solvated PPO block forming the micellar corona. At pH 10 and elevated temperatures, PPO-core micelles were expected to form. Figure 3(D) shows the ^{1}H NMR spectrum of $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ at pH 10 and 45℃, and it was found that the signals due to PPO block almost disappeared and the signals due to the PLGA residues were still prominent, suggesting the formation of PPO-core micelles.

The changes of optical temperature-dependent transmittance at 600 nm in copolymer aqueous solutions are shown in Figure 4. At pH 10 and a concentration of 5 g/L, the transmittance exhibits no changes in the range of 5℃－12℃. Above 13℃, transmittance decreases abruptly from 100% to about 77 % in the range of 15℃－50℃. The decrease of transmittance should be correlated with the increase of scattering light intensity. So the transmittance decrease above 13℃ should be due to the micellization of $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$.

At pH 2, the aqueous solution was bluish at lower temperatures (Figure 4). The transmittance exhibits no changes in the range of 5℃－20℃. Above 20℃, transmittance decreases from 84% to about 30% in the range of 20℃－60℃. The transmittance decrease above 20℃ should be due to the aggregation of PLGA-core micelles.

Dynamic LLS was further employed to detect the thermo-induced formation of PPO-core micelles. Figure 5 shows the temperature dependence of average hydro-

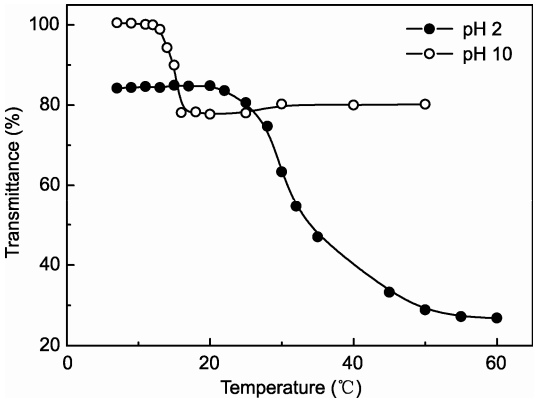

Figure 4 Temperature dependence of transmittance of aqueous solution of $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ at pH 10 and pH 2, respectively. The polymer concentration was fixed at 5.0 g/L.

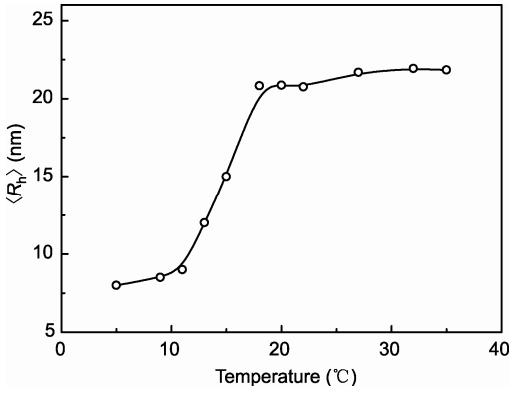

Figure 5 Variation of $\langle R_h \rangle$ as a function of temperature at pH 10. The polymer concentration was 1.0 g/L.

dynamic radius, $\langle R_h \rangle$, for $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ aqueous solutions at pH 10. Obviously, when the temperature is lower than the LCST of PPO, the block copolymer is molecularly soluble and $\langle R_h \rangle$ is typically less than 10 nm. At elevated temperatures, micellization starts to take place, $\langle R_h \rangle$ and the scattering intensity increase dramatically. At even higher temperatures, $\langle R_h \rangle$ keeps constant at ~ 20 nm above 20℃.

For the pH induced micellization, Figure 6 shows the typical plots of the hydrodynamic radius distribution $f(R_h)$ of $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ at different pH. It can be clearly seen that the distribution curve shift to the left with increasing pH value. The $\langle R_h \rangle$ values at pH 2 and 10 are ~53 nm and 8 nm, respectively. This should be ascribed to the coil to helix transition of PLGA block, which turned it hydrophobic. Recently, Lin et al.[31,32] also reported the synthesis and self-assembling behavior of PLGA-b-PPO-b-PLGA triblock copolymer and its application as drug nanocarriers.

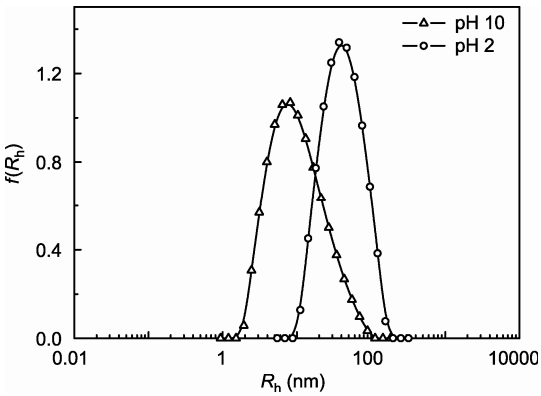

Figure 6 Comparison of hydrodynamic radius distributions, $f(R_h)$, of $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ in water at pH 10 and pH 2 (5℃). The polymer concentration was fixed at 1 g/L.

It is well known that upon pH decrease, the PLGA sequences also exhibit the secondary conformational transition from a charged coil to a more compact α-helical structure at acidic pH[24,27,33,34]. Circular dichroism (CD) was then used to investigate this conformational transition. Figure 7 shows the CD spectra of $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ solution at pH 2 and 10. At low pH, two negative bands at 208 nm and 224 nm can be detected, which can be assigned to π-π* and n-π* transitions, respectively, due to Cotton effects. These features are characteristics of α-helix secondary structures[35,36]. Thus, it clearly shows that the coil-to-helix transition occurs during the pH-induced micellization of $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ triblock copolymer aqueous solutions.

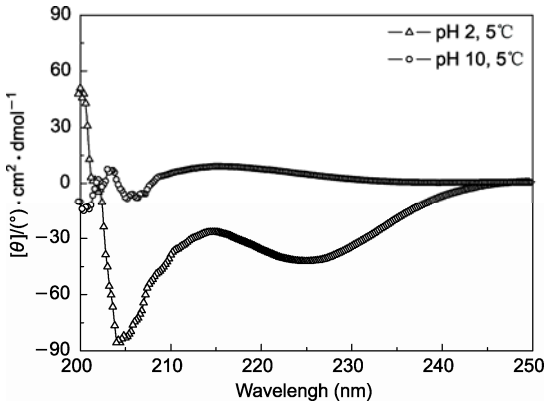

Figure 7 Circular dichroism spectra for $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ aqueous solution at pH 2 and pH 10. The polymer concentration was 1.0 g/L and the temperature was 5℃.

3 Conclusion

The pH- and thermo-responsive "schizophrenic" micellization behavior of a polypeptide hybrid double hydrophilic triblock copolymer, $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$, was investigated via a combination of ^{1}H NMR, optical transmittance, fluorescence probe measurements, laser light scattering (LLS), and circular dichroism (CD) spectroscopy. The triblock copolymer self-assembles into PPO-core micelles at alkaline pH and elevated temperatures, and PLGA-core micelles at acidic pH and low temperature. "Schizophrenic" micellization of $PLGA_{35}$-b-PPO_{33}-b-$PLGA_{35}$ leads to the facile locating of peptide sequence within either micelle cores or stabilizing coronas. The incorporation of polypeptide sequences into DHBCs can endow them with structural versatility, tunable spatial arrangement of chain segments within self-assembled nanostructures, enhanced biocompatibility, and broader applications in the field of biomedicine.

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