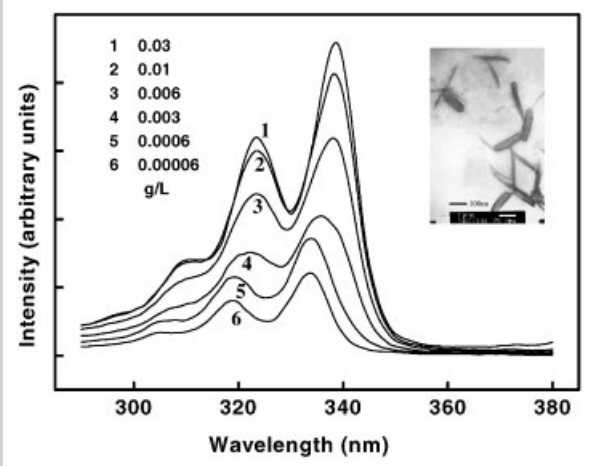
Summary: Self-association behaviors of poly(γ-benzyl L-glutamate)-*graft*-poly(ethylene glycol) (PBLG-*graft*-PEG) and its mixtures with PBLG homopolymer in aqueous media were investigated by fluorescence spectroscopy, transmission electron microscopy (TEM), and nuclear magnetic resonance (NMR) spectroscopy. It was revealed that PBLG-*graft*-PEG could self-assemble to form polymeric micelles with a core-shell structure in the shape of spindle. The introduction of PBLG homopolymer not only decreases the critical micelle concentration, but also changes the morphology of the micelles.



The excitation fluorescence spectra of pyrene as a function of concentrations for the mixture of PBLG-*graft*-PEG with PBLG and a TEM image of the formed micelles.

Self-Assembly of Poly(γ-benzyl L-glutamate)-*graft*-Poly(ethylene glycol) and Its Mixtures with Poly(γ-benzyl L-glutamate) Homopolymer

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Introduction

Because of their amphiphilic characteristics, block and graft copolymers that contain hydrophobic and hydrophilic components can form micelles or nanoparticles with a core-shell structure.[1] The self-assembly of amphiphilic molecules has received much attention both experimentally[2,3] and theoretically.[4–6] The nanoscale structures possess a range of potential applications such as catalysis,[7] drug delivery,[8] nanoreactors etc.[9]

Recently, much interest has been focused on the self-assembly behavior of the block copolymers composed of polypeptide segments and hydrophilic polymer chains.[10–20] An important application of the micelles formed by the polypeptide block copolymer is for drug delivery.[13–20] Kwon et al. have studied the self-assembly of the diblock copolymer composed of poly(β-benzyl L-aspartate) (PBLA) and poly(ethylene glycol) (PEG).[10] It has been demonstrated that PBLA-PEG diblock copolymers could associate to form polymeric micelles in aqueous medium. PBLA chains are restricted within the cores and PEG chains form the water-soluble shells of the micelles. Harada et al. have analyzed the relationship between the conformation of a polypeptide chain and the supramolecular structure of poly(L-lysine)-*block*-poly(ethylene glycol).[11] They concluded that the α-helix structure of the poly(L-lysine) segment tends to be stabilized by the PEG segment through the formation of a dimer with a micelle-like structure in aqueous medium. Using transmission electron microscopy (TEM), fluorescence and NMR spectroscopy, Cho et al. have studied the polymer micelles formed by the diblock copolymer of poly(γ-benzyl L-glutamate) (PBLG) and poly(ethylene glycol) in aqueous medium.[12] It was found that the PBLG-PEG diblock copolymer could form polymeric

micelles with PBLG as the hydrophobic inner core and PEG as the hydrophilic shell. The critical micelle concentration (CMC) of the diblock copolymers, determined by fluorescence measurements, decreases with increasing of PBLG chain length. Studies on the application of the polypeptide-based micelles to drug delivery have also been performed by Cho et al.[14,15,17] The obtained results show that the drug loading contents are dependent on the PBLG chain length of the block copolymer.

However, to our knowledge, no experimental work has so far been reported on the studies of the micelles based on polypeptide graft copolymers and their mixtures with polypeptide homopolymers. It is expected that the character of the polymeric micelles formed by the graft copolymer and its mixtures with polypeptide homopolymer would be different from those formed by polypeptide block copolymers. These related studies would contribute much to widening the research field of self-assembly. In the present work, the self-assembly behaviors of PBLG-*graft*-PEG and its mixtures with PBLG homopolymer in aqueous medium were investigated by fluorescence and NMR spectroscopy, and TEM. The formation of micelles with a core-shell structure was demonstrated.

Experimental Part

Materials

Poly(ethylene glycol methyl ether) (PEG) ($\overline{M}_w = 350$) was purchased from Sigma Inc., and used without further purification. Hexane, tetrahydrofuran (THF), and 1,4-dioxane are of analytical grade and dried with sodium to remove water before use. All other solvents are of analytical grade and used without further purification. Pyrene was purchased from Sigma Inc., and used as received.

Syntheses of PBLG and PBLG-graft-PEG

The PBLG sample was prepared by a standard *N*-carboxyl-γ-benzyl-L-glutamate anhydride (NCA) method as adopted in our previous work.[21,22] Briefly, *N*-carboxyl-γ-benzyl-L-glutamate anhydride (γ-BLG NCA) was prepared according to the method proposed by Goodman and Hutchison.[23] PBLG was obtained by the ring-opening polymerization of γ-BLG NCA initiated by triethylamine in 1,4-dioxane at room temperature for 72 h. The reaction mixture was poured into a large volume of anhydrous ethanol. The precipitated product was dried under vacuum and then purified twice by repeated precipitation from a chloroform solution into a large volume of anhydrous methanol. The molecular weight of PBLG was estimated from the intrinsic viscosity measured in dichloroacetic acid (DCA).[24] The molecular weight of PBLG homopolymer used in the mixed systems is 22 000.

PBLG-*graft*-PEG was prepared by the ester exchange reaction of PBLG with PEG in 1,2-dichloroethane with *p*-toluenesulfonic acid as a catalyst according to the method described in the literature.[25–27] The molecular weight of PBLG used in the reaction is 170 000. The mixture reacted at 55 °C for 72 h and was then precipitated into a large volume of anhydrous ethanol. The resulting product was purified twice from a chloroform solution in a large volume of anhydrous methanol and dried under vacuum. Calculations, according to the results of the NMR measurements, show that the percentage of grafting for the PBLG-*graft*-PEG sample is 25.4%.

Preparation of Stock Solution

A stock solution was prepared by first dissolving PBLG-*graft*-PEG (or mixtures of PBLG-*graft*-PEG with PBLG homopolymer) in a mixture of THF/*N*,*N*-dimethylformamide (DMF) [3:7 (v/v)] in a volumetric flask. The solution was then dialyzed against deionized water using dialysis membrane (3 500 molecular weight cut-off) to remove the organic solvents for about 48 h at room temperature. It was preferred that the deionized water was exchanged at intervals of 3–4 h. The solution was diluted with deionized water to the desired concentration.

Measurements of Fluorescence Spectroscopy

To prove the formation of the micelles, fluorescence measurements were carried out using pyrene as a probe.[10,19,28] The fluorescence spectra of pyrene in aqueous solution were recorded at room temperature on a Varian Cary Eclipse fluorescence spectrophotometer. The sample solutions were prepared by first adding known amounts of pyrene in acetone to a series of flasks. After the acetone had evaporated completely, measured amounts of micelle solutions with various concentrations of PBLG-*graft*-PEG (or mixtures of PBLG-*graft*-PEG with PBLG homopolymer) were added to each of the flasks and mixed by vortexing. The concentration of pyrene in the final solutions was 6×10^{-7} M. The flasks were heated for 3–4 h at 60 °C to equilibrate the pyrene and the micelles. The emission wavelength was 390 nm for excitation spectra and the excitation bandwidth was 5 nm.

Observation of Transmission Electron Microscope

The morphology of the micelles was observed by TEM (JEM-1200-EXII). Drops of micelle solution were placed on a carbon film coated on a copper grid, and then were dried at room temperature. Before the observation, the sample was stained by phosphotungstic acid. The TEM bright field imaging was performed with 120 kV accelerating voltage.

^{1}H NMR Measurements

^{1}H NMR spectra of PBLG-*graft*-PEG were measured in $CDCl_3$ to estimate the percentage of grafting using a NMR instrument (Avance 550) at 500 MHz. The percentage of grafting was calculated from the peak intensities of the methylene proton signal (5.1 ppm) of PBLG and the ethylene proton signal (3.6 ppm) of PEG in the ^{1}H NMR spectrum. To prove the micellar structure, ^{1}H NMR spectra of PBLG-*graft*-PEG and its mixture with PBLG homopolymer were also measured in D_2O.

Results and Discussion

Formation of the Micelles

Excitation spectra of pyrene in the PBLG-*graft*-PEG solutions with various polymer concentrations are shown in Figure 1a. As it can be seen, the fluorescence intensity increases with increasing the concentration of PBLG-*graft*-PEG. Concomitant with the increase in the fluorescence intensity, a red shift of the (0, 0) band from 333 to 338 nm is shown. Such results are explained by, that at a certain concentration, micellization occurs and the pyrene is preferentially partitioned into the hydrophobic part with a corresponding change of the photophysical properties of molecules. Fluorescence studies on the micellization of the mixtures of PBLG-*graft*-PEG and PBLG homopolymer with various ratios have also been carried out. Figure 1b shows typical excitation spectra of pyrene in the various mixtures of different concentrations. Enhanced intensity and a red shift are observed with increasing the concentration of the mixture, indicating that the micellization takes place above the critical polymer concentration.

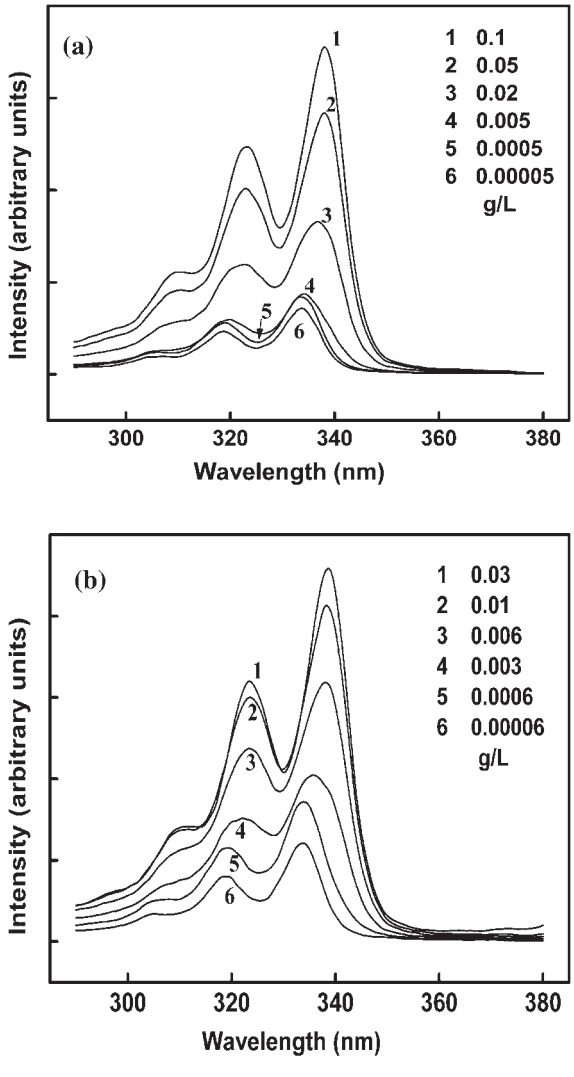

Figure 1. (a) Excitation spectra of pyrene as a function of PBLG-*graft*-PEG concentrations in deionized water. (b) Excitation spectra of pyrene as a function of concentrations for the mixture of PBLG-*graft*-PEG with PBLG in deionized water (the ratio of PBLG-*graft*-PEG to PBLG by weight: 3:7).

Much information about the onset of aggregation can be obtained from the analyses of excitation spectra. According to Wilhelm et al.,[28] the intensity ratio of I_{338}/I_{333} could show the onset of micellization and provide a quantitative method for the determination of the critical micelle concentration. I_{333} was chosen as the wavelength value of the (0, 0) band of pyrene in aqueous medium, while I_{338} was chosen as the wavelength value of pyrene entirely in the hydrophobic domain of polymeric micelles. A plot of I_{338}/I_{333} versus log C for PBLG-*graft*-PEG is shown by curve (a) in Figure 2. At the low concentration of PBLG-*graft*-PEG, negligible changes in the magnitude of I_{338}/I_{333} were observed. As the polymer concentration is increased, at a certain concentration (i.e., the CMC), the value of I_{338}/I_{333} increases dramatically in a sigmoidal manner. A typical plot of I_{338}/I_{333} versus log C for the mixture of PBLG-*graft*-PEG with PBLG homopolymer is shown by curve (b) in Figure 2. Similar to the result obtained for PBLG-*graft*-PEG, the ratio exhibits a flat region in the low concentration extreme and increases abruptly at the value of -3.4 (about 0.0004 $g \cdot L^{-1}$), indicating the onset of aggregation. It is also noted that the micellization tends to occur at a lower concentration for the mixed system.

The critical micelle concentrations of PBLG-*graft*-PEG and its mixtures with PBLG homopolymer were determined according to Ref. [28], and are collected in Table 1. As it can be seen, the CMC values show a tendency of decrease

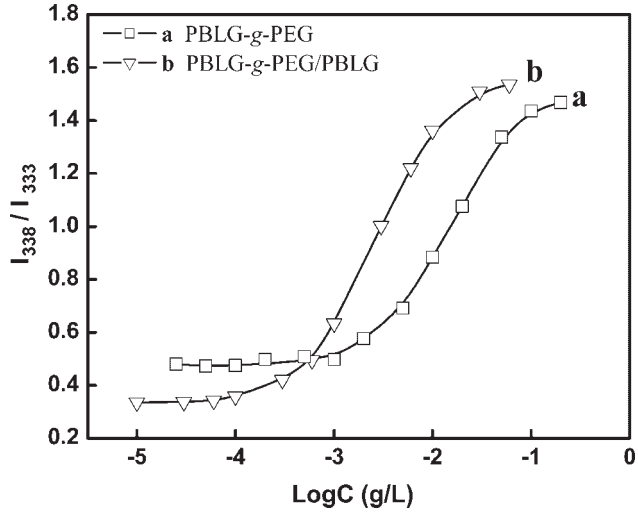

Figure 2. Plots of I_{338}/I_{333} vs log C for PBLG-*graft*-PEG and its mixture with PBLG homopolymer in deionized water (the ratio of PBLG-*graft*-PEG to PBLG by weight: 3:7).

Table 1. The CMC values for PBLG-*graft*-PEG and its mixtures with PBLG homopolymer determined by fluorescence spectroscopy measurements.

Samples	wt.-% of PBLG	wt.-% of PBLG-*graft*-PEG	CMC
			$g \cdot L^{-1}$
1	0	100	2.00×10^{-3}
2	10	90	1.58×10^{-3}
3	30	70	1.12×10^{-3}
4	50	50	7.94×10^{-4}
5	70	30	3.98×10^{-4}

with the increase of PBLG homopolymer content, suggesting that the PBLG homopolymer could promote the micellization. For the mixed systems, when the concentration of the mixture is very low, both PBLG homopolymer and PBLG-*graft*-PEG exist in a no-aggregation fashion. As the concentration increases, the PBLG homopolymer may start to aggregate first because of its stronger hydrophobic property. With a further increase in the concentration of the mixture, the aggregation of the PBLG chains of PBLG-*graft*-PEG could be induced because of the intermolecular interaction between the PBLG homopolymer and the PBLG chain of PBLG-*graft*-PEG. The PBLG homopolymer and PBLG-*graft*-PEG could self-assemble into polymeric micelles with the PBLG chains in the inner of the micelles surrounded by PEG chains. In such a mixed system, the higher the content of PBLG homopolymer, the stronger the hydrophobic property and the smaller the CMC value, as shown in Table 1.

Evidence for the formation of the micelles is also provided by ^{1}H NMR spectra in $CDCl_3$ and D_2O. Curve (a) in Figure 3 shows the ^{1}H NMR spectrum of PBLG-*graft*-PEG in $CDCl_3$ where the micelle formation is not expected. The characteristic peaks of the phenyl protons in the PBLG segment and the methylene protons adjacent to the benzyl group of the PBLG segment appear at 7.2 and 5.1 ppm, respectively. The characteristic peak of ethylene protons of PEG is shown at 3.6 ppm. The ^{1}H NMR spectrum of PBLG-*graft*-PEG in D_2O is shown by curve (b) in Figure 3. It exhibits a striking difference in comparison with the spectrum measured in $CDCl_3$. The peaks of the phenyl protons (7.2 ppm) of the PBLG segment and the methylene protons (5.1 ppm) adjacent to the benzyl group of PBLG disappear. The peak of the ethylene protons of PEG is still visible. Such changes in the ^{1}H NMR spectrum could be attributed to the motions of the protons in the PBLG segments being restricted within the interior of the micelles.[10,18] These results indicate that the graft copolymer could assemble into polymeric micelles with the hydrophobic PBLG segments aggregating as the inner core of the micelles surrounded by PEG. Kwon et al.[10] proved that PBLA-PEG diblock copolymer micelles have a rigid PBLA core and hydrated PEG shell by ^{1}H NMR measurements. Cho et al.[18] also reported that PBLG-PEG diblock copolymer micelles have the rigid PBLG as the core and PEG as the shell according to ^{1}H NMR studies. A typical ^{1}H NMR spectrum of the mixture of PBLG-*graft*-PEG and PBLG homopolymer in D_2O is shown by curve (c) in Figure 3. The peaks at 7.2 and 5.1 ppm also disappear, indicating that in the mixture, both the PBLG homopolymer and the PBLG chains of PBLG-*graft*-PEG exhibit low mobility. The polypeptide chains could be restricted within the interior of the micelles. That is to say, the aggregation of PBLG chains makes the PBLG homopolymer and PBLG-*graft*-PEG assemble to form polymeric micelles with the PBLG chains of both homopolymer and graft copolymer as the interior of the micelles surrounded by PEG chains.

Observations of Transmission Electron Microscopy

Figure 4a presents the morphology of the micelles formed by PBLG-*graft*-PEG. Regular spindly shaped micelles are shown with a length of about 150–200 nm. Because of the difference in molecular architecture, it is a very different morphology compared with the spherical micelles of

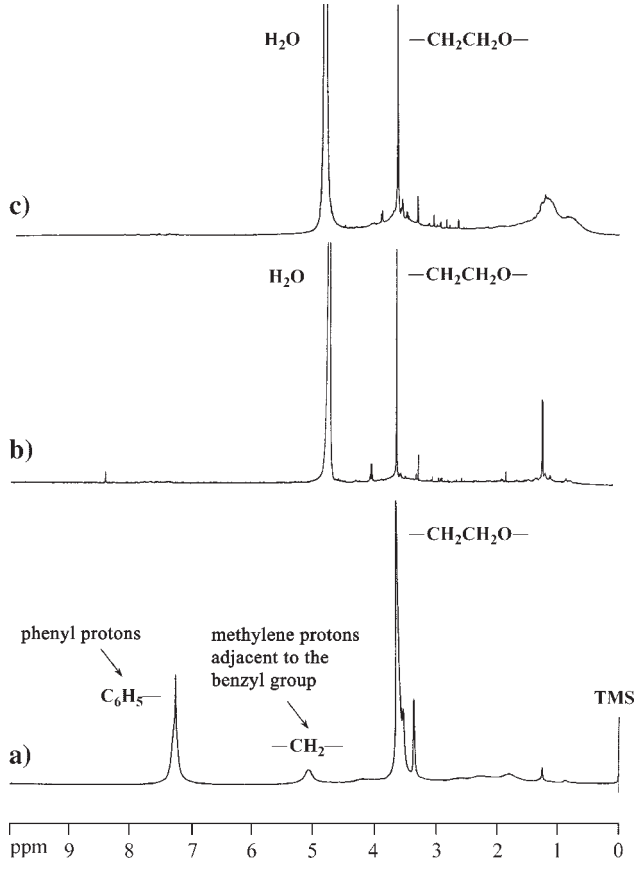

Figure 3. (a) ^{1}H NMR spectrum of PBLG-*graft*-PEG in $CDCl_3$. (b) ^{1}H NMR spectrum of PBLG-*graft*-PEG in D_2O. (c) ^{1}H NMR spectrum of the mixture of PBLG-*graft*-PEG with PBLG in D_2O (the ratio of PBLG-*graft*-PEG to PBLG by weight: 3:7).

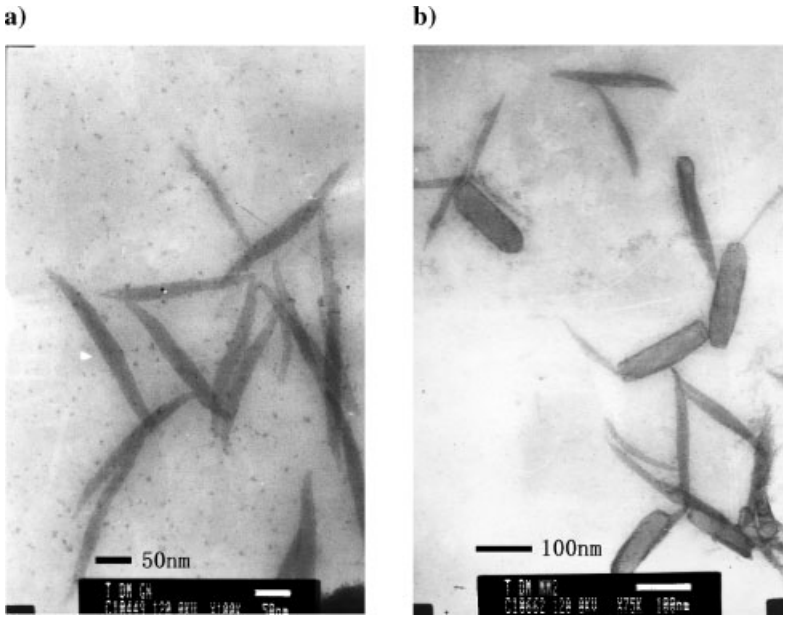

Figure 4. (a) TEM photograph of the micelles formed by PBLG-*graft*-PEG. (b) TEM photograph of the micelles formed by the mixture of PBLG-*graft*-PEG with PBLG (the ratio of PBLG-*graft*-PEG to PBLG by weight: 7:3).

PBLG-PEG block copolymer as described by Cho et al.[8,15] Figure 4b gives an example of the morphology of the micelles based on the mixture of PBLG-*graft*-PEG and PBLG homopolymer. Some micelles change from a spindly shape to a cylindrical shape, while the length of the micelles remains almost the same on the whole. The morphologies of the micelles, formed by the mixtures with other ratios of PBLG-*graft*-PEG to PBLG homopolymer, exhibit similar shapes to that of Figure 4b. The change of the morphology with adding PBLG homopolymer further proves that the PBLG homopolymer could self-assemble into polymeric micelles together with PBLG-*graft*-PEG through the interaction with PBLG chains of PBLG-*graft*-PEG.

Conclusion

The micellization behaviors of PBLG-*graft*-PEG and its mixtures with PBLG homopolymer in aqueous medium were studied by fluorescence spectroscopy, TEM, and NMR spectroscopy. Fluorescence spectroscopy measurements suggest that PBLG-*graft*-PEG associates to form polymeric micelles in water and the addition of PBLG homopolymer could decrease the critical micelle concentration. ^{1}H NMR measurements further prove that PBLG-*graft*-PEG and its mixtures with PBLG homopolymer could assemble into polymeric micelles with hydrophobic PBLG segments aggregating as the interior of the micelles in aqueous medium. The results of the TEM observations show that the micelles of PBLG-*graft*-PEG are almost spindly shaped. The addition of the PBLG homopolymer into the PBLG-*graft*-PEG system changes the morphology of the micelles.

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