pH-responsive Micelles from a Blend of PEG-*b*-PLA and PLA-*b*-PDPA Block Copolymers: Core Protection Against Enzymatic Degradation

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Abstract pH-responsive micelles with a biodegradable PLA core and a mixed PEG/PDPA shell were prepared by self-assembly of poly(ethylene glycol)-*b*-poly(lactic acid) (PEG-*b*-PLA) and poly(2-(diisopropylamino)ethyl methacrylate)-*b*-poly(lactic acid) (PDPA-*b*-PLA). The micellization status with different pH and the enzyme degradation behavior were characterized by ^{1}H-NMR spectroscopy, dynamic light scattering measurement and zeta potential test. The pH turning point of PDPA block was determined to be in the range of 5.5−7.0. While the pH was above 7.0, the PDPA block collapsed onto the PLA core and could protect the PLA core from invasion of enzyme, as a result, the micelle exhibited a resistance to the enzymatic degradation.

Keywords pH-responsive; Enzymatic degradation; Poly(lactic acid)

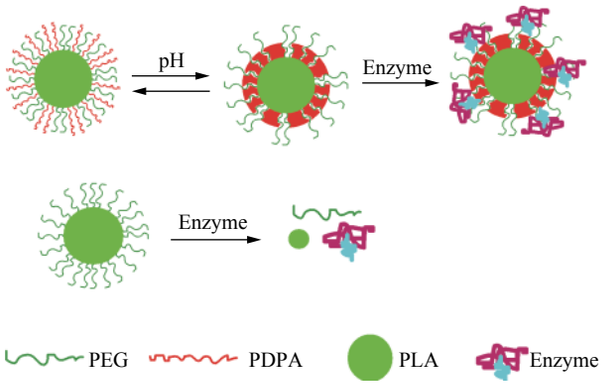
INTRODUCTION

Polymeric micelles fabricated from amphiphilic block copolymers have been extensively studied as drug delivery systems, thanks to their unique properties such as improving the solubility of hydrophobic drugs, increasing the blood circulation time of drugs, and decreasing systemic toxicity of many drugs[1−4]. Among the amphiphilic block copolymers that have been deployed for micelles, poly(ester)s, including poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA) and poly(glycolic acid) (PGA), are the most widely used hydrophobic polymer blocks due to their excellent biodegradability and biocompatibility[5−8]. These poly(ester)s can be used to construct micelle core that serve as a natural reservoir for drugs, peptides, proteins and genes. At the same time, poly(ethylene glycol) (PEG) is usually used as hydrophilic polymer block to stablize the micelle and endow the micelle with the capability of resisting to non-specific adsorption of proteins.

Biodegradability is an advantageous property of poly(ester)s. However, premature biodegradation of the poly(ester) core of micelles by many kinds of enzymes in the human body often leads to the disaggregation of drug carriers[9−11]. Especially at present, more and more drug-carriers are used in the target therapy that demands the drug-carrier can circulate in blood for a very long time before delivering drug to the target site of action at molecular or cellar level in the end[12−15]. Therefore, premature enzymatic degradation is one of the primary barriers for the application of polyester-based drug-carriers[16]. How to obtain a balance between biodegradability and long blood circulation of the drug-loaded micelle is challenging. We have reported a micellar system with a biodegradable PLA core and a mixed PEG/PNIPAM shell to tackle such a problem[17]. The micellar system was prepared by self-assembly of poly(ethylene glycol)-*b*-poly(lactic acid) (PEG-*b*-PLA) and poly(*N*-isopropylacrylamide)-*b*-poly(lactic acid) (PNIPAM-*b*-PLA) in aqueous solution at room temperature, resulting in micelles with a PLA core and a shell consisting of mixed PEG and PNIPAM chains. When the temperature was higher than the lower critical solution temperature (LCST) of PNIPAM, PNIPAM would collapse onto the PLA core and could protect the PLA from the invasion of enzyme. At the sites with a temperature lower than the LCST of PNIPAM, PNIPAM chains in the shell were in the hydrated and extended state and the PLA core could be attacked by enzyme. Such on-demand biodegradation could be realized due to the responsive property of the PNIPAM.

Despite that the developed mixed micelle gives a good drug-carrier prototype, there is still a long way from practical application because the responsive point of the PNIPAM is 32 °C, which is not a typical responsive point of body. Compared to temperature-responsiveness, pH-responsiveness is more helpful during the drug delivery process in body

because a lot of pathological processes exhibit a change in pH[18−21]. For example, the extracellular pH in tumors is lower than that of normal tissues and blood[22], and the intracellular environment of endosomes and lysosomes is more acidic (pH 5.0−6.0)[23]. Herein, we present a new system with a biodegradable PLA core and a mixed PEG/PDPA shell which is prepared by self-assembly of poly(ethylene glycol)-*b*-poly(lactic acid) (PEG-*b*-PLA) and poly(2-(diisopropylamino)ethyl methacrylate)-*b*-poly(lactic acid) (PDPA-*b*-PLA) in aqueous solution. Thanks to the pH-responsive property of PDPA block, core protection against enzymatic degradation can be realized by the change of pH (Scheme 1).



Scheme 1 Schematic illustration of mixed micelles with a pH responsive shell for core-protection against biodegradation

EXPERIMENTAL

Materials

D,L-lactide (LA, 97%) was purchased from Alfa and purified by recrystallization from ethyl acetate. *N*,*N*-diisopropyl-aminoethyl methacrylate (DPA) (97%) was purchased from Sigma-Aldrich and purified by passing through aluminum oxide column to remove the inhibitor. Monomethoxy poly(ethylene glycol) (PEG) with molecular weights of 5000 g/mol was purchased from Aldrich and dried in vacuum for 24 h before use. 2-(Benzylsulfanyl-thiocarbonylsulfanyl) ethanol was synthesized according to a report in literature[24]. 2,2′-Azoisobutyronitrile (AIBN) was recrystallized from ethanol twice. 2-Mercaptoethanol and benzyl bromide were distilled under reduced pressure. Stannous octoate ($Sn(Oct)_2$) (Alfa) was used as received. Proteinase K ($M_n = 2.87 \times 10^4$) was purchased from Merck and used as received. Toluene was distilled before use.

Synthesis of PEG-*b*-PLA and PLA-*b*-PDPA Diblock Copolymers

PEG-*b*-PLA diblock copolymers and PLA macroinitiator were synthesized according to literature and details were included in our previous publication[25]. PLA-*b*-PDPA was synthesized by RAFT methods with PLA as macroinitator and details are given as follows. PLA macroinitor (0.31 g) and AIBN (4.6 mg) were added to a reaction flask followed by vacuum of 2 h. Then, 5 mL of THF and 1.22 g of DPA were added into the flask. The reaction mixture was degassed by three freeze-thaw cycles and flame-sealed under vacuum. Polymerization was performed at 65 °C for 48 h. After the completion of polymerization, the reaction mixture was concentrated by rotary evaporation and dissolved in dichloromethane followed by dialyzed against alcohol for 48 h to remove the copper catalyst. Then the solution was vacuum-dried after a vacuum filtration to get the end product of PDPA-*b*-PLA diblock copolymer.

Preparation of Mixed Micelles with PEG-*b*-PLA and PLA-*b*-PDPA Diblock Copolymers

The PEG-*b*-PLA and PLA-*b*-PDPA block copolymers were first dissolved in acetone to form the original polymer/acetone solutions with a concentration of 1.0 g/L, respectively. Subsequently, the original PEG-*b*-PLA and PLA-*b*-PDPA solutions were mixed with weight ratio of 1:1. Then, distilled water with a pH of 3.0 was added to the mixed acetone solution at a rate of 1 drop per 20 s under vigorous stirring until opalescence appeared. The solution was further strongly stirred overnight and then some amounts of water were added to get a final concentration of 0.1 g/L. The resultant solution with a pH of 3.0 was put into a dialysis bag (molecular weight cut off: 7000 Da) and dialyzed against distilled water (pH = 3) to remove acetone for at least 2 days. The solutions with other pH values were obtained by a further dialysis against distilled water of different pH values, respectively.

Enzymatic Degradation Experiment of Micelles

Proteinase K solution with a concentration of 0.4 g/L was prepared by dissolving proteinase K into a tris-HCl solution at pH 7.4. Proteinase K solution was added to the mixed micelle solution with a pH of 7.4 and the degradation process was monitored *in situ* by measuring light scattering intensity versus time on the laser light scattering instrument. For comparison, the degradation process during which Proteinase K solution was added to PEG-*b*-PLA micelle solution with a pH of 7.4 was also monitored *in situ*.

Drug Release from Micelles

A given amount of indomethacin, PEG-*b*-PLA and PLA-*b*-PDPA were dissolved in acetone together. Then distilled water at a pH of 4 was added dropwise into the mixed solution with vigorous stirring until opalescence appeared. The solutions were strongly stirred overnight and then a certain amount of water was added, and the final concentration of mixed micelle was 0.1 g/L. Then the solution was put into dialysis bags (molecular weight cut off: 7000 Da) and dialyzed against water (pH = 4) for 48 h to remove acetone. Further, the solution was dialyzed against distilled water with pH of 7.4 for another 48 h to get a preferred pH value, and the micelle solution was filtered through a 0.45 μm Millipore filter to remove free indomethacin particles. A solution of single PEG-*b*-PLA micelle with indomethacin loaded was also prepared as the same procedure for comparison. Drug release was monitored on a TU-8110 UV-Vis spectrophotometer. The filtered micelle solution (4 mL) (with or without enzyme) was transferred into a dialysis bag, which was then placed in 20 mL of a phosphate buffer solution (PBS, 0.05 mol/L, pH

7.4) at 37 °C. Aliquots of 4 mL were withdrawn from the buffer solution into containers at a defined interval. The volume of the solution was kept constant by adding 4 mL of the buffer solution after each sampling. The content of indomethacin was measured *via* the absorbance at 350 nm.

Characterizations

^{1}H-NMR spectra were obtained on a Varian UNITY-plus 400M NMR spectrometer using $CDCl_3$ or D_2O with different pH as solvents. The gel permeation chromatography (GPC) was performed with a Waters 1525 chromatograph equipped with a Waters 2414 refractive index detector. Transmission electron microscopy (TEM) measurements were conducted using a JEM-100X electron microscope at an acceleration voltage of 100 kV. Dynamic light scattering (DLS) measurement and zeta potential test were performed on a laser light scattering instrument (BI-200SM) equipped with a digital correlator (BI-9000AT) at 532 nm. Potentiometric titration was performed on a ZD-2 automatic potentiometric titrator.

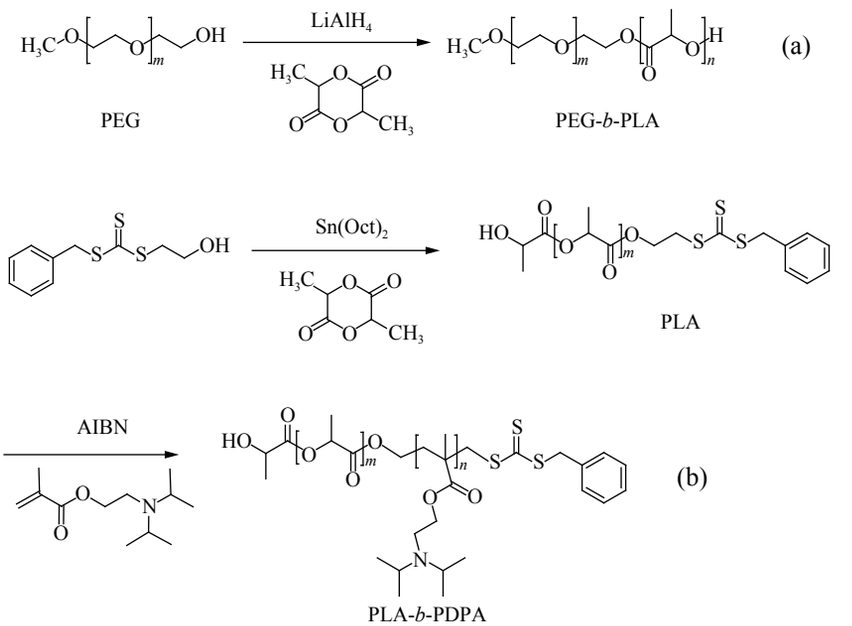
RESULTS AND DISCUSSION

Synthesis of PEG-*b*-PLA and PLA-*b*-PDPA Diblock Copolymers

The block copolymer of PEG-*b*-PLA was synthesized by ring-opening polymerization (ROP) with PEG as a macroinitiator, and the block copolymer of PLA-*b*-PDPA was synthesized by ROP of LA with a RAFT initiator and followed by RAFT polymerization of DPA, as illustrated in Scheme 2. Fig. 1 shows ^{1}H-NMR spectra of PEG-*b*-PLA (Fig. 1A) and PLA-*b*-PDPA (Fig. 1B) in $CDCl_3$. The resonance signals observed in the regions of 5.2 ppm (b, h) and 1.6 ppm (c, g) are attributed to methine protons in the backbone and the pendent methyl protons of PLA, respectively. The signal at 3.6 ppm (a) is attributed to methylene protons of PEG. The resonance signals observed in the regions of 3.8 ppm (i) and 3.0 ppm (d) are attributed to methylene protons between the amino group and the ester group, The signal at 2.6 ppm (e) is attributed to methine protons in the isopropyl group, and the one at 1.0 ppm (f) is attributed to methyl protons in the isopropyl group. The polydispersity of PEG-*b*-PLA, PLA and PLA-*b*-PDPA measured by GPC using THF as the eluent were 1.58, 1.33 and 1.46, respectively (Fig. 2). The number-average molecular weight (M_n) of PEG-*b*-PLA measured by GPC was 2.50×10^4 g/mol, and M_ns of PLA macromolecular initiator and block copolymer PLA-*b*-PDPA measured by GPC were 6.0×10^3 and 1.75×10^4 g/mol, respectively. The composition of the block copolymers was determined by a combination of GPC and ^{1}H-NMR, and the two block copolymers were denoted as PEG_{113}-*b*-PLA_{277} and PLA_{83}-*b*-$PDPA_{94}$ with the subscript indicating the number of repeating units.

Preparation and Characterization of pH-responsive Mixed Micelles

PDPA was a pH-sensitive polymer which was soluble in water as a weak cationic polyelectrolyte in acidic environment, and it became insoluble when the pH was higher than approximately pH 6 due to deprotonation of its tertiary amine groups[26]. When the PEG-*b*-PLA and PLA-*b*-PDPA block copolymers were dissolved in acetone and then with distilled water (pH 3.0) added, a mixed micelle with a PLA core and a PEG/PDPA mixed shell was supposed to be formed. Curve (a) in Fig. 3(A) shows the hydrodynamic diameter (D_h) distribution of mixed micelles with 1:1 ratio of PEG-*b*-PLA to PLA-*b*-PDPA at pH 3 at 25 °C. It is clear that the micelles had a narrow diameter distribution with a mean D_h of 171 nm. When the pH of the solution changed from 3 to 11, the PDPA block turned to hydrophobic from the hydrophilic state due to the deprotonation of the tertiary amine group of PDPA. As a result, the PDPA block



Scheme 2 Synthesis of (a) PEG-*b*-PLA and (b) PLA-*b*-PDPA diblock copolymers

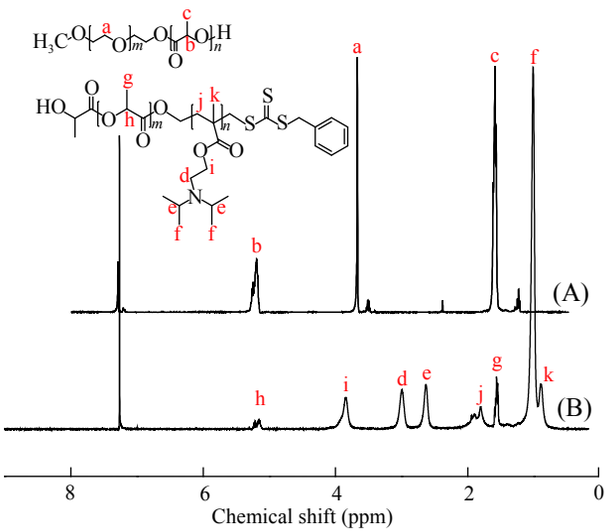


Fig. 1 ^{1}H-NMR spectra of (A) PEG-*b*-PLA and (B) PLA-*b*-PDPA diblock copolymers in $CDCl_3$

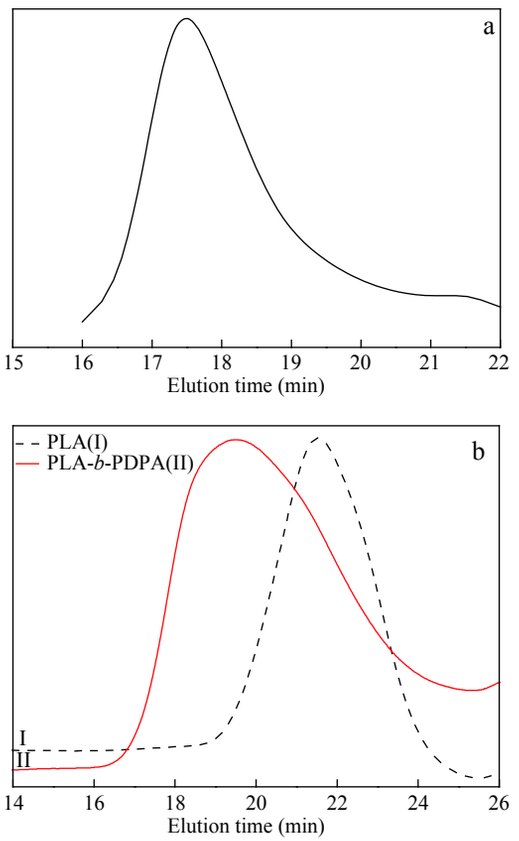


Fig. 2 GPC curves of (a) PEG-*b*-PLA, (b) PLA and PLA-*b*-PDPA using THF as the eluent

collapsed onto the PLA core, leading to a mixed micelle with a PLA core, a PDPA collapsed layer and a PEG shell. The curve (b) in Fig. 3(A) shows the D_h distribution of mixed micelles at pH 11 at 25 °C. It is clear that the micelles also had a narrow diameter distribution, and the mean hydrodynamic diameter decreased to 147 nm from 171 nm. The decreasing diameter of micelles with the change of pH

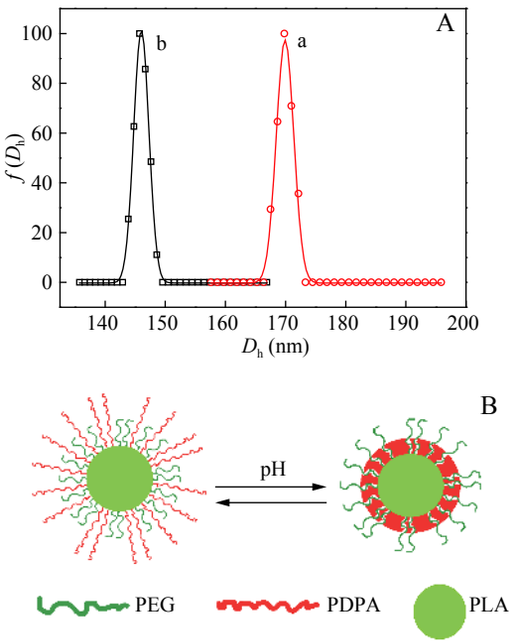


Fig. 3 (A) Hydrodynamic diameter (D_h) distributions of the mixed micelles with 1:1 ratio of PEG-*b*-PLA to PLA-*b*-PDPA at various pH values: (a) pH 3 and (b) pH >> 6; (B) Schematic illustration of the D_h change of the mixed micelles with pH

value can be attributed to the collapse of PDPA block from the stretching conformation. The molecular weight of the PEG is 5.0×10^3 g/mol, and the molecular weight of the PDPA given by GPC is 1.15×10^4 g/mol. When pH is 3, D_h of the mixed micelles is determined by the length of PDPA. By contrast, at pH 11, D_h decreases because of the collapse of PDPA, and it is mainly determined by the length of PEG. The possible process is illustrated in Fig. 3(B). Shown in Fig. 4 is the TEM image of the mixed micelles with a 1:1 PEG-*b*-PLA/PLA-*b*-PDPA ratio at 25 °C at pH 11. The micelles are uniform spheres with a diameter consistent with that obtained by DLS.

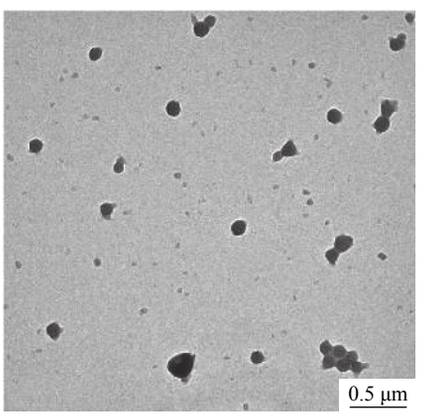


Fig. 4 TEM image of the mixed micelles with a 1:1 PEG-*b*-PLA/PLA-*b*-PDPA ratio at 25 °C at pH 11

The zeta potential test was performed to determine the pH responsiveness of the PDPA block in the micellar shell. Fig. 5(a) shows the zeta potential of the above mixed micelle

at different pH values. It can be seen that when pH was lower than 5.5, the mixed micelle had a positive zeta potential with values ranging from 8 mV to 9 mV due to the protonation of the tertiary amine group of PDPA. When pH was higher than 5.5, the zeta potential decreased rapidly, and at pH 7.0, the zeta potential reached the lowest point with values ranging from 2.5 mV to 3.5 mV. From the zeta potential versus pH, we can determine the pH turning range of PDPA block is about 5.5−7.0. The pK_a value of block of PDPA was also determined by potentiometric titration[27], and the result is given in Fig. 5(b). The pK_a of PDPA was around 6.00, which was in agreement with the result of zeta potential test.

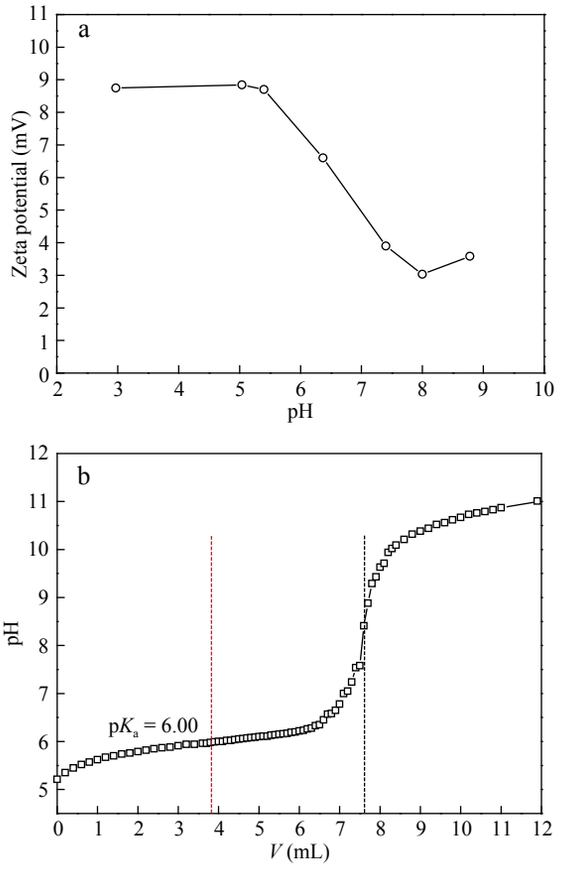


Fig. 5 (a) Zeta potential of the mixed micelles at different pH values; (b) potentiometric titration curve record in aqueous solution

^{1}H-NMR spectroscopy was also used to monitor the collapse of the PDPA block. Fig. 6 shows the ^{1}H-NMR spectra of mixed micelles with a 1:1 ratio of PEG-*b*-PLA/PLA-*b*-PDPA in D_2O at 25 °C at different pH values. At pH 3, the signals due to PEG blocks in the region of 3.3 ppm (a) and the signals due to PDPA blocks in the regions of 3.8 ppm (b), 3.4 ppm (c), 3.1 ppm (d) and 1.0 ppm (e) are visible, indicating that the two blocks had good mobility. The signals attributed to the methine protons in the backbone and the pendent methyl protons of PLA which appear in the regions of 5.2 and 1.6 ppm in Fig. 1 are invisible here, which indicates the PLA core was formed. At pH 7, the signals attributed to PDPA blocks disappear due to the loss of mobility of collapsed PDPA blocks. The intensity of PEG signals remains unchanged, which suggests that the mobility of PEG block was not affected by the collapse of PDPA chains. It should be noted that the chemical shift for methylene in PEG chain shifted slightly as the pH was changed from 3 to 7. Similar phenomenon was also reported by others[28].

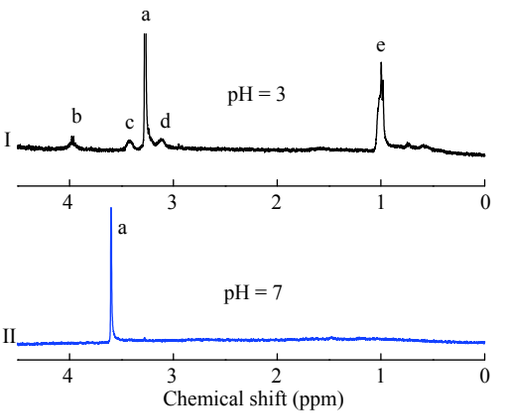


Fig. 6 ^{1}H-NMR spectra of mixed micelles in D_2O with different pH values

From all these evidences, we can draw a conclusion that mixed micelles formed from 1:1 ratio of PEG-*b*-PLA to PLA-*b*-PDPA is pH sensitive and the pH turning range is around 5.5−7.0.

Enzymatic Degradation of Micelles

It is proven that laser light scattering is a good method to study the degradation of polymer. Wu and Jiang have studied polymer degradation by laser light scattering and proved that the decrease in light scattering intensity of the samples is mainly attributed to the decrease in number of the particles[29, 30]. In our previous work, we have prepared a complex micelle with a PLA core and a mixed PEG/PNIPAM shell, and have proven that with the change of temperature, the PNIPAM block can change from the hydrophilic to hydrophobic state. The collapsed PNIPAM layer can prevent the micelle core from degradation by enzymes[17]. Herein, in order to test whether the mixed micelle with a PLA core, a PDPA collapsed layer and a PEG shell can prevent the micelle core from the invasion of enzyme, the process of enzyme degradation was monitored *in situ* by measuring the change of light scattering intensity against time (Fig. 7). As a control, the process of enzyme degradation of simple micelles from only PEG-*b*-PLA was also monitored. The enzyme used here was proteinase K, which could effectively degrade PLA. It was found that for the micelles formed by PEG-*b*-PLA, the relative light scattering intensity dropped quickly after a short platform of 40 min and went to nearly zero in about 80 min. It revealed that the micelles were totally degraded in 80 min at 25 °C. For the mixed micelle with a PLA core, a PDPA collapsed layer and a PEG shell (PLA-*b*-PDPA:PEG-*b*-PLA = 1:1) at pH 7.4, the relative light scattering intensity remained constant, which indicated that the mixed micelle was not affected by the enzyme, thanks to the collapse of PDPA while the environment pH was above 7.0.

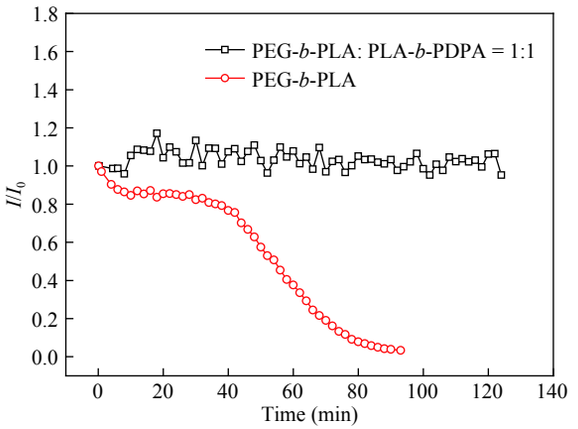


Fig. 7 Degradation time dependence of the relative scattering intensity I/I_0 of micelles formed by single PEG-*b*-PLA block copolymer and 1:1 mixed PEG-*b*-PLA and PLA-*b*-PDPA block copolymer at pH = 7.4, where I_0 is the scattering intensity of micelles tested at $t = 0$

Controlled Drug Release from Micelles

Fig. 8 shows the controlled indomethacin release from micelles in PBS at pH 7.4 with or without enzyme at 37 °C. For both the simple micelle from only PEG-*b*-PLA and the mixed micelle with a PEG-*b*-PLA:PDPA-*b*-PLA ratio of 1:1 in the absence of the enzyme (a and b), the rate of drug release was well controlled and only around 20% of the drug was released in 32 h. However, in the presence of the enzyme, burst release was observed for drug in the simple micelle from only PEG-*b*-PLA (c), and more than 70% of the drug was released in 1 h, but the rate of drug release from the mixed micelle in the presence of enzyme showed only slight change thanks to the protective collapse of the PDPA block (d).

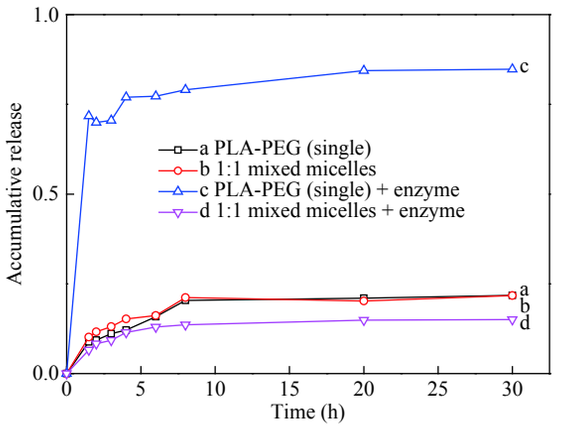


Fig. 8 Drug release profiles of indomethacin from micelles at pH 7.4: (a) simple PEG-*b*-PLA micelles without enzyme; (b) 1:1 mixed micelles without enzyme; (c) simple PEG-*b*-PLA micelles with enzyme; (d) 1:1 mixed micelles with enzyme

CONCLUSIONS

Mixed micelles with a biodegradable PLA core and a mixed PEG/PDPA shell were successfully prepared from PEG-*b*-PLA and PLA-*b*-PDPA. Owing to the pH-responsive property of the PDPA chains, the enzymatic degradation of the mixed micelle can be controlled by changing the pH value of environment. While the pH is increased, the PDPA block collapses onto the PLA core and can protect the PLA core from the invasion of enzyme, and the micelle shows resistance to the enzymatic degradation. This novel type of mixed micelle can serve as a promising drug-carrier for intracellular protein delivery or cancer therapy.

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