Effect of pH and Content of Reduction-Sensitive Copolymer on the Guest Exchange Kinetics of Micelles

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Received 9 May 2018; accepted 7 October 2018; published online 4 November 2018
DOI: 10.1002/polb.24754

ABSTRACT: The micelles formed by reduction-sensitive amphiphilic copolymer have emerged as promising drug nanocarriers due to the controlled drug release and effective anticancer activity triggered by the reducing stimulation. However, the effect of pH on the stability and guest exchange of the micelles formed by reduction-sensitive copolymer have not been systemically investigated. Herein, the micelles formed by a reduction-sensitive copolymer poly(ε-caprolactone)-*b*-poly[oligo(ethylene glycol) methyl ether methacrylate] (PCL–SS–POEGA) with a single disulfide group at different pH values loaded with dyes 3,3′-dioctadecyloxacarbocyanine perchlorate/1,1′-dioctadecyl-3,3,3′, 3′-tetramethylindocarbocyanine perchlorate (DiO/DiI) were prepared through the precipitation-dialysis method. In addition, mixed micelles formed by different contents of reduction-sensitive and reduction-insensitive copolymers encapsulated with DiO/DiI at pH 7.5 were also prepared by the similar approach. The effects of pH and the content of reduction-sensitive copolymer on guest exchange of these micelles were studied by the fluorescence resonance energy transfer method. Results show that the pH value in the environment has great influence on the guest exchange rate of reduction-sensitive micelles in the presence of 10 mM dithiothreitol (DTT) and slight effect on that in the absence of DTT. Under a reducing environment, the guest exchange rate of the micelles containing various contents of disulfide-linked copolymer increases with the increasing content of PCL–SS–POEGA. © 2018 Wiley Periodicals, Inc. J. Polym. Sci., Part B: Polym. Phys. **2018**, *56*, 1636–1644

KEYWORDS: diblock copolymers; fluorescence; micelles; stimuli-sensitive polymers

INTRODUCTION Recently, nanocontainers (e.g., polymeric micelles, liposome, nanogels, and vesicles) used as drug delivery systems are regarded as a promising method to improve the anticancer efficacy of drugs.[1–5] For example, studies have verified that nanoparticles loaded with drugs can reduce drug side effects, prolong circulation time, enhance accumulation in the tumor sites via the enhanced permeability and retention effect and improve drug anticancer activity.[1,6–8] Various environment-sensitive polymeric nanoparticles in response to stimulus such as pH, redox potential, temperature, and light have been developed for the drug delivery to enhance the drug release at the target tumor sites.[9,10] Particularly, reduction-sensitive polymer nanomaterials containing disulfide groups have been designed to promote the efficiency of drug delivery and release, according to the higher concentration of the reducing agent glutathione in cell interior (2–10 mM) than the extracellular environments (2–10 μM).[11–18] For instance, Oh and coworkers have conducted a number of works about reduction-sensitive copolymers used as drug carriers to accelerate the drug release.[19–21] Zhong and coworkers have reported a series of studies about reduction-sensitive nanomaterials for the controlled cytoplasmic delivery of a variety of biomolecules including drugs, DNA, proteins, siRNA, and so forth.[22–27] Their results indicate that reduction-sensitive biodegradable polymer assemblies are highly promising biomaterials which have excellent stability in the circulation and in extracellular fluids but rapidly degrade under the reducing environment presenting in tumor tissues and intracellular apparatus.

Reduction-sensitive polymeric micelles have shown wide applications in the biological field, so it is important to explore the stability of the micelles and guest exchange of the molecules encapsulated in the micelles.[28–30] Our previous work has studied the effect of poly(ε-caprolactone) (PCL) hydrophobic chain length on the stability and guest exchange of micelles formed from disulfide-linked copolymers.[31] We found that the micelles with longer PCL chains have higher stability and slower guest

Additional Supporting Information may be found in the online version of this article.

exchange rate in the aqueous solutions containing 10 mM dithiothreitol (DTT). In addition, Zhong and coworkers demonstrated the colloidal stability, intracellular drug release, and antitumor activity of micellar drugs can be precisely controlled by the content of reduction-triggered shedding of hydrophilic shells.[32] However, the stability and guest exchange of these micelles containing disulfide group at different pH values in the presence and absence of DTT have not been studied previously, yet it is significantly important due to the diversity of pH in the environment of the organs, tissues, cells, and subcellular compartments. In detail, tumor tissue has an extracellular pH value between 6.5 and 7.2, which is slightly lower than the normal blood pH value of 7.4, and intracellular organs endosomes and lysosomes have a pH value of 5.0–6.5 and 4.5–5.0, respectively.[33–35] Furthermore, we are interested in the guest exchange behavior of the mixed micelles formed by different contents of reduction-sensitive copolymer.

Until now, some techniques have been used to investigate the stability of the micelles under various conditions, including dynamic light scattering (DLS),[36] size exclusion chromatography,[37] fluorescence spectroscopy[38] including fluorescence resonance energy transfer (FRET) method. Recently, FRET method is known as a robust method to study the guest exchange behavior of the nanomaterials *in vitro* and *in vivo*.[29,39–52] For an FRET pair, if the fluorescence emission spectrum of the donor overlaps the absorption spectrum of the acceptor, and the distance between donor and acceptor is close to the Förster radius, the energy transfer phenomenon might occur.[53–55] Thayumanavan has conducted a series of researches about the stability and guest exchange of the nanogels.[42–47] For instance, they investigated the effect of Hofmeister ions and crosslinking density on the guest encapsulation stability of a polymeric nanogel.[43,44]

In this study, we evaluated the effect of pH in the environment on the stability and guest exchange of the reduction-sensitive polymeric micelles in the presence and absence of DTT using the FRET-based method. Moreover, the effect of the content of reduction-sensitive copolymer on the stability and guest exchange rate of the mixed micelles was also investigated by the similar method.

EXPERIMENTAL

Materials

Oligo(ethylene glycol) methyl ether acrylate (OEGA, M_n = 480 g mol^{-1}, Sigma-Aldrich) was passed through an alkaline alumina column to remove the inhibitor. CL (Aladdin, 99%) was distilled after dried over calcium hydride (CaH_2). 2-Hydroxyethyl disulfide (Aldrich, 90%), 2-bromo-2-methylpropionyl bromide (Energy Chemical, Shanghai, China, 98%), *N*,*N*,*N*′,*N*′,*N*″-pentomethyldiethylenetriamine (PMDETA, Sigma-Aldrich, Shanghai, China, 99%), stannous octoate ($Sn(EH)_2$, Sigma-Aldrich, Shanghai, China, 95%), and DTT (Aladdin, Shanghai, China, 99%) were used without purification. 3,3′-Dioctadecyloxacarbocyanine perchlorate (DiO, Beyotime, Shanghai, China), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Beyotime), Shanghai, China. Other chemicals were purchased from Sinopharm (Shanghai, China). Dimethyl sulfoxide (97%), dimethylformamide (DMF, 97%) were dried over anhydrous magnesium sulfate and then distilled under reduced pressure. Dichloromethane (97%) and triethylamine (99%) were distilled over CaH_2. Tetrahydrofuran (97%) and toluene (97%) were distilled after refluxing over metal sodium for 24 h. Phosphoric acid (85%), disodium hydrogen phosphate hydrate (99%), and sodium dihydrogen phosphate hydrate (99%) were used to prepare PB solutions with different pH values. The ionic strength for each buffer solution was 10 mM.

Preparation of PCL–R–Poly[oligo(ethylene glycol) methyl ether methacrylate] Copolymer

The reduction-sensitive and reduction-insensitive poly(ε-caprolactone)-*b*-poly[oligo(ethylene glycol) methyl ether methacrylate] (PCL–SS–POEGA/PCL–CC–POEGA) copolymers were synthesized by a combination of ring-opening polymerization and atom transfer radical polymerization (ATRP), as shown in Scheme S1, Supporting Information. The small molecular initiator (HO–SS–Br) and macroinitiator HO–PCL–SS–Br containing a disulfide group were successfully synthesized according to previous work.[56,57] The reduction-responsive copolymer was prepared as follows.[58] A mixture of HO–PCL–SS–Br, 0.25 g, 0.076 mmol, OEGA (0.77 g, 1.6 mmol), PMDETA (15.8 μL, 0.076 mmol) was dissolved in 1.5 mL of purified DMF, and then degassed by three freeze-pump-thaw cycles. Subsequently, CuBr (11 mg, 0.076 mmol) was added into the tube and sealed immediately. The reaction was stopped after stirring at 60 °C for 8 h. Then, the mixture was passed through a column of neutral alumina, concentrated and precipitated into an excess of the mixture of cold diethyl ether/*n*-hexane (1/2, v/v). Sticky solid PCL–SS–POEGA (0.56 g, yield: 77%) was obtained after drying under vacuum for 24 h. The reduction-insensitive polymer PCL–CC–POEGA was synthesized via the similar procedure. The molecular weights of the reduction-sensitive and reduction-insensitive copolymer PCL_i–R–$POEGA_j$ were measured by hydrogen nuclear magnetic resonance (^{1}H NMR), where *i* and *j* are the degrees of polymerization for CL and OEGA, respectively. For PCL_{29}–SS-$POEGA_{13}$ and PCL_{28}–CC–$POEGA_{13}$, M_w/M_ns are 1.15 and 1.14, respectively. ^{1}H NMR for PCL_{29}–SS–$POEGA_{13}$ (δ, ppm, $CDCl_3$): 4.29–4.45 (m, –COO–*CH*$_2$–CH$_2$–SS–CH$_2$–*CH*$_2$–OOC), 4.10–4.28 (m, –COO–*CH*$_2$–CH$_2$–O–CH$_2$–), 4.0–4.09 (m, –OOC–CH$_2$–CH$_2$–CH$_2$–CH$_2$–*CH*$_2$O–), 3.45–3.79 (m, –COO–CH$_2$–*CH*$_2$–O–*CH*$_2$–*CH*$_2$–O–CH$_2$–), 3.32–3.42 (s, –O–C*H*$_3$), 2.87–2.98 (m, –H$_2$C–*CH*$_2$–SS–*CH*$_2$–CH$_2$–), 2.17–2.44 (m, –CH$_2$–*CH*$_2$–COO–CH$_2$, –CH$_2$–*CH*–COO), 1.52–1.95 (m, –OOC–CH$_2$–*CH*$_2$–CH$_2$–*CH*$_2$–CH$_2$O–, –C(CH$_3$)$_2$–*CH*$_2$–CH–COO), 1.29–1.48 (m, OOC–CH$_2$–CH$_2$–*CH*$_2$–CH$_2$–CH$_2$O–), 1.09–1.21 (s, –OOC–*C*(*CH*$_3$)$_2$–CH$_2$).

Preparation of PCL–SS–POEGA Micelles Containing Dyes at Different pH Values

The dyes DiO (2 mg) and DiI (2 mg) were separately dissolved in 4 mL acetone to obtain the dye solutions with a concentration of 0.5 mg mL^{-1}. The copolymer PCL–SS–POEGA (10 mg) was dissolved in 350 μL of acetone and the polymer solution was mixed with 150 μL of dye solution

(DiO or DiI) with a concentration of 0.5 mg mL^{-1}. Then, the mixture was slowly injected into 5 mL of 10 mM PB solutions with different pH values (pH = 4.5, 5.5, 6.5, 7.5, and 8.5). After that, the micellar solution was dialyzed by a dialysis membrane with a molecular-weight cutoff of 14,000 g mol^{-1} against 1 L of PB solution at the same pH value for 48 h to remove the free dye molecules and acetone. The buffer solution was changed every 12 h. For cryogenic transmission electron microscopy (cryo-TEM) experiments, thin films were prepared under controlled temperature and humidity conditions (97–99%) within a custom-built environmental chamber. A total of 3–4 μL of the micelles solution was put on a copper grid coated with carbon film at room temperature. The loaded grid was then plunged into liquid ethane which was cooled by liquid nitrogen. Images were taken by cryo-TEM with a FEI Tecnai G2 F20 Twin instrument at an acceleration voltage of 200 kV and analyzed by ImageJ software.

Preparation of Mixed Micelles Containing Dyes with Different Contents of PCL–SS–POEGA

Similarly, mixed micelles consisting of different contents of PCL–SS–POEGA and PCL–CC–POEGA encapsulated with DiO or DiI in 10 mM PB buffer solution (pH = 7.5) were prepared through the similar method as mentioned above. A typical procedure for preparation of the mixed micelles containing of 50 wt % of PCL–SS–POEGA was conducted as follows. The amphiphilic copolymers PCL–SS–POEGA (5 mg) and PCL–CC–POEGA (5 mg) were dissolved in 350 μL of acetone and mixed with 150 μL of dye acetone solution with a concentration of 0.5 mg mL^{-1} (DiO or DiI), stirred for 30 min, injected into 5 mL of PB solution (pH = 7.5) at a speed of 20 mL h^{-1}, and dialyzed under the same condition.

Guest Exchange Kinetics of PCL–SS–POEGA Micelles and Mixed Micelles

The PCL–SS–POEGA micellar solution containing either DiO or DiI was mixed with the corresponding polymer micelles loaded with the other dye in PB solution at a certain pH value. An amount of degassed DTT buffer solution was added rapidly into the mixed micellar solution and the final concentrations of copolymer and DTT were 1 mg mL^{-1} and 10 mM, respectively. The concentration of the control group without DTT was adjusted by the degassed PB solution. All solutions were filtered through a 450 nm hydrophilic poly(tetrafluoroethylene) filter before mixing. The fluorescence spectroscopy of the micellar solutions as mentioned above were conducted on an F-4600 fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Tokyo Japan). The emission spectra were collected from 490 to 600 nm using a slit width of 2.5 nm and a photomutiplier voltage of 950 V, with a fixed excitation wavelength of 488 nm. The guest exchange experiments of the mixed micelles containing different contents of PCL–SS–POEGA / PCL–CC–POEGA were carried out in the similar way.

RESULTS AND DISCUSSION

Synthesis of PCL–R–POEGA Block Copolymers and Preparation of Micelles

The block copolymers PCL–R–POEGA were synthesized through ring-opening polymerization and ATRP according to previous works.[56,57] As shown in Figure S1, Supporting Information, the integral area ratio of peak (*c*), peak (*f*), and peak (*k*) is 29.0/2.18/13.09, which is consistent with the theoretical ratio. The molecular weights of final products measured by gel permeation chromatography and ^1H NMR are summarized in Table S1. The results indicate reduction-sensitive amphiphilic copolymers were successfully prepared by ATRP using the bifunctional initiator (HO–SS–Br). The micelles formed from PCL–SS–POEGA copolymer were prepared through the precipitation-dialysis method.[31] Similarly, the mixed micelles containing various contents of PCL–SS–POEGA and PCL–CC–POEGA were prepared using the similar approach. The average diameters of these micelles and the mixed micelles measured by a Malvern Zetasizer Nano Series (Malvern Instruments Ltd., Worcestershire, UK) with a 633 nm He–Ne laser as a light source at 25 °C are in the region of 100 ± 15 nm with a low polydispersity index. The hydrodynamic diameter distributions $f(D_h)$ of all micelles loaded with DiI or DiO are shown in Figure S2. Figure S3 shows the cryo-TEM micrograph and the corresponding diameter distribution of the PCL–SS–POEGA micelles. The results show that the diameter of spherical micelles measured by cryo-TEM is (94 ± 20) nm, which is consistent with DLS results. Moreover, the critical micelle concentration (CMC) values of PCL–SS–POEGA micelles at pH 4.5 and pH 7.5 were determined using pyrene as a fluorescence probe on an F-4600 fluorescence spectrophotometer (Hitachi High-Technologies Corporation). The CMC values of PCL–SS–POEGA micelles at pH 4.5 and pH 7.5 are 6.3 and 5.8 mg L^{-1}, as shown in Figure S4.

The Guest Exchange of PCL–SS–POEGA Micelles Containing DiO/DiI at Different pH Values

Here, the stability and guest exchange of PCL–SS–POEGA micelles in PB solutions at various pH values were investigated by FRET method according to previous works.[31,43,46] The hydrophobic dyes, DiO as the donor and DiI as the acceptor, were used as an FRET pair in our study. The fluorescent molecules DiO and DiI were separately encapsulated into reduction-sensitive polymeric micelles in PB solutions with various pH values, respectively. Then, these two kinds of micelle containing DiI or DiO in buffer solutions were mixed with a molar ratio of 1:1, and diluted with DTT solution or PB solution to obtain the polymeric micelles with a polymer concentration of 1 mg mL^{-1} and with DTT concentrations of 0 or 10 mM. If the micelles are intact, there will be no energy transfer from DiO to DiI as the distance between the donor and acceptor is much larger than the Förster radius (R_0 = 5–6 nm).[59,60] However, if the micelles are not stable or the corona are partially destroyed or completely destroyed, the energy transfer phenomenon will occur due to the easy diffusion of dyes through the corona.[61] The time-dependent

fluorescence emission spectra of PCL–SS–POEGA micelles at various pH values after mixing in the presence and absence of DTT were monitored by a fluorescence spectrophotometer.

A series of time-dependent fluorescence spectra of the reduction-sensitive micelles in both reducing and nonreducing conditions at pH 4.5 and pH 8.5 are shown in Figure 1. As shown in Figure 1(a), the fluorescence intensity of DiO (I_{DiO}) at 510 nm decreases slowly and the emission peak of the DiI (I_{DiI}) at 573 nm slightly increases over time in the absence of 10 mM DTT at pH 4.5. The time-resolved spectra of the mixture remain unchanged in buffer solution at pH 8.5 without the addition of DTT, as shown in Figure 1(c), which is consistent with our previous result that the fluorescence spectra of reduction-sensitive micelles in aqueous solutions changed slowly without DTT.[31] The result indicates that pH value has a slight effect on the dyes exchange rate between the micelles in the absence of DTT. In addition, the time-resolved fluorescence spectra of PCL–SS–POEGA micelles in the presence of DTT are shown in Figure 1(b,d). Figure 1(d) shows that the I_{DiO} reduces dramatically, and I_{DiI} increases quickly at pH 8.5 in the presence of 10 mM DTT, but the spectra at pH 4.5 change slowly, as shown in Figure 1(b), indicating that pH value has a significant influence on the stability of PCL–SS–POEGA micelles in the presence of DTT. In detail, Figure 1 (d) shows that I_{DiO} reduces from 3440 to 120 within 120 min and our previous work shows that I_{DiO} approximately reduces from 2500 to 120 within 12 h for PEG_{113}–SS–PCL_{39} micelles in an aqueous solution without buffer agent.[31] Moreover, I_{DiO} decreases from 4050 to 1200 after 1530 min for the reduction-sensitive micelles with 10 mM DTT at pH 4.5, as shown in Figure 1(b). Note that both I_{DiO} and I_{DiI} decrease with time due to the formation of aggregates and attenuation of the incident light; however, we are concerned about the fluorescence intensity ratio $I_{DiI}/(I_{DiO} + I_{DiI})$, so this effect can be eliminated and the ratio $I_{DiI}/(I_{DiO} + I_{DiI})$ levels off. In this study, we systematically studied the effect of pH value on the guest exchange of PCL–SS–POEGA micelles encapsulated with hydrophobic dyes DiI or DiO. The fluorescence spectra of PCL–SS–POEGA micelles at two pH values (pH = 5.5, 6.5) in the presence and absence of DTT are shown in Figure S5, and that of PCL–SS–POEGA micelles at pH 7.5 are shown in Figure 4(a,b).

According to previous work, the FRET ratio $\gamma_{FRET} = I_{DiI}/(I_{DiO} + I_{DiI})$ calculated from the fluorescence spectra of the micelles is related to the stability of the micelles.[43,44,46] Here, the normalized FRET ratios ($\gamma_{FRET,\ t\ =\ t}/\gamma_{FRET,\ t\ =\ 0}$) of the micelles with DTT and without DTT are summarized in Figure 2. As shown in Figure 2(a), the normalized FRET ratios increase slightly over time at various pH values in the absence of DTT, which indicates that the guest molecules escapes from the original micelles into different micelles due to the exchange of copolymer chains.[28,31] It is apparent that the increase of the normalized FRET ratio is the fastest at pH 4.5 in the absence of DTT, presumably due to the reason that the interaction between H^+ and the oxygen atom on POEGA chains at pH 4.5 enhances the hydrogen-bonded interaction between

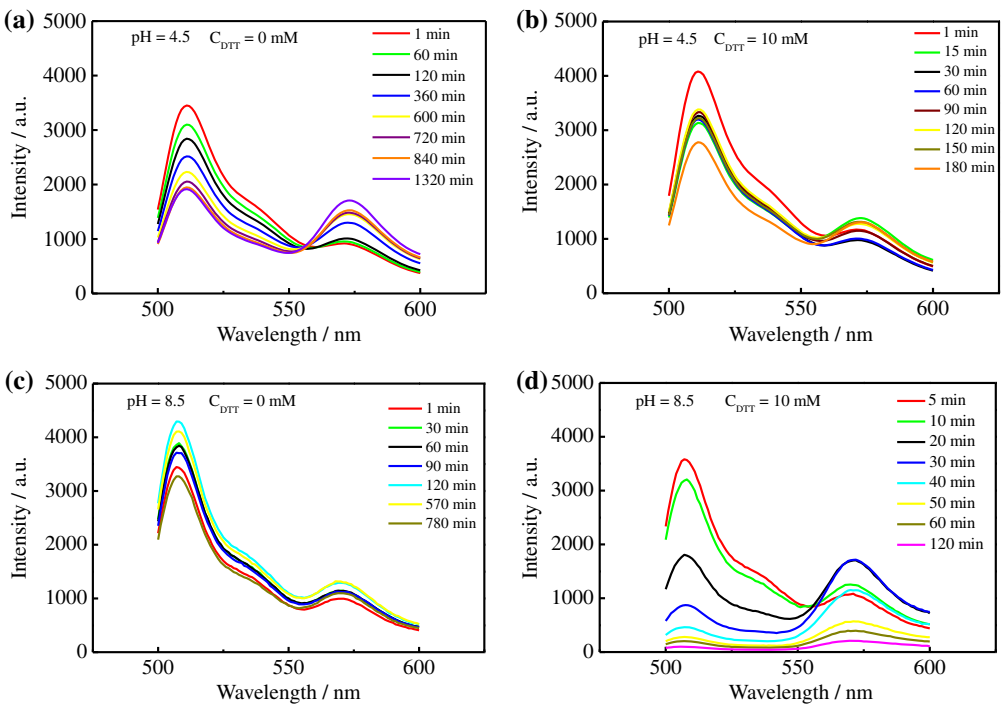

FIGURE 1 Time-dependent fluorescence emission spectra for PCL–SS–POEGA micelles encapsulated with hydrophobic dyes DiI or DiO in PB solutions at different pH values in the presence or absence of 10 mM DTT. (a) pH = 4.5 without DTT; (b) pH = 4.5 containing 10 mM DTT; (c) pH = 8.5 without DTT; and (d) pH = 8.5 containing 10 mM DTT. The concentrations of copolymer were 1 mg mL^{-1}. [Color figure can be viewed at wileyonlinelibrary.com]

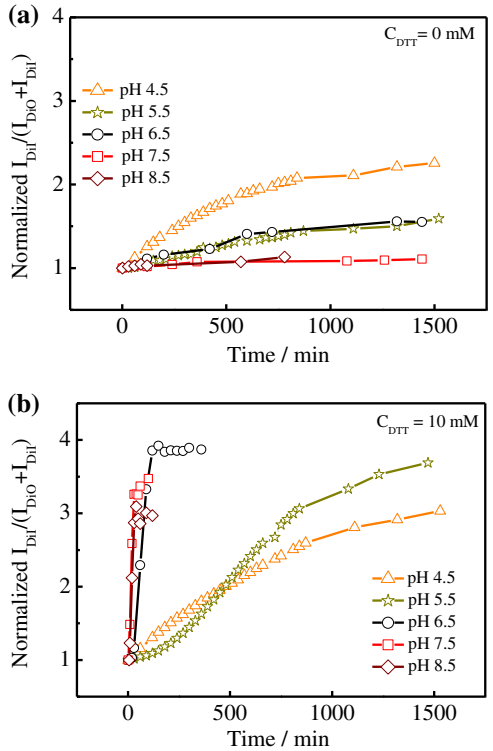

FIGURE 2 Time-dependent curves of normalized FRET ratios ($\gamma_{FRET,\ t=t}/\gamma_{FRET,\ t=0}$) of PCL–SS–POEGA micelles encapsulated with hydrophobic dyes DiI or DiO at different pH values (a) in the absence of DTT, and (b) in the presence of 10 mM DTT. [Color figure can be viewed at wileyonlinelibrary.com]

the polymer and water and increases the solubility of POEGA chains and facilitate the exchange of the copolymer chains.[62] Moreover, the solubility of the dyes molecules might be slightly improved at lower pH to accelerate the exchange rate of dye molecules.[46]

In addition, Figure 2(b) shows the normalized FRET ratios increase more rapidly in the presence of DTT, in comparison with that in the absence of DTT. As shown in Figure 2(b), the normalized FRET ratio at pH 8.5 increases from 1 to 3.1 within 40 min, with the FRET ratio increasing from 0.25 to 0.8. At pH 7.5, the normalized FRET ratio increased from 1 to 3.3 within 40 min and increased to ~3.5 at 100 min. At pH 6.5, the normalized FRET ratio increased to 3.9 within 120 min. However, the normalized FRET ratio at pH 5.5 and at pH 4.5 increased very slowly, only reached to 3.7 after 1440 min and to 3.03 after 1530 min, respectively. We believe that the thiol–disulfide exchange reaction between the reduction-sensitive copolymer and DTT, which induces the formation of the dyes trafficking channels, leads to the faster exchange rate of guest molecules.[32] Since the thiol–disulfide interchange reaction requires thiolate anion and the concentration of thiolate anion highly depends on the pH value, the cleavage of the POEGA depends on the pH value in solutions.[63] Interestingly, the normalized FRET ratio at pH 4.5 is greater than that at pH 5.5 at the same time before 480 min in the presence of DTT. This might be due to the low concentration of thiolate anion at both pH 4.5 and 5.5, but faster exchange of copolymer chains between the micelles at pH 4.5, as shown in Figure 2(a). After 500 min, the normalized FRET ratio at pH 5.5 becomes greater than that at pH 4.5. With the occurrence of thiol and disulfide exchange reaction, the integrity of dense hydrophilic shell of the micelles at pH 5.5 is partially destroyed, leading to the faster diffusion of dyes. In addition, the maximums of normalized FRET ratios for pH 7.5 and 8.5 are lower than that at pH 6.5 and 5.5, presumably due to the fast aggregation rate of the micelles, which prevents further exchange of the dye molecules.[31] The two possible mechanisms for guest exchange between micelles are collision- and diffusion-based pathways. To further understand the guest exchange mechanism of the micellar assemblies, we investigated the dependence of the guest exchange rate on the concentration of the micelles in the presence of DTT. Figure S6 shows the time-dependent normalized FRET ratio curves for two concentrations of the reduction-sensitive micelles in the presence of DTT, and demonstrates that the guest exchange rate is independent on the concentration. Note that for a collision-based mechanism, the guest exchange is dependent on the concentration. Therefore, we can suspect the dominated guest exchange mechanism is not collision-based mechanism but diffusion-based mechanism for the reduction-sensitive micelles in the presence of DTT.

Furthermore, we calculated the guest exchange rate from the time-dependent curves of the normalized FRET ratio of PCL–SS–POEGA micelles against time in the presence of 10 mM DTT at different pH values according to the approach reported by Thayumanavan and coworkers[43,46] The guest exchange rate was obtained from the slope of the linear fit of the linear region points of the normalized FRET ratio versus time in early stage. As shown in Figure 3, the guest exchange rates of the micelles in the presence of 10 mM DTT for the initial stage are 0.002, 0.001, 0.03, 0.08, and 0.08 min^{-1} at pH 4.5, 5.5, 6.5, 7.5, and 8.5, respectively. Obviously, in the presence of DTT, the guest exchange rate is significantly influenced by the pH value in the surrounding environment, which is attributed to the reason that the concentration of thiolate anion can be

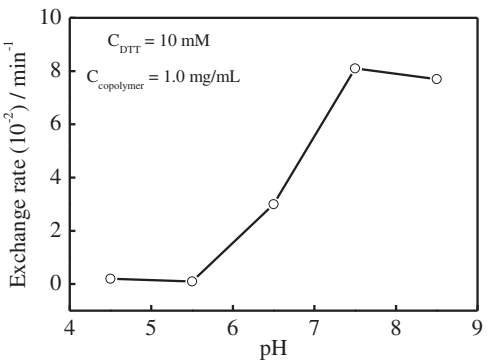

FIGURE 3 Guest exchange rate of PCL–SS–POEGA micelles at different pH values in the presence of 10 mM DTT, where the copolymer concentrations were 1 mg mL^{-1}. The exchange rate was calculated from the initial slope of linear fit (earlier points in the linear regime).

greatly affected by pH value.[63] Accurately, the reaction rate of the thiol–disulfide exchange between reduction-sensitive polymer and DTT decreases with the decreasing pH value, leading to the decreasing diffusion rate of the dyes from the original micelles to other micelles. In comparison, the guest exchange rate of reduction-sensitive micelles in the presence of DTT at pH 4.5 is faster than that at pH 5.5 due to the predominance of the diffusion of dye molecules caused by the exchange copolymer chains between the micelles, where the reaction rate of thiol–disulfide exchange is pretty slow at this pH value. In addition, the guest exchange rate of PCL–SS–POEGA micelles with DTT increases with the increasing pH value from 5.5 to 7.5, where the thiol–disulfide exchange rate is faster at higher pH values. Moreover, the reaction rate of thiol–disulfide exchange at pH 7.5 and 8.5 is similar and quick with excessive DTT due to the fast cleavage rate of the disulfide bond between PCL and POEGA and quick destruction of the hydrophilic shell of the micelles. In this condition, the rate-determining step should be the diffusion process of the fluorescent molecules.[38] Because the pH values in cancer tissues (pH 6.5–7.2) and subcellular organs (endosomes: pH 5.0–6.5; lysosomes: pH 4.5–5.0) are lower than the pH value in blood, it is of high importance to investigate the guest exchange rate of reduction-sensitive polymeric micelles at various pH values under reducing condition due to the broad applications of that in drug delivery.

The Guest Exchange of the Mixed PCL–SS–POEGA/PCL–CC–POEGA Micelles Containing DiO/DiI

Previously, Zhong and coworkers have studied the effect of disulfide content on reduction sensitivity, triggered drug release and the antitumor activity of shell-sheddable micelles. Their results demonstrated that the intracellular drug release from doxorubicin-loaded biodegradable micelles and their therapeutic activity can be precisely controlled by reduction-responsive shedding of hydrophilic shells.[32] However, the effect of content of reduction-sensitive copolymer in the mixed micelles formed by reduction-sensitive and reduction-insensitive copolymer on the guest exchange behavior is still unclear. Using the similar method, we investigated the stability and guest exchange of mixed micelles with different contents of reduction-sensitive polymer in PB solution at pH 7.5. We measured five kinds of mixed micelles containing 100, 75, 50, 25, and 0 wt % reduction-responsive copolymer PCL–SS–POEGA, respectively. The time-dependent fluorescence emission spectra of mixed micelles containing 100 wt % PCL–SS–POEGA and 0 wt % PCL–SS–POEGA encapsulated with hydrophobic dyes DiI or DiO in the presence and absence of DTT, are plotted in Figure 4. It is clear that the fluorescence spectra of the mixed micelles with 100 wt % PCL–SS–POEGA change fast in the presence of DTT, while the fluorescence spectra of that remain unchanged in the absence of DTT. Moreover, the fluorescence spectra for both types of micelles in the absence of DTT remain unchanged at pH 7.5. Others fluorescence spectra of the mixed micelles containing 75, 50, and 25 wt % PCL–SS–POEGA encapsulated with hydrophobic dyes DiI or DiO in the presence and absence of DTT are shown in Figure S7.

Similarly, the normalized FRET ratio was calculated to assess the guest exchange of these mixed micelles as mentioned above. The curves of the normalized FRET ratio versus time for these mixed micelles with different contents of PCL–SS–

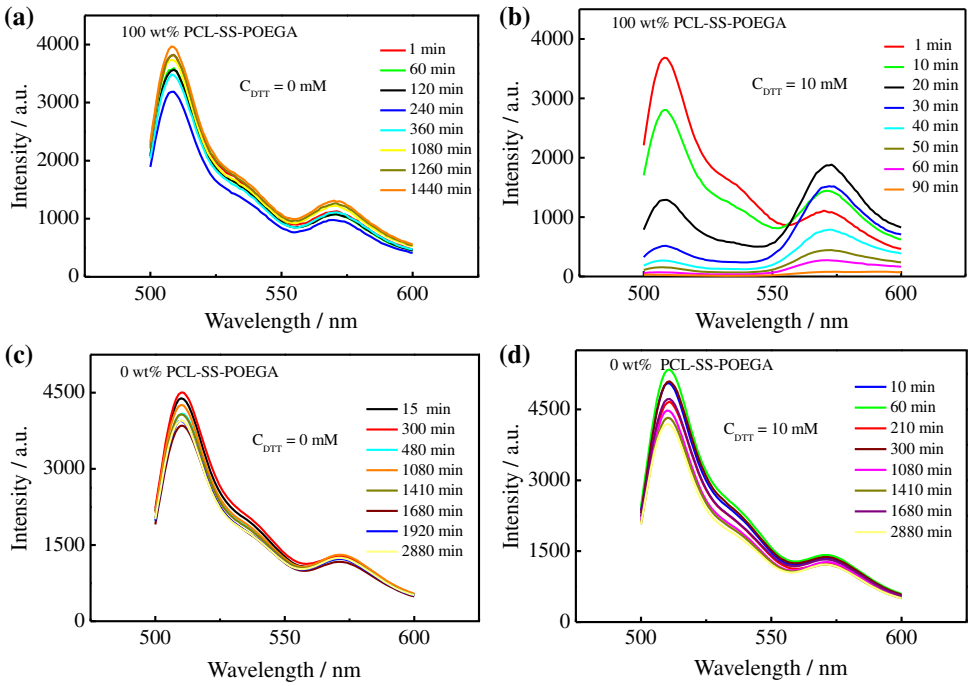

FIGURE 4 Time-dependent fluorescence emission spectra of mixed micelles containing 100 wt % PCL–SS–POEGA (a, b) and 0 wt % PCL–SS–POEGA (c, d) loaded with hydrophobic dyes DiI or DiO in 10 mM PB solution at pH 7.5 without DTT (a, c), and in the presence of 10 mM DTT (b, d). [Color figure can be viewed at wileyonlinelibrary.com]

POEGA and PCL–CC–POEGA in the absence and presence of DTT are shown in Figure 5(a,b). It is clear that the guest exchange rate and the maximums of the normalized FRET ratios for these mixed micelles increase with the increasing content of PCL–SS–POEGA in the presence of DTT. In the absence of DTT, the curves in Figure 5(a) show no significant diversity. Besides, the time required for the normalized FRET ratio to reach the plateau decreases with the increasing PCL–SS–POEGA content in the presence of DTT.

Moreover, the maximum of the normalized FRET ratio against the content of PCL–SS–POEGA in the mixed micelles in the presence and absence of DTT are summarized in Figure 6. The maximums of the normalized FRET ratios for the five kinds of mixed micelles without DTT are approximately 1.0 ± 0.2, indicating the high stability of the mixed micelles in a nonreducing condition at pH 7.5. However, in the presence of DTT, the maximums of the normalized FRET ratios increase with the increasing content of PCL–SS–POEGA in the mixed micelles, which are 1.1, 1.2, 1.7, 3.3, and 3.4 for mixed micelles with 0, 25, 50, 75, and 100 wt % PCL–SS–POEGA, respectively. The results indicate that the amount of guest molecules exchanged between micelles increases with the content of reduction-sensitive copolymer, presumably due to the formation of more dyes trafficking channels after cleaving more POEMA chains in the shells.[32,64] However, the maximum of the normalized FRET ratio for mixed micelles with 25 wt % PCL–SS–POEGA is close to that of micelles with 0 wt % PCL–SS–POEGA, the possible reason is that for mixed micelles with 25 wt % PCL–SS–POEGA after the cleavage of the PCL–SS–POEGA chains, the nearby POEGA chains may change the conformation from extended coil to random coil and cover the empty channels. Therefore, the amount of guest molecules exchanged in these mixed micelles is highly related but not linearly related to the content of PCL–SS–POEGA in the mixed micelles.

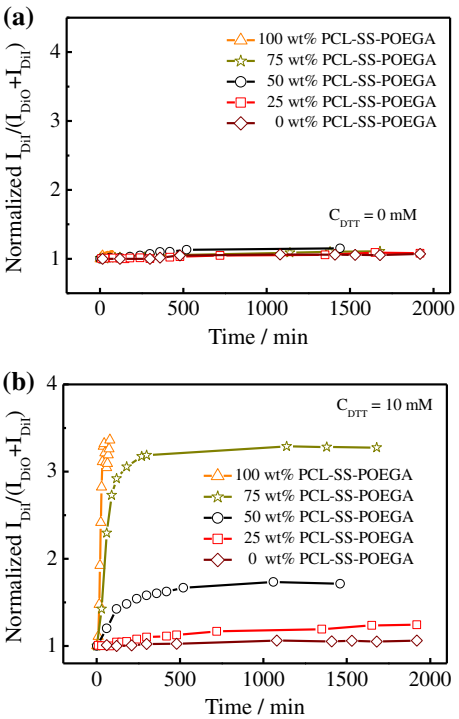

FIGURE 5 Time-resolved curves of the normalized FRET ratios ($\gamma_{FRET,\ t\ =\ t}/\gamma_{FRET,\ t\ =\ 0}$) of mixed micelles with different contents of PCL–SS–POEGA and PCL–CC–POEGA in PB (10 mM, pH = 7.5) solutions (a) without DTT and (b) in the presence of 10 mM DTT. [Color figure can be viewed at wileyonlinelibrary.com]

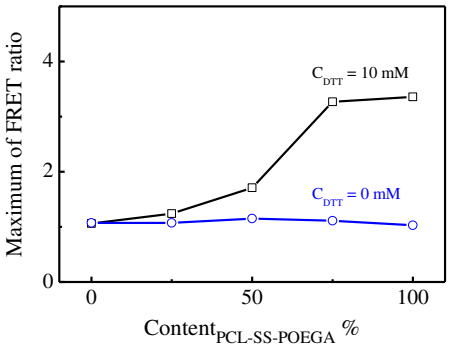

FIGURE 6 The effect of the content of PCL–SS–POEGA in the mixed micelles on the maximum of the normalized FRET ratio, in PB (10 mM, pH = 7.5) solution in the presence and absence of 10 mM DTT. [Color figure can be viewed at wileyonlinelibrary.com]

Finally, the guest exchange rates of all mixed micelles with various contents of PCL–SS–POEGA encapsulated with hydrophobic dyes DiI or DiO in the presence of DTT were calculated using the similar method as mentioned above. We determined the slope values of the plots of the normalized FRET ratio versus time in the initial region, which can indicate the guest exchange rate between the micelles at the beginning. The slope values for the curves of the mixed micelles with 100,75, 50, 25, and 0 wt % PCL–SS–POEGA in the presence of DTT are 0.081, 0.017, 2×10^{-3}, 2.0×10^{-4}, and 5.7×10^{-5} min^{-1}, as shown in Figure 7. The results demonstrate the exchange rate increases dramatically when the content of PCL–SS–POEGA is higher than 50%, presumably due to the reason that cleavage of the POEGA facilitates the diffusion of DTT from the bulk

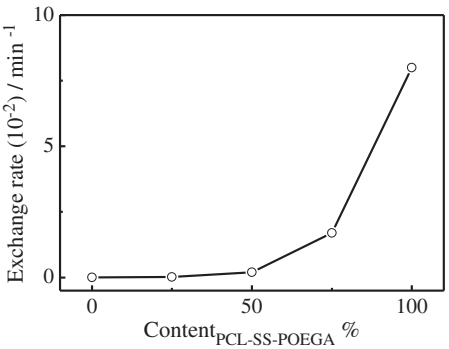

FIGURE 7 Guest exchange rates of mixed micelles with different contents of PCL–SS–POEGA and PCL–CC–POEGA in PB (10 mM, pH = 7.5) solution in the presence of 10 mM DTT, where the total copolymer concentrations were 1 mg mL^{-1}. The exchange rate was also calculated from the initial slope of linear fit (earlier points in the linear regime).

solution to the interface between the hydrophilic corona and the hydrophobic core and further accelerate the cleavage of the POEGA chains. Moreover, the mixed micelles with 100 wt % PCL–SS–POEGA aggregated in half an hour after the addition of 10 mM DTT; however, there was no obvious aggregation for the other four kinds of mixed micelles. These mixed micelles except for the micelles with 100 wt % PCL–SS–POEGA exhibit high stability with the unchanged average hydrodynamic diameter under the reduction condition, as shown in Figure S8, which is consistent with the results reported by Zhong et al. and Kataoka et al.,[32,65] while the guest exchange behavior still exists. According to our results, the stability, the guest exchange rate, and the amount of exchanged guest molecules of these mixed micelles can be tuned through adjusting the contents of reduction-sensitive polymer in the mixed micelles. Figure S9 shows the graphical scheme of the guest exchange kinetics of PCL–SS–POEGA micelles at different pH values and mixed micelles with different contents of PCL–SS–POEGA.

CONCLUSIONS

In conclusion, a reduction-sensitive polymer with one disulfide group between PCL and POEGA has been prepared by a combination of ring-open polymerization and ATRP. The micelles formed by the reduction-sensitive polymer in PB solutions with different pH values and mixed micelles containing different contents of reduction-sensitive and reduction-insensitive polymer at pH 7.5 were prepared through precipitation-dialysis method. The dye exchange behaviors of these micelles in different conditions were investigated using the FRET method. The guest exchange rates of PCL–SS–POEGA micelles in the presence of 10 mM DTT at different pH values follows the order: $5.5 < 4.5 < 6.5 < 7.5 \approx 8.5$. The results illustrate that the pH of the solution has a minor influence on the dye exchange rate of reduction-sensitive micelles in the absence of DTT. In addition, the guest exchange rate of the mixed micelles with different contents of reduction-sensitive polymer was studied by the similar method. Under a reducing environment, the guest exchange rate and the number of exchanged guest molecules in the micelles increase with the increasing content of reduction-sensitive polymer in the mixed micelles.

ACKNOWLEDGMENTS

The financial support of the National Natural Scientific Foundation of China (NNSFC) Projects (21674107) and the Fundamental Research Funds for the Central Universities (WK2340000066) is gratefully acknowledged.

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