In Situ Fabrication of Paclitaxel-Loaded Core-Crosslinked Micelles via Thiol-Ene "Click" Chemistry for Reduction-Responsive Drug Release

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ABSTRACT: In this study, a facile method to fabricate reduction-responsive core-crosslinked micelles via *in situ* thiol-ene "click" reaction was reported. A series of biodegradable poly (ether-ester)s with multiple pendent mercapto groups were first synthesized by melt polycondensation of diol poly(ethylene glycol), 1,4-butanediol, and mercaptosuccinic acid using scandium trifluoromethanesulfonate [$Sc(OTf)_3$] as the catalyst. Then paclitaxel (PTX)-loaded core-crosslinked (CCL) micelles were successfully prepared by *in situ* crosslinking hydrophobic polyester blocks in aqueous media via thiol-ene "click" chemistry using 2,2′-dithiodiethanol diacrylate as the crosslinker. These PTX-loaded CCL micelles with disulfide bonds exhibited reduction-responsive behaviors in the presence of dithiothreitol (DTT). The drug release profile of the PTX-loaded CCL micelles revealed that only a small amount of loaded PTX was released slowly in phosphate buffer solution (PBS) without DTT, while quick release was observed in the presence of 10.0 mM DTT. Cell count kit (CCK-8) assays revealed that the reduction-sensitive PTX-loaded CCL micelles showed high antitumor activity toward HeLa cells, which was significantly higher than that of reduction-insensitive counterparts and free PTX. This kind of biodegradable and biocompatible CCL micelles could serve as a bioreducible nanocarrier for the controlled antitumor drug release. © 2015 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2016**, *54*, 99–107

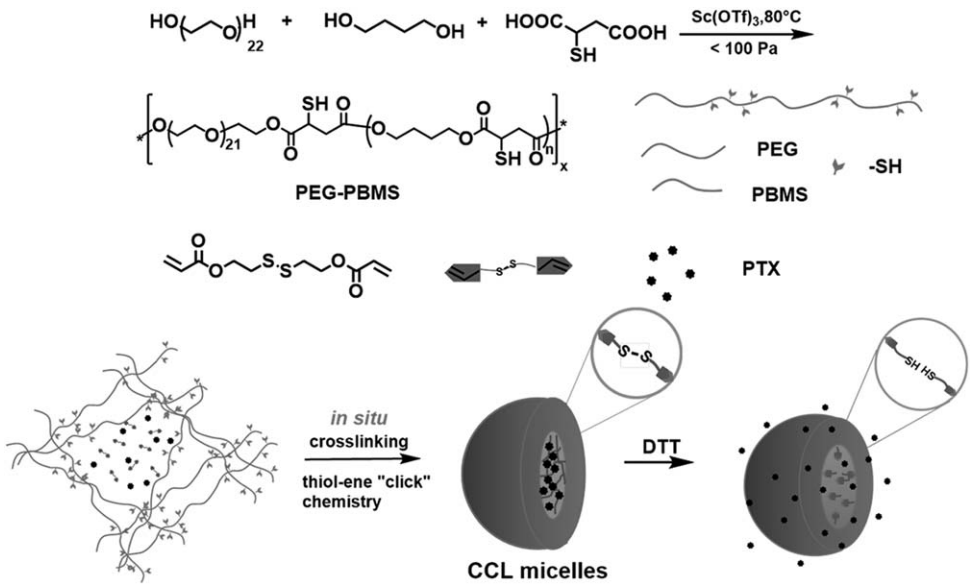
KEYWORDS: antitumor; biodegradability; drug delivery; polycondensation; reduction response; thiol-ene "click" chemistry

INTRODUCTION In the past few decades, great interests have been attracted by micelles self-assembled from amphiphilic block copolymers as nanosized drug carriers.[1–5] However, under extreme dilution conditions, such micelles will disassociate and release the encapsulated drugs at undesired sites. Therefore, one crucial and important question is how to stabilize the polymeric micelles. Nowadays, crosslinking the core or the shell of polymeric micelles after assembly has emerged as a viable strategy.[6–9] By providing covalent linkages between the self-assembled polymer chains, the stability of polymeric micelles has been greatly improved.[10]

For further applications in the clinic, overly stable micelles are not ideal for drug release at desired sites. Many kinds of stimuli-responsive micelles have been developed recently. Stimuli-responsive micelles can release the loaded drugs at target sites in response to some specific stimuli, such as pH, temperature, and reductive environment.[11–14] Disulfide bonds are reductively degradable linkages, known to be destroyed rapidly due to the intracellular reductive environment.[15–17] Hence, the reduction-sensitive disulfide bonds have been applied to nanoparticles for controlled release of loaded drugs.[18–23]

As a FDA approved water soluble polymer, PEG has been widely investigated in both academia and industry for chemical and biological applications.[24–30] The PEG-based drug carriers have a few advantages: a prolonged residence in body, a decreased degradation by metabolic enzymes and a reduction or elimination of protein immunogenicity, etc.[31] Hence,

Ying Huang and Rui Sun contributed equally to this work.



SCHEME 1 *In situ* fabrication of reduction-responsive CCL micelles.

micelles self-assembled from PEG-based aliphatic amphiphilic multifunctional poly(ether-ester)s are attractive candidates for smart drug carriers due to their excellent biodegradability, biocompatibility, and crosslinkability.[32–34] PEG-based multifunctional poly(ether-ester)s are usually synthesized by the ring-opening polymerization of cyclic esters with functional groups in the presence of PEG as a macroinitiator, which always involves burdensome protections and deprotections.[35–37]

Our research group has developed a "one-pot" strategy to synthesize multifunctional biodegradable PEG derivatives by direct polycondensation of PEG diol and functional diacid employing scandium trifluoromethanesulfonate [$Sc(OTf)_3$] as a chemoselective catalyst under mild conditions.[38–41] Recently, we have published a communication, in which 1,4-butanediol was used as hydrophobic monomer to copolymerize with PEG diol and mercaptosuccinic acid, resulting in amphiphilic poly(ethylene glycol)–poly(butylene mercaptosuccinate) (PEG–PBMS) multiblock copolymer containing multiple mercapto groups.[42] This amphiphilic polymer could self-assemble into sphere micelles in aqueous solution for encapsulation of hydrophobic antitumor drugs. The hydrophobic PBMS block with multiple mercapto groups in the micellar core could be crosslinked in the presence of H_2O_2, to give disulfide bonds, which led to reduction-responsive drug release from the micelles. However, this strategy is not suitable for loading oxidation-sensitive drugs into the micelles due to the violent oxidability of H_2O_2. Therefore, it is meaningful to explore new crosslinking agents in order to introduce disulfide crosslinkages into the micelles. The rise of thiol-ene "click" chemistry as a toolbox gathering only simple, high yielding, easily workable transformations, and mild reaction conditions has facilitated an extraordinary increase in the fabrication of biomaterials.[42–49] In this work, 2,2′-dithiodiethanol diacrylate was used to *in situ* crosslink paclitaxel (PTX)-loaded PEG–PBMS micelles in aqueous media via Michael addition type thiol-ene "click" reaction under physiological conditions (Scheme 1). These PTX-loaded CCL micelles with disulfide bonds exhibited reduction-responsive behaviors in the presence of dithiothreitol (DTT). This study provides a universal strategy to prepare drug-loaded CCL micelles, which is suitable for almost all hydrophobic drugs.

EXPERIMENTAL

Materials

Poly(ethylene glycol) (M_n = 1000, Sinopharm Chemical Reagent Co., China) was dried by azeotropic distillation in the presence of dry toluene. Scandium trifluoromethanesulfonate [$Sc(OTf)_3$] was synthesized according to our previous work.[50] 1,4-dithiothreitol (DTT, 99%; Aladdin), mercaptosuccinic acid (98%; Aladdin, China), paclitaxel (PTX, 99.5%; Haoxuan Bio-technique Co., China), 1,4-butanediol (BD, 99.5%; Wulian Chemical Engineering Co., China), 2,2′-dithiodiethanol (90%; Alfa Aesar), acryloyl chloride (98%; Energy Chemical, China), and nile red (99%; Acros Organics) were used as received. All other reagents were purchased from Sinopharm Chemical Reagent Co. and used as received.

Synthesis of Multiblock Copolymers

A series of multiblock copolymers with different PEG and BD molar ratios were synthesized by an improved method according to our previous report.[43] In a 100 mL Schlenk flask equipped with mechanical stirrer, a typical feeding molar ratio of monomers was as follows: PEG diol (6.0 g, 6.0 mmol), BD (2.72 g, 30.0 mmol), mercaptosuccinic acid (5.41 g, 36.0 mmol), and $Sc(OTf)_3$ (0.36 g, 0.72 mmol) were added. The flask was purged with argon and then put into an oil bath thermostated at 80 °C. Vigorous stirring was carried out to ensure the uniformity after PEG was melted. After

esterification for 10 h at 80 °C, polycondensation at 90 °C was carried out under lower pressure for 4 h, and then the mixture continuous reacted at high vacuum (< 100 Pa) for another 6 h. Thereafter, the reaction was stopped and the products were dissolved in a small amount of THF and then precipitated in cold diethyl ether. Yield: 8.5 g (66%).

Synthesis of 2,2′-Dithiodiethanol Diacrylate and 1,6-Hexamethylene diacrylate

The 2,2′-dithiodiethanol (3 g, 17.5 mmol) was dissolved in 30 mL of THF in a 100 mL Schlenk flask. Then excess triethylamine (9.76 mL, 70 mmol) was added to the flask, and the mixture was stirred for 20 min at 0 °C. Thereafter, acryloyl chloride (2 equal, 5.72 mL, 70 mmol) was introduced by dripping slowly and the reaction mixture was stirred at room temperature for 12 h. THF was evaporated and then 30mL of dichloromethane was added. The solution was poured into a separatory funnel and washed with saturated $NaHCO_3$ and NaCl solutions for three times separately. The organic layer was dried by anhydrous $MgSO_4$, evaporated to give yellow liquids, which was dried under reduced pressure for 12 h. Yield: 2.6 g (59%). 1,6-hexamethylene diacrylate was synthesised by the same method. Yield: 1.8 g (53%).

Preparation of PTX- or NR-Loaded CCL Micelles

The PTX-loaded CCL micelles were *in situ* prepared by the solvent evaporation method. CCL micelles with disulfide bond (crosslinked by 2,2′-dithiodiethanol diacrylate) was named as CCL micelles-SS, CCL micelles crosslinked by 1,6-hexamethylene diacrylate was named as CCL micelles-CC. Briefly, 5 mg of PTX, 50 mg of PEG–PBMS, and crosslinker (2,2′-dithiodiethanol diacrylate or 1,6-hexamethylene diacrylate, the molar ratio of mercapto groups and double bonds is 1:1) were dissolved in 30 mL of THF, and 30 mL of phosphate buffer solution (PBS, pH = 7.4) was added dropwise into the solution under vigorous stirring to induce the hydrophobic PTX and crosslinker incorporated into the hydrophobic micellar core. After stirring for 2 h, the solution was evaporated to remove most of the THF. Thereafter, the solution was stirred for another 2 h to make the "click" reaction complete. The solution was dialyzed against distilled water for 24 h using a tubular dialysis membrane (MWCO = 3500 Da) and filtrated to remove residual THF and unloaded PTX. The final concentration of PTX-loaded CCL micelles aqueous solution was adjusted to 1.0 mg/mL. The solid state of PTX-loaded CCL micelles was recovered by lyophilization. The drug-loading capacity of the CCL micelles was investigated by RP–HPLC analysis. The drug loading content (DLC) and the drug loading efficiency (DLE) were calculated as below:

$$\mathrm{DLC}\,(\%) = (\text{weight of loaded drug} / \text{weight of polymer}) \times 100$$

$$\mathrm{DLE}\,(\%) = (\text{weight of loaded drug} / \text{weight of drug in feed}) \times 100$$

NR was loaded into the micelles using the same strategy in the dark. NR-loaded CCL micelles-SS and NR-loaded CCL micelles-CC were prepared separately. Briefly, 0.2 mL of NR's DMF solution (1.0 mg/mL), 10 mg of PEG–PBMS, and crosslinker (2,2′-dithiodiethanol diacrylate or 1,6-hexamethylene diacrylate, the molar ratio of mercapto groups and double bonds is 1:1) were dissolved in 5 mL of THF, and 5 mL of phosphate buffer solution (PBS, pH = 7.4) was added dropwise into the solution under vigorous stirring to induce NR and crosslinker incorporated into the hydrophobic micellar core. After stirring for 2 h, the solution was evaporated to remove most of the THF. Thereafter, the solution was stirred for another 2 h to make the "click" reaction completely. The solution was dialyzed against distilled water for 24 h using a tubular dialysis membrane (MWCO = 3500 Da) and filtrated to remove residual THF, DMF (all of the added NR was loaded into the CCL micelles).

Determination of Crosslinking Degrees of the CCL Micelles

The crosslinking degree of CCL micelles was determined using Ellman's reagent as described in literature.[51] Briefly, 1 mg/mL solution of polymer or CCL micelles was prepared in PBS (pH 7.4). Then Ellman's reagent (0.5 mL, 10 mM) was added into the solutions and the reactions were allowed to proceed for 2 h at room temperature. Afterward, the absorbance was measured at a wavelength of 412 nm. The amount of thiols was calculated from the corresponding standard curve elaborated between 0.25 and 2 mM of L-cysteine solution in PBS (pH 7.4). The crosslinking degrees were calculated at last.

In Vitro Drug Release

In vitro release profiles of PTX from the CCL micelles were investigated by dialysis method. Concretely, 4 mL of PTX-loaded CCL micelles solution was introduced into a dialysis bag (MWCO = 14 kDa) against 20 mL PBS buffer solution (pH 7.4, 10 mM) containing 10 mM DTT (mimicking intracellular reductive condition). Another group was conducted without DTT (nonreductive condition). Tween 80 (0.1 wt %) was introduced into the PBS buffer solution to solubilize released PTX. At desired time intervals, 5 mL of release mediums were taken out and replenished with an equal volume of corresponding fresh media. The amounts of released PTX were determined by RP–HPLC analysis.

Cell Uptake and Internalization Analysis

NR-loaded CCL micelles-SS and NR-loaded CCL micelles-CC were prepared separately. The internalization and intracellular distribution of NR-loaded CCL micelles in HeLa cells were monitored using confocal laser scanning microscopy (BX61W1-FV1000; OLYMPUS, Japan). Autoclave sterilized coverslips were placed in 6-well plate. The HeLa cells were seeded in 6-well plates with a concentration of 1×10^5 cells per well in 1.8 mL of complete α-MEM and cultured for 24 h. Thereafter, 200 μL NR-loaded CCL micelles-SS, NR-loaded CCL micelles-CC, or free NR suspension were added and cultured for another 1 h and 4 h. Then the culture media was removed, followed by washing the cells thrice with PBS and fixed with4 % paraformaldehyde for 15 min. Cell nucleus were stained by Hochest 33324 (Sigma-Aldrich, blue fluorescence). After being mounted with neutral balsam, samples

were observed with 60× confocal laser scanning microscopy (CLSM).

Microplate reader was used for quantitative determination of cellular uptake of NR-loaded CCL micelles. 180 μL suspension of the HeLa cells were seeded into each well of 96-well plate (5×10^3 cells) and incubated for 24 h. Then 20 μL PBS containing NR-loaded CCL micelles-SS, NR-loaded CCL micelles-CC, or free NR solutions were added. After incubated for 1 h and 4 h, cells were washed with PBS twice, and finally 100 μL PBS was added. Cells incubated with equivalent amount PBS was used as negative control. Fluorescence intensity was measured with a microplate reader (SpectraMax, molecular devices, AmericaX) with excitation wavelength 550 nm and emission wavelength of 605 nm. Each group was tested in quintuplicate.

Cell Viability Assay

The *in vitro* relative cytotoxicities of PEG–PBMS against HeLa cells were evaluated by the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Japan). Appropriate amounts of PEG–PBMS solution were dissolved in PBS to obtain their extracts with concentration of 100, 10, 1, and 0.1 mg/L. HeLa cells were seeded into 96-well plates at a density of 10,000 cells per well in 180 μL of culture medium. Twenty-four hours later, 20 μL extracts of various concentrations were added to the well. Cells were exposed to the gel precursor for 48 h. Before 100 μL fresh medium and 10 μL CCK-8 reagent were added, each well was washed three times with PBS. After a further incubation of 1 h, the optical density (OD) of the wells was measured at 450 nm with a Microplate Spectrophotometer (SpectraMax M5). Nontreated cells were used as a negative control. Relative cell viability was calculated as following equation:

$$\text{Relative cell viability}\ (\%) = \left[\frac{(\text{OD450}_{\text{sample}} - \text{OD450}_{\text{blank}})}{(\text{OD450}_{\text{control}} - \text{OD450}_{\text{blank}})}\right] \times 100$$

The cytotoxicities of PTX-loaded CCL micelles-SS and PTX-loaded CCL micelles-CC were also evaluated by CCK-8 assay. Micelles were diluted to final PTX concentrations of 5, 1, 0.1, 0.05, and 0.005 mg/L. Similarly, after HeLa cells incubated for 24 h in 96-well plates, the cells were incubated with various concentrations of PTX-loaded CCL micelles for another 48 h. As a comparison, another HeLa cell line was incubated with PTX stock solutions (free PTX) under the same conditions. PTX stock solutions were prepared according to a reference method.[52]

Characterization

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) spectrum was recorded on a Bruker Avance DMX500 spectrometer in $CDCl_3$ with tetramethylsilane as internal standard. The molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC). The GPC system consisted of a Waters degasser, a Waters 1525 HPLC pump with 717 plus autosampler, Waters 2410 RI detector and columns: Styragel, HT 3, HT 4. The calibration was performed with commercial polystyrene standards.

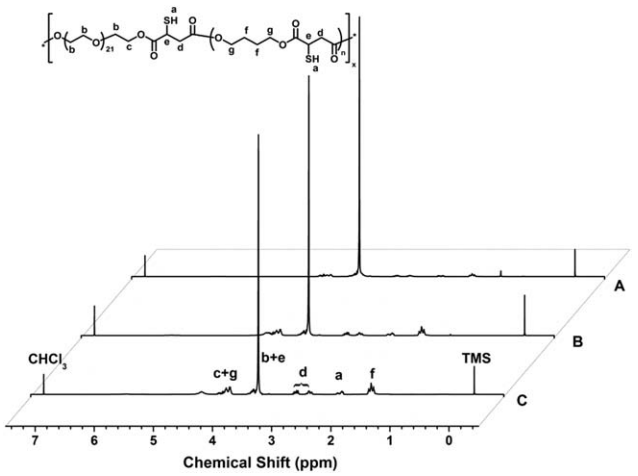


FIGURE 1 ^{1}H NMR spectra (400MHz, $CDCl_3$) of PEG–PBMS multiblock copolymers. The feed molar ratio of PEG and BD is 1:3 (A), 1:4 (B), and 1:5 (C).

Tetrahydrofuran (THF) was used as the mobile phase at a flow rate of 1.0 mL/min at 40 °C. The hydrodynamic diameter and size distribution of micelles were determined by dynamic light scattering (DLS) at 173 ° angle to the incident beam and at 37 °C on a Malvern 2000 zetasizer analyzer. All micelles solutions had a final polymer concentration of 1.0 mg/mL and were filtered through a 0.45 μm filter. Transmission electron microscopy (TEM) images were obtained using JEM-1230 operating at an acceleration voltage of 60 kV. A drop of 1.0 mg/mL micelles solution was dropped onto the surface of Formvar-carbon film-coated copper grids. Excess solution was quickly wicked away with a filter paper. All grids were finally stained by 2 wt % phosphotungstic acid. The amount of released PTX was investigated by reverse-phase high-performance liquid chromatography (RP–HPLC) analysis. A mobile phase of methanol/water (70/30, v/v) was used, and the flow rate was set at 1 mL/min (35 °C).

RESULTS AND DISCUSSION

Synthesis and Characterization of PEG–PBMS Multiblock Copolymers

PEG–PBMS multiblock copolymers with different PEG and BD molar ratios were synthesized by one-pot melt polycondensation reaction in the presence of $Sc(OTf)_3$. The chemical composition of multiblock copolymers was characterized by proton nuclear magnetic resonance (^{1}H NMR). As shown in Figure 1, the characteristic proton signals of PEG and PBMS segments are presented and marked. All peaks can be well assigned with their chemical structures. Notably, the characteristic signal of mercapto proton (H^a) was clearly detected at 2.38 ppm, demonstrating the stability of mercapto groups during the polycondensation process. The molar ratios of butyl units to PEG segments could be calculated by the integral of corresponding proton (H^b and H^f), which is very close to the initial feed ratio (Table 1), indicating the similar activity of butanediol and diol PEG in polycondensation with mercaptosuccinic acid. Moreover, the M_n of PEG–PBMS measured by GPC analysis was also summarized in Table 1.

TABLE 1 Multiblock Copolymers With Various PEG and BD Molar Ratio

	PEG:BD								
Copolymers	In Feed[a]	In Polymer[b]	Yield (%)	M_n (g/mol)[c]	MWD[c]	DLE[d] (%)	DLC[d] (%)	Diameter[e] (nm)	PDI[e]
1	1:3	1:3.1	77	6100	1.31	73.0	6.8	127.2	0.425
2	1:4	1:3.9	72	6300	1.34	74.1	6.9	129.7	0.316
3	1:5	1:4.9	66	6900	1.41	75.5	7.0	119.5	0.185

[a] Feeding molar ratio.
[b] Measured by ^{1}H NMR.
[c] From GPC using polystyrene standards.
[d] Measured by HPLC.
[e] Measured by DLS.

Synthesis and Characterization of the Crosslinkers

The 2,2′-dithiodiethanol diacrylate and 1,6-hexamethylene diacrylate were synthesized through the acylation reaction. The ^{1}H NMR spectra of 2,2′-dithiodiethanol diacrylate and 1,6-hexamethylene diacrylate are shown in Figure 2, in which the characteristic proton signals are presented and marked.

Preparation and Characterization of PTX-Loaded CCL Micelles

The PTX-loaded CCL micelles were prepared by the solvent evaporation method and dialysis. The PTX-loaded micelles were *in situ* crosslinked via thiol-ene "click" reaction under physiological conditions. The drug-loading capacity of the PTX-loaded CCL micelles was investigated by HPLC. The DLC and DLE were calculated as summarized in Table 1. The DLC and DLE of the PTX-loaded CCL micelles did not changed much upon the varying of PEG/BD molar ratio. The diameters of the PTX-loaded CCL micelles were measured by DLS, which also did not changed much upon the varying of PEG/BD molar ratio. However, the PTX-loaded CCL micelles with lowest PDI were obtained using copolymer 3 with longer average PBMS chain length. More stable and uniform PTX-loaded CCL micelles could be prepared from PEG–PBMS multiblock copolymer with high hydrophobic content. PTX-loaded CCL micelles based on copolymer 3 were chosen to perform the follow-up experiments.

The crosslinking degrees of CCL micelles were measured by using Ellman's reagent, which were 97.3% for CCL micelles-SS and 94.8% for CCL micelles-CC. The high crosslinking degrees demonstrate high efficiency of thiol-ene "click" reaction in micellar cores and the successful syntheses of CCL micelles. To investigate whether the disulfide bond could be degraded under reductive conditions, the changes of CCL micelles sizes in response to 10 mM DTT was monitored by DLS measurement at time intervals. As shown in Figure 3(A), the size of CCL micelles-SS increased slightly with 10 mM DTT during the experimental period. This phenomenon should be attributed to the gradual decrosslinking of the CCL micelles-SS caused by the cleavage of disulfide bonds, resulting in larger micelles with looser structure. Besides, the size of CCL micelles-SS in PBS did not change during the time intervals, which indicated the stability of micelles. However, the size of CCL micelles-CC did not increase sharply even after 30 h with 10 mM DTT [Fig. 3(B)], which could be attributed to stability of carbon–carbon bonds in reductive environment. The sizes and morphologies of PTX-loaded CCL micelles were further characterized by TEM measurements as shown in Figure 3(C,D). The self-assembled micelles are well dispersed as individual nanoparticles with regularly

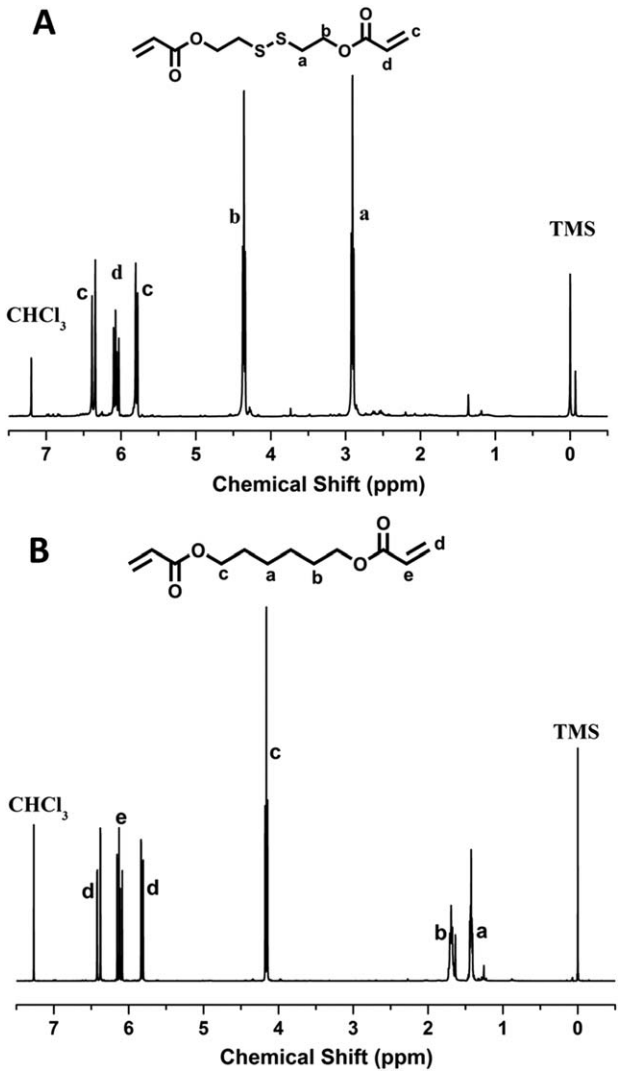


FIGURE 2 ^{1}H NMR spectra (400MHz, $CDCl_3$) of 2,2′-dithiodiethanol diacrylate (A) and 1,6-hexamethylene diacrylate (B).

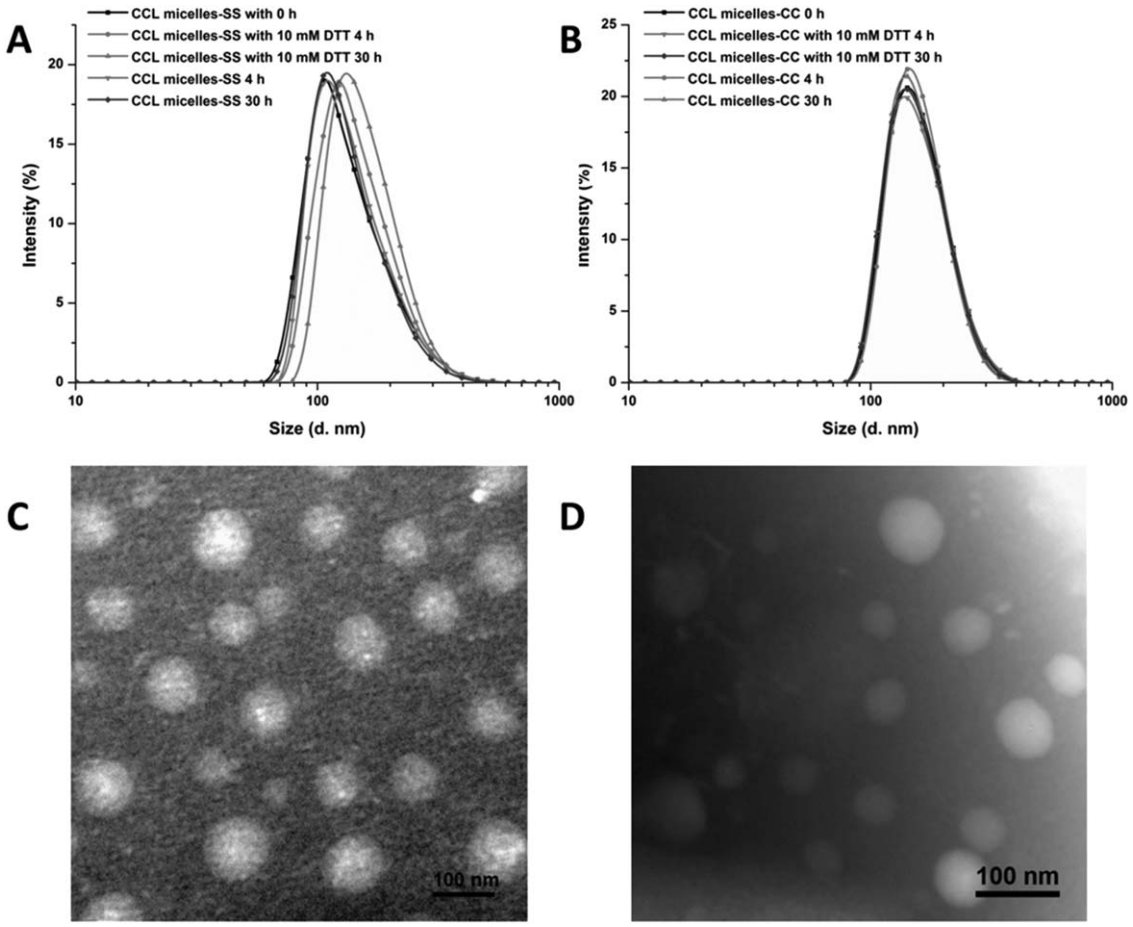


FIGURE 3 Size distribution and TEM images of PTX-loaded CCL micelles. Change in the size distribution of PTX-loaded CCL micelles in response to 10 mM DTT, determined by DLS at 37 °C, CCL micelles-SS (A) and CCL micelles-CC (B). TEM images of CCL micelles-SS (C) and CCL micelles-CC (D).

spherical shapes. The diameters of PTX-loaded CCL micelles observed from TEM images are smaller than those by DLS, which is due to the dehydration of micelles during the preparation process of TEM samples.[53,54]

In Vitro PTX Release From CCL Micelles

The PTX release profiles from CCL micelles in phosphate buffer solution (PBS, pH = 7.4) with or without 10 mM DTT were obtained using dialysis method. Tween 80 (0.1 wt %) was introduced into the PBS buffer solution to solubilize released PTX. As shown in Figure 4, the release of PTX from PTX-loaded CCL micelles-SS in PBS with 10 mM DTT was rapid and about 80% of loaded PTX was released within 10 h. In contrast, PTX release from the CCL micelles-SS in PBS without DTT was much slower and only about 20% of loaded PTX was released even after 60 h. Furthermore, the PTX release from CCL micelles-CC was also carried out either in pure PBS or in PBS with 10 mM DTT. The release profiles are very similar to that using PTX-loaded CCL micelles-SS in pure PBS, indicating that PTX-loaded CCL micelles-CC have no reductive-responsibility. It should be noted that the concentration of glutathione (GSH) in tumor cells (8–10 mM) is about 2-fold higher than that in normal cells (2–8 mM) and much

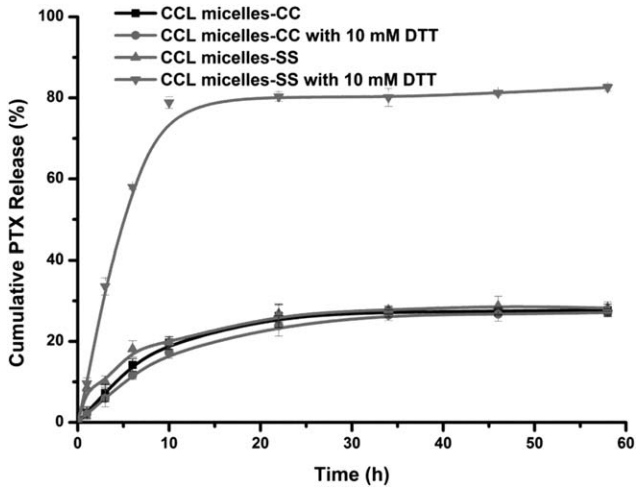


FIGURE 4 PTX release kinetics from PEG–PBMS CCL micelles in 10 mM PBS (pH = 7.4, containing 0.1% Tween 80) with and without 10 mM DTT at 37 °C. In the presence of a reducing agent (DTT, 10 mM), the hydrophobic drug molecules can be easily released from the core due to the cleavage of the disulfide bonds.

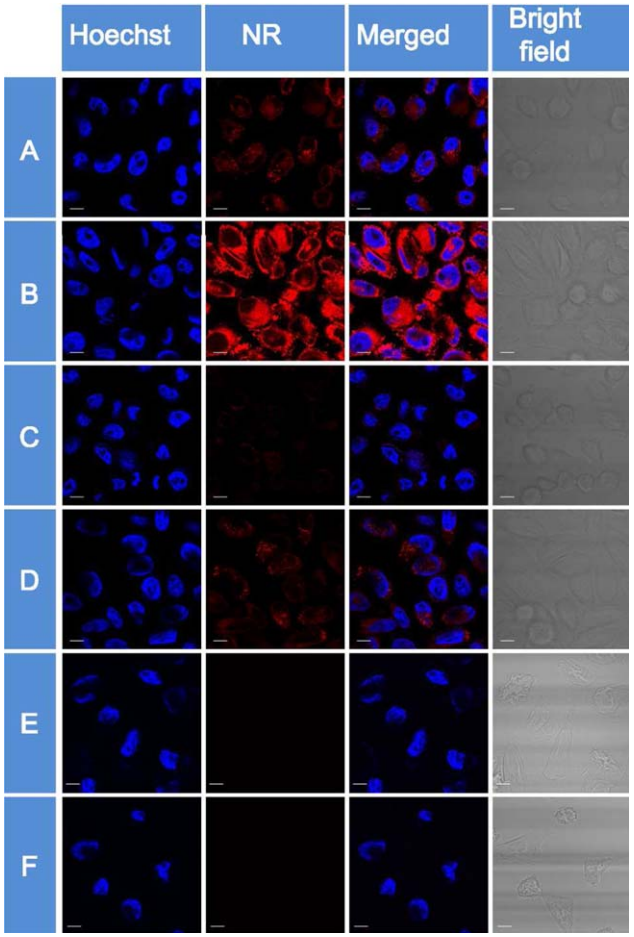


FIGURE 5 CLSM images of HeLa cells after 1 or 4 h incubation with free NR or NR-loaded CCL micelles. For each panel, the images from left to right showed cell nuclei stained by Hoechst 33342 (blue), NR fluorescence in cells (red), overlays of two left images and bright field. The scale bars correspond to 10 μm in all the images. (A) NR-loaded CCL micelles-SS, 1 h. (B) NR-loaded CCL micelles-SS, 4 h. (C) NR-loaded CCL micelles-CC, 1 h. (D) NR-loaded CCL micelles-CC, 4 h. (E) Free NR, 1 h. (F) Free NR, 4 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

higher than that in blood plasma (1–2 μM)[55]. The PTX-loaded CCL micelles-SS could be stable enough in simulative human blood plasma environment, passively targeted to the tumor tissue through the EPR effect, and disassociated in tumor cells upon the reductive environment to release PTX.

Cell Uptake and Internalization Analysis

In order to investigate the cell uptake and internalization of CCL micelles into HeLa cells, NR was used as a fluorescence probe to replace PTX for preparation of CCL micelles. NR-loaded CCL micelles were incubated in HeLa cells for 1 h and 4 h, which were monitored using CLSM. As shown in Figure 5(A), after incubating NR-loaded CCL micelles-SS in HeLa cells for 1 h, weak fluorescence of NR was accumulated around the nuclei of HeLa cells. Then, after 4 h incubation, strong red fluorescence was observed even in the cell nuclei, indicating that NR has been efficiently delivered into HeLa cells and released in cell nuclei [Fig. 5(B)]. As a comparison, the fluorescence of NR was much weaker when HeLa cells were incubated with NR-loaded CCL micelles-CC than those incubated with CCL micelles-SS for the same time [Fig. 5(C,D)]. Moreover, no fluorescence was detected when incubating free NR with HeLa cells even after 4 h [Fig. 5(F)], which indicates that free NR could not be taken in cells. All of the results by CLSM imply that PEG–PBMS CCL micelles-SS could be used as drug carriers to delivery hydrophobic drugs into cells, and the loaded drug could be rapidly released in response to the high GSH concentration. Microplate reader was used for quantitative determination of cellular uptake of NR-loaded CCL micelles. As shown in Figure 6, NR-loaded CCL micelles-SS showed much higher fluorescence intensity than NR-loaded CCL micelles-CC and free NR, which further confirms that PEG–PBMS CCL micelles-SS is an efficient nanocarrier to deliver hydrophobic molecules into tumor cells, and quickly release them upon the intracellular reductive environment.

Relative Cell Viabilities of PEG–PBMS and PTX-Loaded CCL Micelles

To evaluate the cytotoxicities of PEG–PBMS, a HeLa cell line was exposed to PEG–PBMS solutions at a series of concentrations ranging from 0.1 to 100 mg/L for 48 h by CCK-8 assay. As shown in Figure 7(A), compared with the control, the presence of all the tested samples did not significantly inhibit cell growth, which suggested that PEG–PBMS was almost nontoxic against HeLa cells. Even when the concentration was as high as 100 mg/L, the relative cell viability still remained over 90% after 48 h incubation. The nontoxicity of PEG–PBMS may arise from the biocompatibility of PEG.[56]

Furthermore, the antitumor activity of the PTX-loaded CCL micelles against HeLa cells upon their reduction-responsive was also investigated CCK-8 assay, and free PTX was used as a control. As shown in Figure 7(B), the cytotoxicities of free

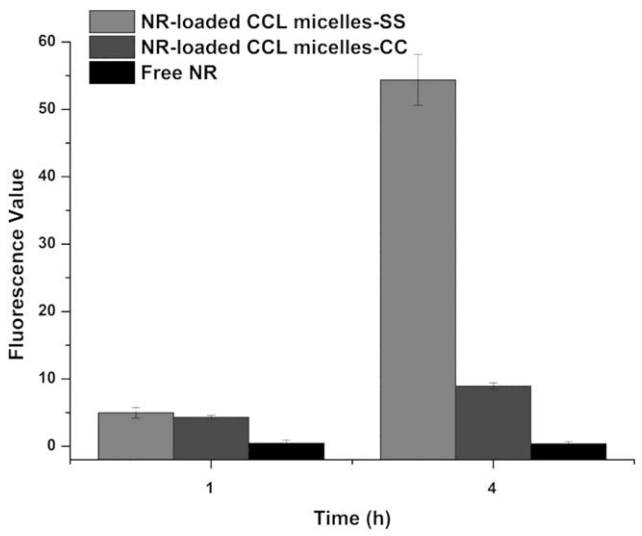


FIGURE 6 Quantitative determination of cellular uptake of NR-loaded CCL micelles and free NR by microplate reader.

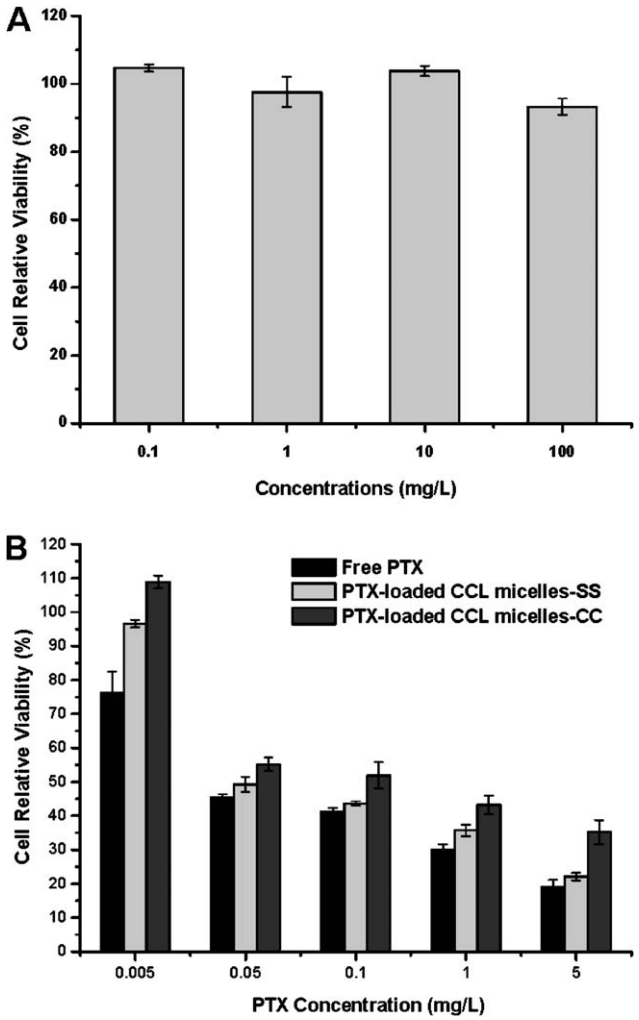


FIGURE 7 Relative cell viabilities of PEG–PBMS (A) and PTX stock solutions, PTX-loaded CCL micelles (B) against HeLa cells.

PTX and PTX-loaded CCL micelles all increased with the increase of dose. Nevertheless, PTX-loaded CCL micelles show lower cytotoxicity than free PTX. This phenomenon could be attributed to the fact that the loaded PTX was released after micelles were endocytosed to enter the cells, which was a delayed process compared to the rapid diffusion of free PTX.[57] Moreover, the cytotoxicity of PTX-loaded CCL micelles-SS toward HeLa cells was significantly higher than that of PTX-loaded CCL micelles-CC. The high antitumor efficiency of PTX-loaded CCL micelles-SS might be due to the fast release of PTX under the intracellular reductive environment.

CONCLUSIONS

In this study, we developed a facile method to prepare amphiphilic PEG–PBMS multiblock copolymers by "one pot" polycondensation of diol poly (ethylene glycol), 1,4-butanediol, and mercaptosuccinic acid using $Sc(OTf)_3$ as a chemoselective catalyst under mild conditions. Then reduction-responsive PTX-loaded CCL micelles-SS could be prepared easily by *in situ* crosslinking hydrophobic PBMS segments with 2,2′-dithiodiethanol diacrylate via thiol-ene "click" reaction under physiological conditions. PTX-loaded CCL micelles-SS could be stable during blood circulation, efficiently be taken up by tumor cells through EPR effect, quickly release antitumor drugs in response to high GSH concentration in tumor cells, and finally kill the tumor cells, which made it a promising candidate as a biodegradable and biocompatible nanocarrier for antitumor chemotherapy.

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