Introduction

In the past decade, polymeric micelles self-assembled from amphiphilic block copolymers have been intensively investigated as nano-carrier systems for tumor-targeted drug delivery to achieve improved cancer chemotherapy. In aqueous solutions, wherein a critical micelle concentration (CMC) of 16 mg L\(^{-1}\) was determined. The micelles were readily crosslinked by adding 7.6 mol % of dithiothreitol (DTT) relative to the lipoyl groups. Notably, micelles after crosslinking demonstrated a markedly enhanced stability against dilution, physiological salt concentration, and organic solvent. In the presence of 10 × 10\(^{-3}\) M DTT, however, micelles were subject to rapid de-crosslinking.

In vitro release studies showed minimal release of DOX from crosslinked micelles at a concentration of 10 mg L\(^{-1}\) (C < CMC, analogous to intravenous injection), wherein less than 15% of the DOX was released in 10 h. In contrast, rapid release of DOX was observed for DOX-loaded non-crosslinked micelles under otherwise the same conditions (~80% release in 0.5 h). In the presence of 10 × 10\(^{-3}\) M DTT mimicking an intracellular reductive environment, sustained release of DOX from crosslinked micelles was achieved, in which 75% of the DOX was released in 9 h. These novel reduction-sensitive reversibly crosslinked biodegradable micelles are highly promising for targeted intracellular delivery of anticancer drugs.
tumor sites by the enhanced permeability and retention (EPR) effect.[7] Moreover, micelles are able to effectively enhance the aqueous solubility and bioavailability of hydrophobic drugs including paclitaxel and doxorubicin (DOX).[4–6]

Biodegradable micelles based on block copolymers of poly(ethylene glycol) (PEG) and aliphatic biodegradable polyesters such as poly(lactic acid) (PLA), poly[(lactic acid)-co-(glycolic acid)] (PLGA), and poly(e-caprolactone) (PCL) have been most frequently studied[2,8–12] because of their approved use in medical devices by the US FDA. It should be noted that a couple of micellar anti-cancer drug formulations, e.g., NK911 and Genexol-PM, have already advanced to clinical trials.[13,14]

One practical issue with micelles is their spontaneous dissociation at concentrations below their critical micelle concentration (CMC).[15] For instance, it was reported that micelles rapidly dissociate upon intravenous administration as a result of large volume dilution, which leads to premature drug release and diminished drug targetability.[16,17] In the past decade, different types of crosslinked block copolymer micelles have been developed to overcome the instability problem.[15,18,19] For instance, Wooley and co-workers reported various forms of shell-crosslinked micelles[20–25] Armes and co-workers reported core-crosslinked stimuli-sensitive micelles.[26] The groups of Kissel and Hennink separately reported photo-polymerized core-crosslinked biodegradable micelles for the delivery of anticancer drugs.[27,28] Stable micelles were also obtained by crosslinking at the core–shell interface based on ABC triblock copolymers,[29,30] This interfacial crosslinking approach combines the advantages of both core and shell crosslinking methods, in which the crosslinking reaction may be performed at high concentrations without intermicellar crosslinking, and drug release may be retarded by the crosslinking layer. However, for drug delivery applications, overly stable micelles are not ideal either because the release of drug is prohibited after the micelles arrive at the target sites. It should also be noted that most crosslinked micelles reported so far are not biodegradable and/or biocompatible.

In this paper, we report on reduction-sensitive interfacially crosslinked biodegradable micelles for the controlled release of DOX. These disulfide crosslinked micelles show good stability against dilution and salt, but are prone to dissociation under a reductive environment that mimics intracellular conditions. In the past years, various types of reduction-sensitive polymers and bioconjugates have been developed for the targeted intracellular delivery of drugs and biopharmaceuticals.[31] These novel biodegradable micelles have the following features: i) all the copolymer components (PEG, PCL, 1,4-dithio-2,3-threitol (DTT), and lipoic acid) are biocompatible and widely applied in medicine. Lipoic acid is a naturally occurring pro-vitamin for anti-oxidation, showing no cytotoxicity even at high concentrations.[32] The therapeutic application of lipoic acid has been approved by the FDA; ii) the crosslinking reaction readily takes place with addition of a catalytic amount of DTT under aqueous conditions; iii) crosslinked micelles, although they remain stable at concentrations below the CMC, are readily dissociated in a mildly reductive environment under otherwise the same conditions; iv) remarkably, these micelles display minimal release of DOX below the CMC, but rapid and sustained release of DOX is achieved in the presence 10 × 10⁻³ M DTT. These reduction-sensitive interfacially crosslinked biodegradable micelles are highly promising for targeted cancer therapy.

**Experimental Part**

**Materials**

e-Caprolactone (e-CL, 99%, Alfa Aesar) was dried over CaH₂ and distilled under reduced pressure prior to use. Monomethoxy PEG (M₉₈ = 5,000, Fluka), acryloyl chloride (96%, Alfa Aesar), triethylamine (99%, Alfa Aesar), triethylamine (99%, Alfa Aesar), DTT (99%, Merck), lipoic acid (98%, Acros), 4-(dimethylamino)pyridine (DMAP), and DOX hydrochloride (>99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd.) were used as received. PEG acrylate[33] and lipoic acid anhydride[34,35] were prepared according to reported procedures.

**Synthesis of PEG-SH**

To a 100 mL three-necked flask equipped with a magnetic stirrer and a 50 mL dropping funnel under a N₂ atmosphere, were charged with DTT (1.5 g, 9.7 mmol) and methanol (10 mL). PEG acrylate (2.19 g, 0.4 mmol) in dry methanol (30 mL) was then added dropwise over a time span of 1 h at room temperature. The reaction was allowed to proceed for 24 h. The product was isolated by precipitation into cold diethyl ether, filtration, and drying under vacuum for 2 d. Yield: 70.0%.

**Synthesis of PCL-AC**

To a 100 mL three-necked flask equipped with a magnetic stirrer and a 25 mL dropping funnel, were introduced the PCL prepolymer (M₉₈ = 5,400, PDI = 1.24, 5.4 g, 1 mmol), triethylamine (0.58 mL, 4 mmol), and dry dichloromethane (DCM, 40 mL). The flask was placed in an ice bath and 0.25 mL of acryloyl chloride (3 mmol) in 10 mL of DCM was added dropwise under vigorous stirring over 30 min. The reaction was allowed to proceed in the dark at room temperature for 16 h. The product was isolated by filtration to remove insoluble byproducts, evaporation of all solvents, re-dissolving in 10 mL of DCM, precipitating in cold diethyl ether, filtration, and drying under vacuum for 2 d. Yield: 95.2%.

**Synthesis of PEG-DTT-PCL by Michael Addition Chemistry**

To a 50 mL three-necked flask equipped with a magnetic stirrer under a N₂ atmosphere, were introduced PCL-AC (1.0 g, 0.20 mmol), PEG-SH (1.24 g, 0.25 mmol), and dry acetonitrile (20 mL). The pH of...
the solution was adjusted to 8.0 using triethylamine and the reaction was allowed to proceed at 30 °C for 4 d. The product was isolated by precipitation in cold diethyl ether, filtration, and drying under vacuum for 2 d. Yield: 75%.

Synthesis of PEG-L2-PCL

To a DCM solution (2.5 mL) of lipoic acid anhydride (0.1 mmol) and DMAP (5.6 mg, 0.044 mmol), was added PEG-DTT-PCL (0.2 g, 0.02 mmol). The mixture was stirred under a N2 atmosphere at room temperature for 2 d. The product was isolated by precipitation in cold diethyl ether, filtration, and drying under vacuum for 2 d. Yield: 60%.

Characterization

The 1H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterated chloroform (CDCl3) as a solvent. The chemical shifts were calibrated against the residual solvent signal of CDCl3. The molecular weight and polydispersity of the copolymers were determined by gel permeation chromatography (GPC) using a Waters 1515 instrument. The molecular weight and polydispersity of the copolymers were determined by gel permeation chromatography (GPC) using a Waters 1515 instrument equipped with two linear Plgel columns following a guard column and a differential refractive-index detector. The measurements were performed using THF as the eluent at a flow rate of 1.0 mL/min at 30 °C and a series of narrow poly(ethylene oxide) standards for the calibration of the columns. The size of the micelles was determined using dynamic light scattering (DLS). The micellar solutions were filtered through a 450 nm syringe filter before measurements. Measurements were carried at 25 °C using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He-Ne laser using back-scattering detection. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 µL of a 0.1 mg mL−1 micellar solution on a copper grid followed by staining with phosphotungstic acid.

Formation of PEG-L2-PCL Micelles and their CMC

Typically, micelles were prepared under stirring by dropwise addition of 0.5 mL of phosphate buffer (PB, pH 7.4, 20 × 10−3 M) to 0.5 mL of a PEG-L2-PCL block copolymer solution (1 mg mL−1) in tetrahydrofuran (THF) at room temperature followed by dialysis against PB.

The CMC was determined using pyrene as a fluorescent probe. The concentration of the block copolymer was varied from 1.0 × 10−4 to 0.5 mg mL−1 and the concentration of pyrene was fixed at 1.0 × 10−6 M. The fluorescence spectra were recorded using an Edinburgh FLS920 fluorescence spectrometer with an excitation wavelength of 330 nm. The emission fluorescence at 373 and 383 nm was monitored. The CMC was estimated as the cross-point when extrapolating the intensity ratio I373/I383 at low and high concentration regions.

Crosslinking and De-Crosslinking of PEG-L2-PCL Micelles

To 0.9 mL of a PEG-L2-PCL micelle solution (0.31 mg mL−1, 2.58 × 10−3 mmol) in a Schlenk bottle under a N2 atmosphere, was introduced 0.1 mL of a PB solution of DTT (0.3 µg, 1.95 × 10−6 mmol, 7.6 mol-% relative to lipooyl units). The mixture was stirred at room temperature for 1 d. Thus crosslinked micelles were studied in terms of size, morphology, and stability against extensive dilution, physiological salt concentration, and organic solvent.

The de-crosslinking of PEG-L2-PCL micelles in response to 10 × 10−3 M DTT was investigated at 37 °C and pH 7.4 at concentrations below the CMC using DLS. In brief, under a N2 atmosphere, crosslinked micelles as prepared above were diluted 100- to 1000-fold. DTT was added to reach a final concentration of 10 × 10−3 M. The change of the micelle sizes over time was followed by DLS.

Loading and in vitro Release of DOX from Crosslinked Micelles

DOX.HCl and 2 equiv. of triethylamine were dissolved in dry dimethyl sulfoxide (DMSO) and added to a THF solution of PEG-L2-PCL copolymer at a theoretical drug loading content of 5 wt.%. Under vigorous stirring, a two-fold volume of PB (20 × 10−3 M, pH 8.5) was added dropwise. After most of the THF was evaporated, the micelle solution was dialyzed against 500 mL of the same buffer (MWCO 3500). The micelles were crosslinked as described previously. For the determination of drug loading content, DOX-loaded crosslinked or non-crosslinked micelles were freeze-dried, dissolved in DMSO, and analyzed by fluorescence spectroscopy, wherein a calibration curve was obtained with DOX/DMSO solutions with different DOX concentrations.

The release profiles of DOX from the crosslinked micelles were studied at ultra-low block copolymer concentrations (< CMC) in PB (20 × 10−3 M, pH 7.4) at 37 °C and pH 7.4, either in the presence or absence of 10 × 10−3 M DTT. In brief, a DOX-loaded crosslinked micelle solution (75 µL, 0.3 mg mL−1) was transferred into a dialysis tube (MWCO 12 000−14 000) and diluted with 2.1 mL of PB to yield a block copolymer concentration of ≈0.01 mg mL−1. To investigate drug release behaviors under a reductive environment, DTT was introduced to the dialysis tube to reach a final DTT concentration of 10 × 10−3 M. The dialysis tube was immersed into 43 mL of the corresponding buffer (with 10 × 10−3 M DTT) and continuously shaken (200 rpm) at 37 °C. At desired time intervals, 10 mL of the release media was taken out for fluorescence measurement and replenished with an equal volume of fresh media. In a similar way, the release of DOX from DOX-loaded crosslinked micelles or DOX-loaded non-crosslinked micelles in the absence of DTT under otherwise the same conditions was also determined and used as a control.

Results and Discussion

Synthesis of the PEG-L2-PCL Diblock Copolymer

The synthesis of the PEG-L2-PCL diblock copolymer is illustrated in Scheme 1. The PCL prepolymer was prepared by the ring-opening polymerization of ε-CL using isopropyl alcohol as an initiator and Sn(oct)2 as a catalyst. 1H NMR
**Reduction-Sensitive Reversibly Crosslinked Biodegradable...**

End-group analysis showed an $M_n$ of 5400, which is close to that designed ($M_n$ theory = 5000). GPC revealed a unimodal distribution with a PDI of 1.24. The subsequent reaction of the hydroxy end-groups of PCL with acryloyl chloride yielded PCL acrylate with over 95% functionality, as determined by $^1$H NMR spectroscopy by comparing the intensities of signals at $delta$ 5.8–6.5 (acryloyl end group) and 1.24 (methyl end group) (Figure S1, see Supporting Information). PEG-DTT was readily synthesized by a Michael addition reaction between PEG acrylate and 22-fold DTT in methanol. $^1$H NMR spectra indicated a quantitative derivatization (Figure S2).

A PEG-DTT-PCL block copolymer was prepared by the Michael addition between PCL acrylate and excess PEG-DTT in acetonitrile using triethylamine as a catalyst. The product was isolated by precipitation into cold diethyl ether. The unreacted PEG-DTT was removed by extensive washing with cold methanol. The $^1$H NMR spectrum showed that signals assignable to acryloyl protons had completely disappeared, and furthermore that the ratio between the PEG and PCL blocks was close to 1:1 (Figure S3). GPC measurement revealed a unimodal distribution with an $M_n$ of 9400 and a PDI of 1.34 (Figure S4). These results confirmed the quantitative coupling and successful synthesis of the PEG-DTT-PCL block copolymer.

**Micelle Formation from PEG-L2-PCL Diblock Copolymers**

Micelles were prepared by adding water dropwise to the PEG-L2-PCL block copolymer solution in THF under stirring at 25°C, followed by dialysis against PB (pH 7.4, 20 x 10^{-3} M). DLS measurements showed that PEG-L2-PCL formed micelles with sizes that ranged from 20 to 150 nm and polydispersities of 0.1–0.2 (Figure 2A). A TEM micrograph revealed that these micelles had a spherical morphology with particle sizes in close agreement with those determined by DLS (Figure 2B). Using pyrene as a fluorescence probe, the CMC of the PEG-L2-PCL block...
Table 1. Molecular characteristics of the PEG-L2-PCL copolymer.

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>$M_n$ NMR $^a$</th>
<th>$M_n$ GPC $^b$</th>
<th>PDI $^b$</th>
<th>CMC $^c$</th>
<th>mg L$^{-1}$</th>
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<tbody>
<tr>
<td>PEG-L2-PCL</td>
<td>5 000–5 400</td>
<td>9 600</td>
<td>1.36</td>
<td>16</td>
<td></td>
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$^a$ Calculated from $^1$H NMR spectra by comparing the intensities of signals at $\delta$ 4.0 (PCL main chain) and $\delta$ 3.68 (PEG main chain); $^b$ Determined by GPC using PEG standards; $^c$ Determined using pyrene as a fluorescence probe.

copolymer was determined to be $\approx 0.016$ mg mL$^{-1}$ (Figure 3, Table 1).

Crosslinking and De-Crosslinking of PEG-L2-PCL Micelles

PEG-L2-PCL micelles were conveniently crosslinked in PB (pH 7.4, $20 \times 10^{-3}$ M) by introducing 7.6 mol-% of DTT relative to lipoyl moieties (Scheme 2). Lipoyl groups have previously been exploited to obtain crosslinked liposomes$^{[34,35]}$ and to yield a reversibly polymerized lipid for gene delivery.$^{[36]}$ The crosslinking/polymerization mechanism is based on thiol–disulfide exchange. Under the catalysis of DTT, part of the lipoyl rings is opened to yield dihydrolipoyl groups (i.e., the reduced form of lipoyl groups). The dihydrolipoyl groups readily exchange with the disulfide bonds of other lipoyl rings successively, which results in formation of linear disulfide bonds between different lipoyl groups. DLS and TEM measurements showed that the crosslinked micelles had similar particle sizes to the parent non-crosslinked micelles. The stability of the crosslinked micelles was investigated using DLS against extensive dilution, physiological salt, as well as organic solvent. As shown in Figure 4A, crosslinked micelles showed a slight increase in size and similar PDI after a 1 000-fold dilution ($C \ll C_{MC}$), while unimers were observed for the non-crosslinked micelles upon the same volume dilution. The crosslinked micelles also exhibited enhanced stability against salt as compared to their non-crosslinked counterparts. For example, the presence of 150 $\times 10^{-3}$ NaCl caused little size change for the crosslinked micelles, while it resulted in the formation of large aggregates for the non-crosslinked micelles with sizes changing from $\approx 30$ nm to 300 nm (Figure 4B). Moreover, addition of 10-fold of THF to the crosslinked micelles yielded swollen particles with sizes of $\approx 400$ nm and a narrow distribution (Figure 4C). In contrast, the non-crosslinked micelles were dissociated to unimers. This largely improved micelle stability strongly indicates successful ‘locking’ of the micellar structures.

To investigate whether the crosslinked micelles can be un-crosslinked under a reductive environment analogous to that of the cytoplasm and cell nucleus, the change in micelle size in response to $10 \times 10^{-3}$ M DTT was monitored over time in PB (pH 7.4, $20 \times 10^{-3}$ M) at a concentration below the CMC (Figure 5). Interestingly, the results showed that the micelle size increased from $\approx 40$ nm to over 160 nm in 18 min, most probably as a result of partial cleavage of the disulfide bonds (partial de-crosslinking). After 50 min, two populations at $\approx 50$ and 180 nm were clearly observed. After a longer time of 8 h, a large portion of particles had sizes of $\approx 20$ nm. We have also checked the influence of stronger reductive conditions using $100 \times 10^{-3}$ M DTT on the crosslinked micelles, in which the micelles were completely dissociated to unimers within 1 h.

It appears that the rate of de-crosslinking for the crosslinked PEG-L2-PCL micelles is slower than that of PEG-b-poly(acrylic acid)-b-poly(N-isopropylacrylamide) (PEG-PAA-PNIPAM) polymersomes crosslinked by cystamine, which showed complete disruption within 30 min at pH 7.4 in the presence of $10 \times 10^{-3}$ M DTT.$^{[37]}$ But, it is significantly faster compared to cystamine-crosslinked poly(ethylene oxide)-b-poly[(N,N-dimethylacrylamide)-stat-(N-acryloxyxysuccinimide)]-b-poly(N-isopropylacrylamide) [PEO-b-P(DMA-stat-NAS)-b-PNIPAM] micelles, which required 0.7 M DTT at 45 °C for 10 h to accomplish disassembly.$^{[26]}$

Figure 2. Size distribution of the PEG-L2-PCL micelles measured by DLS at an angle of 173° (A) and by TEM (B).
DOX Loading and Release

DOX is a potent anticancer drug widely used for treatment of various types of solid malignant tumors.\cite{38,39} However, cardiotoxicity has been one of the life-threatening side effects of DOX-based therapy.\cite{40,41} In the past decade, tremendous efforts have been directed to develop nanovehicles, such as micelles and polymersomes, for the tumor-targeted delivery of DOX.

DOX was loaded into the micelles by a solvent evaporation and dialysis method. The theoretical drug loading content was set at 5 wt.-%. The results showed a drug loading efficiency of approximately 46%. DLS and TEM measurements revealed that DOX-loaded crosslinked micelles have a similar size and shape relative to the parent micelles (data not shown).

The release of DOX from the crosslinked micelles was studied using a dialysis tube (MWCO 12 000) at a low micelle concentration of 10 mg·L\(^{-1}\), which was below the CMC (16 mg·L\(^{-1}\)). DOX-loaded micelles, either crosslinked or non-crosslinked, were prepared at a concentration of 0.3 mg·mL\(^{-1}\). The release of DOX was followed immediately after dilution with 30-fold PB buffer. As shown in Figure 6, rapid release of DOX from the non-crosslinked micelles was observed, wherein approximately 80% of the DOX was released in 20 min. This fast drug release upon a large volume dilution has been commonly encountered in micellar systems, as a result of spontaneous dissociation of micelles at concentrations below the CMC. Insufficient stability against dilution is a major problem for micelles to accomplish tumor-targeted drug delivery in vivo. In contrast, the release of DOX from the crosslinked micelles was minimal (<15%) over a period of 10 h under otherwise the same conditions (Figure 6). This remarkably enhanced stability of the release of drugs from the micelles is most likely because of crosslinks locating at the core–shell interface, which not only prevents micelle dissociation but also forms an effective physical barrier to impede DOX diffusion. This interfacial crosslinking approach is advantageous over shell- or core-crosslinking in that the crosslinking reaction can proceed at high concentrations without formation of inter-micellar crosslinks and drug release is significantly retarded by crosslinking. Importantly, in the presence of 10 × 10\(^{-6}\) M DTT analogous to the intracellular reductive environment, the sustained release of DOX from the crosslinked micelles was achieved, in which about 75% of the DOX was released in 9 h (Figure 6). These disulfide crosslinked micellar drugs may ideally solve the problem of the low colloidal stability of micelles and premature drug release upon intravenous (i.v.) administration as well as slow release of drugs inside targeted cells. We are convinced that these reversibly crosslinked biodegradable micelles will have tremendous potential for targeted cancer chemotherapy.

Conclusion

We have demonstrated that reduction-responsive reversibly crosslinked biodegradable micelles are stable against extensive dilution and physiological salt conditions, retain most drugs even at concentrations below the CMC, but release DOX in a rapid and controlled manner under a reductive environment mimicking that of the intracellular compartments. This represents a first report on stimuli-sensitive reversibly crosslinked biodegradable micelles.
which may elegantly solve the dilemma of the spontaneous dissociation of micelles in circulatory systems and slow drug release at the sites of action. Notably, these micelles are based on solely biocompatible materials, and the crosslinking reaction readily takes place with the assistance of DTT under aqueous conditions. We are convinced that these novel multifunctional biodegradable micelles will have tremendous potential for targeted intracellular release of anticancer drugs.

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Keywords: anticancer drug; biodegradable; drug delivery systems; micelles; reduction-sensitive