



## Biodegradable micelles with sheddable poly(ethylene glycol) shells for triggered intracellular release of doxorubicin

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### ABSTRACT

Biodegradable micelles with sheddable poly(ethylene glycol) shells were developed based on disulfide-linked poly(ethylene glycol)-*b*-poly( $\epsilon$ -caprolactone) (PEG-SS-PCL) diblock copolymer and applied for rapid intracellular release of doxorubicin (DOX). PEG-SS-PCL was prepared with controlled block lengths via exchange reaction between PEG orthopyridyl disulfide and mercapto PCL. The micelles formed from PEG-SS-PCL, though sufficiently stable in water, were prone to fast aggregation in the presence of 10 mM dithiothreitol (DTT), due to shedding of the PEG shells through reductive cleavage of the intermediate disulfide bonds. Interestingly, the *in vitro* release studies revealed that these shell-sheddable micelles released DOX quantitatively within 12 h under a reductive environment analogous to that of the intracellular compartments such as cytosol and the cell nucleus. In contrast, minimal drug release (<20%) was observed within 24 h for the reduction insensitive PEG-PCL micelles under the same conditions as well as for PEG-SS-PCL micelles under the non-reductive conditions. Remarkably, cell experiments showed that these shell-sheddable micelles accomplished much faster release of DOX inside cells and higher anticancer efficacy as compared to the reduction insensitive control. These shell-sheddable biodegradable micelles are highly promising for the efficient intracellular delivery of various lipophilic anticancer drugs to achieve improved cancer therapy.

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### 1. Introduction

In the past decades, polymeric micelles self-assembled from amphiphilic block copolymers have emerged as one of the most promising nano-carrier systems for the controlled release of various hydrophobic anticancer drugs including DOX and paclitaxel [1–3]. Micelles are usually associated with several merits such as largely enhanced drug solubility in water, prolonged circulation time, passive targeting to the tumor tissues via the enhanced permeability and retention (EPR) effect, decreased side effects, and improved drug bioavailability [4–6]. It should be noted that a couple of micellar anticancer drug formulations, *e.g.* NK911<sup>®</sup> and Genexol-PM<sup>®</sup>, have already advanced to the clinical trials [7,8].

Biodegradable micelles based on block copolymers of PEG and aliphatic biodegradable polyesters such as poly(lactic acid) (PLA), poly(lactic acid-co-glycolic acid) (PLGA), and poly( $\epsilon$ -caprolactone) (PCL) are among the most frequently studied, due to their approved

use in medical devices by the US Food and Drug Administration (FDA) [9–12]. Aliphatic polyesters display gradual degradation kinetics inside body with degradation times ranging from days to weeks to months [13–15]. Usually, sustained release of drugs over a period of days to weeks via a diffusion-controlled mechanism is observed [16]. However, for cancer therapy, it is often more desirable to accomplish rapid drug release after micelles arriving at the pathological site, which may enhance the therapeutic efficacy as well as reduce probability of drug resistance in cells.

In recent years, stimuli-sensitive micelles and polymersomes have been reported to release drugs quickly in response to an appropriate stimulus including temperature and pH [17–20]. The applied copolymers, nevertheless, are mostly based on non-degradable polymers such as poly(*N*-isopropyl acrylamide) [21–24], poly(diethylaminoethyl methacrylate) [25], and poly(2-(diisopropylamino) ethyl methacrylate) [26,27]. The other interesting approach to enhance drug release rate is by utilizing sheddable PEG coatings, in which shedding of the ‘stealth’ polymer in response to a stimulus such as pH, reduction and proteolytic enzyme would result in destabilization of the nano-vehicles [28]. In particular, reduction-sensitive polymers have received a tremendous amount of interest for intracellular drug delivery due to the existence of a high difference

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in the redox potential between the mildly oxidizing extracellular milieu and the reducing intracellular fluids [29]. For example, reduction-sensitive polymer/DNA complexes [30], polyion complex micelles for siRNA delivery [31,32], polymersomes [33], micelles [34], crosslinked micelles [35], crosslinked polymersomes [36], and degradable nanogels [37] have been reported to achieve fast release of DNA, siRNA or drugs inside the cells or under a reductive condition mimicking that of the intracellular compartments.

In this study, we were set to develop reduction-sensitive biodegradable micelles with sheddable PEG shells to achieve efficient intracellular release of DOX, for which a new PEG–PCL diblock copolymer containing a single disulfide linkage (PEG–SS–PCL) was designed. The effective shedding of the PEG shells and triggered release of DOX in response to 10 mM DTT, analogous to the intracellular redox potential, were demonstrated. The intracellular release of DOX from shell-sheddable micelles was studied using mouse leukemic monocyte macrophage cell line (RAW 264.7) and the results were compared with those for the reduction insensitive PEG–PCL micelles.

## 2. Materials and methods

### 2.1. Materials

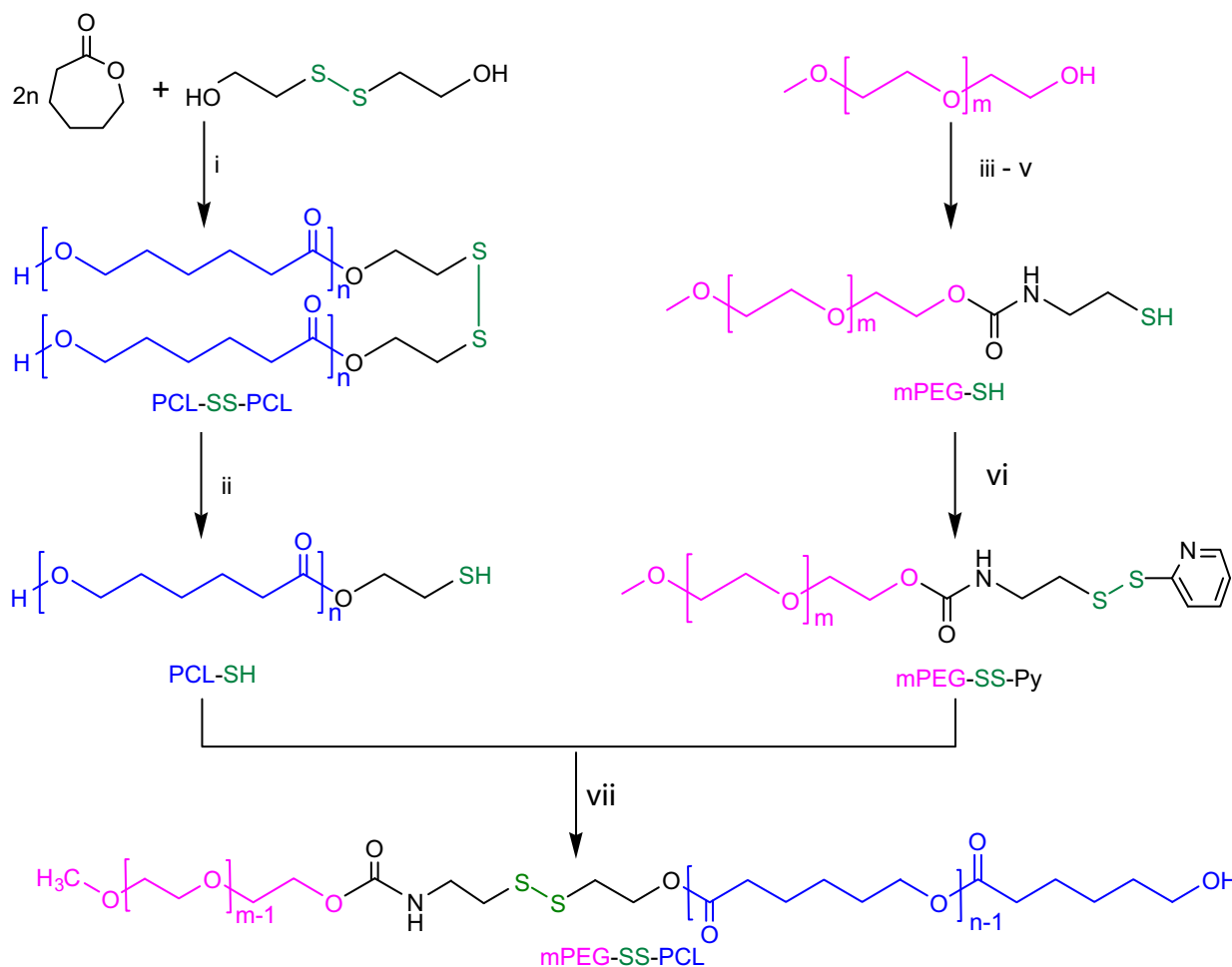
$\epsilon$ -Caprolactone ( $\epsilon$ -CL, 99%, Alfa Aesar) and bis(2-hydroxyethyl)disulfide (HES, 98%, ABCR) were dried over CaH<sub>2</sub> and distilled under reduced pressure prior to use. Poly(ethylene glycol) monomethyl ether (CH<sub>3</sub>O–PEG,  $M_n$  = 5000, Fluka) was dried

by azeotropic distillation from toluene. Toluene and tetrahydrofuran (THF) were dried by refluxing over sodium wire and distilled prior to use. Dichloromethane (DCM), *N,N*-dimethyl formamide (DMF), and dimethyl sulfoxide (DMSO) were dried by refluxing over CaH<sub>2</sub> and distilled before use. Stannous octoate (Sn(Oct.)<sub>2</sub>, 95%, Sigma), 2-mercaptopyridine (Py-SH, 99%, Sigma), dithiothreitol (DTT, 99%, Merck), sodium methoxide (CH<sub>3</sub>ONa, 98%, Alfa Aesar), *p*-nitrophenyl chloroformate (*p*-NPC, 97%, Alfa Aesar), cystamine dihydrochloride (Cystamine·2HCl, >98%, Alfa Aesar), triethylamine (Et<sub>3</sub>N, 99%, Alfa Aesar), 2,2'-dithiodipyridine (Py-SS-Py, 99%, Fluka), cysteamine (NH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>SH, 95%, J&K), doxorubicin hydrochloride (>99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd.), pyridine (Py, 99.5%), calcium hydride (CaH<sub>2</sub>), and anhydrous methanol were used as-received.

### 2.2. Synthesis of PCL-SH

PCL-SH was synthesized by ring-opening polymerization of  $\epsilon$ -CL using HES as an initiator followed by reduction with DTT. Briefly, under an argon atmosphere, to a reaction vessel equipped with magnetic stirring bar, HES (212 mg, 1.375 mmol), Sn(Oct.)<sub>2</sub> (96 mg, 0.237 mmol),  $\epsilon$ -CL (8.569 g, 75.08 mmol) and toluene (50 mL) was charged. The vessel was closed with a stopper and immersed in an oil bath thermostated at 100 °C. The polymerization was allowed to proceed under stirring for 24 h. The reaction was terminated by adding excess HCl. A sample was taken to determine the monomer conversion by <sup>1</sup>H NMR. The resulting PCL-SS-PCL polymer was isolated by precipitation in cold diethylether, filtration, and drying in vacuo at 40 °C. Yield: 77%, monomer conversion >95%,  $M_n$  (GPC) 9300, PDI (GPC) 1.42.

To a THF solution (5 mL) of PCL-SS-PCL (0.4991 g, 0.096 mmol) and DTT (0.157 g, 1.02 mmol), catalytic amount of CH<sub>3</sub>ONa (2.4 mg, 0.044 mmol) in anhydrous methanol was added under stirring. The reaction was allowed to proceed for 30 h at 25 °C. The resulting polymer PCL-SH was precipitated in cold diethylether, filtrated under a N<sub>2</sub> flow, and dried in vacuo. Yield: 76%,  $M_n$  (GPC) 6600, PDI (GPC) 1.30.



**Scheme 1.** Synthetic pathway for PEG–SS–PCL. Conditions: (i) Sn(Oct.)<sub>2</sub>, toluene, 100 °C, 24 h; (ii) DTT, CH<sub>3</sub>ONa, THF, 25 °C, 30 h; (iii) *p*-NPC, pyridine, DCM, r.t., 10 h; (iv) cystamine dihydrochloride, Et<sub>3</sub>N, DMSO, r.t., 24 h; (v) DTT, H<sub>2</sub>O, r.t., 24 h; (vi) Py-SS-Py, H<sub>2</sub>O, pH 1.5, 30 °C, 36 h; (vii) DCM, pH 2.5, r.t., 48 h.

### 2.3. Synthesis of PEG-SS-Py

PEG-SS-Py was synthesized according to the following procedure: (i) activating PEG with *p*-NPC, (ii) reacting with cystamine dihydrochloride in the presence of Et<sub>3</sub>N, (iii) reducing with DTT to yield PEG-SH, and (iv) exchanging with Py-SS-Py. In brief, under a nitrogen atmosphere, to a DCM solution (70 mL) of mPEG (10.0 g, 2 mmol) and pyridine (0.791 g, 10 mmol), a solution of *p*-NPC (1.614 g, 8 mmol) in 15 mL DCM was added dropwise at 0 °C. After completion of addition, the reaction

mixture was warmed to r.t. and the reaction proceeded for another 20 h. The resulting polymer PEG-NPC was isolated by precipitation in cold diethylether and dried in vacuo. Yield: 94.9%. <sup>1</sup>H NMR showed NPC functionality close to 100%.

Under a nitrogen atmosphere, to a DMSO solution (5 mL) of cystamine dihydrochloride (0.612 g, 2.72 mmol) and Et<sub>3</sub>N (0.568 g, 5.61 mmol), a solution of PEG-NPC (2.787 g, 0.540 mmol) in DMSO (11 mL) was added dropwise at r.t. The reaction was allowed to continue for 24 h. The resulting PEG-cystamine conjugate was isolated by precipitation in cold diethylether twice and dried in vacuo. Yield: 99%. <sup>1</sup>H

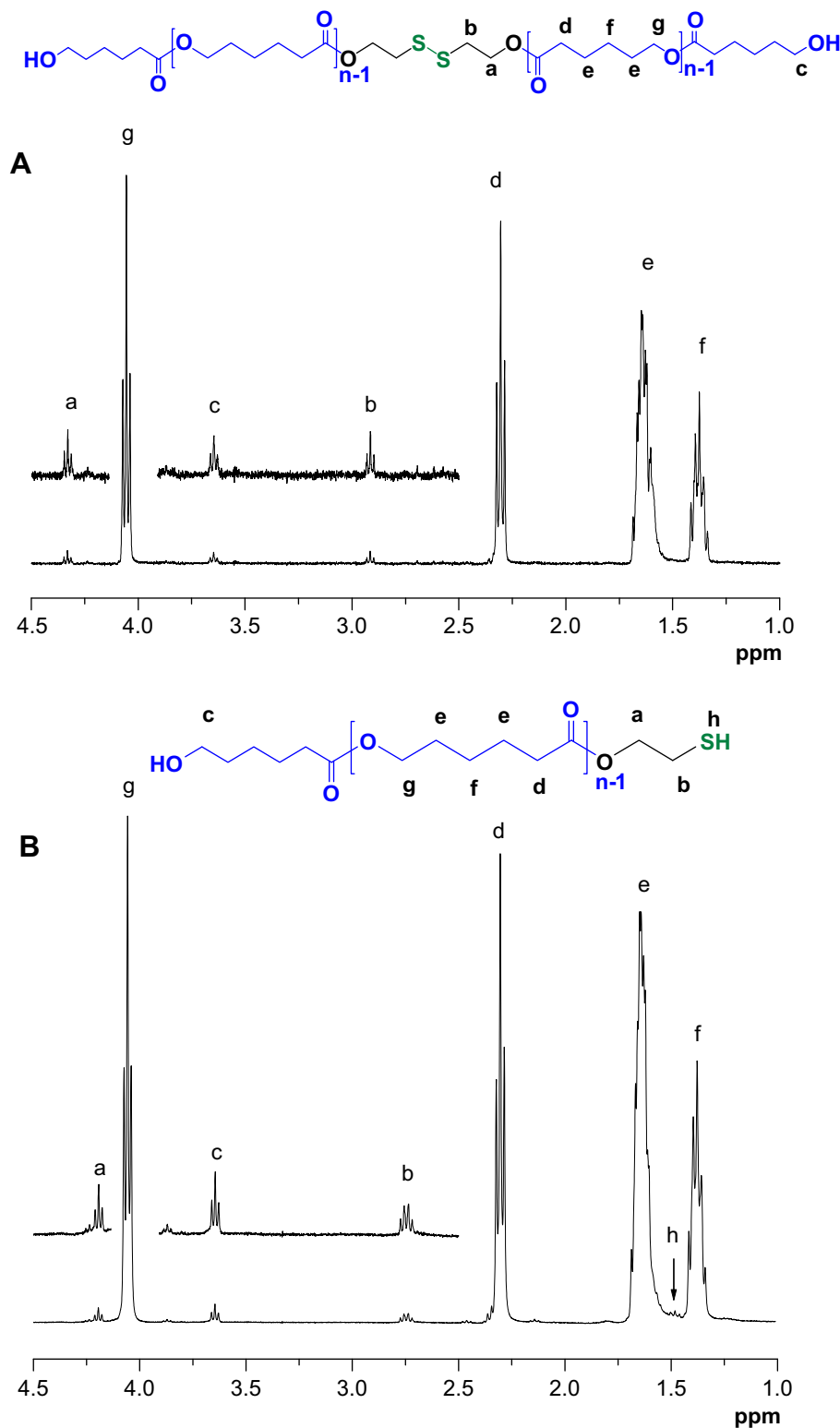


Fig. 1. <sup>1</sup>H NMR spectra (400 MHz, CDCl<sub>3</sub>) of PCL-SS-PCL (A) and PCL-SH (B).

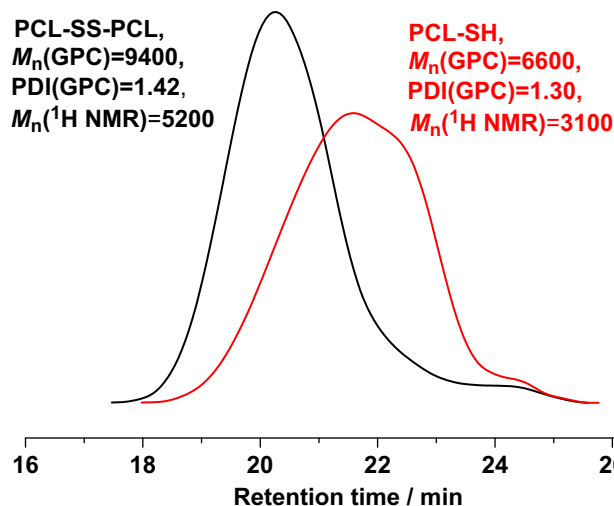


Fig. 2. GPC traces of PCL-SS-PCL and PCL-SH.

NMR showed quantitative conversion of *p*-NPC to cystamine. PEG–cystamine (2.763 g, 0.535 mmol) was treated with DTT (0.616 g, 3.99 mmol) in water (17 mL) for two days, to yield PEG-SH. The polymer was isolated by ultrafiltration (MWCO 1000) and freeze-drying.  $^1\text{H}$  NMR and Ellman assay showed high thiol functionality (>98%).

Under an argon atmosphere, to a suspension of PEG-SH (1.222 g, 0.24 mmol), Py-SS-Py (0.106 g, 0.48 mmol) and catalytic amount of 2-mercaptopyridine (5.8 mg) in 40 mL of water was added HCl solution (37 wt%) to adjust the pH to 1.5. The reaction mixture was stirred at r.t. for 36 h before adjusting its pH to 7.0 using NaOH solution (1.0 M). The solution was filtered to remove excess Py-SS-Py. PEG-SS-Py was isolated by ultrafiltration and freeze-drying. Yield: 82%. The orthopyridyl disulfide functionality was determined to be 85% ( $^1\text{H}$  NMR).

#### 2.4. Synthesis of PEG-SS-PCL and PEG-PCL

Under an argon atmosphere at r.t., to a DCM solution (8 mL) of PEG-SS-Py (0.298 g, 0.057 mmol) was added acetic acid to adjust the pH to 2.5 followed by addition of PCL-SH (0.138 g, 0.046 mmol) in DCM. The reaction was allowed to

proceed under stirring for 48 h. The resulting PEG-SS-PCL conjugate was isolated by precipitation in cold diethylether, filtration, extensive washing with cold methanol to remove free PEG if present, and drying in vacuo. Yield: 32%.  $^1\text{H}$  NMR showed equivalent coupling of PEG and PCL.  $M_n$  (GPC) 12 300, PDI (GPC) 1.14.

PEG-PCL diblock copolymer, used as a reduction insensitive control, was synthesized by ring-opening polymerization of  $\epsilon$ -CL in toluene at 100 °C using  $\text{CH}_3\text{O}-\text{PEG}$  as an initiator and  $\text{Sn}(\text{Oct})_2$  as a catalyst. The polymerization was carried out at an  $[\text{M}]_0/[\text{I}]_0$  molar ratio of 26.6. The resulting polymer was isolated by precipitation in cold diethylether, filtration, and drying in vacuo at 40 °C. Monomer conversion > 95%,  $M_n$  ( $^1\text{H}$  NMR) 7900,  $M_n$  (GPC) 12600, PDI (GPC) 1.08.

#### 2.5. Characterization

The  $^1\text{H}$  NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterated chloroform ( $\text{CDCl}_3$ ) as a solvent. The chemical shifts were calibrated against residual solvent signals of  $\text{CDCl}_3$ . The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) 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 polystyrene standards for the calibration of the columns. The size of micelles was determined using dynamic light scattering (DLS). The micellar solutions were filtered through a 450 nm syringe filter before measurements. Measurements were carried out at 25 °C using 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  $\mu\text{L}$  of 0.1 mg/mL micellar solution on the copper grid followed by staining with phosphotungstic acid.

#### 2.6. Micelle formation and critical micelle concentration

Typically, micelles of PEG-SS-PCL and PEG-PCL were prepared under stirring by dropwise addition of 1.0 mL PB (50 mM, pH 7.4) to 1.0 mL of block copolymer solution (0.1 wt%) in THF at r.t. followed by extensive dialysis against PB for 24 h.

The critical micelle concentration (CMC) was determined using pyrene as a fluorescence probe. The concentration of block copolymer was varied from  $6.0 \times 10^{-4}$  to 0.15 mg/mL and the concentration of pyrene was fixed at 0.6  $\mu\text{M}$ . The fluorescence spectra were recorded using FLS920 fluorescence spectrometer with the excitation wavelength of 330 nm. The emission fluorescence at 372 and 383 nm was monitored. The CMC was estimated as the cross-point when extrapolating the intensity ratio  $I_{372}/I_{383}$  at low and high concentration regions.

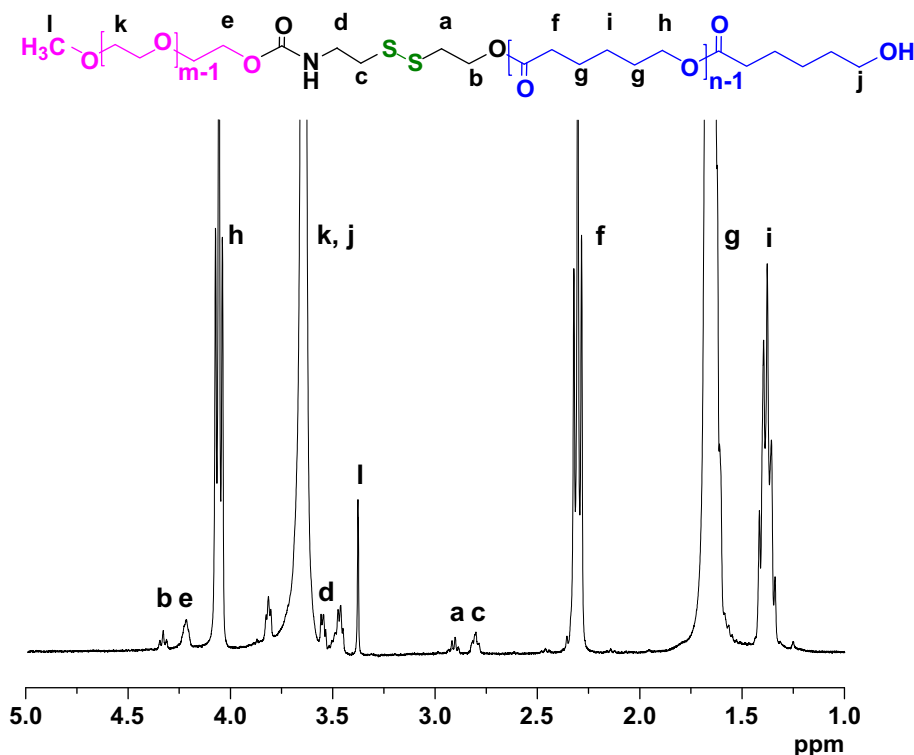


Fig. 3.  $^1\text{H}$  NMR spectrum (400 MHz,  $\text{CDCl}_3$ ) of PEG-SS-PCL.

**Table 1**  
Characteristics of PEG-SS-PCL and PEG-PCL block copolymers.

Block copolymer	$M_n$ (design)	$M_n^a$ ( $^1\text{H}$ NMR)	$M_n^b$ (GPC)	PDI <sup>b</sup> (GPC)	CMC <sup>c</sup> (mg/L)
PEG-SS-PCL	5000–3000	5000–3100	12 300	1.14	6.67
PEG-PCL	5000–3000	5000–2900	12 600	1.08	18.42

<sup>a</sup> Determined by  $^1\text{H}$  NMR.

<sup>b</sup> Determined by GPC (THF as the eluent, 1.0 mL/min, 30 °C, polystyrene standards).

<sup>c</sup> Determined using pyrene as a fluorescence probe.

### 2.7. Reduction-triggered destabilization of PEG-SS-PCL micelles

The size change of micelles in response to 10 mM DTT in PB buffer (pH 7.4, 50 mM) was followed by DLS measurement. Briefly, to 1.5 mL solution of PEG-SS-PCL micelles in PB (50 mM, pH 7.40) was added 10 mM DTT. The solution was placed in a shaking bed at 37 °C with a rotation speed of 200 rpm. At different time intervals, the size was determined using DLS.

### 2.8. Encapsulation of DOX

DOX was loaded into micelles by dropwise addition of 0.8 mL PB (50 mM, pH 7.4) to a mixture of 0.8 mL PEG-SS-PCL or PEG-PCL block copolymer solution in THF (5 mg/mL) and 80  $\mu\text{L}$  DOX solution in DMSO (5 mg/mL) under stirring at room temperature, followed by dialysis against PB (50 mM, pH 7.4) for 24 h at r.t. (MWCO of 3500). The dialysis medium was changed four or five times. The contents inside the dialysis tube were filtered and lyophilized. The whole procedure was performed in the dark. The amount of DOX was determined using fluorescence (FLS920) measurement (excitation at 480 nm). For determination of drug loading content, DOX-loaded micelles were dissolved in DMSO and analyzed with fluorescence spectroscopy, wherein calibration curve was obtained with DOX/DMSO solutions with different DOX concentrations.

Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following formula:

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

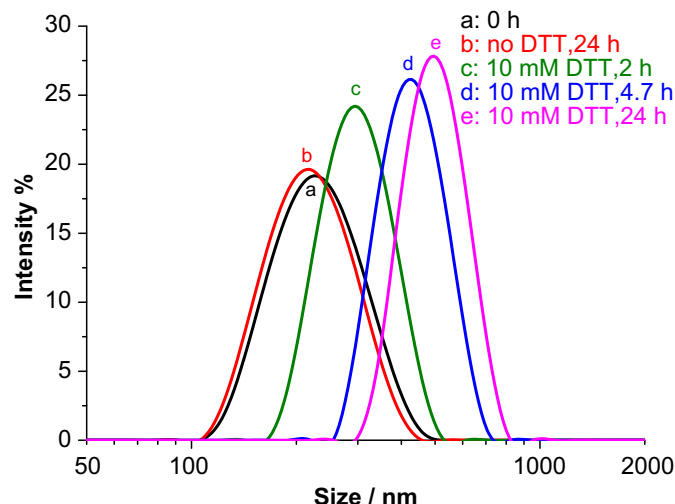
$$\text{DLE}(\%) = (\text{weight of loaded drug}/\text{weight of drug in feed}) \times 100\%$$

### 2.9. Reduction-triggered release of DOX from PEG-SS-PCL micelles

The release profiles of DOX from PEG-SS-PCL micelles were studied using a dialysis tube (MWCO 12 000) at 37 °C in two different media, i.e. PB (50 mM, pH 7.4) with 10 mM DTT or PB (50 mM, pH 7.4) only. In order to acquire sink conditions, drug release studies were performed at low drug loading contents (ca. 0.5 wt%) and with 0.7 mL of micelle solution dialysis against 20 mL of the same medium. At desired time intervals, 6 mL release media was taken out and replenished with an equal volume of fresh media. The amount of DOX released was determined by using fluorescence (FLS920) measurement (excitation at 480 nm). The release experiments were conducted in triplicate. The results presented are the average data.

### 2.10. Intracellular release of DOX

The cellular uptake and intracellular release behaviors of DOX-loaded PEG-SS-PCL micelles were followed with fluorescence microscopy using mouse leukemic



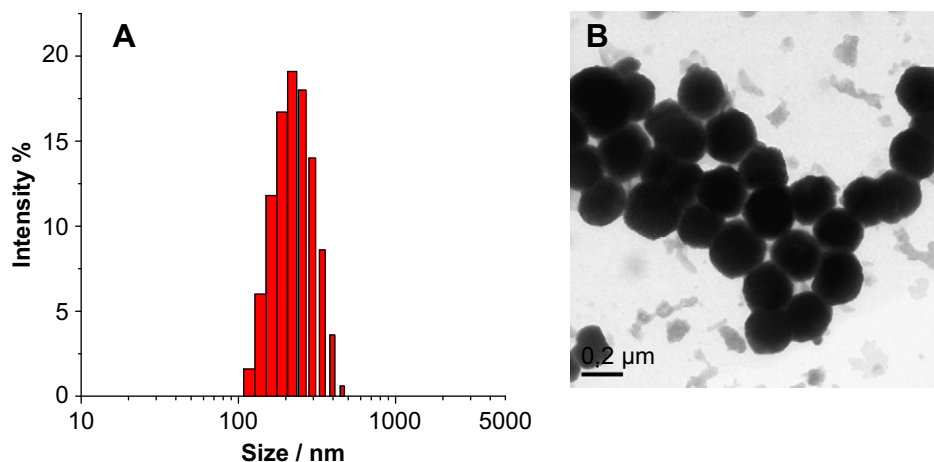
**Fig. 5.** The size change of PEG-SS-PCL micelles in response to 10 mM DTT in PB buffer (pH 7.4, 50 mM) determined by DLS measurement.

monocyte macrophage cell line (RAW 264.7). RAW 264.7 cells were cultured in a disc containing 2 mL of DMEM media for two days to  $\sim 70\%$  confluency ( $\sim 2 \times 10^5$  cells/disc), 100  $\mu\text{L}$  of PB (50 mM, pH 7.4) solution of DOX-loaded PEG-SS-PCL micelles or DOX-loaded PEG-PCL micelles containing 15  $\mu\text{g}$  DOX was added. The cells were incubated with DOX-loaded micelles for 2 or 24 h at 37 °C in a humidified 5%  $\text{CO}_2$ -containing atmosphere. The culture media were removed and the cells were rinsed two times with DMEM prior to the fluorescence observation.

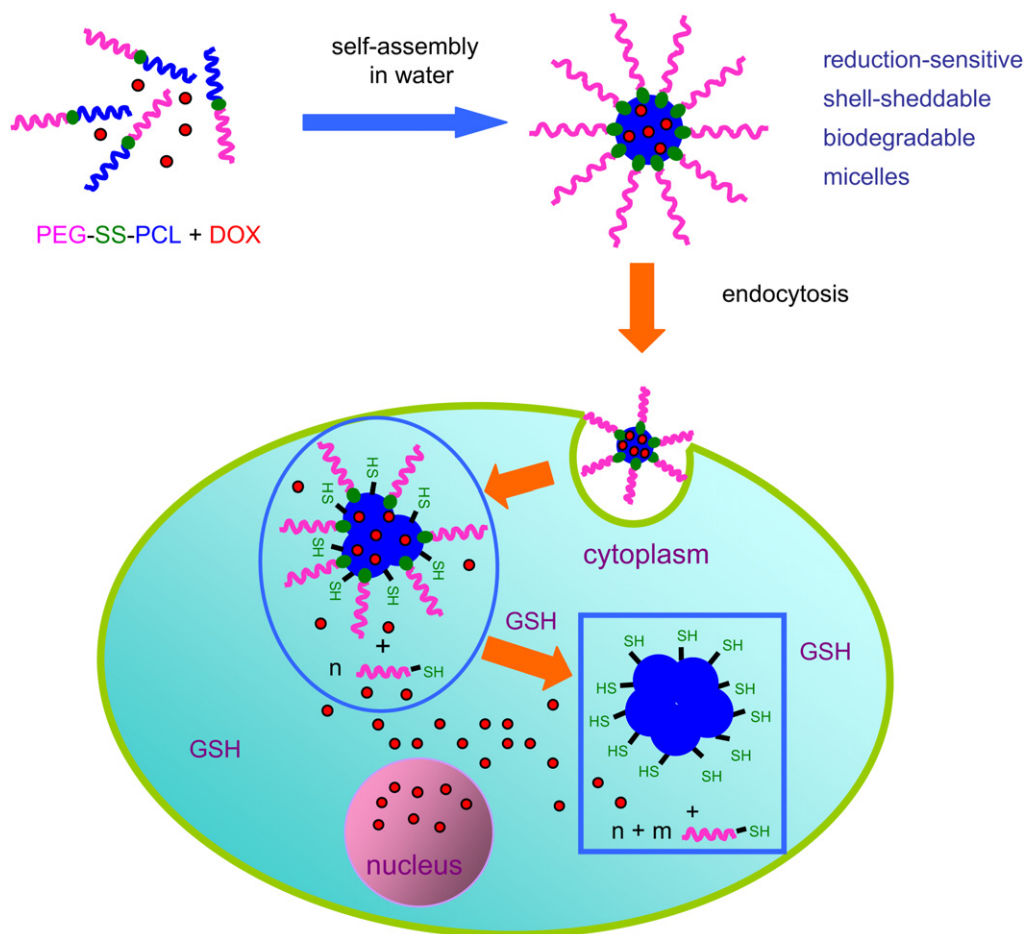
## 3. Results and discussion

### 3.1. Synthesis of PEG-SS-PCL diblock copolymer

PEG-SS-PCL was readily prepared via exchange reaction between PEG orthopyridyl disulfide (PEG-SS-Py) and mercapto PCL (PCL-SH). The synthetic approach is illustrated in Scheme 1. The ring-opening polymerization of  $\epsilon$ -caprolactone ( $\epsilon$ -CL) at 100 °C using bis(2-hydroxyethyl)disulfide as an initiator and stannous octoate as a catalyst yielded PCL-SS-PCL polymer with a defined structure. The  $^1\text{H}$  NMR end group analysis showed that PCL-SS-PCL had an  $M_n$  of 5200 (Fig. 1A). Gel permeation chromatography (GPC) measurement revealed a unimodal distribution with an  $M_n$  of 9400 (polystyrene standards) and a moderate polydispersity index (PDI) of 1.42 (Fig. 2). The reduction of PCL-SS-PCL was carried out in THF at room temperature using DTT as a reducing agent and sodium methoxide as a catalyst.  $^1\text{H}$  NMR and GPC measurements confirmed



**Fig. 4.** The size distribution determined by DLS (A) and TEM micrograph (B) of PEG-SS-PCL micelles.



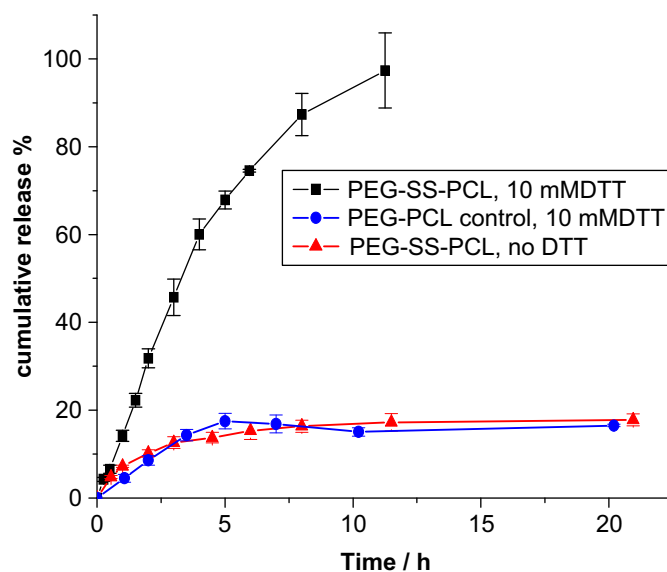
**Scheme 2.** Illustration of reduction-sensitive shell-sheddable biodegradable micelles based on PEG-SS-PCL block copolymer for efficient intracellular release of anticancer drugs triggered by glutathione (GSH) tripeptide.

successful cleavage of disulfide bonds to yield PCL-SH. The resonances at  $\delta$  2.92 attributable to the methylene protons neighboring to the disulfide bond ( $-\text{CH}_2-\text{SS}-\text{CH}_2-$ ) disappeared completely and new peaks at  $\delta$  2.75 characteristic of methylene protons next to the thiol end group ( $-\text{CH}_2-\text{SH}$ ) were detected (Fig. 1B). The  $^1\text{H}$  NMR end group analysis showed an  $M_n$  of 3100, which is close to half of that of PCL-SS-PCL. Moreover, GPC revealed an  $M_n$  of 6600 with a PDI of 1.30 (Fig. 2). The slightly higher molecular weight of PCL-SH compared to half of the parent PCL-SS-PCL is most likely due to loss of low molecular weight fractions during precipitation.

PEG-SS-Py was synthesized by activating PEG with 4-nitrophenyl chloroformate (4-NC), reacting with cystamine dihydrochloride in the presence of triethylamine, reducing with DTT to yield PEG-SH, and followed by exchanging with 2,2'-dithiodipyridine.  $^1\text{H}$  NMR and Ellman assay showed high thiol functionality in PEG-SH. The exchange reaction between PEG-SH and 2,2'-dithiodipyridine

was carried out at pH 2–3 in water.  $^1\text{H}$  NMR measurement revealed that PEG-SS-Py had a functionality over 95%.

The exchange reaction between PEG-SS-Py and PCL-SH was performed in DCM at pH 2.5 using a PCL-SH/PEG-SS-Py molar ratio



**Fig. 6.** Reduction-triggered release of DOX from PEG-SS-PCL micelles (pH 7.4, 50 mM). PEG-PCL micelles was used as a reduction insensitive control.

**Table 2**

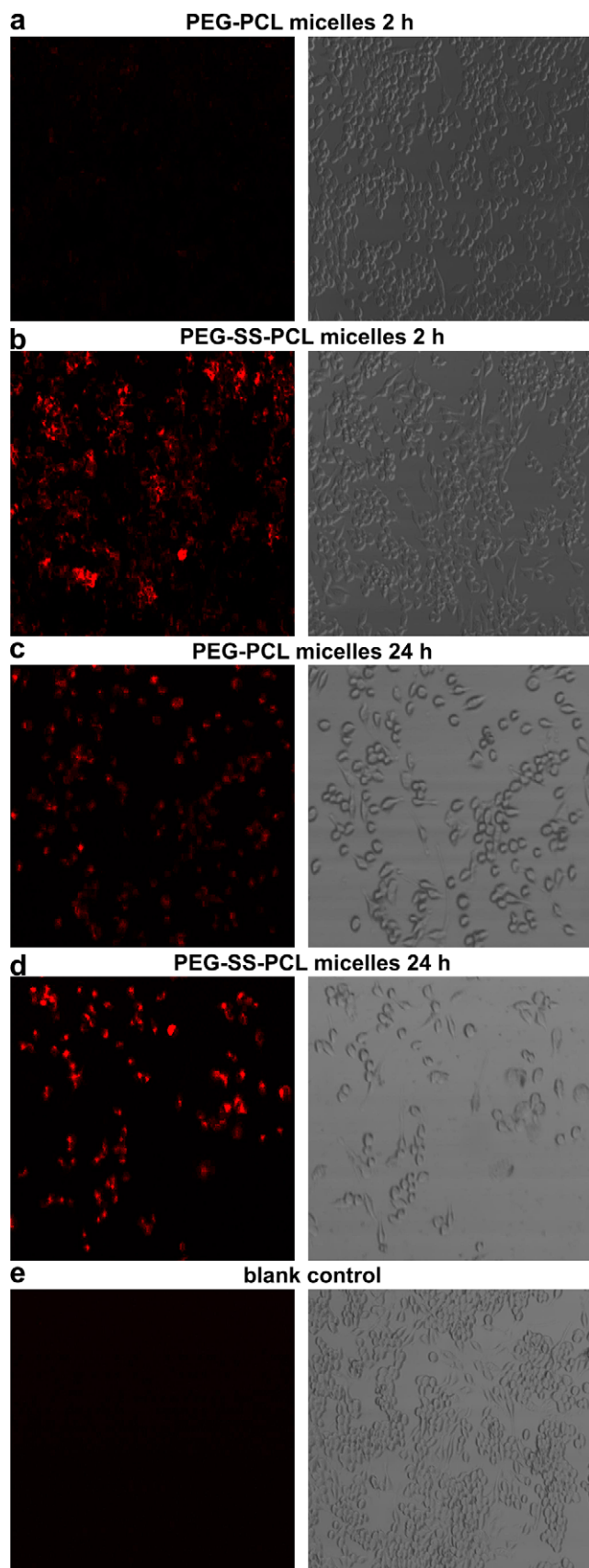
Drug loading content and drug loading efficiency for DOX with PEG-SS-PCL micelles and PEG-PCL micelles.

Sample	Micellesize <sup>a</sup> (nm)	PDI <sup>a</sup>	Theoretical drug loading content (wt%)	Drug loading content <sup>b</sup> (%)	Drug loading efficiency (wt%)
PEG-SS-PCL	56.3	0.27	10	6.0	60
PEG-PCL	37.7	0.35	10	7.0	70

<sup>a</sup> Size and PDI of DOX-loaded micelles were determined by DLS.

<sup>b</sup> Determined by fluorescence measurement.





of 1/1.2. The resulting PEG-SS-PCL block copolymer was purified by extensive washing with cold methanol to remove free PEG if present. As shown in Fig. 3, peaks assignable to both PEG ( $\delta$  3.63) and PCL ( $\delta$  4.10, 2.30, 1.65, and 1.35) were present in the  $^1\text{H}$  NMR spectrum. Importantly, comparing the integrals of signals at  $\delta$  3.63 and 4.10 pointed to an equivalent coupling of PEG and PCL. Furthermore, GPC curve showed a unimodal distribution with a PDI of 1.14 (Table 1). These results supported successful synthesis of PEG-SS-PCL diblock copolymer. In this study, PEG-PCL diblock copolymer with a similar composition was also prepared and used as a control (Table 1).

### 3.2. Micelle formation and reduction-responsive destabilization

Micelles of PEG-SS-PCL and PEG-PCL block copolymers were prepared by dialysis method. Dynamic light scattering (DLS) measurements showed that PEG-SS-PCL formed micelles with sizes of  $\sim 240$  nm (Fig. 4A). TEM micrograph revealed that these micelles had a spherical morphology with an average size of  $\sim 200$  nm (Fig. 4B). The smaller size observed by TEM as compared to that determined by DLS is most likely due to shrinkage of the PEG shell. The critical micelle concentration (CMC) was determined using pyrene as a probe. PEG-SS-PCL showed a CMC of  $\sim 6.6$  mg/L, which is lower than that of PEG-PCL control (CMC  $\sim 18$  mg/L).

The size change of micelles in response to 10 mM DTT in PB buffer (pH 7.4, 50 mM) was followed by DLS measurement. Notably, fast aggregation was observed for PEG-SS-PCL micelles, in which micelle size increased from 230 nm to 329 nm in 2 h, reaching over 500 nm after 24 h (Fig. 5). The aggregates were formed due to most probably reductive cleavage of the intermediate disulfide bonds, which results in shedding of the PEG shells (Scheme 2). In contrast, no change in micelle sizes was discerned after 24 h in the absence of DTT under otherwise the same conditions.

### 3.3. Loading and reduction-triggered release of DOX

DOX is one of the most potent anticancer drugs and used widely in the treatment of different types of solid malignant tumors [38,39]. DOX is known to interact with DNA by intercalation and inhibition of macromolecular biosynthesis [40]. It is crucial, therefore, to deliver and release DOX in the cytoplasm and/or right into the cell nucleus. The aim of this study was to develop triggered intracellular delivery systems for DOX, which may lead to enhanced cancer chemotherapy.

DOX was loaded into micelles by dialysis of a polymer/DOX solution in DMSO against PB buffer. The theoretical drug loading content was set at 10 wt%. The results showed that the drug loading efficiencies were approximately 60% and 70% for PEG-SS-PCL micelles and PEG-PCL micelles, respectively (Table 2). It should be noted that after loading of DOX, PEG-SS-PCL micelles had smaller sizes of ca. 56.3 nm. The release of DOX from PEG-SS-PCL micelles was investigated using a dialysis tube (MWCO 12000) in pH 7.4 PB buffer at 37 °C in the presence or absence of 10 mM DTT. In order to

**Fig. 7.** The intracellular release of DOX from the reduction-sensitive shell-shedtable PEG-SS-PCL micelles followed by fluorescence microscopy using macrophage RAW 264.7 cells. The reduction insensitive PEG-PCL micelles were used as a control. RAW 264.7 cells were cultured in a disc containing 2 mL of DMEM media for two days to  $\sim 70\%$  confluency ( $\sim 2 \times 10^5$  cells/disc). The cells were incubated with DOX-loaded micelles (15  $\mu\text{g}$  DOX) for 2 or 24 h at 37 °C in a humidified 5%  $\text{CO}_2$ -containing atmosphere. The culture media were removed and the cells were rinsed two times with PBS prior to the fluorescence observation. (a) after 2 h incubation with DOX-loaded PEG-PCL micelles; (b) after 2 h incubation with DOX-loaded PEG-SS-PCL micelles; (c) after 24 h incubation with DOX-loaded PEG-PCL micelles; (d) after 24 h incubation with DOX-loaded PEG-SS-PCL micelles; (e) cells cultured for 24 h without DOX-loaded micelles (blank control).

acquire sink conditions, drug release studies were performed at low drug loading contents (ca. 0.5 wt%) and with 0.7 mL of micelle solution dialysis against 20 mL of the same medium. Samples of 6 mL were taken at desired time intervals, and replaced by an equal volume of fresh buffer. Remarkably, the results showed that PEG-SS-PCL micelles released DOX rapidly in the presence of 10 mM DTT, a reductive environment analogous to that of the intracellular compartments such as cytosol and the cell nucleus. For example, ca. 60% DOX was released in 4 h and virtually quantitative release was observed after 12 h (Fig. 6). In contrast, minimal drug release (<20%) was observed within 24 h for the reduction insensitive PEG-PCL micelles under the same conditions as well as for PEG-SS-PCL micelles in the absence of DTT (Fig. 6). Gao and coworkers reported that less than 20% DOX is released from PEG-PCL micelles at neutral pH in one month [16]. Therefore, it is evident that fast drug release from PEG-SS-PCL micelles is triggered by reduction. This is in line with the previous observation that PEG-SS-PCL micelles were destabilized and formed aggregates in response to 10 mM DTT. It should be further noted that no burst release was observed and DOX was released from PEG-SS-PCL micelles in a zero order manner up to 60% release. This constant release rate indicates that release of DOX is controlled most likely by a combination of diffusion and degradation. Therefore, shedding of PEG shells represents a highly promising approach to achieve fast yet controlled drug release.

#### 3.4. Intracellular release of DOX

The cellular uptake and intracellular release behaviors of DOX-loaded PEG-SS-PCL micelles were followed with fluorescence microscopy using mouse leukemic monocyte macrophage cell line (RAW 264.7). After RAW cells were cultured in a disc to ~70% confluency ( $\sim 2 \times 10^5$  cells/disc), 100  $\mu$ L of PB solution of DOX-loaded micelles (15  $\mu$ g DOX per disc) was added. After 2 and 24 h incubation, the culture medium was removed and the cells were rinsed two times with DMEM prior to the fluorescence assessment. The results are shown in Fig. 7. Remarkably, strong DOX fluorescence was observed in the cells after 2 h incubation with DOX-loaded PEG-SS-PCL micelles. In contrast, negligible DOX fluorescence was shown for the RAW cells incubated with DOX-loaded PEG-PCL micelles. It has been reported that PEG-PCL micelles can be rapidly uptaken by the cells via endocytosis [41]. The difference in DOX fluorescence is most likely due to significantly faster intracellular release of DOX from PEG-SS-PCL micelles as compared to PEG-PCL micelles. It has been shown that disulfide linkages could be rapidly cleaved in the intracellular compartment caused by the presence of comparatively high glutathione concentrations (2–10 mM), and based on this intriguing phenomenon, intracellular delivery systems for drugs, DNA and siRNA have successfully been developed [29,36]. Notably, the number of viable cells at this time point was comparable. After a longer incubation time of 24 h, DOX fluorescence was also observed for the RAW cells incubated with DOX-loaded PEG-PCL micelles. The fluorescence intensity, however, was obviously lower than the cells treated with DOX-loaded PEG-SS-PCL micelles. It should further be noted that smaller number of viable cells were observed for DOX-loaded PEG-SS-PCL micelles as compared to the PEG-PCL micelles, indicating a greater drug efficacy for shell-sheddable micelles. Cytotoxicity studies revealed that PEG-SS-PCL micelles, similar to PEG-PCL micelles, were nontoxic. Hence, PEG-SS-PCL micelles boast many favorable properties of an ideal drug carrier, which include excellent biocompatibility and biodegradability, adequate drug loading capacity, minimal drug release under extracellular settings (non-reductive), and rapid drug release in response to the intracellular level of reducing potential.

#### 4. Conclusions

We have demonstrated that reduction-responsive, shell-sheddable and biodegradable PEG-SS-PCL micelles are capable of rapidly releasing DOX inside the cells to yield significantly enhanced drug efficacy as compared to the “traditional” reduction insensitive PEG-PCL micelles. The resulting micelles are nontoxic. The findings that shedding of PEG shells could induce rapid drug release from the micelles are striking. We are convinced that reduction-responsive shell-sheddable biodegradable micelles hold great promise for efficient cytoplasmic delivery and release of potent hydrophobic anticancer drugs.

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#### Appendix

Figures with essential color discrimination. Certain figures in this article are difficult to interpret in black and white. The full color schemes can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.07.051.

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