A Convenient Scheme for Synthesizing Reduction-Sensitive Chitosan-Based Amphiphilic Copolymers for Drug Delivery

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Received 21 February 2011; accepted 23 May 2011
DOI 10.1002/app.34968
Published online 2 September 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: A novel type of reduction-sensitive graft copolymers, chitosan-*S*-S-poly(ε-caprolactone) (CS-*S*-S-PCL, here -*S*-S- means PCL was conjugated onto chitosan backbone through disulfide linkage), was synthesized through a convenient route using dithiodipropionic anhydride (DTDPA) as a disulfide donor. Reaction of hydroxy-terminated poly(ε-caprolactone) (PCL) with DTDPA quantitatively yielded DTDPA functionalized PCL (PCL-*S*-S-COOH). The disulfide-containing polyester was regioselectively conjugated onto the hydroxy groups of chitosan under mild and homogeneous conditions, utilizing dodecyl sulfate-chitosan complexes (SCC) as an intermediate. The self-assembly and Doxorubicin (Dox) release behavior of the copolymers were investigated. Spherical micelles could be formed through self-assembly of CS-*S*-S-PCL in aqueous media. The reduction-sensitive behavior of CS-*S*-S-PCL micelles was investigated by using Dithiothreitol (DTT) as a reductive reagent. In the presence of 10 m*M* DTT, the micelles gradually lost their aggregation stability and were precipitated out after four days. In addition, the Dox release was accelerated when the micelles were treated with DTT. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 3137–3144, 2012

Key words: stimuli-sensitive polymers; graft copolymers; chitosan; drug delivery systems

INTRODUCTION

Within the last two decades, polymeric micelles have emerged as a powerful delivery vehicle for tumor targeting due to their high stability,[1,2] suitable diameter for extravasation through enhanced permeation and retention effect (EPR effect),[3,4] high capacity of loading hydrophobic drugs[2,5] and ease of functionalization.[6–8] By tailoring the chemical structure of amphiphilic copolymers, polymeric micelles which can respond to various biological stimuli, such as tumoral acidic microclimate,[9–11] reductive cytoplasm,[12–17] and intracellular enzyme[18] etc., can be constructed to further improve the therapeutic effect of the incorporated payloads. Specifically, reduction-sensitive polymeric micelles were paid wide interests due to reductive characteristics of cytoplasm and oxidative nature of both extracellular matrix and blood.[19]

Up to date, the synthesis of reduction-responsive polymers was mainly based on the strategy that disulfide groups were integrated into the polymers through either crosslinking or conjugation methods.[12–14,16,17,20] Zhong et al. synthesized a novel type of poly(ethylene glycol)-b-PCL block copolymers, where poly(ethylene glycol) (PEG) and PCL blocks were linked through disulfide groups. It was found that Doxorubicin (Dox) release from the micelles could be accelerated in the presence of Dithiothreitol (DTT) which was used as a reductive reagent.[13] Thayumanavan et al. synthesized a series of amphiphilic copolymers with alkyl chains conjugated onto the polymer backbone through disulfide groups.[15,21] Fan et al.[14] proposed a different concept for constructing a reduction-responsive delivery vehicle. A novel type of graft copolymers which were composed of disulfide-containing poly(amino acid) main chain and PEG grafts was developed. It was found that the copolymer micelles could be disassembled at relatively low DTT concentration (1 m*M*). Very recently, Wang et al. constructed a reduction-sensitive polymeric micelles by crosslinking the mercapto-containing poly(phosphoester) shell layer via hydrogen peroxide treatment.[16] In general, disulfide

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Contract grant sponsor: National Natural Science Foundation of China; contract grant numbers: 20974097, 20774081.
Contract grant sponsor: National Basic Research Program of China; contract grant number: 2009CB930104.
Contract grant sponsor: Fundamental Research Funds for the Central Universities; contract grant number: 2010QNA4012.

bond exchange reaction was utilized for integrating disulfide groups into amphiphilic copolymers. However, it was found that the disulfide groups were difficult to be completely exchanged. In addition, the synthetic scheme was always very tedious.

Dithiodipropionic acid (DTDP) is a cost-effective and stable disulfide-containing reagent. It can be conveniently transformed into dithiodipropionic anhydride (DTDPA) through dehydration reaction.[22] To our best knowledge, the utilization of DTDPA for preparing reduction-responsive polymers has not been reported.

In this study, carboxy-terminated poly(ε-caprolactone) (PCL) with disulfide group as a spacer was conveniently synthesized using DTDPA as a precursor. The polymer was further conjugated onto the hydroxy groups of chitosan, using the SCC-based regioselective modification strategy of chitosan which was developed previously in our research group.[23,24] Chitosan was specifically selected as the hydrophilic component in this study due to its biodegradability, biocompatibility, and the availability of abundant reactive groups for diverse functionalization.[25] The biocompatibility of chitosan scaffold was evaluated by VandeVord et al.[26] No pathological inflammatory responses were observed during the implantation of the chitosan scaffold. Similar results were also reported for other types of chitosan-based formulations.[25] Compared with other reduction-responsive drug delivery systems, CS-*S*-S-PCL could not only be prepared under very mild reaction conditions by using the synthetic scheme developed in this manuscript and our previous works, but also have the potential to be diversely modified due to the presence of abundant reactive amino groups. For example, the copolymers could be facilely conjugated with bioactive moieties such as cell-penetrating ligands and endosome-disrupting agents to enhance the endocytosic ability of the copolymer micelles. In addition, the copolymer micelles could also be conveniently crosslinked to improve their stability when diluted below critical micellization concentration. Doxorubicin is one of the most popular chemotherapeutic agents which were commonly used in the treatment of hematological malignancies, carcinoma, and soft tissue sarcomas. It works by intercalating DNA. The ideal vehicles for delivering Dox should be able to site-specifically release the agent within the cells due to the serious heart-damage side effect of Dox. For this reason, Dox was adopted as a model chemotherapeutic agent in this study for evaluating the reduction-sensitivity of CS-*S*-S-PCL.[13,16] The chemical structure of the resultant graft copolymers was characterized by IR and ^{1}H-NMR. The self-assembly, Dox entrapment, and release behavior of the copolymers were studied.

EXPERIMENTAL

Materials

Chitosan was purchased from Aoxing Corp. (Zhejiang, China). The degree of deacetylation (DD) which was determined by ^{1}H-NMR analysis was 88%, and the viscosity-average molecular weight (MW) was 150 kDa. SDS was purchased from Shantou Xilong Chemical Company (Shantou, China). PCL was synthesized in our laboratory, using $Sn(Oct)_2$ as a catalyst and benzyl alcohol as an initiator. 3,3′-Dithiodipropionic acid (DTDP), *N*,*N*-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were obtained from Acros (Shanghai, China). All other chemicals and solvents were of analytical grade and used as received.

Synthesis of disulfide-functionalized PCL

Five grams of DTDP was refluxing in 15 mL of acetyl chloride at 65°C for 2 h. After distilling most of acetyl chloride under vacuum, the solution was precipitated into excess ethyl ether to remove the remaining acetyl chloride. The product was vacuum dried over sodium hydroxide to give DTDPA.

Eight grams of PCL (M_w = 4000 g/mol) dissolved in 20 mL of dried dimethylformamide (DMF) was added with 0.57 g (1.5 equiv.) of DTDPA and 0.25 g (1 equiv.) of *N*,*N*-dimethyl-4-aminopyridine (DMAP). After all the chemicals were completely dissolved, 0.3 mL (1 equiv.) of triethylamine was added. The reaction proceeded at 35°C for 24 h. Afterwards, the solution was precipitated into excess ethyl ether, washed with methanol and vacuum dried to give PCL-*S*-S-COOH.

Degradation of chitosan

Chitosan was degraded according to the procedure reported by Du et al.[27] with some modification. In brief, 10 g of chitosan was dispersed in 200 mL of deionized water under stirring. After the reaction temperature reached 50°C, 6.7 mL of 30% hydrogen peroxide solution was added and the reaction continued for 4 h. Afterwards, the reaction mixture was filtered to remove the water-soluble components. The residue was further treated with salicylic acid solution to quench the rest free radicals, washed with acetone, and dried under vacuum.

Synthesis of Chitosan-*S*-S-PCL (CS-*S*-S-PCL)

PCL-*S*-S-COOH (0.25×10^3 mol), NHS (0.3×10^3 mol) and DCC (0.5×10^3 mol) were codissolved in 10 mL of DMF. The mixed solution was stirred at room temperature for 24 h, and then filtered to remove the byproduct, *N*,*N*′-dicyclohexylurea

(DCU). The filtrate was precipitated into anhydrous diethyl ether, and vacuum dried to yield the active ester derivative of PCL-*S*-S-COOH (PCL-*S*-S-NHS). Dodecyl sulfate-chitosan complexes (SCC) (0.5 g), which was prepared according to the procedure previously reported,[23,24] was dissolved in 10 mL of dimethylsulfoxide (DMSO). A certain amount of PCL-*S*-S-NHS was added. The clear mixed solution was stirred at 40°C for 72 h, and then precipitated into 15% Tris-HCl solution (pH 9.0) to remove sodium dodecyl sulfate (SDS). The unreacted PCL was removed by Soxhlet extraction with acetone for 48 h.

Characterization

Infrared (IR) spectra were obtained on a Bruker Vector Spectrometer. Samples were pressed into KBr pellets. 1H NMR spectra were recorded on a Bruker DMX-500 NMR Spectrometer operating at 500 MHz. UV-Vis spectra were recorded on a Shimadzu UV-1800 Spectrometer. The size and size distribution of the micelles were analyzed by Brookhaven 90 Plus particle size analyzer. Each analysis lasted for five runs of 1 min and was performed at 25°C with an angle detection of 90°. Zeta potential was recorded with a Zetasizer Nano (Malvern Instruments, Britain). All micelle solutions to be tested had a final polymer concentration of approximate 1 mg/mL. Prior to the light scattering measurements, all the sample solutions were filtered through 0.45 μm cellulose filters. Transmission electron microscopy (TEM) images were obtained on a JEOL JEM-1230 electron microscope operating at an acceleration voltage of 80 kV. Samples were deposited from the micelle solutions (1 mg/mL) onto copper grids coated with carbon. The micelle solution was allowed to stick onto copper grid at atmospheric pressure and room temperature for 1 h, and then removed by pipette.

Micellization of CS-S-S-PCL

The micelle solutions were prepared by a dialysis procedure described by Eisenberg et al.[28] In brief, the graft copolymer (25 mg) was first dissolved in 6 mL of the admixture of trifluoroethanol (TFE) and acetic acid (HAc) (v/v, 5/1) under stirring, and then filtered to remove possible dust. The solution was dialyzed against distilled water with a cellulose membrane (MW cut off 3500) at room temperature for three days to yield a transparent solution.

Fluorescence measurements

Fluorescence spectra were recorded on a HITACHI F-4500 fluorescence spectrophotometer (Hitachi High-Technologies, Tokyo, Japan). Pyrene was used as a fluorescence probe to analyze the self-assembly of CS-*S*-S-PCL in distilled water. Samples for fluorescence measurement were prepared according to the literature[28] and the concentration of the aqueous solutions ranged from 1.0×10^6 to 1.0 mg/mL. The pyrene concentration in the solution was fixed at 6.0×10^7 *M*. For the measurement of pyrene excitation spectra, the slit widths for both excitation and emission sides were maintained at 6 nm, and an emission wavelength of 372 nm was used.

Encapsulation of Dox into the CS-*S*-S-PCL micelles

Dox was loaded into the CS-*S*-S-PCL micelles through an oil-in-water (O/W) emulsion method.[29] Totally, 10 mg of Dox in the form of HCl salt was dispersed in 2 mL of chloroform, and 8 μL of triethylamine was added dropwisely to neutralize excess HCl. The admixture was vigorously stirred overnight and emulsified into the micelle solution to form an O/W emulsion. The emulsion was stirred for 2 h, evaporated under vacuum to form a transparent solution, and finally filtered through 0.45-μm filters. The micelle solution was freeze dried, and the amount of the incorporated Dox was quantified with UV-Vis at 483 nm. The loading percent and entrapment efficiency of Dox were calculated according to the Eq. (1), where W_{loaded} and W_{added} are the weight of Dox incorporated within the micelles and that initially added respectively, and W_{micelle} is the weight of the micelles.

$$\text{Loading percent} = W_{\text{loaded}}/W_{\text{micelle}} \times 100\% \quad (1)$$

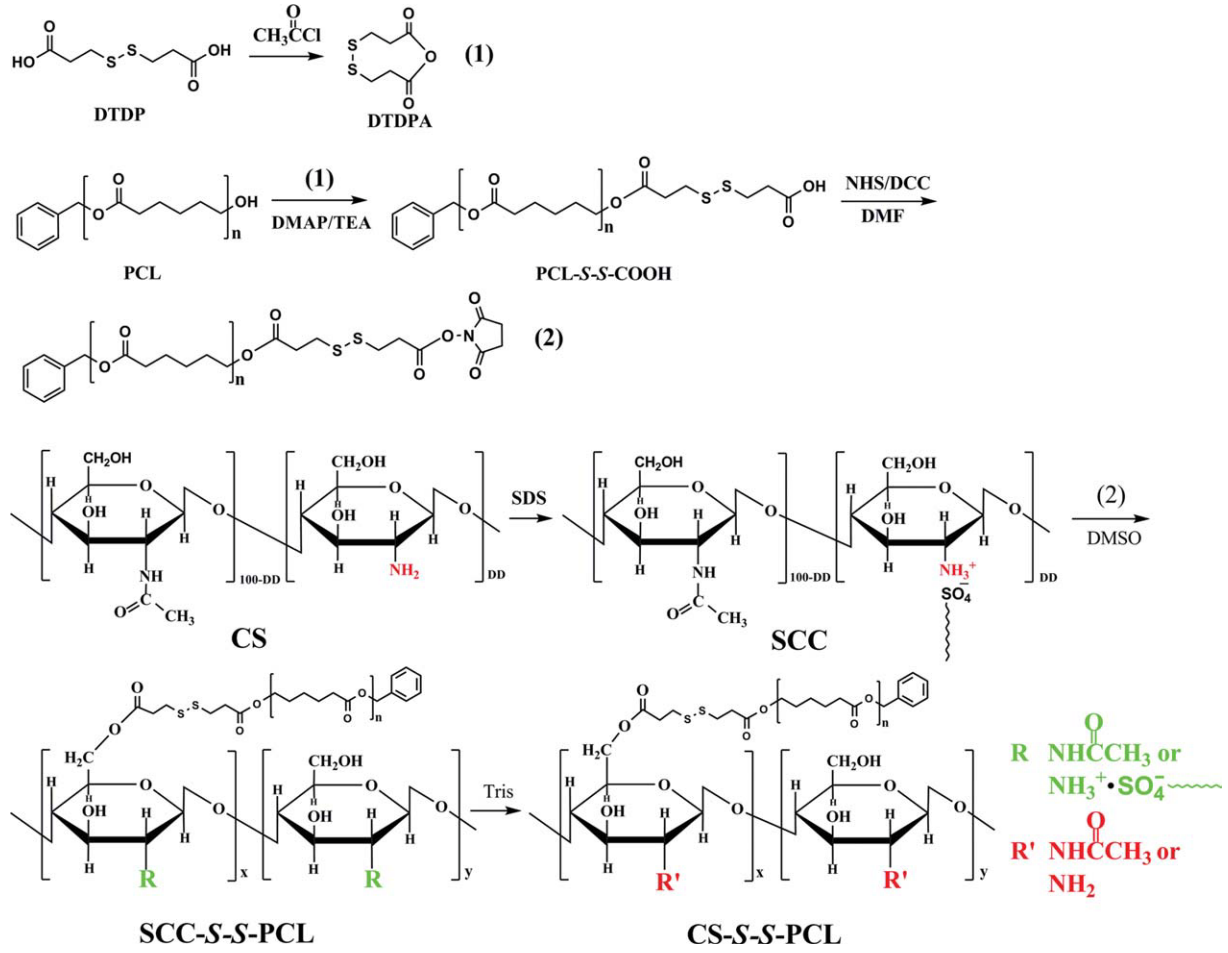
$$\text{Entrapment efficiency} = W_{\text{loaded}}/W_{\text{added}} \times 100\% \quad (2)$$

Changes in the turbidity of the micelle solution

Totally 4 mL of Dox-loaded CS-*S*-S-PCL micelle solution (1 mg/mL) was dialyzed with a cellulose tube (Cut off MW 3500) against 100 mL of pH 6.0, 50 m*M* acetate buffer. The turbidity of the micelle solution in the presence of 10 m*M* DTT was recorded with UV-Vis spectrophotometer at 700 nm. At appropriate intervals, the micelle solution was withdrawn to record the turbidity, and then recharged into the dialysis tube. The micelle solution without DTT treatment was used as a control.

Dox release from the micelles

Totally, 5 mL of Dox-loaded CS-*S*-S-PCL micelle solution (1 mg/mL) was introduced in a cellulose dialysis tube (Cut off MW 3500). The tube was then put in a vial containing 20 mL of pH 6.0, 50 m*M* acetate buffer and incubated at 37°C and 60 rpm. At appropriate intervals, 5 mL of the solution was withdrawn



Scheme 1 Synthetic scheme to CS-S-S-PCL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and replaced with 5 mL of fresh buffer. Dox content in the release medium was quantified with UV-Vis at 483 nm.

RESULTS AND DISCUSSION

Synthesis of CS-S-S-PCL

The synthetic route to CS-*S*-S-PCL was shown in Scheme 1. DTDP was selected as a disulfide donor due to its cost-effectiveness and capability to be conveniently converted to DTDPA which has high reactivity toward the reagents carrying either hydroxy or amino groups. The reaction of hydroxy-terminated PCL with DTDPA was studied by ^{1}H-NMR (Fig. 1). The quantitative conversion of hydroxy groups could be verified by the following two facts. One is the disappearance of the peak at 3.7 ppm, which is originated from the terminal methylene group of the pristine PCL. Another is the emergence of the peaks at 2.9 and 2.7 ppm, which belong to the two methylene groups of DTDPA. No obvious difference of IR spectra between the pristine PCL and PCL-*S*-S-COOH could be distinguished because the symmetric vibration of disulfide bond was not infrared active.

According our unpublished results, it was found that the molecular weight of chitosan backbone had a significant effect on the organo-solublity of CS-O-PCL. Low molecular weight of (<20 kDa) facilitates the dissolution of the copolymers in common organic solvents. As a result, chitosan was first degraded through treatment with hydrogen peroxide.

Both small hydrophobic molecules (such as cholesterol and stearic acid[30]) and hydrophilic polymers (such as PEG[31] and Polyacrylamide[32]) can be directly conjugated onto chitosan. However, the modification of chitosan with hydrophobic polymers which, compared with small molecules, can provide higher stability of the copolymer micelles, was always tedious due to the poor solubility of chitosan in common organic solvents.[33–35] Phthaloylation of chitosan was frequently used for enhancing its organo-solublity.[33] However, the very high reducibility of hydrazine which was used for deprotection of phthaloyl groups will definitely lead to the cleavage of disulfide bonds. In addition, PCL grafts will

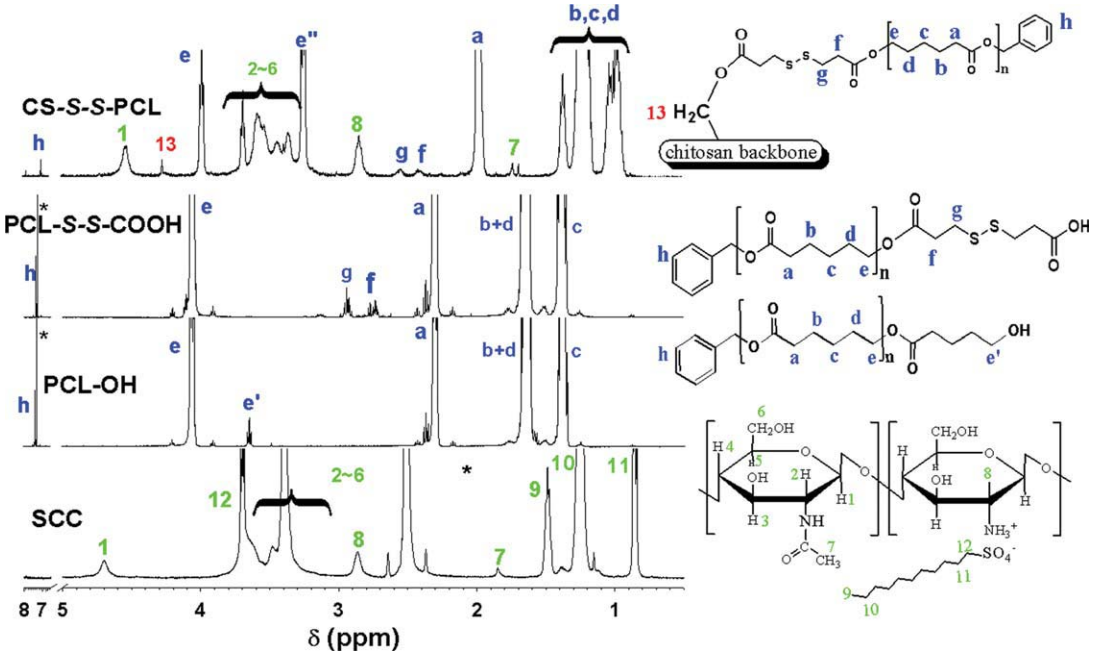


Figure 1 ^{1}H NMR spectra of SCC in d6-DMSO; PCL-OH in CDCl3; PCL-S-S-COOH in CDCl3 and CS-S-S-PCL in CF3COOD/D2O (1:2). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

also be severely degraded in the presence of the strong alkaline hydrazine. In our previous study, a facile scheme based on SCC was proposed for regioselective modification of chitosan.[23,24] In this study, SCC was used as an intermediate so that the disulfide functionality could maintain intact throughout the reaction process. The successful conjugation of PCL onto CS was demonstrated by ^{1}H-NMR and IR. The peaks of the methylene groups from DTDP moiety in ^{1}H-NMR spectrum of CS-*S*-S-PCL appeared at 2.9 and 2.7 ppm. The appearance of ester band at 1730 cm^{1} also indicated successful conjugation of PCL onto chitosan (Fig. 2). PCL grafting level was calculated according to the integral area ratio of the peak at 2.3 ppm (CH_2 of DTDP moiety) to that at 2.8 ppm (H-8 of chitosan), as shown in Table I. The amino group content was determined according to the integral area ratio of the peak at 3.2 ppm (H-8 of chitosan) to that at 1.8 ppm (H-7 on acetyl group of chitosan). It can be observed that the amino groups in the copolymers maintained intact, suggesting that PCL was conjugated onto chitosan at hydroxy sites.

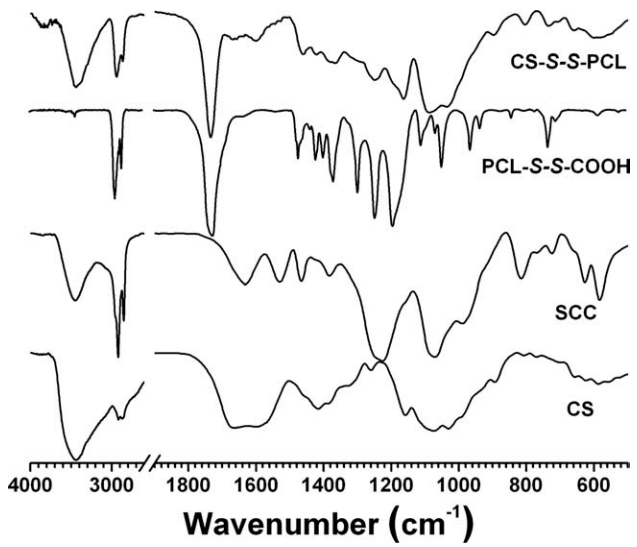


Figure 2 IR spectra of CS, SCC, PCL-S-S-COOH and CS-S-S-PCL.

TABLE I
Effect of Feed Ratio on the Characteristics of CS-*S*-*S*-PCL

Copolymers	CPS-4	CPS-8	CPS-12
Feed ratio[a]	4	8	12
PCL (%)[b]	2.89	5.22	7.38
Amino group (%)[c]	96	96	95
Diameter (nm)	23.9[d](0.36[f])	20.7(0.277)	21.2(0.301)
	39.9[e](0.287)	32.8(0.302)	32.5(0.306)
Entrapment (%)	65.3[g]/9.8[h]	76.3/11.45	88.3/13.25
Zeta potentials(mV)	+33.6[I]	+32.7	+31.7

[a] The feed weight ratio of SCC to PCL.
[b] The grafting level of PCL.
[c] The remained amino groups of chitosan after conjugation with PCL, determined by ^{1}H-NMR analysis.
[d] The average diameter of the blank CS-*S*-*S*-PCL micelles.
[e] The polydispersity of the micelles.
[f] The average diameter of Dox-loaded CS-*S*-*S*-PCL micelles.
[g] The entrapment efficiency of Dox in the CS-*S*-*S*-PCL micelles.
[h] The loading percent of Dox in the CS-*S*-*S*-PCL micelles.
[I] Measured in 50 m*M* acetate buffer (pH = 6.0).

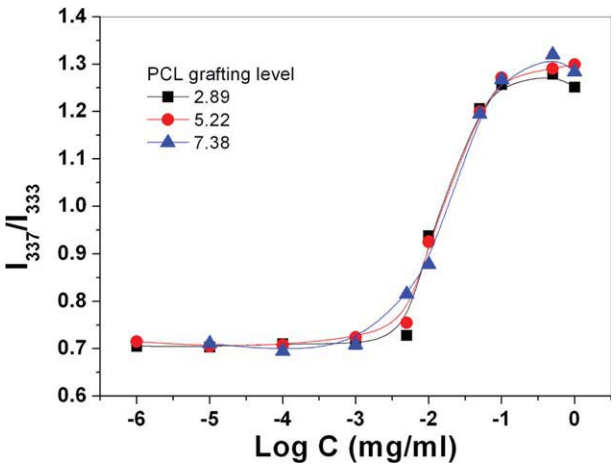


Figure 3 Plot of the I337/I333 ratio of pyrene excitation spectra in water as a function of CS-S-SPCL concentration. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Comparing with the IR and ^{1}H-NMR spectra of SDS, a decrease in the intensity of the peaks of SDS segments that 1250 cm^1 and the disappearance of methylene groups of SDS at 0.8 ppm indicated that SDS was completely removed after the process of deprotection. The copolymers can be dissolved in the mixed solvent of TFE and HAc.

Self-assembly behaviors of CS-*S*-S-PCL

The self-assembly behavior of CS-*S*-S-PCL was studied by fluorescence probe methods (Fig. 3). The critical aggregation concentration (CAC) of the copolymers ranged from 3×10^3 to 5×10^3 mg/mL. With the increase of PCL grafting level, there was no apparent change in the CAC of the copolymers. The CAC of CS-*S*-S-PCL was comparable with that of CS-O-PCL, indicating the presence of disulfide bonds has little effect on the self-assembly behavior of the copolymers.

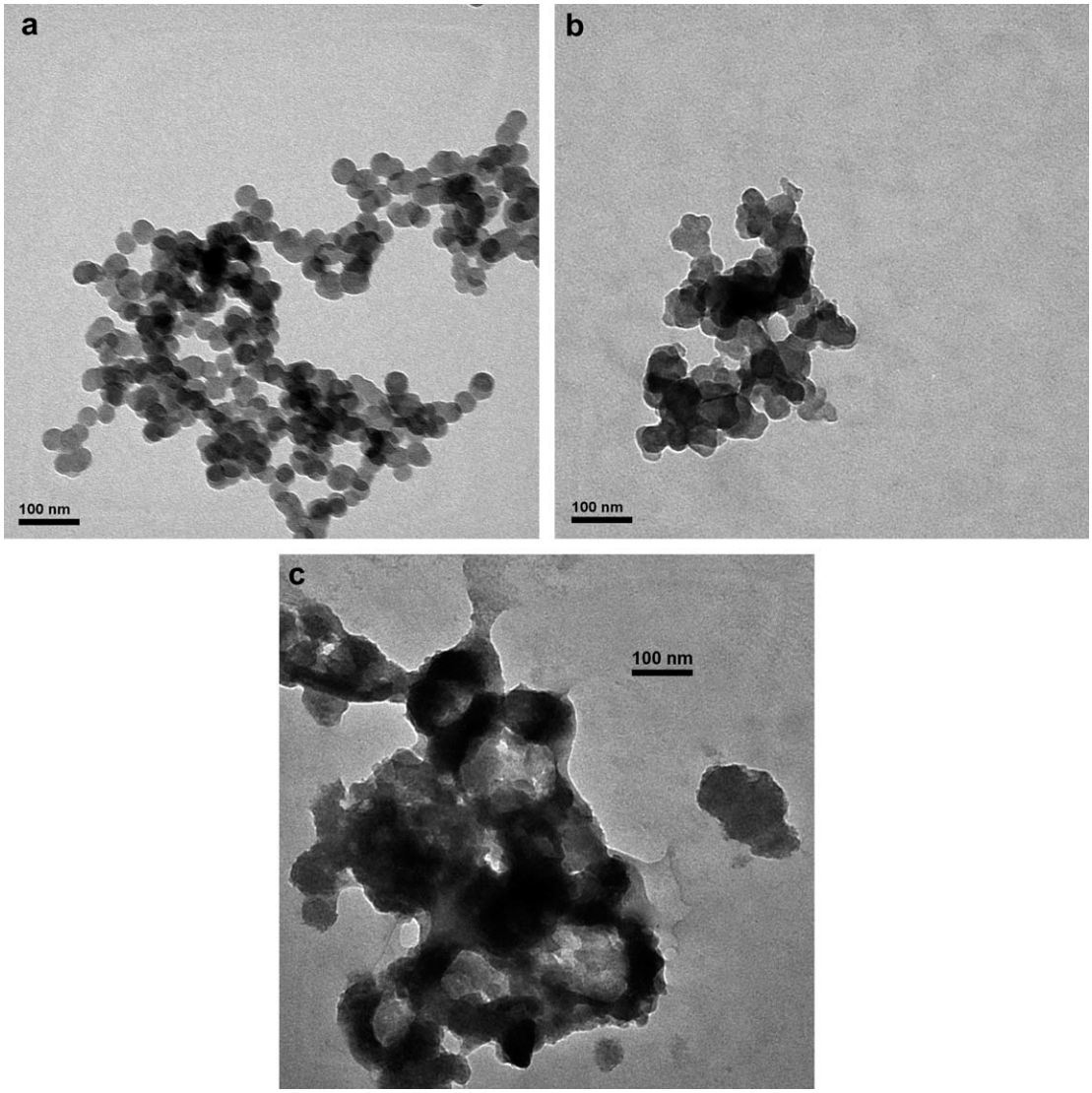


Figure 4 TEM micrographs of the CS-S-S-PCL micelles. (A) Blank CPS-8, (B) Dox loaded CPS-8, (C) CPS-8 micelles after 48 h degradation.

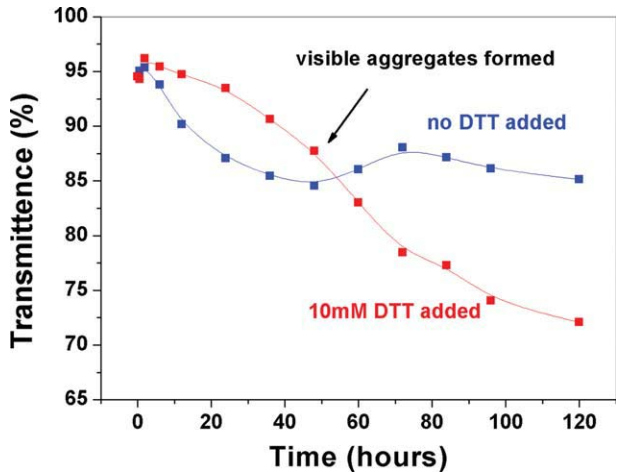


Figure 5 The turbidity curve of Dox-loaded CS-*S*-*S*-PCL micelles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The morphologies of the copolymer micelles were observed with TEM (Fig. 4). In all the cases, the micelles displayed spherical morphology. The size and size distribution of the micelles were determined by dynamic light scattering (DLS), as shown in Table I. The number mean diameter of the micelles was nearly independent of PCL grafting levels. In addition, the size distribution was relatively narrow. The zeta potential of the micelles was determined in 50 m*M* buffer solution. The value of zeta potential was over + 30 mV, irrespective of the grafting level of PCL.

Release behavior of Dox from the CS-*S*-S-PCL micelles and turbidity test

In this work, Dox was used as a model drug for *in vitro* release study. It was incorporated into CS-*S*-S-PCL micelles by O/W emulsion method[29] to improve the entrapment efficiency (Table I). It could be observed that the entrapment efficiency increased with PCL grafting levels. In addition, Dox incorporation enlarged the micelles.

The reduction-triggered disassembly of the CS-*S*-S-PCL micelles was preliminarily evaluated by monitoring the turbidity of Dox-loaded micelle solution in the presence of 10 m*M* DTT which is similar to the intracellular condition (Fig. 5). The turbidity of the solution subjected to DTT treatment gradually increased. Visible aggregates could be distinguished after 48 h and obvious precipitates were generated after 96 h, while no apparent change in turbidity could be observed when no DTT was added. Compared with disulfide-containing block copolymers such as PEG-*S*-S-PCL,[13] the disassembly rate of CS-*S*-S-PCL was much slower. This could be explained as following. Once disulfide linkage was cleaved, block copolymer will lose the hydrophilic segment (such as PEG[13,20]) immediately. After a period of degradation, the content of the hydrophilic block was too low to maintain the aggregation stability of the micelles. On the contrary, the graft copolymers still maintain associated when one of the disulfide bonds is cleaved. For example, one molecule of CPS-4 contains three PCL-*S*-S-COOH grafts. Only if all the three disulfide linkage was cleaved, the chitosan backbone will be detached to destabilize the micelles. Similar results were also reported on the reduction-triggered disassembly of the amphiphilic copolymers with small molecules as the pendent hydrophobic groups.[15]

The release profiles of Dox from the micelles were shown in Figure 6. It can be clearly observed that Dox release in presence of DTT was faster than that absent of DTT. It was also found that DTT addition has a neglected effect on the Dox release from CS-O-PCL micelles. Such results suggested the cleavage of disulfide linkages can enhance the diffusion rate of drug and accelerate the release. This result was similar to that previously reported.[13] The release profile was fitted by the eq. (3) which describes the relationship between the cumulative release of drugs from microparticles and the release time.[36] In the equation, M_t and M_{max} represent the cumulative release amount of drug at $t = 0$ and $t = \max$, respectively, R_s the mean radius of the microparticles and D the diffusion coefficient of the incorporated drugs. The apparent diffusion coefficient of Dox from the micelles was calculated to be 1.132×10^{17} cm^2/s in the absence of DTT, while increased to be 1.542×10^{17} cm^2/s when DTT was added into the release medium. Such results demonstrated the release rate of Dox could be accelerated in the reduction environment.

$$\frac{M_t}{M_{\max}} = 1 - \frac{6}{\pi^2} \cdot \sum_{n=1}^{\infty} \frac{1}{n^2} \cdot \exp\left(-\frac{D \cdot n^2 \cdot \pi^2 \cdot t}{R_8^2}\right) \quad (3)$$

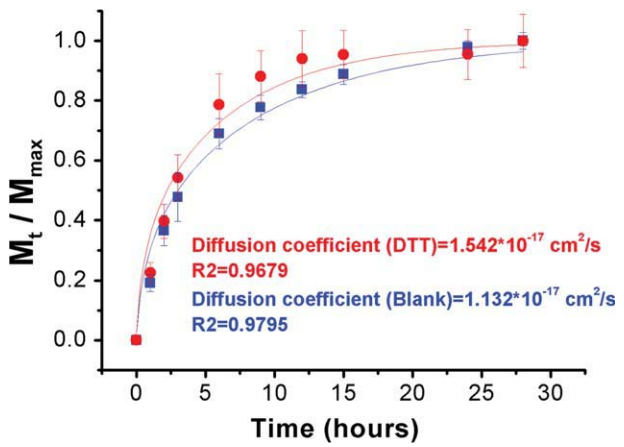


Figure 6 The release profiles of Dox from the micelles in the presence or absence of DTT in pH 6.0 acetate buffer (n = 3). The solid line represents the fitted results according to equation (3). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSIONS

CS-*S*-S-PCL with well-defined structures could be conveniently synthesized by using DTDPA as a disulfide donor and SCC as an intermediate. The copolymers could self-assemble into spherical micelles which were gradually disassembled in the presence of reductive DTT and eventually precipitated out. Dox release from the micelles could be accelerated in the presence of DTT.

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