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Intracellular release of doxorubicin from core-crosslinked polypeptide micelles triggered by both pH and reduction conditions

Liangliang Wu, Yan Zou, Chao Deng*, Ru Cheng, Fenghua Meng, Zhiyuan Zhong*

Biomedical Polymers Laboratory, and Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, People's Republic of China

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ABSTRACT

Reduction and pH dual-sensitive reversibly core-crosslinked polypeptide micelles were developed from lipoic acid (LA) and cis-1,2-cyclohexanedicarboxylic acid (CCA) decorated poly(ethylene glycol)-*b*-poly(L-lysine) (PEG-P(LL-CCA/LA)) block copolymers for active loading and triggered intracellular release of doxorubicin (DOX). PEG-P(LL_{18}-CCA_4/LA_{14}) and PEG-P(LL_{18}-CCA_8/LA_{10}) ($M_{n\ PEG}$ = 5.0 kg/mol) formed nano-sized micelles that were readily crosslinked in the presence of a catalytic amount of dithiothreitol (DTT) in phosphate buffer (pH 7.4, 10 mM). PEG-P(LL_{18}-CCA_4/LA_{14}) micelles displayed an elevated DOX loading over PEG-P(LL_{14}-LA_{14}) controls likely due to presence of ionic interactions between DOX and CCA. These core-crosslinked polypeptide micelles while exhibiting high stability against extensive dilution and high salt concentration were quickly dissociated into unimers in the presence of 10 mM DTT. The *in vitro* release studies showed that DOX release from PEG-P(LL_{18}-CCA_4/LA_{14}) micelles at pH 7.4 and 37 °C was significantly inhibited by crosslinking (*i.e.* less than 20% release in 24 h). The release of DOX was, however, doubled under endosomal pH of 5.0, possibly triggered by cleavage of the acid-labile amide bonds of CCA. In particular, rapid DOX release was observed under a reductive condition containing 10 mM glutathione (GSH), in which 86.0% and 96.7% of DOX were released in 24 h at pH 7.4 and 5.0, respectively, under otherwise the same conditions. MTT assays demonstrated that these core-crosslinked polypeptide micelles were practically non-toxic up to a tested concentration of 1.0 mg/mL, while DOX-loaded micelles caused pronounced cytotoxic effects to HeLa and HepG2 tumor cells with IC_{50} (inhibitory concentration to produce 50% cell death) of ca. 12.5 μg DOX equiv/mL following 48 h incubation. Confocal microscopy observations revealed that DOX-loaded crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles more efficiently delivered and released DOX into the nuclei of HeLa cells than PEG-P(LL_{14}-LA_{14}) counterparts. These dual-bioresponsive core-crosslinked polypeptide micelles have appeared as an advanced platform for targeted cancer therapy.

1. Introduction

Biodegradable polymeric micelles have emerged as one of the most promising platforms for targeted cancer therapy [1–5]. The preclinical and clinical studies have shown that micellar drug formulations bring about several benefits such as enhancing drug water solubility, prolonging drug circulation time, passive tumor-targeting via the enhanced permeability and retention (EPR) effect, decreasing systemic side effects and improving drug tolerance [6,7]. However, therapeutic effects of current micellar drugs are far from optimal due to presence of several practical challenges including low drug loading levels, poor *in vivo* stability leading to premature drug release and reduced tumor targetability [8–10], and slow/deficient drug release in tumor tissue and/or inside the tumor cells [11,12].

To restrain premature drug release and improve drug accumulation in the tumor tissues, core and shell-crosslinked micelles have been developed [13–18]. Especially, disulfide crosslinking that is prone to cleavage in intracellular environment due to the existence of a high reducing potential in the cytoplasm and cell nucleus [19–21], has recently been explored for reversible stabilization of polymeric micelles. For example, several groups reported that polymeric micelles crosslinked via disulfide-containing crosslinkers such as cystamine 3,3′-dithiobis(sulfosuccinimidylpropionate), N,N-bis(acryloyl)cystamine, and cystine N-carboxyanhydride (Cystine-NCA) showed enhanced stability, while rapidly released encapsulated drugs under an intracellular reductive environment [22–27]. Disulfide-crosslinked micelles have also been prepared by

* Corresponding authors. Tel./fax: +86 512 65880098.
E-mail addresses: cdeng@suda.edu.cn (C. Deng), zyzhong@suda.edu.cn (Z. Zhong).

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oxidizing free thiol groups in shell-forming poly(L-cysteine) block [28] or in core-forming polyphosphoester, polycarbonate, polyacrylate, polypeptide and dendritic block [29–33]. The *in vivo* studies showed that disulfide-crosslinked micelles had enhanced tumor specificity and therapeutic efficacy in the ovarian cancer xenograft mice as compared to both free PTX and the non-crosslinked counterparts [32]. We found that disulfide-crosslinked micelles could be readily obtained from lipoic acid (LA) conjugates [34–36]. LA is a naturally occurring compound generated by human body. The disulfide-containing lipoic ring is prone to ring-opening polymerization in the presence of a catalytic amount of DTT under aqueous conditions to form a linear polydisulfide. Our studies have shown that disulfide-crosslinked micelles based on LA conjugates had excellent biocompatibility, inhibited drug release under a physiological environment, and fast intracellular drug release, giving rise to high anti-tumor activity. This disulfide-crosslinking approach has elegantly resolved the extracellular stability and intracellular drug release dilemma.

In the past years, introduction of ionic interactions between drug and polymer has shown to improve drug loading capacity. For example, micelles based on PEO-poly(methacrylic acid) (PEO-PMA) and PEO-polycarbonate were reported to provide a high drug loading level of DOX [37–39]. It should be noted, however, that strong ionic interaction between drug and micelles might on the other hand inhibit drug release in target tumor cells, resulting in compromised therapeutic effect. Recently, Kataoka et al. developed charge-conversion micelles based on PEG-poly((N′-citraconyl-2-aminoethyl)aspartamide) (PEG-pAsp(EDA-Cit)) that are negatively charged under physiological pH allowing efficient loading of positively charged proteins while become positively charged at endosomal pH due to cleavage of the labile amide bond facilitating intracellular protein release [40]. Shen et al. demonstrated that charge-conversion polymeric micelles based on 1,2-cyclohexanedicarboxylic anhydride modified polyethylenimine or polylysine were effective for nuclear drug release [41,42]. Wang et al. reported that charge-conversion nanoparticles based on 2,3-dimethylmaleic anhydride modified poly(2-aminoethyl methacrylate hydrochloride) (PAMA) promoted tumoral-cell uptake and drug release in slightly acidic tumor extracellular environment [43].

In this paper, we report on reduction and pH dual-sensitive core-crosslinked polypeptide micelles based on lipoic acid (LA) and cis-1,2-cyclohexanedicarboxylic acid (CCA) decorated poly (ethylene glycol)-*b*-poly(L-lysine) (PEG-P(LL-CCA/LA)) block copolymers for active loading and triggered intracellular release of DOX (Scheme 1). These crosslinked micelles were designed based on the following considerations: (i) polypeptide micelles that are intrinsically non-cytotoxic and biodegradable have been used for delivery of various anticancer drugs including DOX, paclitaxel (PTX), 7-ethyl-10-hydroxy-camptothecin (SN-38) and cis-platin [6,44–47]. Besides, polypeptides compared to traditional biodegradable polymers such as aliphatic polyesters and polycarbonates are inherently functional, allow precise control over polarity, charges and architecture, show excellent stability against hydrolysis, and are prone to rapid biodegradation *in vivo* by specific enzymes [48,49]; (ii) the conjugation of LA, which is a natural antioxidant produced by human body, would transform hydrophilic poly(L-lysine) (PLL) block into hydrophobic facilitating micelle formation as well as core-crosslinking in the presence of a catalytic amount of DTT. The resulting disulfide-crosslinked micelles while possessing enhanced stability under the extracellular environment are prone to rapid de-crosslinking and drug release under an intracellular reductive condition; and (iii) the conjugation of CCA to PLL would not only facilitate active DOX loading through ionic interactions at neutral pH but also promote drug release at endosomal pH as CCA is linked to PLL via an acid-labile amide bond. In this study, preparation of reduction and pH dual-sensitive core-crosslinked polypeptide micelles, loading and *in vitro* release of DOX, as well as anti-tumor activity of DOX-loaded PEG-P(LL-CCA/LA) micelles was investigated.

2. Materials and methods

2.1. Materials

α-Methoxy-ω-amine-poly(ethylene glycol) (PEG-NH_2, M_n = 5.0 kg/mol, Suzhou PegBio Co., Ltd.), N^{ε}-benzyloxycarbonyl-L-lysine (H-Lys(Z)-OH, GL Biochem (Shanghai) Ltd.), lipoic acid (LA, 98%, Acros), 1,4-dithio-DL-threitol (DTT, 99%, Merck), 1.3-dicyclohexyl carbodiimide (DCC, 99%, Alfa Aesar), 1,2-cyclohexanedicarboxylic acid anhydride (CCAA, 97%, Alfa Aesar) was used as received. Triphosgene (BTC, Shanxi Jiaocheng Jingxin Chemical Factory) was re-crystallized with ethyl acetate prior to use. Tetrahydrofuran (THF) was dried by refluxing over sodium wire and distilled prior to use. N,N-dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) were dried by CaH_2 and distilled under reduced pressure before use. Ethyl acetate, petroleum ether (b.p. 60–90 °C), methylene dichloride (DCM), pyridine (Py) were refluxed by CaH_2 and distilled prior to use. Methanol, anhydrous diethyl ether were used as received. Lipoic acid anhydride (LAA) was synthesized according literature [35]. *N*-benzyloxycarbonyl-L-lysine *N*-carboxyanhydride (ZLL-NCA) was synthesized according to the Fuchs–Farthing method using triphosgene [50,51].

2.2. Characterization

^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using $CDCl_3$, D_2O or DMSO-d_6 as a solvent. The chemical shifts were calibrated against solvent signals. The molecular weight and polydispersity of copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with MZ-gel SDplus columns (500 Å, 10E3 Å, 10E4 Å) following a differential refractive-index detector (RI 2414). The measurements were performed using DMF with 0.05 mol/L LiBr as the eluant at a flow rate of 0.8 mL/min at 25 °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). Measurements were carried out at 25 °C by a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He–Ne laser using back-scattering detection. The zeta potential of the micelles was determined with a Zetasizer Nano-ZS from Malvern Instruments. 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 0.1 mg/mL micelles suspension on the copper grid followed by staining with 1 wt.% phosphotungstic acid.

2.3. Synthesis of PEG-*b*-PZLL diblock copolymers

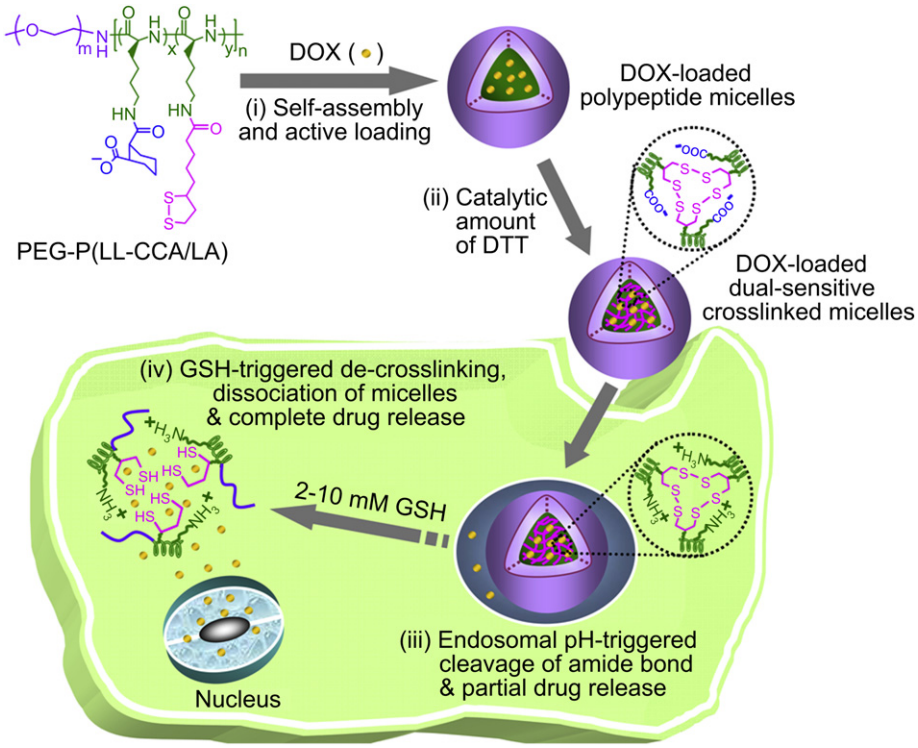
PEG-*b*-PZLL diblock copolymers were synthesized by ring-opening polymerization of ZLL-NCA using PEG-NH_2 as a macroinitiator. The following is a typical example on synthesis of PEG-*b*-$PZLL_{14}$ copolymer. Under a nitrogen atmosphere, to a solution of ZLL-NCA (920 mg, 3.0 mmol) in DMF (9.2 mL) under stirring was quickly added the stock solution of PEG-NH_2 (1.0 g, 0.20 mmol, monomer to initiator ratio of 15) in DMF (9.2 mL). The reaction mixture was stirred for 72 h at 40 °C and then precipitated with excess diethyl ether to obtain white solid. The resulting copolymer was purified by dissolving in chloroform and then precipitating with diethyl ether for three times. The PEG-*b*-$PZLL_{14}$ product was dried at room temperature in vacuo for 24 h. Yield: 90%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.66(s, –CO–N**H**–), 7.35(s, –C_6**H_5**), 5.10(s, –**CH_2**C_6H_5), 4.45(–COC**H**NH–), 3.70(m, –OC**H_2**C**H_2**O–), 3.31(s, –OC**H_3**), 3.31(s, –CONHC**H_2**–), 1.18–1.75(m, –CHC**H_2**C**H_2**C**H_2**C**H_2**NH–). PEG-*b*-$PZLL_{18}$ copolymer was synthesized at a monomer to initiator ratio of 20.

2.4. Synthesis of PEG-*b*-PLL diblock copolymers

PEG-*b*-PLL copolymer was obtained through the deprotection of Z group in PEG-*b*-PZLL using HBr. In a typical example, HBr (33 wt.% in HOAc, 1.31 mL, 7.2 mmol) was added to the solution of PEG-*b*-$PZLL_{14}$ (970 mg, 0.1 mmol) in 9.7 mL CF_3COOH. The reaction mixture was vigorously stirred for 1 h at 0 °C, and then precipitated in excess diethyl ether. The product was further purified by dissolving in DMF and precipitating in diethyl ether twice, and then was dried at room temperature in vacuo for 24 h. Yield: 95%. ^{1}H NMR (400 MHz, D_2O): δ 4.45(s, –COC**H**NH–), 3.75(m, –OC**H_2**C**H_2**O–), 3.41(s, –OC**H_3**), 3.10(s, –C**H_2**NH_3Br), 1.38–1.90(m, –CHC**H_2**C**H_2**C**H_2**CH_2–). ^{1}H NMR analysis revealed that PEG-*b*-PLL_{14} was prepared. PEG-*b*-PLL_{18} copolymer was obtained in a similar way.

2.5. Synthesis of PEG-P(LL-CCA/LA)

PEG-P(LL-CCA/LA) was obtained by conjugating CCA and LA to PEG-*b*-PLL through sequential amidation with 1,2-cyclohexanedicarboxylic acid anhydride (CCAA) and lipoic acid anhydride (LAA) in pyridine. The following is a typical example on the synthesis of PEG-P(LL_{18}-CCA_4/LA_{14}). Briefly, CCAA (6.5 mg,



Scheme 1. Illustration of reduction and pH dual-sensitive core-crosslinked PEG-P(LL-CCA/LA) micelles for active loading and triggered intracellular release of DOX. (i) Self-assembly of PEG-P(LL-CCA/LA) copolymer and active loading of DOX; (ii) core-crosslinking of micelles under catalytic amount of DTT to yield reduction and pH dual-sensitive crosslinked micelles, (iii) endosomal pH-triggered cleavage of amide bond of CCA and partial drug release; and (iv) GSH-triggered de-crosslinking, micelle dissociation and complete drug release.

0.04 mmol) was added under stirring to a solution of PEG-PLL_{18} (90 mg, 0.01 mmol) in pyridine (30 mL) at room temperature. The reaction was allowed to proceed at 30 °C for 24 h. LAA (360 mg, 0.90 mmol, 5 equiv. to total primary amine) was then added. The reaction was continued at 40 °C for another 48 h. The resulting conjugate was isolated by precipitation in excess diethyl ether. The polymer was further purified by dissolving in DMF and then precipitating in diethyl ether for three times. Yield: 85%. ^{1}H NMR (400 MHz, DMSO-d_6): PEG block: δ 3.70; PLL block: δ 0.80–1.95, 3.10, 4.20; CCA moieties: δ 1.40–1.98, 2.65; LA moieties: 1.40–1.69, 2.15, 1.89/2.43, 3.02, and 3.74. PEG-P(LL_{18}-CCA_8/LA_{10}) copolymer was prepared using a CCAA/PEG-PLL_{18} molar feed ratio of 8/1. PEG-P(LL_{14}-LA_{14}) copolymer was synthesized by directly adding LAA (280 mg, 0.70 mmol, 5 equiv. to total primary amine) into a solution of PEG-PLL_{14} (79 mg, 0.01 mmol) in pyridine (30 mL) and then stirring at 40 °C for 48 h.

2.6. Micelle formation and critical micelle concentration (CMC)

Micelles were prepared by dropwise addition of distilled water to a DMF solution of PEG-P(LL-CCA/LA) under stirring at room temperature, followed by extensive dialysis against deionized water for 48 h using a membrane (MWCO 3500 Da). The water was refreshed every 8 h.

The CMC was determined using pyrene as a fluorescence probe. The concentration of polymer was varied from 6.0×10^{-4} to 0.15 mg/mL and the concentration of pyrene was fixed at 0.6 μM. The fluorescence spectra were recorded using a FLS920 fluorescence spectrometer with the excitation wavelength of 330 nm. The emission fluorescence at 372 and 383 nm was monitored. The CAC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

2.7. Crosslinking and de-crosslinking of micelles

The crosslinked micelles were prepared using a catalytic amount of DTT as reported previously [35]. Briefly, the above-prepared micelles solution was bubbled with N_2 for 20 min, and then 10 mol% DTT relative to the amount of lipoyl units was added to the solution under a N_2 atmosphere. After stirring for 24 h at room temperature, the micelles solution was dialyzed against distilled water for one day. The colloidal stability of the crosslinked micelles and the non-crosslinked micelles against large volume dilution and high salt concentration was investigated using DLS.

The de-crosslinking of crosslinked micelles in responsive to a reductive environment was monitored by DLS measurement. Briefly, crosslinked micelles solution in a vial was bubbled with N_2 for 20 min, and then predetermined amount of DTT was added to yield the final DTT concentration of 10 mM. The vial was sealed and placed in a shaking bed at 200 rpm and 37 °C. The size of the micelles was determined by DLS at different time intervals.

2.8. pH-Induced degradation of amide bond between CCA and PLL

The degradation of amide bond between CCA and PLL was studied by ^{1}H NMR spectroscopy using PEG-P(LL-CCA) as a model. Briefly, PEG-P(LL-CCA) was dissolved in D_2O, and the solution pH was adjusted to 5.0 using DCl. The solution was placed in a shaking bed at 200 rpm and 37 °C, and was monitored by ^{1}H NMR spectroscopy at different time intervals.

2.9. Encapsulation of DOX

DOX was loaded into micelles by dropwise addition of distilled water to a DMF solution of DOX and PEG-P(LL-CCA/LA) (Theoretical drug loading contents = 9.1–23.1 wt.%) under stirring at room temperature, followed by dialysis against distilled water for 12 h. The water was refreshed 5 times. The whole procedure was performed in the dark. The DOX-loaded micelles were crosslinked as described above using a catalytic amount of DTT (10 mol% relative to the lipoyl units).

The amount of DOX was determined using fluorescence (FLS920) measurement (excitation at 480 nm). For determination of drug loading content, the DOX-loaded non-crosslinked micelles were lyophilized and 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:

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

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

2.10. In vitro release of DOX

The release profiles of DOX from crosslinked micelles as well as non-crosslinked micelles were studied using a dialysis tube (MWCO 12,000) under shaking (200 rpm) at 37 °C in four different media, *i.e.* NaAc/HOAc (10 mM, pH 5.0) with 10 mM GSH, PB (10 mM, pH 7.4) with 10 mM GSH, NaAc/HOAc (10 mM, pH 5.0) and PB

(10 mM, pH 7.4) only. The release studies were performed at a low micelle concentration of 25 mg/L. Typically, 0.5 mL DOX-loaded micelles solution, either crosslinked or non-crosslinked, was dialyzed against 25 mL of release media. 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 and the results presented were the average data with standard deviations.

2.11. MTT assays

The anti-tumor activity of DOX-loaded crosslinked and non-crosslinked micelles was evaluated in HeLa and HepG2 cells by MTT assays. The cells were plated in a 96-well plate (5×10^3 cells/well) using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL) for 1 day. The medium was aspirated and replaced by 80 μL of fresh medium supplemented with 10% FBS. 10 μL of DOX-loaded crosslinked or non-crosslinked micelles in PB (10 mM, pH 7.4) was added to yield final micelle concentrations of 0.1, 0.25, 0.5, 0.75, and 1.0 mg/mL. The cells were cultured at 37 °C in an atmosphere containing 5% CO_2 for 2 day. The medium was aspirated and replaced by 100 μL of fresh medium. 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) solution (5 mg/mL) was added. The cells were incubated for another 4 h. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 200 μL of DMSO, and the absorbance at a wavelength of 490 nm of each well was measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 490 nm with control wells containing only cell culture medium. Data are presented as average ± SD ($n = 4$). The cytotoxicity of blank crosslinked and non-crosslinked micelles was determined in a similar way.

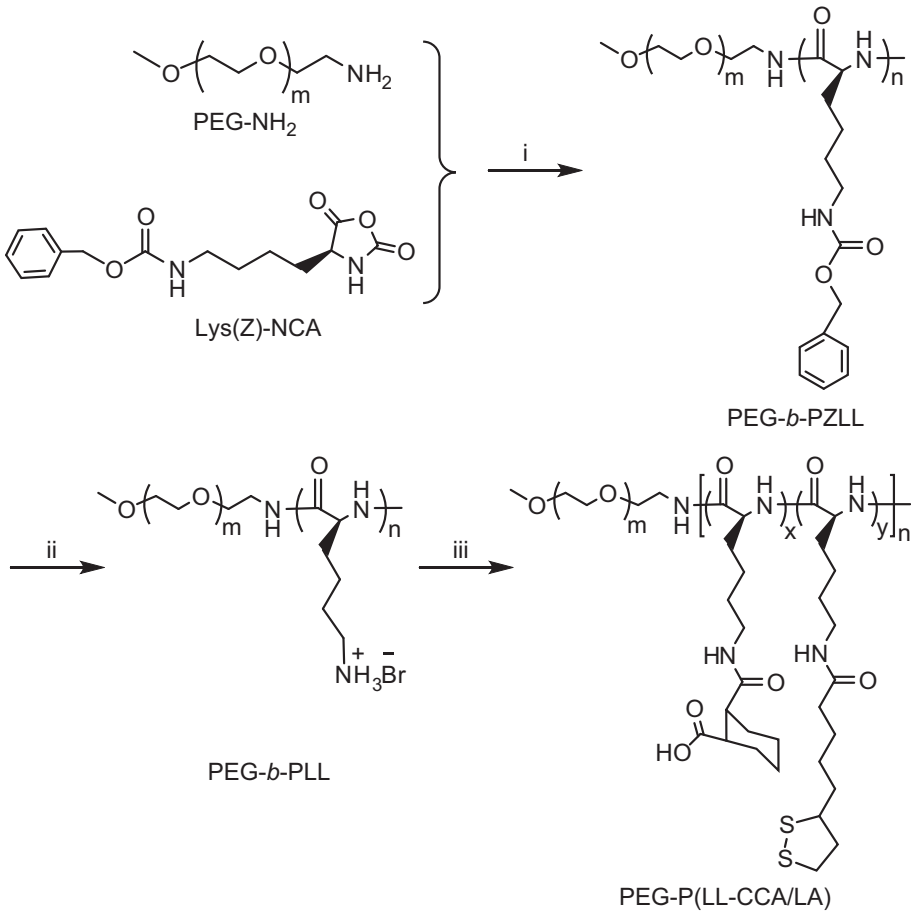
2.12. Intracellular release of DOX

The cellular uptake and intracellular release behaviors of DOX-loaded crosslinked micelles were followed with confocal laser scanning microscopy (CLSM) using HeLa cells. The cells were cultured on microscope slides in a 6-well plate (5×10^5 cells/well) using RPMI-1640 medium supplemented with 10% FBS, 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL). The cells were incubated with DOX-loaded micelles for 4 or 12 h at 37 °C in a humidified 5% CO_2-containing atmosphere. The culture medium was removed and the cells were rinsed three times with PBS. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The fluorescence images were obtained using confocal microscope (TCS SP2).

3. Results and discussion

3.1. Synthesis of PEG-b-PLL(CCA/LA) copolymers

PEG-P(LL-CCA/LA) conjugates were synthesized through three steps (Scheme 2). Firstly, PEG-*b*-PZLL copolymers were synthesized in high yields through ring-opening polymerization of ZLL-NCA in DMF using PEG-NH_2 as a macroinitiator. ^{1}H NMR showed characteristic signals of PEG (δ 3.31 and 3.70) and PZLL (δ 1.18–1.75, 3.10, 4.45, 5.10, 7.35, and 7.66) (Fig. 1A). The degree of polymerization (DP) of PZLL was calculated, by comparing signal intensities of PEG methylene protons (δ 3.70) and benzyl methylene protons of PZLL (δ 5.10), to be 14 and 18. The molecular weights determined by ^{1}H NMR were close to the design (Table 1). GPC measurements showed that PEG-*b*-PZLL copolymers had a low polydispersities of 1.05–1.11 (Table 1). The molecular weights determined from GPC, though increased in parallel with the design, deviated from those determined by ^{1}H NMR, likely because polystyrene was used as a reference. Secondly, acid deprotection of PEG-*b*-PZLL in CF_3COOH with HBr/HOAC at 0 °C for 1 h yielded water soluble PEG-*b*-PLL copolymers. The disappearance of signals at δ 7.35 and 5.10 attributable to the Z group confirmed complete deprotection (Fig. 1B). Notably, ^{1}H NMR showed that DP of PLL block was not



Scheme 2. Synthetic route for PEG-P(LL-CCA/LA) conjugates. Conditions: (i) DMF, 40 °C, 72 h; (ii) HBr/HOAc, CF_3COOH, 0 °C, 1 h; (iii) CCAA, Py, 30 °C, 24 h; LAA, Py, 40 °C, 48 h.

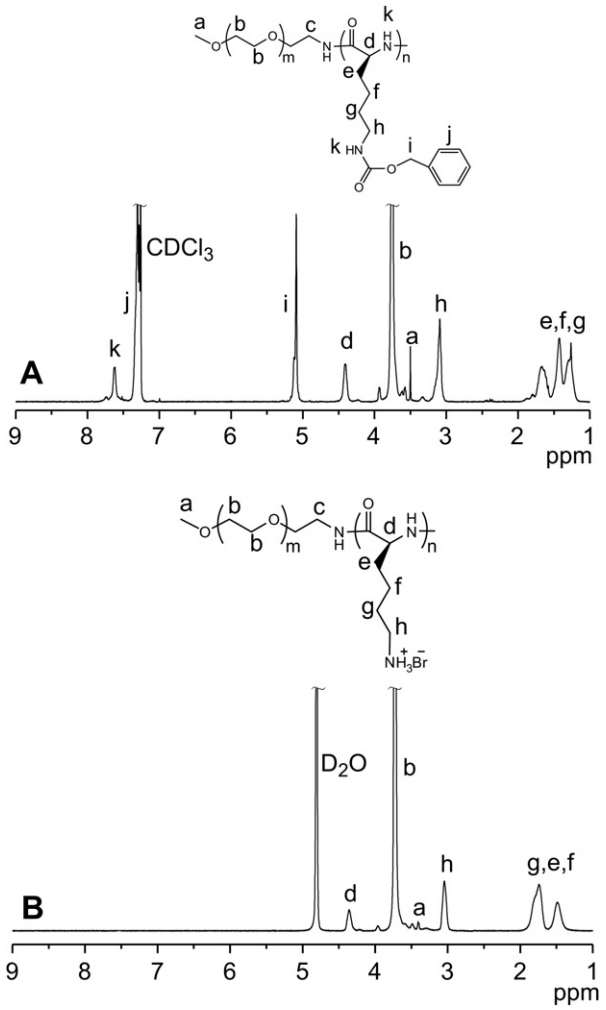


Fig. 1. ^{1}H NMR spectra of (A) PEG-*b*-PZLL$_{18}$ (400 MHz, $CDCl_3$) and (B) PEG-*b*-PLL$_{18}$ (400 MHz, D_2O).

changed, indicating that polypeptide main chain was intact during deprotection.

Finally, PEG-*b*-P(LL-CCA/LA) copolymers were obtained by sequential amidation of PLL with 1,2-cyclohexanedicarboxylic acid anhydride (CCAA) and lipoic acid anhydride (LAA) in pyridine. ^{1}H NMR displayed besides resonances owing to PEG and PLL also signals attributable to CCA (δ 2.65) and LA (δ 2.15) moieties (Fig. 2A and B). The average number of CCA substituents per polymer chain could be determined by comparing the peak intensities of CCA methine protons (δ 2.65) and PEG methylene protons (δ 3.70) (Fig. 2A). The average number of LA substituents per polymer chain could be calculated by comparing the intensities of signals at δ 2.15 (α-methylene protons neighboring to carbonyl group of LA) and δ 0.80–1.95 (methylene protons of PLL, CCA and LA) (Fig. 2B). The results showed that numbers of CCA and LA substitutions in PEG-P(LL-CCA/LA) copolymer were 4 and 14, respectively (denoted as PEG-P(LL$_{18}$-CCA$_4$/LA$_{14}$)). Using a CCA/PEG-*b*-PLL molar feed ratio of 8/1, PEG-P(LL$_{18}$-CCA$_8$/LA$_{10}$) was prepared. In order to investigate the influences of CCA units on drug loading and release behaviors, PEG-P(LL$_{14}$-LA$_{14}$) was synthesized as a control.

Table 1
Synthesis of PEG-*b*-PZLL copolymers.

Entry	Copolymers	M_n (kg/mol)			M_w/M_n[b]	Yield (%)
		Design	^{1}H NMR[a]	GPC[b]		
1	PEG-*b*-PZLL$_{14}$	5–3.9	5–3.7	15.9	1.05	96
2	PEG-*b*-PZLL$_{18}$	5–5.2	5–4.7	19.6	1.11	95

[a] Calculated from ^{1}H NMR by comparing intensities of signals at δ 5.0 and 3.1 which were assignable to benzyl methylene protons of PZLL and methylene protons of PEG, respectively.

[b] Determined by GPC measurements using DMF as an eluant at a flow rate of 0.8 mL/min (standards: polystyrene, 25 °C).

3.2. Preparation and reduction-triggered destabilization of crosslinked micelles

PEG-P(LL-CCA/LA) micelles were readily prepared by solvent exchange method. Dynamic light scattering (DLS) measurements showed that all three copolymers formed small-sized micelles with

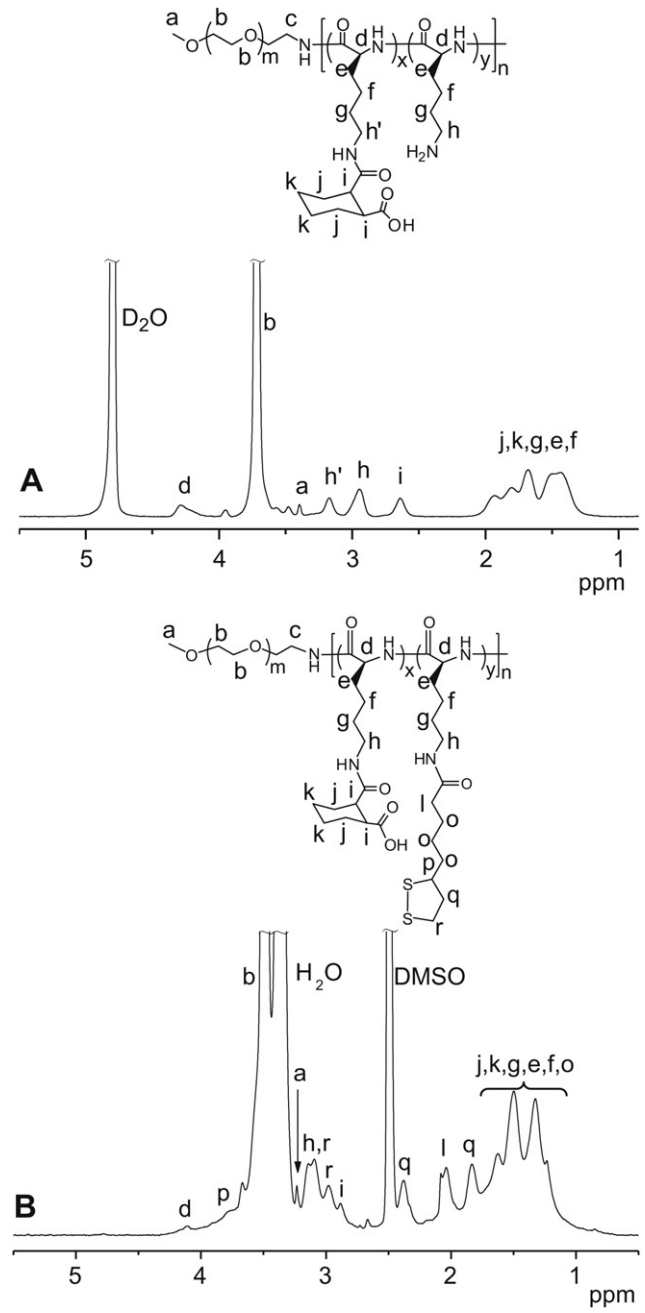


Fig. 2. ^{1}H NMR spectra of (A) PEG-P(LL$_{18}$-CCA$_4$) (400 MHz, D_2O) and (B) PEG-P(LL$_{18}$-CCA$_4$/LA$_{14}$) (400 MHz, DMSO-d_6).

Table 2
Characteristics of non-crosslinked and crosslinked PEG-P(LL-CCA/LA) micelles.

Entry	Polymers	Non-crosslinked micelles			Crosslinked micelles		
		Size[a] (nm)	PDI[a]	CAC[b] (mg/L)	Size[a] (nm)	PDI[a]	Zeta[a] (mV)
1	PEG-P(LL_{14}-LA_{14})	67.4	0.13	4.3	66.5	0.15	−13.6
2	PEG-P(LL_{18}-CCA_4/LA_{14})	45.6	0.11	6.7	41.8	0.12	−16.0
3	PEG-P(LL_{18}-CCA_8/LA_{10})	50.1	0.12	7.8	49.9	0.12	−19.4

[a] Determined by DLS using Zetasizer Nano-ZS (Malvern Instruments) at 25 °C in water.
[b] Determined using pyrene as a fluorescence probe.

average hydrodynamic diameters of 45.6–67.4 nm and low polydispersities (PDI) of 0.11–0.13 (Table 2). The critical micelle concentration (CMC) determined using pyrene as a fluorescence probe showed that PEG-P(LL_{14}-LA_{14}), PEG-P(LL_{18}-CCA_4/LA_{14}) and PEG-P(LL_{18}-CCA_8/LA_{10}) conjugates had a low CMC of 4.3, 6.7 and 7.8 mg/L, respectively, which increased with increasing CCA contents (Table 2).

The crosslinking of PEG-P(LL-CCA/LA) micelles was carried out by introducing 10 mol% dithiothreitol (DTT) relative to the lipoyl units in the micelles, similar to our previous reports [34–36]. DLS measurements revealed that PEG-P(LL_{18}-CCA_4/LA_{14}) micelles following crosslinking had slightly reduced sizes while maintaining a low PDI (Fig. 3A). The circular dichroism analyses showed that core-forming polypeptide segments in both crosslinked and non-crosslinked micelles adopted an α-helical conformation (Fig. 3B). The zeta potential measurements showed that PEG-P(LL-CCA/LA) micelles had modest negative surface charges ranging from −13.6 mV to −19.4 mV (Table 2). TEM micrograph revealed that crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles had a spherical morphology and a size distribution close to that determined by DLS (Fig. 3C).

The colloidal stability of crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles against extensive dilution and high salt condition was investigated using DLS measurements. Notably, crosslinked micelles following 1000-fold dilution (C < CMC) showed only slight increase in hydrodynamic size and maintained a low PDI, while the parent non-crosslinked micelles were completely dissociated into unimers under otherwise the same conditions (Fig. 4A). The addition of 2 M NaCl while had little influence on crosslinked micelles resulted in significant aggregation of non-crosslinked micelles (Fig. 4B). These combined results indicated that crosslinked PEG-P(LL-CCA/LA) micelles have excellent colloidal stability.

The reduction-sensitivity of crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles was examined by monitoring change of micelle sizes in time in response to 10 mM DTT in PB buffer (pH 7.4, 10 mM) at 37 °C. The results showed that DTT caused rapid dissociation of crosslinked micelles to less than 10 nm in 8.5 h (Fig. 5A), indicating complete de-crosslinking of micelles. The fast dissociation of

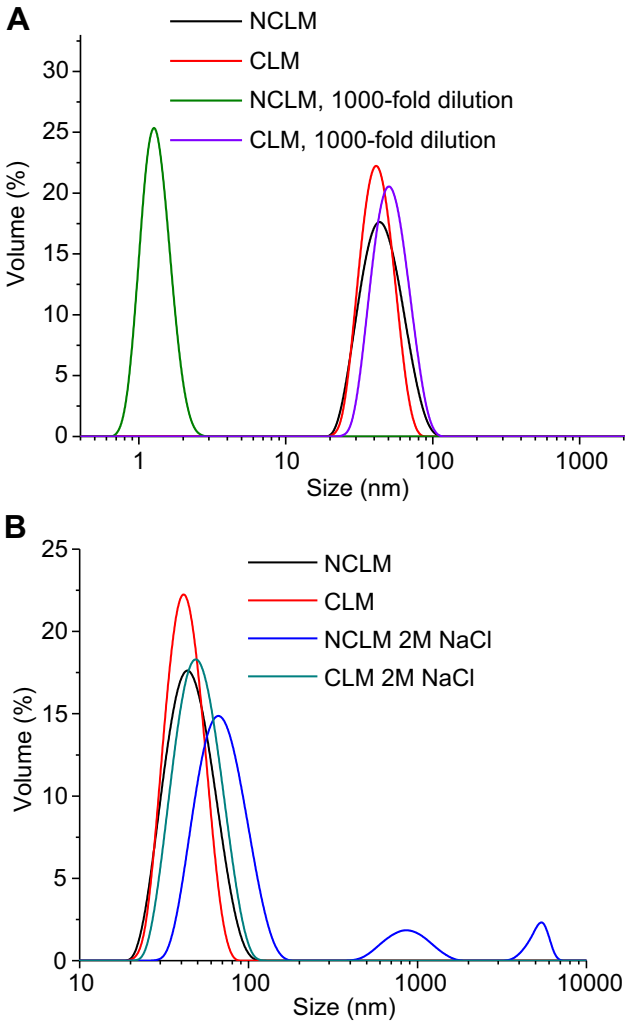


Fig. 4. Colloidal stability of crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles against 1000-fold dilution (A) and 2 M NaCl (B). The corresponding non-crosslinked micelles were used as a control.

crosslinked micelles in response to 10 mM DTT was also confirmed by diminishing light scattering intensity following 8.5 h incubation (Fig. 5B). The de-crosslinking of micelles under reductive conditions leads to cleavage of disulfide crosslinks into two hydrophilic thiol groups (*i.e.* dihydrolipoic moiety) (Scheme 1). In contrast, little size change was observed in 24 h for crosslinked micelles in the absence of DTT under otherwise the same conditions (Fig. 5A). Therefore, core-crosslinked PEG-P(LL-CCA/LA) micelles while

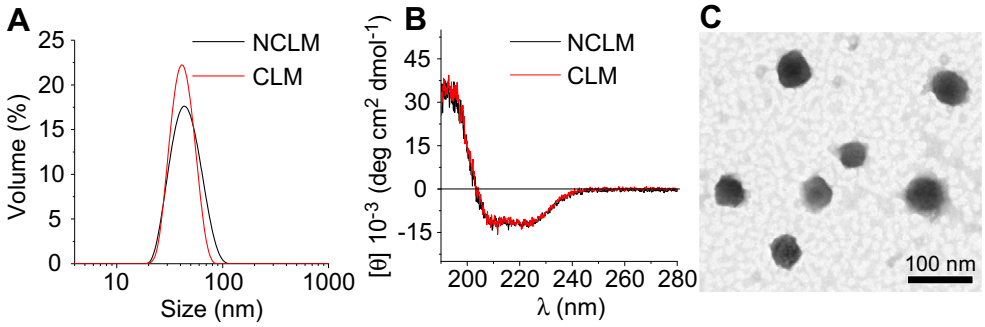


Fig. 3. Characterization of crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles. (A) DLS measurements; (B) CD analyses; and (C) TEM.

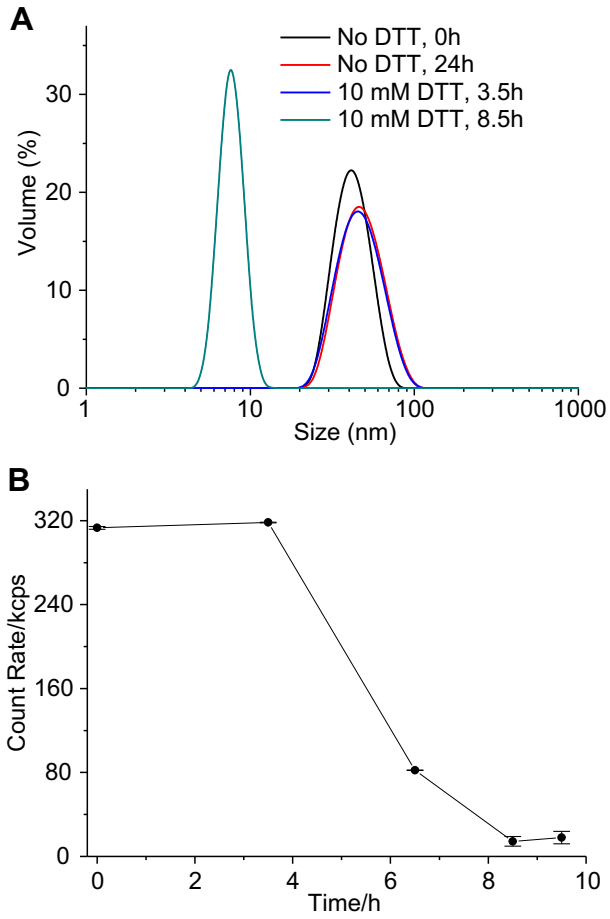


Fig. 5. Change of size distributions (A) and count rates (B) in time of crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles in response to 10 mM DTT.

possess superior colloidal stability under extracellular conditions are prone to rapid dissociation under a reductive condition mimicking that of the cytoplasm and cell nucleus.

3.3. pH-Induced hydrolysis of PEG-P(LL_{18}-CCA_{18})

It has been demonstrated that amide bond formed between an amino group and CCA exhibits pH-dependent hydrolysis [42]. Here, we have synthesized PEG-P(LL_{18}-CCA_{18}) as a model polymer to investigate acid-sensitive hydrolysis of CCA by ^{1}H NMR (Fig. 6A). The results showed that upon hydrolysis, intensity of methylene protons adjacent to the amide (H_a) decreased while that of methylene protons next to the amine (H_b) increased. The percentage of amide hydrolysis calculated by comparing intensities of peaks H_a and H_b was ca. 40% and 60% at pH 5.0 in 24 h and 48 h, respectively (Fig. 6B). These results confirmed that the amide bond between CCA and PLL is prone to acid hydrolysis.

3.4. Loading and in vitro release of DOX

DOX-loaded micelles were prepared at a polymer concentration of 5 mg/mL and theoretical DOX loading contents of 9.1–23.1 wt.%. The results showed that PEG-P(LL_{18}-CCA_4/LA_{14}) micelles had in general higher drug loading efficiency (DLE) than PEG-P(LL_{14}-LA_{14}) controls (Table 3), likely due to presence of ionic interactions between the carboxylic groups of the CCA and the amino groups of DOX [37,52]. The crosslinked PEG-P(LL_{18}-CCA_8/LA_{10}) micelles, however, demonstrated decreased DLE in comparison to PEG-P(LL_{18}-CCA_4/LA_{14}) micelles (Table 3). This is because incorporation of CCA while increasing the ionic interactions with DOX also decreases hydrophobicity of micellar cores and their hydrophobic interactions with drugs that would lower drug loading. Therefore, the balance between ionic and hydrophobic interactions is crucial to DOX loading into these dual-sensitive micelles. Notably, PEG-P(LL_{14}-LA_{14}) and PEG-P(LL_{18}-CCA_4/LA_{14}) micelles displayed no

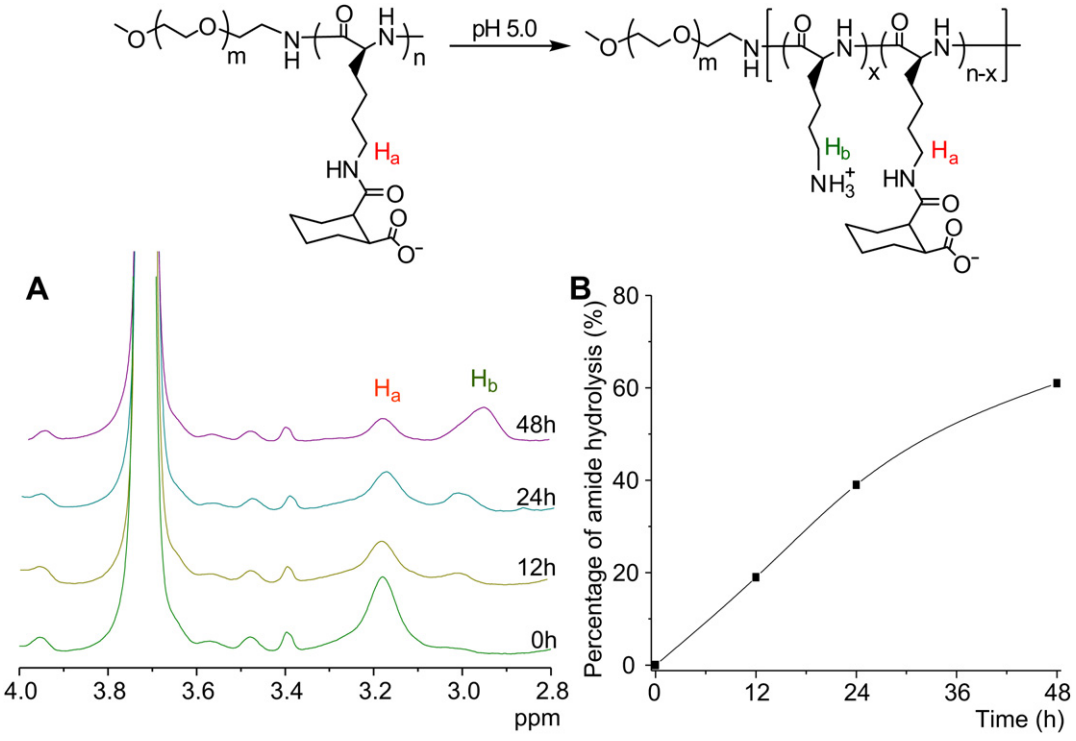


Fig. 6. Hydrolysis of PEG-P(LL_{18}-CCA_{18}) at pH 5.0 in DCl/D_2O at 37 °C.

Table 3
Characteristics of DOX-loaded crosslinked PEG-P(LL-CCA/LA) micelles.

Copolymers	DLC (wt.%) theory	Non-crosslinked micelles		Crosslinked micelles		DLC (wt.%)	DLE (%)
		Size (nm)	PDI	Size (nm)	PDI		
PEG-P(LL_{14}-LA_{14})	9.1	56.5	0.14	55.7	0.20	6.6	71
	16.7	77.6	0.20	66.9	0.17	11.8	67
	23.1	87.5	0.21	78.2	0.18	15.0	59
PEG-P(LL_{18}-CCA_4/LA_{14})	9.1	44.9	0.20	44.7	0.20	7.7	83
	16.7	125.3	0.20	94.2	0.15	13.3	77
	23.1	199.5	0.18	144.1	0.16	15.9	63
PEG-P(LL_{18}-CCA_8/LA_{10})	9.1	88.3	0.22	78.7	0.24	6.4	68

obvious size change at low DOX loading of 6.6 and 7.7 wt.% as well as subsequent crosslinking with catalytic amount of DTT (Table 3). The sizes of PEG-P(LL_{14}-LA_{14}) and PEG-P(LL_{18}-CCA_4/LA_{14}) micelles increased, however, with increasing DOX loading contents at higher DOX loading contents of 11.8 and 13.3 wt.%, respectively. Moreover, DOX-loaded micelles shrank by 9–55 nm following crosslinking (Table 3).

The *in vitro* drug release studies were carried out at a low micelle concentration of 20 μg/mL at 37 °C using a dialysis tube (MWCO 12,000) under four different conditions: (i) pH 7.4, (ii) pH 5.0, (iii) pH 7.4 and 10 mM glutathione (GSH), and (iv) pH 5.0 and 10 mM GSH. As shown in Fig. 7, release of DOX from crosslinked PEG-P(LL_{14}-LA_{14}) and PEG-P(LL_{18}-CCA_4/LA_{14}) micelles was largely inhibited, in which less than 20% drug was released in 24 h at pH 7.4. In contrast, 57.2% and 68.3% DOX were released from corresponding non-crosslinked counterparts under otherwise the same conditions. It is interesting to note that release of DOX from crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles was obviously faster at pH 5.0 than at pH 7.4, possibly triggered by cleavage of the acid-labile amide bonds between PLL and CCA, whereas pH had little influence on DOX release from crosslinked PEG-P(LL_{14}-LA_{14}) micelles. Most remarkably, fast drug release was observed for crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles under a reductive condition containing 10 mM GSH, in which 86.0% and 96.7% of DOX were released in 24 h at pH 7.4 and 5.0, respectively (Fig. 7A). In comparison, 89.4% and 79.5% of DOX were released from crosslinked PEG-P(LL_{14}-LA_{14}) micelles under otherwise the same reductive conditions at pH 7.4 and 5.0, respectively (Fig. 7B). Hence,

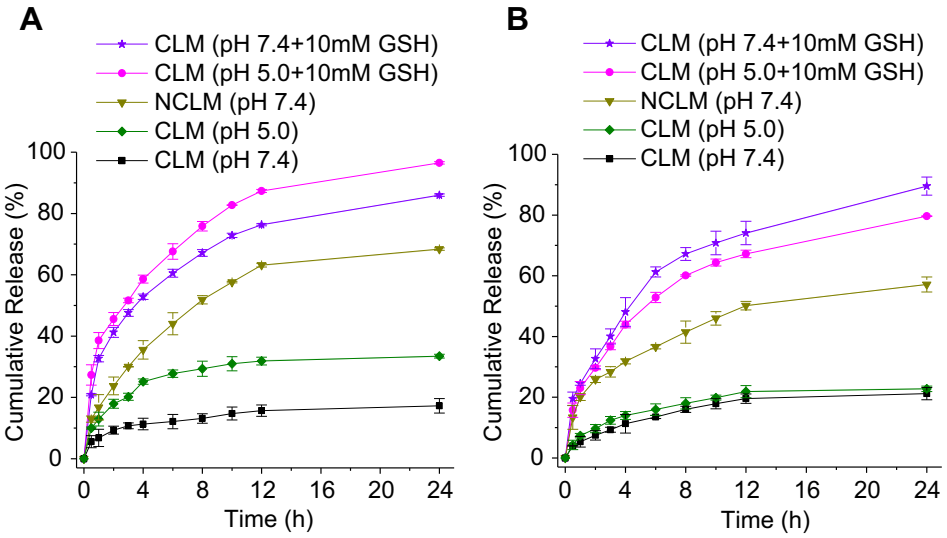


Fig. 7. *In vitro* release of DOX at pH 7.4 or 5.0 in the presence or absence of 10 mM GSH from crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles (A) and crosslinked PEG-P(LL_{14}-LA_{14}) micelles (B). The parent non-crosslinked micelles were used as a control. The drug release studies were performed at a low micelle concentration of 20 μg/mL. Data are presented as mean ± SD (n = 3).

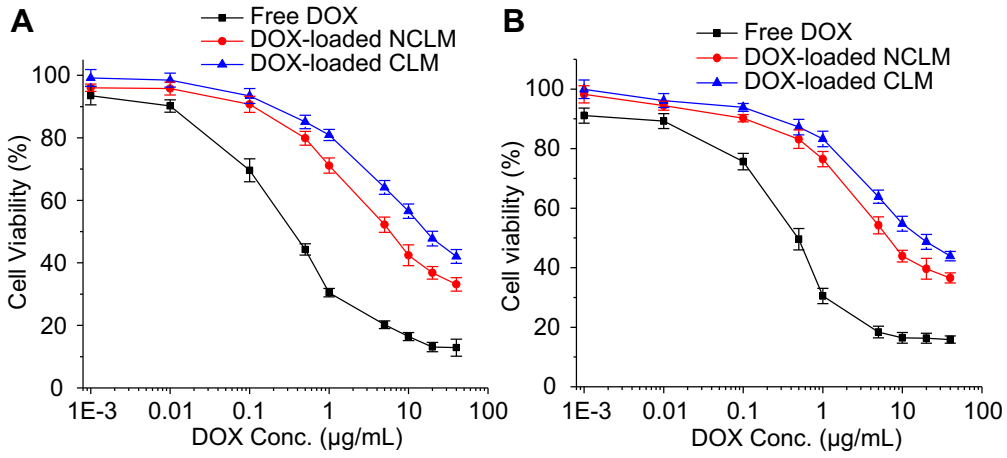


Fig. 8. Anti-tumor activity of DOX-loaded core-crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles in HeLa cells (A) and HepG2 cells (B). DOX-loaded non-crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles were used as controls. The cells were treated with DOX-loaded micelles or free DOX for 48 h. Data are presented as mean ± SD (n = 3).

introduction of CCA has not only resulted in higher DOX loading levels but also faster drug release at endosomal pH either in the presence or absence of GSH.

3.5. Anti-tumor activity of DOX-loaded core-crosslinked micelles

The drug activity of DOX-loaded crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles was studied in HeLa and HepG2 cells and compared with DOX-loaded crosslinked PEG-P(LL_{14}-LA_{14}) micelles. The results showed that DOX-loaded crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles induced pronounced anti-tumor effect with a cytotoxicity profile close to that of DOX-loaded non-crosslinked counterparts (Fig. 8). The IC_{50} (*i.e.* inhibitory concentration to produce 50% cell death) of DOX-loaded crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles was determined to be 12.7 and 12.4 μg/mL for HeLa and HepG2 cells, respectively. Notably, higher IC_{50} of 21.2 and 20.9 μg/mL for HeLa and HepG2 cells, respectively, was determined for DOX-loaded crosslinked PEG-P(LL_{14}-LA_{14}) micelles (Fig. S1), further confirming that introduction of acid-labile CCA is beneficial in improving anti-tumor activity of DOX-loaded crosslinked PEG-P(LL-CCA/LA) micelles. Moreover, the anti-tumor activity of DOX-loaded crosslinked PEG-P(LL_{14}-LA_{14}) micelles can further be

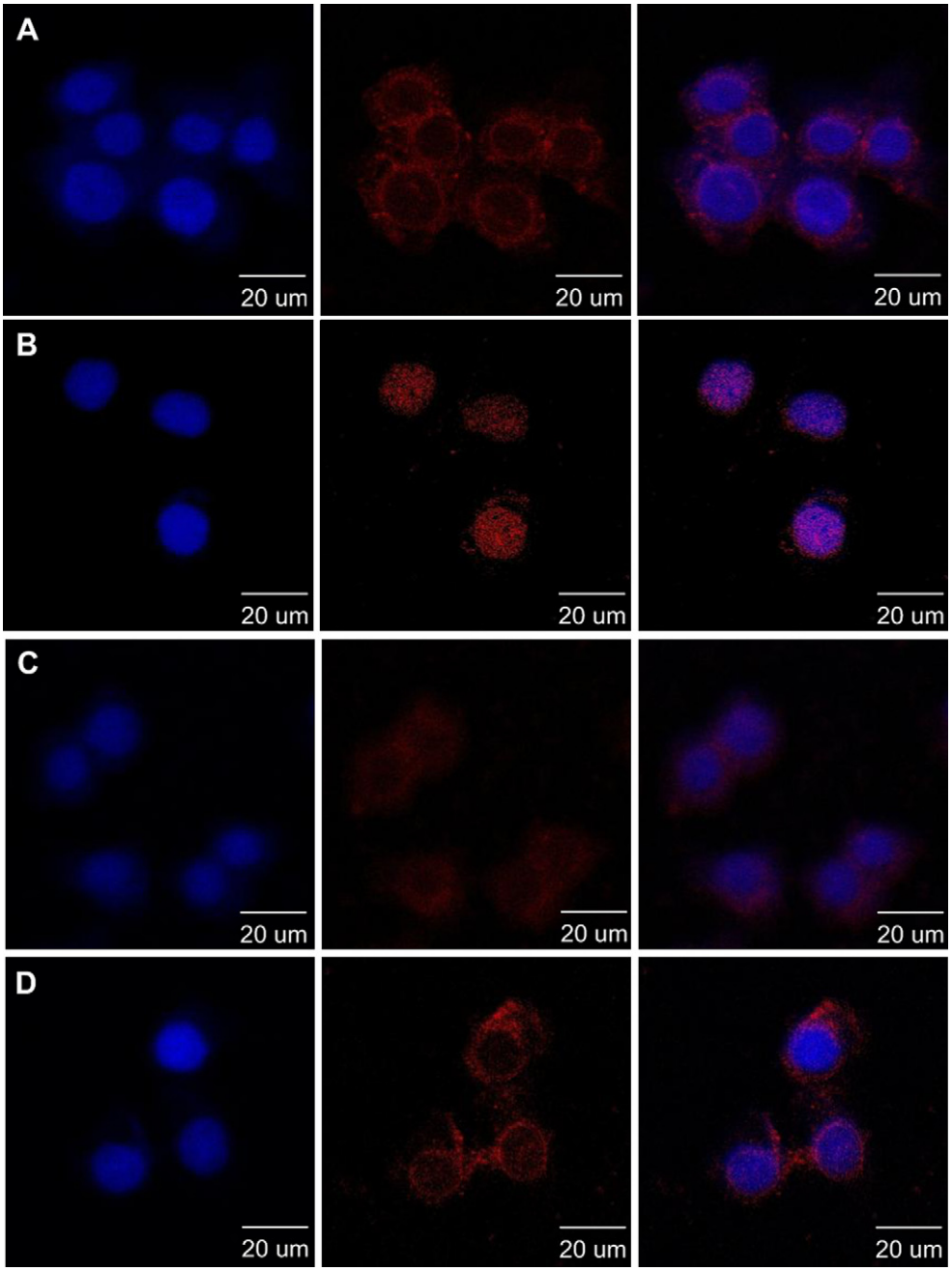


Fig. 9. CLSM images of HeLa cells incubated with DOX-loaded crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) and PEG-P(LL_{14}-LA_{14}) micelles (dosage: 10 μg DOX equiv./mL). For each panel, the images from left to right show cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), and overlays of the two images. The scale bars correspond to 20 μm in all the images. (A) DOX-loaded crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles, 4 h incubation; (B) DOX-loaded crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles, 12 h incubation; (C) DOX-loaded crosslinked PEG-P(LL_{14}-LA_{14}) micelles, 4 h incubation; and (D) DOX-loaded crosslinked PEG-P(LL_{14}-LA_{14}) micelles, 12 h incubation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

boosted by conjugating a tumor-specific ligand such as cyclic RGD peptide, TAT, folic acid, aptamer, antibody and antibody fragment [53–55]. MTT assays showed that both non-crosslinked and crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles were practically non-toxic (cell viabilities: 80.1%–106.3%) up to a tested concentration of 1.0 mg/mL (Fig. S2), supporting that PEG-P(LL-CCA/LA) micelles possess good biocompatibility.

3.6. Intracellular drug release of DOX-loaded core-crosslinked PEG-P(LL-CCA/LA) micelles

HeLa cells were used to investigate the cellular uptake and intracellular drug release behaviors of DOX-loaded crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles using confocal laser scanning microscopy (CLSM). DOX fluorescence was observed in HeLa cells following 4 h incubation with DOX-loaded crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles (Fig. 9A). Notably, DOX was delivered and released into the nuclei of HeLa cells following a prolonged incubation time of 12 h (Fig. 9B). In comparison, DOX-loaded crosslinked PEG-P(LL_{14}-LA_{14}) micelles released drug mainly to the perinuclei region of HeLa cells after 12 h (Fig. 9C and D). This different intracellular distribution of drug is likely due to the reason that cleavage of acid-labile amide bonds of CCA in endosomes facilitates the endosomal escape of dual-sensitive micelles via increasing the osmotic pressure and/or interaction with endosomal membrane. These intracellular drug release observations were in line with higher anti-tumor activity of DOX-loaded crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles as compared to PEG-P(LL_{14}-LA_{14}) controls, confirming that pH and reduction dual-sensitive crosslinked PEG-P(LL_{18}-CCA_4/LA_{14}) micelles promote fast intracellular drug release.

4. Conclusions

We have demonstrated that reduction and pH dual-sensitive reversibly core-crosslinked polypeptide micelles based on PEG-P(LL-CCA/LA) block copolymers efficiently load and deliver doxorubicin into the nuclei of cancer cells, affording high anti-tumor efficacy. These dual-responsive degradable micelles have uniquely resolved following two dilemmas encountered by current micellar drug formulations: (i) active drug loading *versus* intracellular drug release. Our results have shown that introduction of CCA has not only improved drug loading efficiencies and contents at physiological pH, likely via its ionic interactions with doxorubicin, but also promoted drug release under endosomal pH conditions due to cleavage of acid-labile amide bond between CCA and PLL; and (ii) stability *versus* intracellular drug release. These disulfide-crosslinked micelles have on one hand demonstrated excellent stability with inhibited drug release under extracellular environments and on the other hand displayed fast micelle dissociation and drug release in response to cytoplasmic glutathione. Notably, these dual-sensitive polypeptide micelles are highly versatile and can be readily prepared with tunable micellar core properties such as hydrophobicity, carboxylic groups, and crosslinking density.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.03.035.

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