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Reduction/temperature/pH multi-stimuli responsive core cross-linked polypeptide hybrid micelles for triggered and intracellular drug release

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ABSTRACT

The high toxicity, poor stability, premature drug release, and lack of intracellular stimuli responsibility of current polymeric micelles still hinder them for potential clinical applications. To address these challenges, a novel type of multi-stimuli responsive, core cross-linked polypeptide hybrid micelles (CCMs) was developed for triggered anticancer drug delivery in tumor microenvironment. The CCMs was prepared via free radical copolymerization by using N,N'-methylene-bis-acylamide (BACy) as the cross-linking agent, 2,2-azobisisobutyronitrile (AIBN) as the initiator, where poly (γ-benzyl-L-glutamate) (PBLG) and N-isopropylacrylamide (NIPPAM) as comonomers. The doxorubicin (DOX) was then introduced into the CCMs by hydrazone bond to prepare the drug-incorporated core cross-linked micelles (CCMs-DOX). By the experimental results, the CCMs showed reduction responsibility due to the degradable disulfide bond in the polymer network. The hydrazone bond can be broken under acidic condition causing a controllable drug release for CCMs-DOX. Compared to only 7.7% DOX release under pH 7.4 at 37°C, a much higher DOX release rate up to 85.3% was observed under 10 mM GSH (pH 5.0, 42°C). *In vitro* cell assays showed that the blank CCMs showed almost no toxicity against HUVEC cells while the CCMS-DOX exhibited significant cancer cell killing effect. These experimental results suggested that the prepared multi-stimuli responsive polymeric micelles could serve as a smart and promising drug delivery candidate for anti-cancer therapy.

1. Introduction

Recently, stimuli-responsive polymeric micelles serving as functional drug vehicles have emerged as the most promising technology platform for various drug delivery system (DDS) due to their small size, core-shell structure, drug-loading capacity, and triggered drug release in response to biological stimuli such as differences in redox potential, pH, and temperature between normal cells and tumor cells [1–5]. The stimuli-responsive polymeric micelles consist of a hydrophobic core and a hydrophilic shell, and possess the functional group for controlled drug release [6]. Therefore, various anti-cancer drugs can be incorporated in the micellar core such as paclitaxel or doxorubicin (DOX), and the hydrophilic shell can protect them from degradation by enzymes, prevent micelle aggregation, improve the bioavailability of drugs in water, and decrease side effects on healthy cells [7–10]. Therefore, the stimuli-responsibility can realize the triggered release of anti-cancer drugs in cancer tissue for targeted delivery [11–14].

However, for the conventional polymeric micelles from linear amphiphilic block copolymers, the micellar instability caused by plasma proteins, high electrolyte concentration, variation of pH and temperature, massive dilution, and mechanical shear forces in the blood circulation still remains challenges for DDS. The premature drug release caused by micellar instability may cause serious toxicity problems and hinder the effect of drug delivery at aimed sites, which would extremely limit their application if used in vivo. Therefore, it is of great importance for drug delivery vectors to endure the complex biological environment. Covalent cross-links in micelles can remarkably enhance the structural stability rather than the weak noncovalent intermolecular interactions in linear polymer micelles. Therefore, the high stability of polymer micelles with cross-linked structure have promising applications in DDS [15,16]. However, the permanently cross-linked micelles cannot be disintegrated and may accumulate in the host cells or tissues causing a long-term toxicity. In order to address the dilemma, in the last decade various strategies have been developed for the fabrication of degradable crosslinked micelles, which can maintain their nano-structures in the complicated extracellular environment, but undergo the biodegradation process in response to intracellular microenvironment [17].

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On the other hand, although synthesis of tailored biopolymers with controllable architectures and compositions has been facilitated by new polymerization process, comparing to the proteins or peptides, synthetic biopolymers have not yet match the natural structures and biofunctional diversity so far. Due to the good biodegradability, biocompatibility, biofunctionality, and structural analogous to natural biomolecules, synthetic polypeptides have gained great interest for DDS [18]. Recently, various kinds of polypeptides with stimuli responsibility have been developed, such as reduction, temperature, pH, and enzyme as well [19–25]. Owing to good colloidal stability, the polypeptide based cross-linked polymeric micelle has been intensively studied for drug, protein, and gene delivery. For example, by using a ring-opening polymerization, Zhang et al. reported a reduction-sensitive shell-crosslinked polyglutamate-*b*-polysaccharide micelles for DOX intracellular delivery [26]. Ren et al. reported the preparation of a pH/sugar-sensitive, core-cross-linked, polyion complex micelles based on poly glutamic acid via a phenylboronic acid–catechol interaction for protein intracellular delivery [27]. Gao et al. developed a reduction- and temperature-sensitive core-cross-linked polyglutamate hybrid micelle with pendant diethylene glycol with lower critical aggregation concentration for DOX delivery against HeLa cells [28]. Via a reduction-responsive disulfide cross-linked stearyl-peptide-based micelle system, Yao et al. achieved co-delivery of DOX and microRNA-34a for prostate cancer therapy [29]. Recently, our group reported a reduction-responsive core cross-linked polyethylene glycol-polypeptide hybrid micelle with high loading capacity and efficient intracellular drug release [30]. However, due to the difficulty in fabrication schemes that involve complex and multi-step synthesis procedures, it is still challenging to integrate reduction, temperature, and pH stimuli-responsibility into one cross-linked micelle based on polypeptide for intracellular and efficient drug delivery [31].

Herein, we developed a simple approach to fabricate the core cross-linked polypeptide hybrid micelles (CCMs) via free radical copolymerization, and the anticancer drug, DOX, was then introduced to the CCMs through an acid-sensitive hydrazone bond to prepare a novel type of reduction, temperature, and pH multi-stimuli responsive core cross-linked micelles (CCMs-DOX). Their chemical structure, size, and morphology were fully characterized, and the release experiments demonstrated that the CCMs-DOX exhibited multi-stimuli responsive drug release. The blank CCMs showed almost no toxicity against HUVEC cells (normal cell) while the CCMs-DOX showed great promise of anti-tumor efficacy and intracellular drug delivery against HeLa cells (cancer cell) by the CCK8 assay and CLSM analysis. Scheme 1 illustrates the preparation of the CCMs, CCMs-DOX, and the overall mechanism of the controlled drug release in response to the simulated tumor microenvironment.

2. Experimental

2.1. Materials

N-isopropylacrylamide (NIPPAM) (98%) and Acryl amide (99%) were obtained from Best Reagent Ltd. 2,2-azobisisobutyronitrile (AIBN) (99%) purchased from Kemio Chemical Reagent Ltd. Hydrazino (80%) were obtained from Zhiyuan Chemical Reagent Ltd. Glutathione (GSH) (98%) and DOX (98%) purchased from Hua Feng Chemical Materials Ltd. N,*N*-dimethylformamide (DMF), toluene, dichloromethane, tetrahydrofuran (THF), and ethyl acetate were used after distilled.

2.1.1. Synthesis of N,N'-methylene-bis-acylamide (BACy)

Cysteamine hydrochloride (2.30 g, 10.2 mmol) was dissolved in 18 mL water and cooled in ice-water bath for 30 min. Acryloyl chloride (1.8 mL, 21.5 mmol) was dissolved in 3 mL of THF. Both above solutions were added slowly into three-necked flask within 5 min; at the same time, NaOH solution (1.60 g, 40 mM) was also drop-wisely added. The mixture was stirred for 3 h in ice-water bath, and then at room temperature for another 12 h. The organic phase was extracted with dichloromethane, and then dried over anhydrous $MgSO_4$. The BACy was finally purified by recrystallization from ethyl acetate/hexane mixture (1:1, v/v). The ^{1}H spectrum of BACy in $CDCl_3$ was shown in Fig. 1a. 1.60 g (61 wt%). ^{1}H-NMR (400 MHz): δ (ppm) = 2.89 (t, 4H, –CONHCH$_2$C***H$_2$***S–), 3.68 (t, 4H, –CONHC***H$_2$***CH$_2$S–), 5.66–6.31 (m, 6H, C***H$_2$***=C***H***CO–), and 6.61 (brs, 2H, –CON***H***–).

2.1.2. Synthesis of vinyl poly (γ-benzyl-L-glutamate) (PBLG)

γ-Benzyl-L-glutamate N-carboxyanhydride (BLG-NCA) was prepared according to a reported literature procedure [32]. Allylamine (5 μL, 66.2 μmol), BLG-NCA (0.70 g, 2.7 mmol), and 3 mL of DMF were added to a Schlenk flask. After degassed by three freeze-thaw cycles, the mixture solution was reacted for 24 h at room temperature by gently stirring under N_2 atmosphere. The product was precipitated in ice-cooled ether and dried under vacuum. The ^{1}H spectrum of PBLG in $CDCl_3$ was show in Fig. 1b. Yield: 0.90 g (79 wt%). ^{1}H-NMR (400 MHz): δ (ppm) = 1.80–2.16 (–COOCH$_2$C***H$_2$***–), 2.31–2.52 (–COOC***H$_2$***CH$_2$–), 2.54 (CH$_2$=CHC***H$_2$***–), 4.48–4.67 (–CONHC***H***–), 4.99–5.19 (C$_6$H$_5$C***H$_2$***–), 5.46 ~ 6.67 (C***H$_2$***=C***H***CH$_2$–), 7.21–7.39 (C$_6$***H$_5$***–), and 7.72–7.92 (–CON***H***CH–).

2.1.3. Preparation of CCMs

In a typical experiment, NIPPAM (0.61 g, 5.3 mmol), PBLG (0.20 g, 0.1 mmol), BACy (0.04 g, 0.2 mmol), AIBN (0.04 g, 0.2 mmol), and 150 mL of toluene were added into a three-necked flask, and the mixture solution was stirred for 12 h at 85℃ under N_2 atmosphere. The product was precipitated in a mixture of ice-cooled ether/ tetrahydrofuran solution. The product was then filtrated and dried under vacuum. The ^{1}H spectrum of the cross-linked copolymer was shown in Fig. 1c. Yield: 1.50 g (70 wt%). ^{1}H-NMR (400 MHz): δ (ppm) = 0.99 ~ 1.30 (–C***H***$_3$, in NIPPAM unit), 1.77–2.15 (–COOCH$_2$C***H$_2$***–, in PBLG unit), 2.32–2.53 (–COOC***H$_2$***CH$_2$–, in PBLG unit), 3.66–3.78 (–CONHC***H$_2$***CH$_2$S–, in BACy unit), 3.89–4.02 (–C***H***–, in NIPPAM unit), 4.52–4.67 (–C***H***–, in PBLG polymer backbone), 4.96–5.20 (C$_6$H$_5$C***H$_2$***–, in PBLG unit), 7.18 ~ 7.37 (C$_6$***H$_5$***–, in PBLG unit), 7.70 ~ 7.98 (–CON***H***CH–, in PBLG unit and NIPPAM unit).

The CCMs were prepared by a dialysis method. 10 mg of cross-linked copolymer was dissolved in 4 mL of DMF. The solution was drop-wisely added to 5 mL of deionized water and stirred for 1 h. After that, dialysis method was used to remove the unbonded molecules and organic solvents with a dialysis tube with molecular weight cut-off (MWCO) of 12,000 for 3 days. Finally, the CCMs were freeze-dried into white powder.

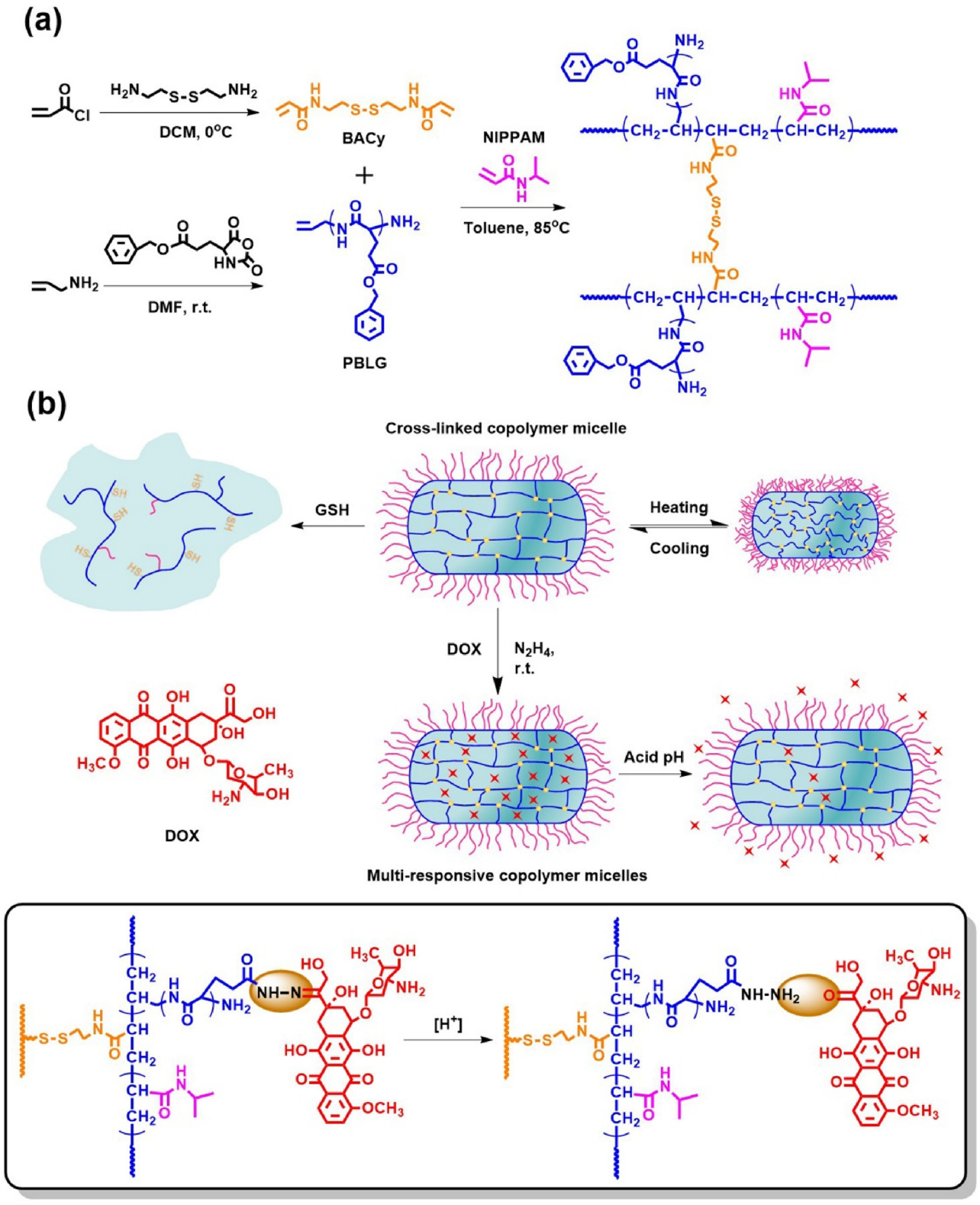
2.1.4. Preparation of CCMs-DOX

Briefly, 0.50 g of CCMs was dissolved in 50 mL of DMF, and then 10 mL of anhydrous hydrazine and excessive DOX (0.10 g, 0.2 mmol) were added into the mixture solution under stirring for 24 h at room temperature with the exclusion of light and protection of N_2 atmosphere. The product solution was dialyzed against PBS buffer (pH 7.4, 10 mM) with a dialysis tube (MWCO 12,000), and the PBS buffer was refreshed every 5 h for 3 days to remove the excess DOX. Finally, the product solution was freeze-dried into a red powder.

The CCMs-DOX were prepared by a dialysis method. 10 mg of as prepared product was dissolved in 4 mL of DMF. The solution was drop-wisely added to 5 mL of deionized water and stirred for 1 h. Then the solution was transferred into a dialysis tube (MWCO 12,000) and dialyzed against PBS buffer (pH 7.4, 10 mM) for 3 days to prepare the CCMs-DOX. Finally, the product was freeze-dried into a brown powder.

2.2. Characterization

^{1}H-NMR spectra of BACy, PBLG, and CCMs were obtained using a Bruker 400-MHz spectrometer with deuterated chloroform ($CDCl_3$) as solvent using tetramethylsilane (TMS) as the internal standard. FTIR



Scheme 1. (a) Synthetic route to the CCMs. (b) Schematic illustration of the multi-stimuli responsiveness of CCMs and triggered drug release process of the CCMs-DOX.

spectra of BACy, PBLG, and CCMs were gathered in solid state on a PE Spectrum One FTIR spectrophotometer under room temperature at 4 cm^{-1} resolution from 400 to 4000 cm^{-1}. The micelle diameter of CCMs were determined by Dynamic light scattering (DLS, Malvern Zetasizer Nano-ZS90). Each sample of 1 mg/mL was added into a 2.0 mL quartz cuvette and measured under ambient in three times. The turbidity measurement was carried out to monitor the degradation behavior of the micelles by UV-vis spectroscopy, and the relative turbidity of sample solution was determined by calculating the ratio of the absorbance of the micelles in presence of GSH to that of the initial non-degraded micelles. GPC measurements were performed to obtain the number average molecular weight (M_n) on a Waters 1515 solvent pump

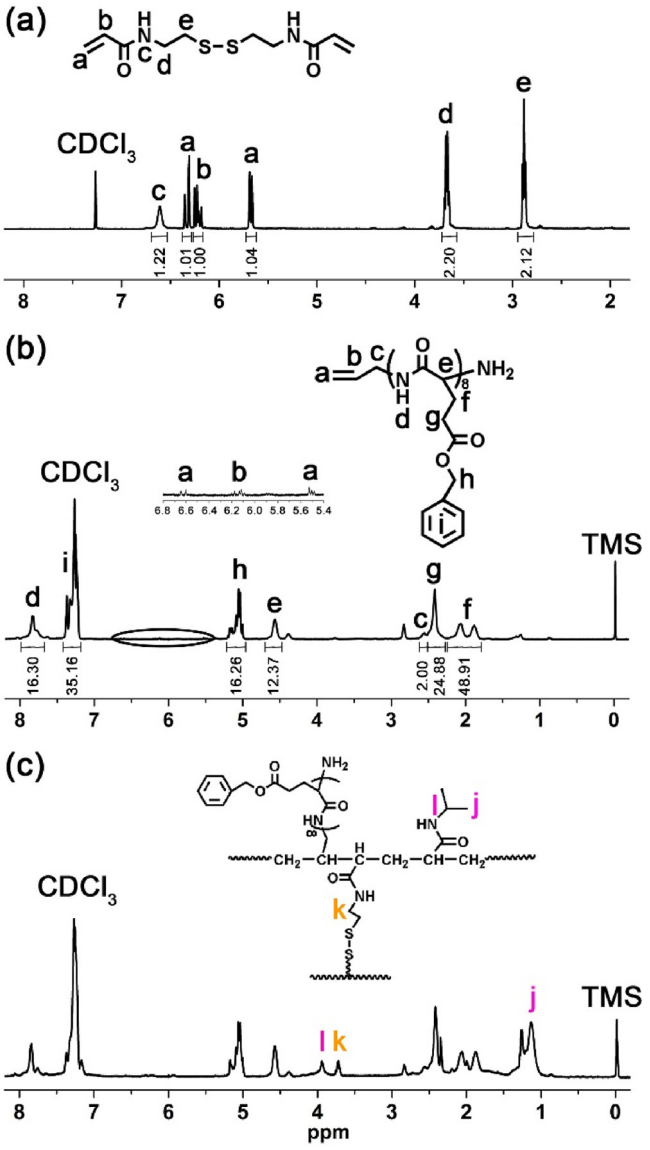


Fig. 1. ^{1}H NMR spectra of BACy (a), PBLG (b), and CCMs (c) in $CDCl_3$.

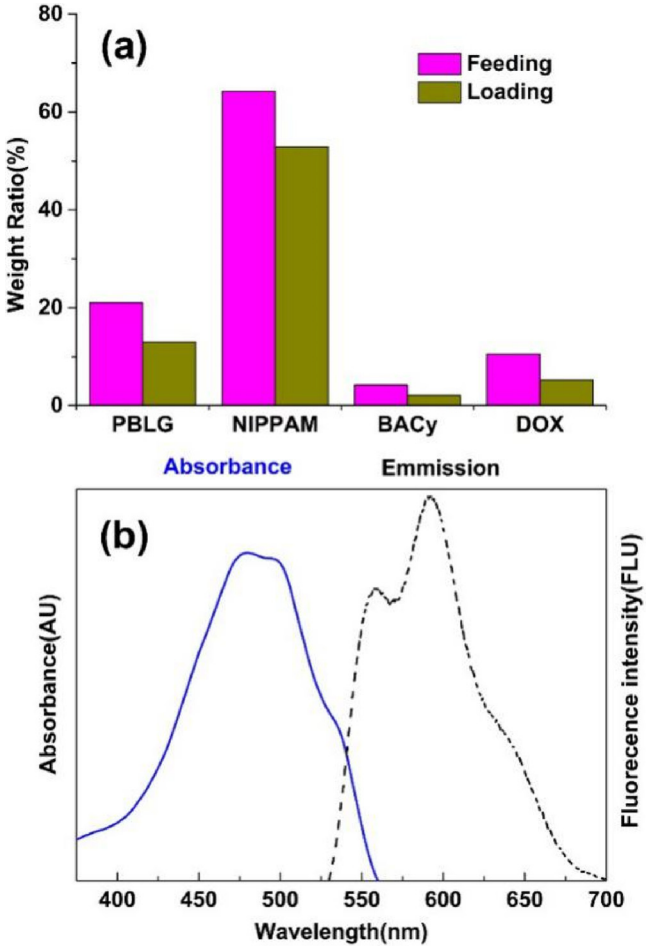


Fig. 2. Properties of CCMs-DOX. (a) Comparison chart of PBLG, NIPPAM, BACy, and DOX feeding and loading ratios in copolymer. (b) Absorbance and fluorescence spectra of DOX in CCMs-DOX.

with a Waters 2410 refractive index detector. HPLC grade DMF was chosen as the eluent at a flow rate of 1.0 mL/min. The linear polystyrene was chosen as standards to obtain the calibration curve. Morphologies of micelles were observed by transmission electron microscopy (Hitachi H-600, Japan). Each sample was prepared by dropping the solution of micelles onto carbon-coated copper grids and negatively stained with 1% phosphotungstic acid for TEM observation. Raman spectroscopy was used to confirm the disulfide bond in polymer network on a LabRam confocal Raman microscope (Renishaw CR-IM16-139, USA). UV–vis spectrometer (Mapada TU1950, China) was used to investigate the pH-responsibility of the micelles by tracing the absorbance of sample solution in different pH from 0.5 to 7.4 at a concentration of 1.0 mg/mL under room temperature.

2.2.1. Determination of critical micelle concentration (CMC)

The CMC value of the CCMs were determined using fluorescence spectroscopy (RF-5301PC, Shimadzu, Japan) using pyrene as fluorescent probe. Briefly, a large number of scans for different concentrations of the CCMs (5×10^{-4} to 1 mg/mL) were gathered from fluorescence spectroscopy, in which the final concentration of pyrene in each sample was set to 6.0×10^{-6} mol/L. According to the excitation spectra of pyrene, the ratios of pyrene probe fluorescence intensity at 373 and 384 nm (I_{373}/I_{384}) were calculated and plotted against the logarithm of the micelle concentrations. Finally, the CMC value was obtained from the intersection of the tangent to the curve at the inflection with the horizontal tangent at low concentration.

2.2.2. LCST measurement and temperature-responsibility of CCMs

The lower critical solutions temperature (LCST) of the CCMs was obtained by measuring the optical absorbance of the samples in aqueous solutions at various temperatures at 500 nm with a UV–vis spectrometer. Prior to measurements the sample solution was thermo-stated at different temperatures, and the LCST is defined as the temperature exhibiting a 50% decrease of the total decrease in transmission rate.

2.2.3. In vitro drug release

According to a standard curve obtained from DOX/DMF solutions, the drug loading and encapsulation efficiency was measured on a UV–vis spectrophotometer at the absorbance of 480 nm. The drug loading was defined as the weight ratio of loaded drug to the drug-entrapped polymer micelles. The encapsulation efficiency of DOX was obtained from the weight ratio of the drug loaded in micelles to that used in the preparation. The in *vitro* DOX drug release study was performed at 37℃ and 42℃. 2 mL of CCMs-DOX at a concentration of 1 mg/mL in a dialysis tube (MWCO 12,000) were incubated in different media: 1) PBS (pH 7.4, 37 ˚C) without GSH, 2) PBS (pH 7.4, 37 ˚C) with 10 mM GSH, 3) PBS (pH 5.0, 37 ˚C) without GSH, 4) PBS (pH 5.0, 37 ˚C) with 10 mM GSH, and 5) PBS (pH 5.0, 42 ˚C) with 10 mM GSH under gentle stirring, respectively. At each time interval, 1.5 mL of medium

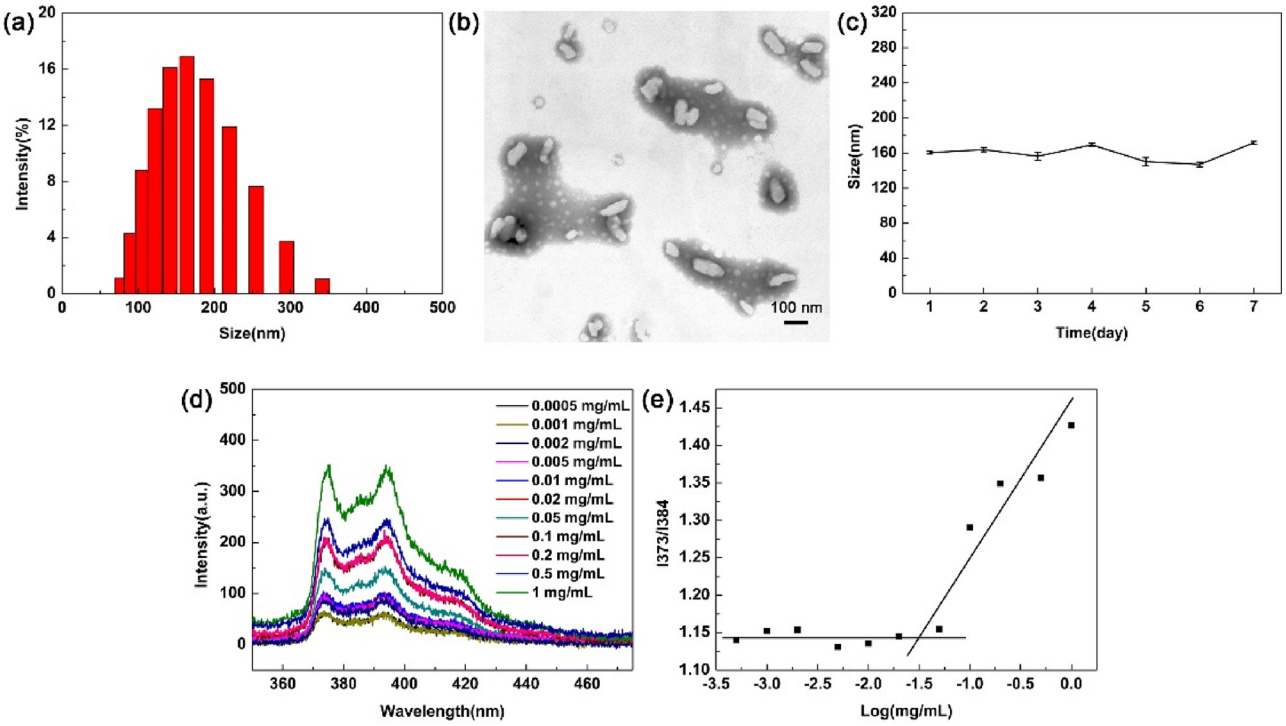


Fig. 3. (a) Size distribution of copolymer micelles by DLS measurement, and their TEM photo (b). (c) Hydrodynamic size of CCMs in cell culture media (DMEM with 10% FBS) as a function of time. (d) Fluorescence emission spectrum of pyrene at 335 nm, and (e) plots of the intensity ratio vs log C.

was used for measuring and replaced with 1.5 mL of fresh medium.

2.2.4. In vitro cell assay

Cancer cell viability of the CCMs and CCMs-DOX were determined by Cell Counting Kit-8 (CCK-8) assay. The HeLa cells (human cervical carcinoma cells, cancer cells) and HUVEC cells (human umbilical vein endothelial cells, normal cells) were cultured in DMEM (dulbecco's modified eagle medium) at 37 °C in a humidified atmosphere with 5% CO_2 and supplemented with 10% heat-inactivated fetal bovine serum. The logarithmic growth phase cells were washed with PBS, digested with trypsin, and centrifugalized to remove liquid supernatant. Cells were seeded into 96-well plate at a density of 5×10^5 cells/well, and incubated for 24 h to allow cell attachment. After that, the medium was removed and replaced with 100 μL of different concentrations of free DOX, blank CCMs, or CCMs-DOX. The cells were incubated with different samples for several hours. At predetermined intervals, 10 mL of CCK-8 solution were added into each well, and the cells were incubated for 3 h. After incubation, the absorbance was measured at 450 nm to evaluate the cell viability by a micro-plate reader (Thermo Fisher Multlskan FC, USA).

2.2.5. Confocal laser scanning microscopy

Confocal Laser Scanning Microscope (CLSM) was used to investigate the cellular uptake of DOX. The cells were incubated in an UV disinfected 6-well plate with cover glass at a density of 5×10^5 cells/well. After 24 h incubation period, samples of the free DOX or CCMs-DOX were added into each well. At 2 h and 24 h, the suspension was removed, and the samples were washed with fresh DMEM and PBS before fluorescence observation. The samples were then mixed with fluorescent mounting medium to be observed by CLSM (Motic AE31, Japan) with an imaging software.

3. Results and discussion

3.1. Preparation and structural characterization of the CCMs and CCMs-DOX

In this work, to prepare the CCMs-DOX, CCMs was firstly synthesized via free radical copolymerization by using BACy as crosslinker and AIBN as radical initiator, where PBLG and NIPPAM as comonomers. The preparation of CCMs was a simple single-step procedure where all the comonomers were directly mixed by using toluene as the solvent. Since PBLG, NIPPAM, and BACy contain vinyl groups, they can be directly incorporated into the backbone of CCMs (Scheme 1a). Thus, comparing to the conventional method where each component is individually attached to polymer network, this single-step method eliminates the need of multiple conjugation steps, and the amount of each component can be easily controlled.

The DOX was then introduced into the CCMs by hydrazone bond to prepare the multi-responsive core cross-linked micelles, CCMs-DOX, by the dialysis method. The CCMs-DOX could keep stable in aqueous solution due to the formation of hydrogen bonds between hydrophilic PNIPPAM outer shell and water molecules, which acted as a hydration barrier. In brief, the CCMs-DOX contains three basic units: BACy as a disulfide bonded cross-linker to provide reduction-responsibility, the biocompatible polypeptide was used to bond DOX to provide pH-responsivity, and NIPPAM as the hydrophilic unit to provide temperature-responsibility. The synthetic route and stimuli-responsive drug release process of the micelles were illustrated in Scheme 1.

^{1}H NMR spectra were used to confirm the chemical structures of the synthesized BACy, PBLG, and CCMs. The representative ^{1}H NMR spectrum of BACy (in $CDCl_3$) was depicted in Fig. 1a. The resonance signals of $–CH_2–$ (e and d) neighboring to the disulfide bond of the BACy molecule, vinyl group (a and b), and –NH– (c) was observed at δ = 2.89 (e), 3.68 (d), 5.66–6.31 (a and b), and 6.61 (c), respectively, as described in experimental section. As shown in Fig. 1b, the chemical structure of PBLG was evidenced by ^{1}H NMR in $CDCl_3$ with 15% trifluoracetic acid. The resonance signals of protons of β- and γ-methylene

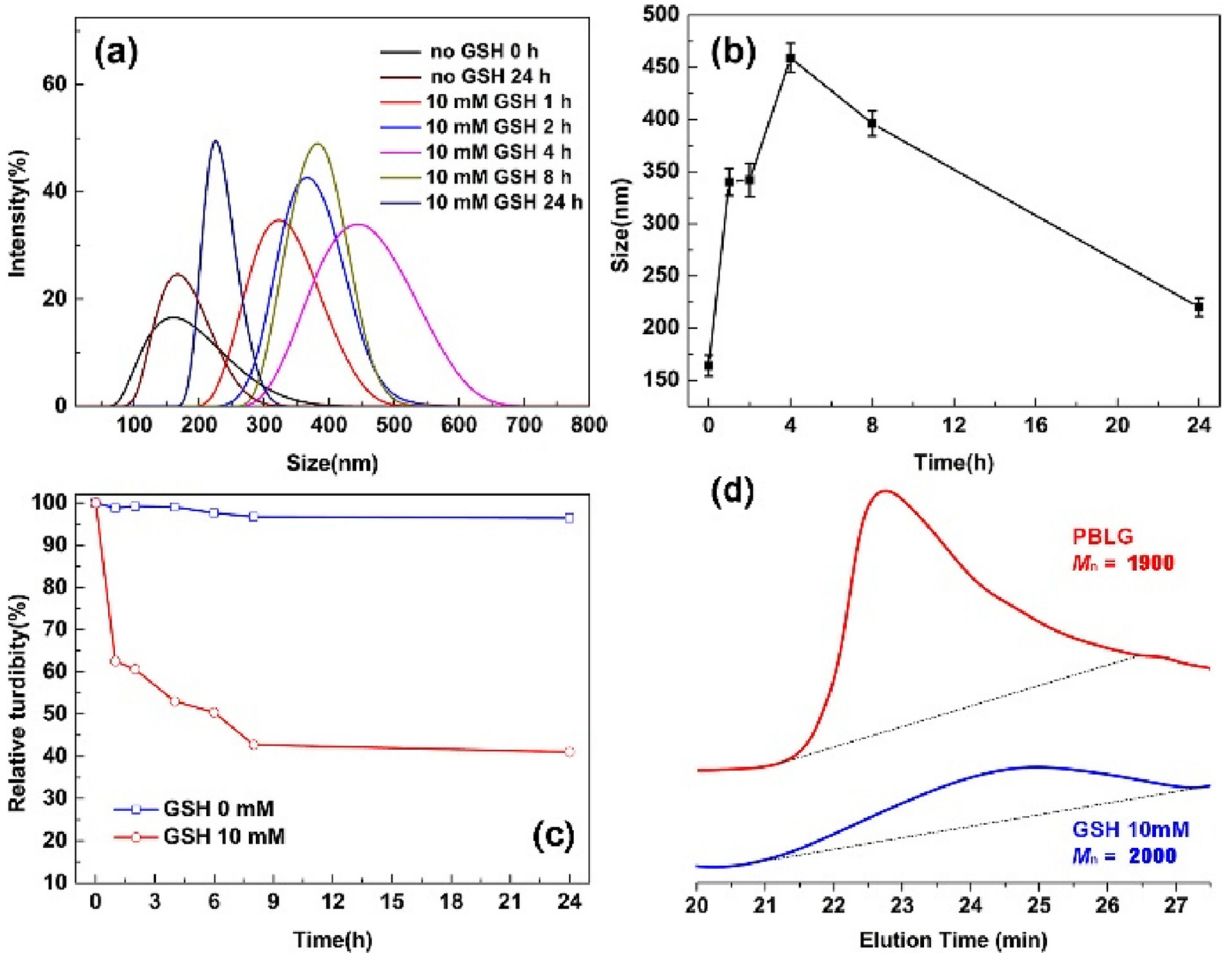


Fig. 4. Investigations on the redox-induced micelle degradation: (a) and (b)The size change of copolymer micelles in response to GSH by DLS measurement. (c) The relative turbidity in the presence and absence of 10 mM GSH. (d) Investigation on the reduction-responsibility by GPC measurement of PBLG (up, before degradation) and copolymer micelle (down, after degradation).

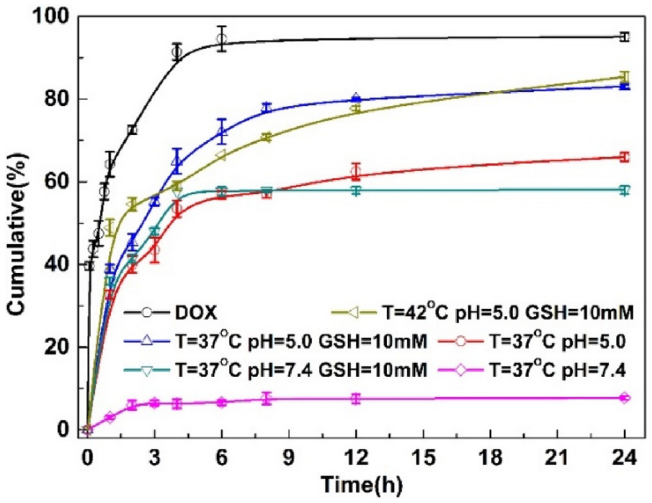


Fig. 5. *In vitro* DOX release profiles of CCMs-DOX under different simulated physiological conditions.

groups (f and g), $-CH_2-$ in initiator residue (c), α-methine group (e) in polymer backbone, methylene group of benzyl (h), phenyl group (i), and amide group (d) appeared at δ = 1.80–2.16 (f), 2.31–2.52 (g), 2.54 (c), 4.48–4.67 (e), 4.99–5.19 (h), 7.21–7.39 (i), and 7.72–7.92 (d), respectively. The resonance signals of vinyl group (a and b) in the initiator residue at δ = 5.46–6.67 indicated the successfully synthesis of PBLG. The ^{1}H NMR spectrum of CCMs (in $CDCl_3$) was depicted in Fig. 1c. The resonance signals at δ = 0.99–1.30 (j), 3.66–3.78 (k), and 3.89–4.02 (l) were belong to the methyl group in NIPPAM units (j), $-CH_2-$ group (k) of the BACy crosslinker, and methine group in NIPPAM units (l). As shown in Fig. 1c, the appearance of the benzyl unit in PBLG (δ = 7.18–7.37), $-CH_2-$ group (k) signals in BACy, and methine group (l) in NIPPAM unit, and disappearances of the vinyl group in PBLG unit (δ = 5.46–6.67) indicated the successfully synthesis of the CCMs. The FTIR and Raman spectra of crosslinked micelles were shown in Figure S1.

The CCMs-DOX were prepared by introducing DOX molecule into the CCMs via pH-responsive hydrazone bond by the dialysis method. As shown in Fig. 2a, herein, the polymer component weight ratios were referred to the feeding and loading ratios before and after polymerization, respectively. The small difference between the feeding and loading of monomers indicated efficient conversion during the polymerization reaction. Therefore, the drug/polymer composition in the end product can be precisely controlled by altering the feeding ratio of monomers. CCMs-DOX were also characterized by UV–vis and fluorescence spectroscopy (Fig. 2b) to further confirm and quantify the DOX loading. Free DOX emits red fluorescence at 590 nm and the spectrum depicted in Fig. 2b confirms that CCMs-DOX conserved the fluorescent property of DOX with the absorbance and emission maxima at 480 and 590 nm, respectively. By using the absorbance measured from CCMs-DOX, the drug loading was quantified that the weight of DOX accounts for 6.3% of the total drug entrapped polymer weight, and 56.0% for

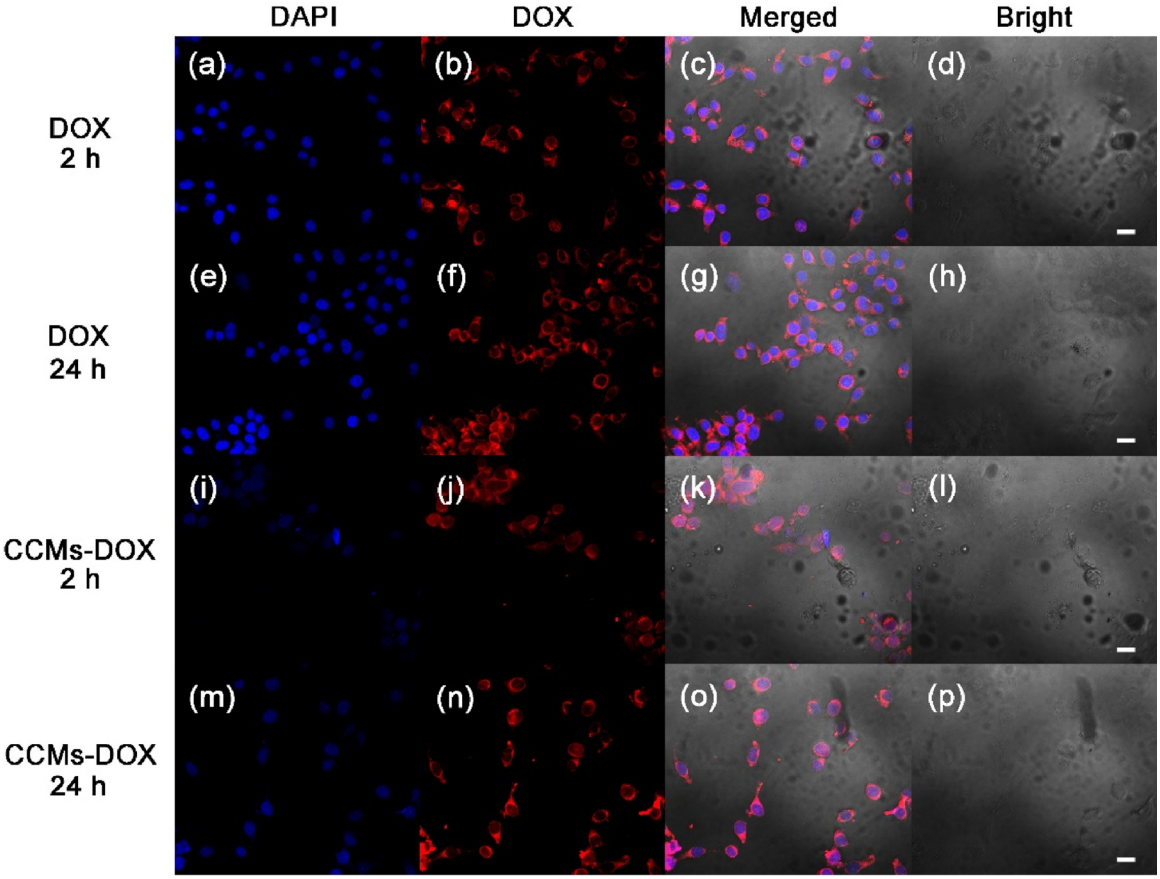


Fig. 6. CLSM images of HeLa cells cultured with free DOX over incubation: (a–d) 2 h and (e–h) 24 h; Cells cultured with CCMs-DOX: (i–l) 2 h and (m–p) 24 h. I, stained by DAPI; II, DOX fluorescence images; III, merged images; IV the bright field. The scale bar corresponds to 25 μm.

encapsulation efficiency.

The CCMs were prepared by dialysis method and formed a core-shell structure, which consisted of a hydrophobic polymer network inner core and a hydrophilic PNIPPAM outer shell as a hydration barrier. DLS measurements showed that the cross-linked copolymer formed micelles with average sizes of about 160 nm and a narrow unimodal distribution with a PDI of 0.24 as shown in Fig. 3a. As shown in Fig. 3b, TEM micrograph revealed that these micelles had an oval morphology with an average size of about 100 nm. The smaller size (about 100 nm) observed by TEM as compared to that determined by DLS is probably due to shrinkage of the hydrophilic shell through overnight dehydration before TEM observation. After 7 days, no significant changes in size were observed in DMEM media, indicating that the micelles retained good stability due to the presence of hydrophilic PNIPPAM outer shell and the covalent cross-linked polymer network. Therefore, the CCMs could enable long circulation time in blood and reduce liver and macrophage uptake when used in vivo. As shown in Fig. 3d and e, the CMC values of CCMs were determined to be 31.6 mg/L by the intersection of the tangent to the curve of I_{373}/I_{384} at the inflection with the horizontal tangent. Because of the low CMC value, it was indicated that the CCMs could provide excellent colloidal stability in very dilute aqueous solution such as bloodstream and body fluids.

3.2. Redox degradation of the CCMs

To monitor the redox-responsive behavior of the micelles, the degradation experiments of the micelles with redox agent were carried out by DLS, UV–vis, and GPC. GSH was chosen as the reducing agents to study the degradation behavior of CCMs, and the size change of CCMs in response to 10 mM GSH in PBS buffer (pH 7.4) at 37 °C was followed by DLS measurements. In Fig. 4a and b, a rapid degradation was observed for CCMs in the first 2 h, in which the average micelle size increased from about 160 nm to around 350 nm, reaching over 450 nm after 4 h. It was indicated that the reductive cleavage of the disulfide bonds had resulted in the degradation of the cross-linked micelles and formation of a swelling structure. When proceeding to over 8 h, a remarkable size decrease of the nanoparticles was observed, which can be explained by the disassembly of the micelles into linear polymer chains, finally reaching 220 nm after 24 h degradation. Fig. 4c shows the degradation extent of the CCMs by relative turbidity in PBS buffer 7.4 at 37 °C in the presence of 10 mM GSH. In the first 2 h after the addition of GSH, the relative turbidity changed very quickly and then the relative turbidity decreased gradually as shown due to the degradation of CCMs. When proceeding to 24 h, the translucent emulsion of micelles changed into a clear solution indicating that the CCMs were degraded into linear polymeric chains. However, the CCMs kept stable and no significant change in relative turbidity was observed in the solution without GSH. In Fig. 4d, the GPC experiments indicated that the PBLG (before copolymerization) had a similar number-average molecular weight (M_n = 1900) to that of the degraded linear polymer chains (M_n = 2000). It was suggested that the cross-linked polymer network in micelles were chemically cleaved through disulfide-thiol exchange reaction, leading to the formation of the short linear polymers. Additionally, the temperature- and pH-responsibility of the CCMs-DOX was also investigated and shown in Figure S2 and S3).

3.3. In vitro drug release

To investigate the potential application of CCMs-DOX as multi-responsive drug delivery vehicle in chemical therapy, the DOX releasing

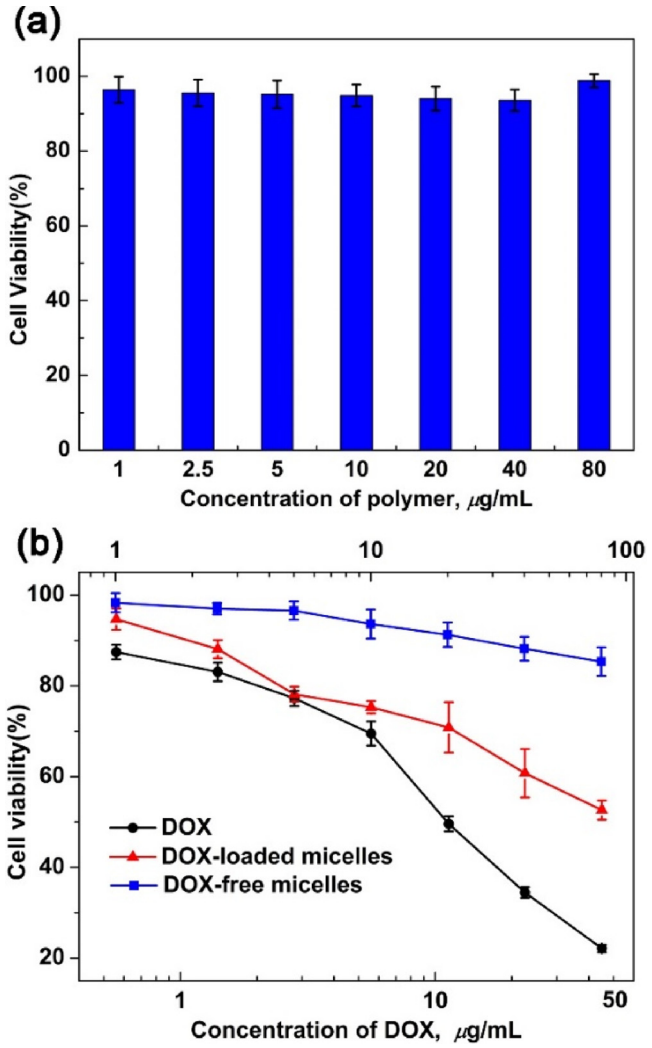


Fig. 7. Cell survival assay of cells. (a) Cytotoxicity of blank CCMs to HUVEC cells following 24 h incubation. (b) HeLa cells cultured with free DOX, blank CCMs, and CCMs-DOX for 24 h. The concentration of blank CCMs was shown on the top x-axis.

capacity was tested. As shown in Fig. 5, the release behavior of the free DOX in PBS (pH 7.4) and the incorporated DOX from CCMs-DOX was monitored under different conditions as shown in the experimental section. In contrast with the burst release of free DOX in the first 6 h, the sustained drug release from CCMs-DOX was observed. For CCMs-DOX at 37 °C, it was indicated that the DOX cumulative release was less than 7.7% at pH 7.4 for 24 h, indicating that the micelles were remarkably stable with minimum premature drug release under physiological conditions. On the other hand, the release rate of DOX was much faster and had a higher cumulative release in stimuli conditions. In the presence of 10 mM GSH, the cumulative release of DOX increased to 58% at pH 7.4, while reached 83.1% at pH 5.0 after 24 h. When the temperature was set to 42 °C, the cumulative release of CCMs-DOX reached a maximum of 85.3% at pH 5.0 in the presence of 10 mM GSH after 24 h. It could be explained by that the collapse of PNIPPAM units and formation of defects in the micellar shell helped DOX to diffuse from the inner micellar core to the outer medium, so the cumulative drug release became higher above the LCST of the CCMs at 42 °C. Overall, the release behavior of the CCMs-DOX suggested that the as prepared polymeric micelles with multi-responsibility could be used as a promising drug delivery candidate for cancer therapy and potentially facilitated in the tumor microenvironment.

3.4. In vitro cell assays

To confirm our expectation and evaluate the therapeutic properties, the *in vitro* cell assays were carried out to investigate the intra-cellular drug release of CCMs-DOX by CLSM. Fig. 6 showed the CLSM images of the cells incubated for 2 and 24 h with free DOX (Fig. 6a–h) and CCMs-DOX (Fig. 6i–p) solution. As shown in Fig. 6b and j, comparing to the free DOX that a weaker DOX fluorescence was observed in the HeLa cells after 2 h incubation with CCMs-DOX. It was indicated that only small amount of released DOX from CCMs-DOX entered cells via endocytosis, which could be explained by the slow release kinetics of CCMs-DOX and the quicker diffuse rate of the free DOX. When HeLa cells cultured with CCMs-DOX for 24 h, the gradually bright red fluorescence was observed within the cell nuclei in Fig. 6n indicating that more CCMs-DOX entered cells via endocytosis and more DOX was released. Based on the experimental results it was suggested that it took period of time for CCMs-DOX to be endocytosed in to cell and release incorporated drugs into the cells comparing to the quicker diffuse of free DOX. On the anther hand, the *in vitro* cell assays showed that CCMs-DOX release DOX at a slow rate for the first 2 h, that is, the reasonable stability of disulfide bonds would allow sufficient time for CCMs-DOX to reach the tumor site and be internalized by tumor cells, without premature release of DOX. After 24 h, the release rate of DOX increases, suggesting that drug release would be greatly accelerated after CCMs-DOX are endocytosed by cancer cells where a lower pH and a high concentration of GSH environment in endosomes or lysosomes enhances the cleavage of hydrazone bonds and disulfide bonds. In addition, for *in vivo* medical applications the nanoparticles should have a desirable range of hydrodynamic size from 10 nm (avoiding elimination by kidneys) to 200 nm (minimizing liver and spleen uptake) and keep their stability in the presence of salts with high concentrations to prevent phagocytosis. Herein, the hydrodynamic sizes of CCMs-DOX are about 160 nm and this kind of nanoparticle shows excellent stability and minor aggregation in culture media (Fig. 3c), which can be attributed to the presence of PNIPPAM units in the nanoparticle surface coating, providing excellent steric stabilization. In brief, the CLSM experiment confirmed the successfully triggered release of DOX from the CCMs-DOX and its efficient drug delivery to the cell nuclei, demonstrating the expected results.

The *in vitro* cell assays were also carried out for evaluation of the cytotoxic activity for free DOX, blank CCMs, and CCMs-DOX within HUVEC and HeLa cells. As shown in Fig. 7a, it should be noted that the blank CCMs showed almost non-toxicity against HUVEC cells up to sample concentration of 80 μg/mL following 24 h incubation. In Fig. 7b, free DOX and CCMs-DOX effectively reduced viability of HeLa cells. It was shown that the cell viability was dose-dependent, and CCMs-DOX had a lower cytotoxicity to HeLa cells comparing with that of the free DOX. The amount of DOX from CCMs required to achieve IC50 for HeLa cells was 49.8 μg/mL at 24 h after incubation and was higher than that of free DOX (19.6 μg/mL), which was likely due to the quicker diffuse rate of the free DOX through cell membrane and slow release kinetics of CCMs-DOX. However, the CCMs-DOX exhibited significant cancer cell killing effect that 52.6% of the cells remained viable at a DOX dose of 45 μg/mL, while 22.1% viability of the cells remained for free DOX at the same concentration.

4. Conclusions

In summary, we demonstrated a simple method to prepare a multi-stimuli responsive micelle, CCMs-DOX, for triggered anticancer drug delivery in tumor microenvironment. The CCMs-DOX exhibited a minimal drug release in physiological environment without stimuli comparing with an effective drug release in response to the simulated tumor microenvironment. The cellular uptake and cytotoxicity of the micelles demonstrated that the CCMs showed excellent biocompatibility, and the CCMs-DOX could be internalized by HeLa cells and

demonstrated killing capacity toward cancer cells. Based on the above discussions, we are convinced that this as prepared multi-functional micelle with excellent stability, good biocompatibility, and multi-stimuli responsibility holds great promise for constructing a safe and effective triggered drug release system in cancer therapy.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2018.06.015.

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