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Redox and pH-responsive degradable micelles for dually activated intracellular anticancer drug release

Wei Chen [a,b], Ping Zhong [a], Fenghua Meng [a,*], Ru Cheng [a], Chao Deng [a], Jan Feijen [a,b], Zhiyuan Zhong [a,*]

[a] Biomedical Polymers Laboratory, and Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, Department of Polymer Science and Engineering, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou, 215123, P. R. China

[b] Department of Polymer Chemistry and Biomaterials, Faculty of Science and Technology, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

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ABSTRACT

Redox and pH dual-responsive biodegradable micelles were developed based on poly(ethylene glycol)-SS-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) (PEG-SS-PTMBPEC) copolymer and investigated for intracellular doxorubicin (DOX) release. PEG-SS-PTMBPEC copolymer with an M_n of 5.0–4.1 kg/mol formed micellar particles with an average diameter of 140 nm and a low polydispersity of 0.12. DOX was loaded into PEG-SS-PTMBPEC micelles with a decent drug loading content of 11.3 wt.%. The *in vitro* release studies showed that under physiological conditions only ca. 24.5% DOX was released from DOX-loaded micelles in 21 h. The release of DOX was significantly accelerated at pH 5.0 or in the presence of 10 mM glutathione (GSH) at pH 7.4, in which 62.8% and 74.3% of DOX was released, respectively, in 21 h. The drug release was further boosted under 10 mM GSH and pH 5.0 conditions, with 94.2% of DOX released in 10 h. Notably, DOX release was also facilitated by 2 or 4 h incubation at pH 5.0 and then at pH 7.4 with 10 mM GSH, which mimics the intracellular pathways of endocytosed micellar drugs. Confocal microscopy observation indicated that DOX was delivered and released into the nuclei of HeLa cells following 8 h incubation with DOX-loaded PEG-SS-PTMBPEC micelles, while DOX was mainly located in the cytoplasm for reduction-insensitive PEG-PTMBPEC controls. MTT assays revealed that DOX-loaded PEG-SS-PTMBPEC micelles had higher anti-tumor activity than reduction-insensitive controls, with low IC_{50} of 0.75 and 0.60 μg/mL for HeLa and RAW 264.7 cells, respectively, following 48 h incubation. PEG-SS-PTMBPEC micelles displayed low cytotoxicity up to a concentration of 1.0 mg/mL. These redox and pH dual-bioresponsive degradable micelles have appeared as a promising platform for targeted intracellular anticancer drug release.

1. Introduction

In the past decades, biodegradable nanoparticles and micelles have emerged as one of the most promising nanosystems for the controlled and targeted delivery of potent anticancer drugs [1–6]. The PEGylated nanoparticles and micelles have demonstrated several interesting features including prolonged circulation time, better pharmacological profiles, enhanced accumulation in the tumor sites via the enhanced permeability and retention (EPR) effect, decreased adverse effects, and improved drug tolerance [7]. It is noted, however, that current nanosystems often suffer sluggish drug release at the tumor site as well as in the cancer cells that has become a critical account for the compromised treatment benefits in the clinical settings.

In recent several years, intracellular environment-sensitive nanosystems that release payloads in response to an intrinsic biological signal, in particular endosomal pH and cytoplasmic glutathione (GSH), have been designed and explored for enhanced cancer therapy [2,8–11]. For example, pH-sensitive micelles have been developed based on acid labile ortho ester [12,13], hydrazone [14,15], cis-aconityl [16,17], and acetal [18,19] bonds. Fréchet et al. reported that trimethoxybenzylidene acetals exhibit a great pH sensitivity [18,20,21]. Taking advantage of rapid pH-sensitive hydrolysis of trimethoxybenzylidene acetals, we have designed poly(ethylene glycol)-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) (PEG-PTMBPEC) block copolymer and prepared pH-responsive biodegradable micelles and polymersomes [22,23]. The *in vitro* drug release studies showed that release of paclitaxel (PTX) and/or DOX was highly pH-dependant. More recently, we have prepared pH-sensitive degradable chimeric polymersomes based on trimethoxybenzylidene acetals for high loading and triggered release of doxorubicin hydrochloride [24], as well as trimethoxybenzylidene acetal-modified low molecular weight polyethylenimine for active intracellular DNA release and improved *in vitro* transfection [25]. It should be noted, nevertheless, that intracellular drug release from these pH-responsive degradable nanocarriers, though improved, is not fast due to a small difference in endosomal and physiological pHs. For example, ca. 36% of PTX and

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

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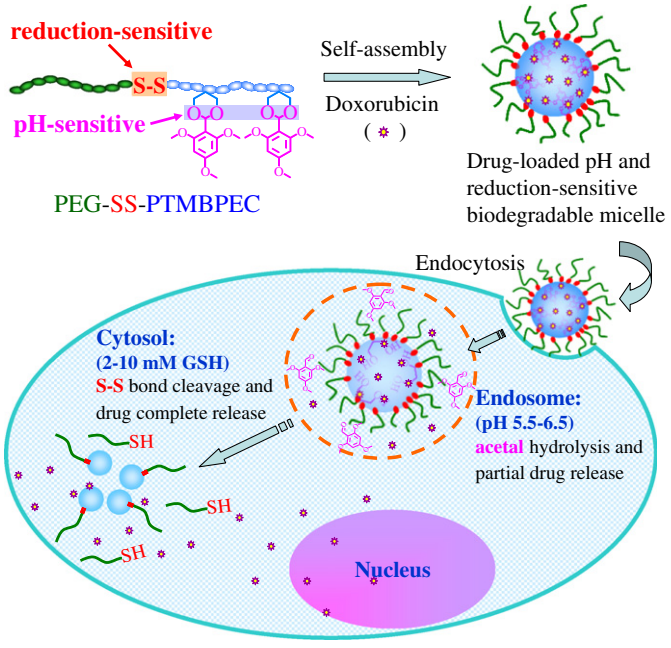
58% of DOX was released from PEG-PTMBPEC micelles in 12 h at pH 5.0 and 37 °C [23].

In contrast to pH-sensitive micelles that promote drug release in endosomes, redox-responsive micelles and nanoparticles are intended to disassemble and release drugs into the cytosol which contains 100 to 1000-fold higher concentration of reducing GSH tripeptide (approximately 2–10 mM) than the extracellular fluids (approximately 2–20 μM) [26,27]. We recently found that reduction-sensitive shell-sheddable biodegradable micelles based on poly(ethylene glycol)-SS-poly(ε-caprolactone) (PEG-SS-PCL) or dextran-SS-PCL copolymers efficiently released DOX under a reductive condition containing 10 mM dithiothreitol (DTT) in phosphate buffer at pH 7.4 and achieved significantly enhanced anticancer efficacy [28,29]. This reduction-sensitive shell-shedding approach has been adopted by several different groups to boost the intracellular drug release from various micellar anticancer drugs [30–36]. The highly hydrophobic and slow degradation nature of core-forming polyesters like PCL, however, might hamper quick and complete drug release inside the cancer cells.

In this study, we report on novel redox and pH-responsive micellar nanoparticles based on PEG-SS-PTMBPEC block copolymer for dually-triggered intracellular release of DOX in cancer cells (Scheme 1). It was assumed that DOX-loaded PEG-SS-PTMBPEC micelles, like DOX-loaded PEG-PTMBPEC counterparts, following endocytosis would result in partial release of DOX in mildly acidic endosomal compartments due to occurring hydrolysis of acetal bonds. Once DOX-loaded PEG-SS-PTMBPEC micelles escaped from endosomes, cleavage of the intervening disulfide bonds in response to a high GSH concentration would lead to further thorough release of DOX into the cytosol. Notably, core-forming PTMBPEC block following partial acetal hydrolysis might facilitate reduction-triggered drug release inside the cells owing to improved hydrophilicity of the micellar core. It should be noted that we and other groups have reported pH and reduction dual-sensitive nanoparticles based on polybases with low pK_a such as poly(2-(diethyl amino)ethyl methacrylate) that become water soluble at low pH instead of pH induced degradation[37–39]. In this paper, synthesis, *in vitro* drug release and antitumor activity of DOX-loaded PEG-SS-PTMBPEC micellar nanoparticles were investigated and the results were compared with those obtained using DOX-loaded PEG-PTMBPEC counterparts.



Scheme 1. Illustration of reduction and pH-sensitive biodegradable micelles based on PEG-SS-PTMBPEC copolymer for dually activated intracellular release of anticancer drugs.

2. Experimental section

2.1. Materials

Dicyclohexyl carbodiimide (DCC, 99%, Alfa Aesar), N-hydroxysuccinimide (NHS, 98%, Alfa Aesar), cystamine dihydrochloride (98%, Alfa Aesar), glutathione (GSH, 99%, Roche), *p*-nitrophenyl chloroformate (NPC, 97%, Alfa Aesar), cystamine dihydrochloride (cystamine · 2HCl, >98%, Alfa Aesar), zinc bis[bis(trimethylsilyl)amide] (97%, Aldrich), and doxorubicin hydrochloride (DOX · HCl, 99%, Beijing Zhong Shuo Pharmaceutical Technology Development Co. Ltd.) were used as received. Allyl alcohol (98%, Alfa Aesar) was distilled under reduced pressure prior to use. 2,2′-Azobisisobutyronitrile (AIBN, 98%, J&K) was re-crystallized twice from hexane and methanol, respectively. Methoxy poly(ethylene glycol) (PEG, M_n = 5.0 kg/mol) was purchased from Fluka and dried by azeotropic distillation from anhydrous toluene. Dichloromethane (DCM) was dried by refluxing over CaH_2 under an argon atmosphere. Mono-2,4,6-trimethoxybenzylidene-pentaerythritol carbonate (TMBPEC) was synthesized according to our previous report [23].

2.2. Synthesis of PEG-Cystamine (PEG-Cys)

PEG-Cys was obtained as reported previously [40]. Briefly, to a solution of cystamine · 2HCl (1.8 g, 8 mmol) and Et_3N (2.02 g, 20 mmol) in DMSO (15 mL) at room temperature (r.t.) was dropwise added a DMSO solution of PEG-NPC (2.0 g, 0.4 mmol) that was prepared by treating PEG (M_n = 5.0 kg/mol) with NPC in DCM. The reaction mixture was stirred at r.t. for 20 h. PEG-Cys was isolated by extensive dialysis against water (MWCO 1000) followed by lyophilization. Yield: 92%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.95 (–CH_2–SS–CH_2–), 3.19 (–CH_2NH_2), 3.38 (–OCH_3), 3.56 (–CH_2–NH–C(O) –), 3.64 (PEG), 4.23 (–CH_2–O–C(O) –).

2.3. Synthesis of allyl-PTMBPEC

In the glove-box under a nitrogen atmosphere, to a stirred solution of TMBPEC (0.50 g, 1.47 mmol) and allyl alcohol (5.8 mg, 0.1 mmol) in DCM (4.0 mL) was quickly added zinc bis[bis(trimethylsilyl)amide] (20 mg, 0.05 mmol). The reaction vessel was sealed and placed in an oil-bath thermostated at 50 °C. The polymerization was allowed to proceed with magnetic stirring for 4 days. The resulting Allyl-PTMBPEC polymer was isolated by twice precipitation from cold diethyl ether and dried at r.t. *in vacuo*. Yield: 80%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.77–3.82 (Ar–OCH_3), 4.10 (–CH(OCH_2–)$_2$), 4.63 (allyl–CH_2–), 4.74 (– (O)C–O–CH_2CCH_2–O–C(O)–), 5.30 (vinyl protons), 5.98 (Ar–C*H*–), 6.08 (aromatic protons).

2.4. Synthesis of HOOC-PTMBPEC

Under a N_2 atmosphere, to a solution of Allyl-PTMBPEC (0.40 g, 0.1 mmol) in THF (10 mL) was added 3-mercaptopropanoic acid (0.212 g, 2 mmol) and AIBN (0.082 g, 0.5 mmol). The mixture was stirred for 24 h at 65 °C. The resulting polymer was isolated by precipitation from diethyl ether and dried at r.t. *in vacuo*. Yield: 95%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.95 (–CH_2–CH_2–O–C(O)–), 2.76 (–CH_2–COOH), 2.95 (–CH_2–S–CH_2–), 4.25 (–CH_2–CH_2–O–C(O) –).

2.5. Synthesis of amphiphilic PEG-SS-PTMBPEC block copolymer

Under a N_2 atmosphere, to a stirred solution of HOOC-PTMBPEC (70 mg, 17 μmol) and NHS (8.0 mg, 70 μmol) in DCM (10 mL) in an ice bath was dropwise added a DCC (21 mg, 102 μmol) solution in DCM. The reaction was allowed to proceed at r.t. for 20 h. Then, a

solution of PEG-Cys (70 mg, 14 μmol) in DCM was added. The reaction was continued for another 28 h at r.t.. The polymer was isolated by precipitation from cold diethyl ether and washed with ethanol for five times. Finally, PEG-SS-PTMBPEC block copolymer was dried at r.t. *in vacuo*. Yield: 95%. ^{1}H NMR (400 MHz, $CDCl_3$): δ, 2.80–3.0 (–CH_2–SS–CH_2–, –CH_2–S–CH_2–), 3.64 (PEG), 3.77–3.82 (Ar–OCH_3), 4.10 (–CH(OCH_2–)$_2$), 4.74 (– (O)C–O–CH_2CCH_2–O–C(O) –), 5.98 (Ar–C*H*–), 6.08 (aromatic protons).

2.6. Characterization

^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterated chloroform ($CDCl_3$) as a solvent. The chemical shifts were calibrated against residual solvent signals of $CDCl_3$. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using THF as the eluent at a flow rate of 1.0 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns. The size of the micelles was determined by dynamic light scattering (DLS) at 25 °C using a Zetasizer Nano-ZS (Malvern Instruments) equipped with a 633 nm He–Ne laser. The micelles suspension was filtered through a 450 nm syringe filter before measurements. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of a 0.2 mg/mL suspension of the micelles on the copper grid followed by staining with phosphotungstic acid.

2.7. Micelle formation and critical micelle concentration (CMC)

Micelles were prepared under stirring by dropwise adding 2.0 mL of phosphate buffer (PB, 10 mM, pH 7.4) to 0.20 mL of block copolymer solution in THF (0.5 wt.%) at r.t.. The resulting suspension was stirred overnight under reduced pressure to thoroughly remove THF.

CMC was determined using pyrene as a fluorescence probe. The concentration of block copolymer varied from 2.0×10^{-5} to 0.2 mg/mL and the concentration of pyrene was fixed at 1.0 μM. Fluorescence spectra were recorded using a FLS920 fluorescence spectrometer and an excitation wavelength of 330 nm. Fluorescence emissions at 372 and 383 nm were monitored. The CMC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

2.8. pH-dependent hydrolysis of acetal groups in the micelles

The hydrolysis of acetal groups in PEG-SS-PTMBPEC micelles was followed by UV/vis spectroscopy by measuring the absorbance at 290 nm, according to our previous reports [22,23]. PEG-SS-PTMBPEC micelle suspension (0.2 mg/mL) prepared as described above was divided into four aliquots of 2.0 mL, which were adjusted into four different conditions: i.e. (i) PB buffer (100 mM, pH 7.4), (ii) acetate buffer (100 mM, pH 5.0), (iii) PB buffer containing 10 mM GSH (100 mM, pH 7.4), and (iv) acetate buffer containing 10 mM GSH (100 mM, pH 5.0). The suspensions were shaken at 37 °C. At the desired time intervals, 80 μL aliquots were removed and diluted with 3.5 mL PB buffer (100 mM, pH 7.4) and the absorbance at 290 nm was measured. At the end, all the samples were completely hydrolyzed by the addition of two drops of concentrated HCl and measured again to determine the absorbance at 100% hydrolysis, which was used to calculate the extent of acetal hydrolysis.

2.9. pH and redox-triggered change of micelle sizes

The change of micelle sizes in response to pH and/or redox was followed by DLS measurements at 37 °C. PEG-SS-PTMBPEC micelle suspension (0.2 mg/mL) was divided into four aliquots of 1 mL, which were adjusted into four different conditions: *i.e.* (i) PB buffer (100 mM, pH 7.4), (ii) acetate buffer (100 mM, pH 5.0), (iii) PB buffer containing 10 mM GSH (100 mM, pH 7.4), and (iv) acetate buffer containing 10 mM GSH (100 mM, pH 5.0). The suspensions were gently stirred at 37 °C and micelle sizes were monitored in time by DLS. PEG-PTMBPEC micelles were used as a reduction-insensitive control.

2.10. Loading and release of DOX

DOX-loaded micelles were prepared by dropwise adding 5.0 mL of PB buffer (10 mM, pH 7.4) to a mixture of 0.5 mL of copolymer solution in DMF (5.0 mg/mL) and 25, 50 or 100 μL of DOX solution in DMSO (5.0 mg/mL) under stirring at r.t. followed by ultrasonication for 0.5 h and extensive dialysis against PB buffer (10 mM, pH 7.4) (MWCO 3500) at r.t. in the dark.

The *in vitro* release of DOX from PEG-SS-PTMBPEC micelles was investigated at 37 °C under five different conditions, *i.e.* (i) PB buffer (100 mM, pH 7.4), (ii) acetate buffer (100 mM, pH 5.0), (iii) PB buffer containing 10 mM GSH (100 mM, pH 7.4), and (iv) acetate buffer containing 10 mM GSH (100 mM, pH 5.0), and (v) 2 or 4 h in acetate buffer (100 mM, pH 5.0) followed by PB buffer containing 10 mM GSH (100 mM, pH 7.4). DOX-loaded micelle suspension was divided into six aliquots and immediately transferred to a dialysis tube with a MWCO of 12000–14000. The dialysis tube was immersed into 20 mL of appropriate buffer (0.1 M) and shaken at 37 °C. At desired time intervals, 5.0 mL of the release medium was taken out and replenished with an equal volume of fresh medium. To avoid oxidation of GSH, the release media were perfused with nitrogen gas. The concentration of DOX was determined by fluorescence (FLS920) measurements (excitation at 480 nm).

To determine drug loading content (DLC), DOX-loaded micelle suspensions were freeze-dried, dissolved in DMSO and analyzed with fluorescence spectroscopy. A calibration curve was obtained using DOX/DMSO solutions with different DOX concentrations. To determine the amount of DOX released, calibration curves were run with DOX/corresponding buffer solutions with different DOX concentrations at pH 5.0 and 7.4, respectively. The emission at 600 nm was recorded. The release experiments were conducted in triplicate. The results are presented as the average ± SD. The DLC and drug loading efficiency (DLE) were calculated according to the following formula:

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

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

2.11. MTT assay

The cytotoxicity of empty and DOX-loaded PEG-SS-PTMBPEC micelles and PEG-PTMBPEC micelles was studied by MTT assay using HeLa and RAW 264.7 cells. Cells were seeded onto a 96-well plate at a density of 1×10^4 cells per well in 100 μL of Dulbecco's Modified Eagle medium (DMEM) containing 10% FBS and incubated for 24 h (37 °C, 5% CO_2). The medium was removed and replenished by 80 μL of fresh DMEM medium containing 10% FBS. 20 μL of micelle suspensions at different concentrations in PB buffer (10 mM, pH 7.4) were added. The cells were incubated for another 48 h, the medium was aspirated and replaced by 100 μL of fresh medium, and 10 μL of MTT solution (5 mg/mL) was added. The cells were incubated for 4 h, and then 100 μL of DMSO was added to dissolve the resulting purple crystals. The optical densities at 570 nm were measured

using a BioTek microplate reader. Cells cultured in DMEM medium containing 10% FBS (without micelles) were used as controls.

2.12. Confocal microscopy observation of HeLa cells incubated with DOX-loaded micelles

HeLa cells were plated on microscope slides in a 24-well plate (5×10^4 cells/well) using DMEM medium containing 10% FBS. The cells were incubated with prescribed amounts of DOX-loaded micelles or free DOX at 37 °C and 5% CO_2. After incubation for 4 and 8 h, the culture medium was removed and the cells on microscope plates were washed three times with PBS. The cells were fixed with 4% paraformaldehyde and the cell nuclei were stained with Hoechst 33342. Fluorescence images of cells were obtained with a Nikon Digital Eclipse C1si Confocal Laser Scanning Microscope (Nikon).

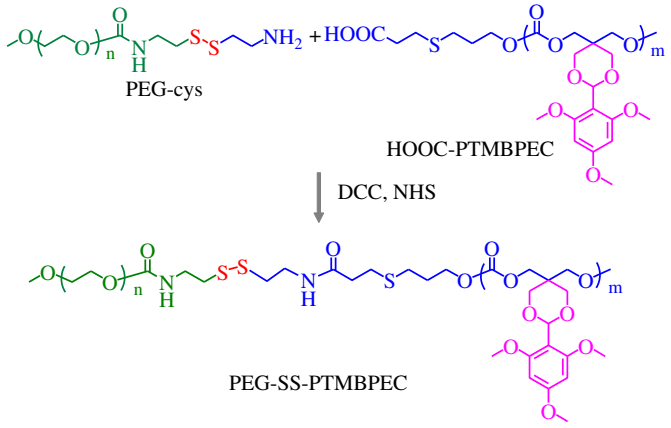
3. Results and discussion

The present study was set to develop reduction and pH dual-bioresponsive biodegradable micelles and investigate their intracellular anti-cancer drug release behaviors in cancer cells. To this end, PEG-SS-PTMBPEC block copolymer micelles were designed, in which pendant acetal bonds in core-forming PTMBPEC block are prone to hydrolysis under endosomal pH conditions while the intermediate disulfide bond linking hydrophilic PEG and hydrophobic PTMBPEC blocks is subject to reductive degradation in the cytosol due to its high reducing potential. We hypothesized that these micellar nanoparticles upon endocytosis would swell due to acetal hydrolysis, triggering partial drug release in endosomes. The swollen nanoparticles following further trafficking to cytoplasm would rapidly collapse leading to complete release of payloads. Thus, reduction and pH dual-bioresponsive biodegradable micelles might induce synergistic effects in enhancing drug release and anti-tumor activity in target cells.

3.1. Synthesis of amphiphilic PEG-SS-PTMBPEC copolymer

PEG-SS-PTMBPEC block copolymer was prepared by coupling reaction between PEG cystamine (PEG-Cys) and carboxyl PTMBPEC (HOOC-PTMBPEC) using carbodiimide chemistry (Scheme 2). PEG-Cys was synthesized by activating hydroxyl end group of PEG (M_n = 5.0 kg/mol) with *p*-nitrophenyl chloroformate (NPC) followed by treating with an excess of cystamine in DMSO (Scheme S1A). ^{1}H NMR indicated quantitative transformation of hydroxyl end into cystamine, as revealed by an integral ratio of close to 4:3 between signals at δ 2.95 (methylene protons of cystamine moiety neighboring to the disulfide bond) and 3.38 (methoxy protons of PEG) (Fig. S1). HOOC-PTMBPEC was obtained by ring-opening polymerization of TMBPEC using allyl alcohol as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst followed by conjugation with 3-mercaptopropanoic acid in the presence of AIBN in THF (Scheme S1B). ^{1}H NMR showed that PTMBPEC with an allyl functional group was successfully synthesized, as revealed by presence of signals at δ 5.30 and 4.6 attributable to vinyl protons and ethylene protons of allyl moieties, respectively (Fig. 1A). ^{1}H NMR end group analysis suggested that allyl-PTMBPEC had an M_n of 4.1 kg/mol, which was close to the theoretical value (4.3 kg/mol). Fig. 1B showed that allyl group has been quantitatively converted into carboxylic group following treatment with 3-mercaptopropanoic acid as revealed by complete disappearance of vinyl protons and detection of new signals at δ 2.95 and 2.76 assignable to the methylene protons next to the thioether and methylene protons neighboring to the carboxylic group, respectively.

The coupling reaction of PEG-Cys and HOOC-PTMBPEC was carried out in DCM at r.t., in the presence of DCC and NHS. The resulting PEG-SS-PTMBPEC block copolymer was isolated by precipitation in



Scheme 2. Synthesis of PEG-SS-PTMBPEC by coupling reaction between PEG-Cys and HOOC-PTMBPEC in DCM at r.t. for 48 h in the presence of DCC and NHS.

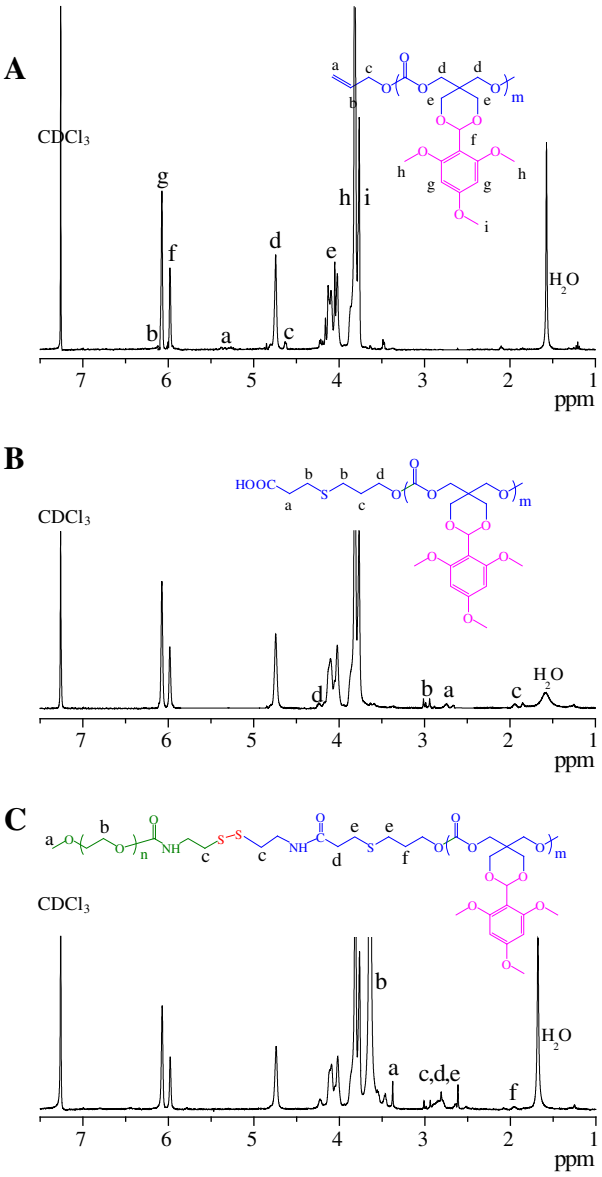


Fig. 1. ^{1}H NMR spectra (400 MHz, $CDCl_3$) of allyl-PTMBPEC (A), HOOC-PTMBPEC (B), and PEG-SS-PTMBPEC (C).

Table 1
Synthesis of PEG-SS-PTMBPEC and PEG-PTMBPEC block copolymers.

Copolymer	Conv.[a] (%)	M_n (kg/mol)			PDI (GPC[b])	CMC[c] (mg/L)
		Design	^{1}H NMR	GPC[b]		
PEG-SS-PTMBPEC	86	5.0–5.0	5.0–4.1	12.8	1.16	0.81
PEG-PTMBPEC	84	5.0–5.0	5.0–3.9	12.3	1.09	0.64

[a] Determined by ^{1}H NMR.
[b] Determined by GPC (eluent: DMF containing 1 wt.% LiBr, flow rate: 0.8 mL/min, standards: polystyrene).
[c] Critical micelle concentration determined using pyrene as a fluorescent probe.

diethyl ether followed by extensive washing with ethanol to remove excess PEG. The results of synthesis are given in Table 1. ^{1}H NMR clearly showed signals characteristic of both PEG and PTMBPEC blocks (Fig. 1C). The integral ratio between resonances at δ 5.98 (acetal protons of PTMBPEC) and 3.38 (methoxy protons of PEG) was close to the theoretical value of 4:1, indicating equivalent coupling of PEG and PTMBPEC. Importantly, gel permeation chromatography (GPC) measurements revealed a unimodal distribution with an M_n of 12.8 kg/mol (polystyrene standards) and a low polydispersity index (PDI) of 1.16 (Table 1), confirming successful synthesis of well-defined PEG-SS-PTMBPEC block copolymer.

PEG-PTMBPEC block copolymer, which was used as a reduction-insensitive control, was synthesized according to our previous report [23], using methoxy PEG-OH (M_n = 5.0 kg/mol) as an initiator and zinc bis[bis(trimethylsilyl)amide] as a catalyst in CH_2Cl_2 at 50 °C. The results showed that PEG-PTMBPEC had a low PDI of 1.09 and an M_n of 5.0–3.9 kg/mol, close to that of PEG-SS-PTMBPEC (Table 1).

3.2. Micelle formation and pH-triggered acetal hydrolysis

Micelles were prepared by solvent exchange method. Dynamic light scattering (DLS) measurements showed that PEG-SS-PTMBPEC formed micellar nanoparticles with an average diameter of ca. 140 nm and a narrow size distribution in phosphate buffer (PB, 10 mM, pH 7.4) (Fig. 2A). TEM revealed a homogeneous distribution of spherical nanoparticles with sizes in accordance with those determined by DLS (Fig. 2B). The critical micelle concentration (CMC) was determined to be 0.81 and 0.64 mg/L for PEG-SS-PTMBPEC and PEG-PTMBPEC block copolymers, respectively, by fluorescence measurements using pyrene as a probe (Table 1).

The hydrolysis of acetals in PEG-SS-PTMBPEC micelles was studied at 37 °C at pH 7.4 or pH 5.0, either in the absence or presence of 10 mM GSH. The extent of acetal hydrolysis was determined according to our previous reports [22,23], using UV/vis spectroscopy by monitoring the absorbance at 290 nm, which is the characteristic absorbance of the hydrolysis product, 2,4,6-trimethoxybenzaldehyde. The results showed that the hydrolysis rate of acetals in PEG-SS-PTMBPEC micelles was highly pH-dependant (Fig. 3). Rapid hydrolysis took place at pH 5.0 with a half-life of 6.5 h, while negligible hydrolysis (less than 10%) was observed at pH 7.4 even after 24 h. It should be noted that a similar acetal half-life of 6.0 h was observed for reduction-insensitive PEG-PTMBPEC micelles at pH 5.0 [23], indicating that introduction of disulfide bond has little influence on pH-sensitive degradation of acetals in micelles. The results showed that presence of GSH (10 mM) had little effect on the rate of acetal hydrolysis in micelles (Fig. 3), implying that PEG shedding caused by cleavage of the disulfide bonds does not alter acetal hydrolysis kinetics.

3.3. Redox and pH-induced size change of PEG-SS-PTMBPEC micelles

The size change of PEG-SS-PTMBPEC micelles in response to acidic pH and 10 mM GSH was studied by DLS. The results showed that PEG-SS-PTMBPEC micelles swelled from 140 nm to about 300 nm in

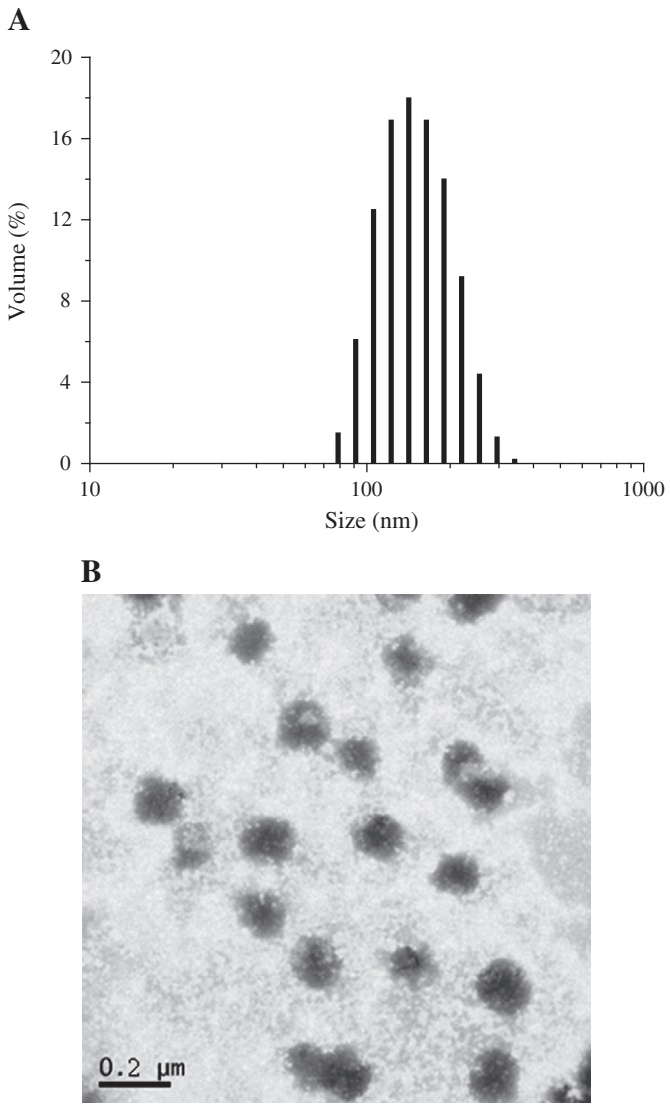


Fig. 2. Size distribution of PEG-SS-PTMBPEC micelles determined by DLS (A) and TEM after staining with phosphotungstic acid (B).

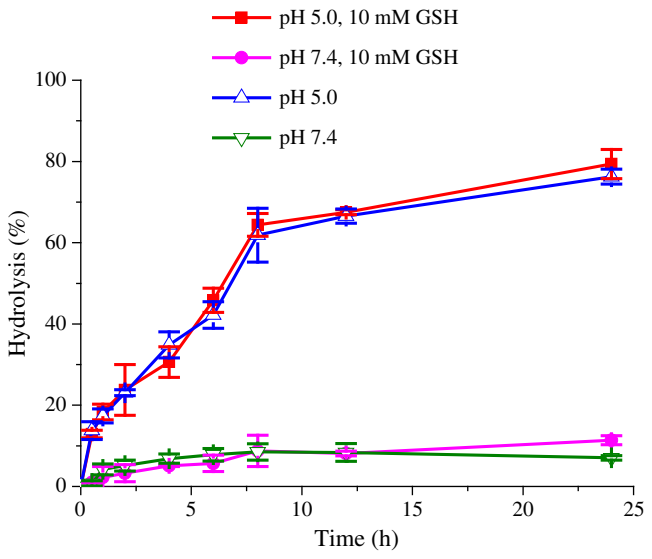


Fig. 3. pH-dependant hydrolysis of acetals in PEG-SS-PTMBPEC micelles with or without 10 mM GSH.

6 h and to over 700 nm in 16 h at pH 5.0 (Fig. 4A), which was similar to that observed for PEG-PTMBPEC micelles (Fig. S2A). This swelling of micelles is due to increased hydrophilicity of micellar core as a result of acetal hydrolysis [22,23]. In contrast, no change of micelle size was observed over 24 h at pH 7.4. Interestingly, significant change of micelle sizes was also observed for PEG-SS-PTMBPEC micelles at pH 7.4 in the presence of 10 mM GSH, wherein micelle sizes increased from 140 nm to 450 nm in 6 h and to over 1000 nm in 16 h (Fig. 4B). This reduction induced fast aggregation behavior has been reported for other reduction-sensitive shell-sheddable micelles such as PEG-SS-PCL and dextran-SS-PCL micelles [28,29]. In comparison, no obvious size change was discerned for reduction-insensitive PEG-PTMBPEC micelles in 16 h under otherwise the same conditions (Fig. S2B).

It should be noted that the fastest size change of PEG-SS-PTMBPEC micelles was observed at pH 5.0 in the presence of 10 mM GSH, in which large aggregates with a diameter over 1000 nm were formed in 6 h (Fig. 4A). These results indicate that redox and pH dual-sensitive micelles likely induce synergistic effects for intracellular drug release. It should further be noted that no precipitation was observed under these conditions, most probably due to enhanced hydrophilicity of core-forming PTMBPEC following acetal hydrolysis [41].

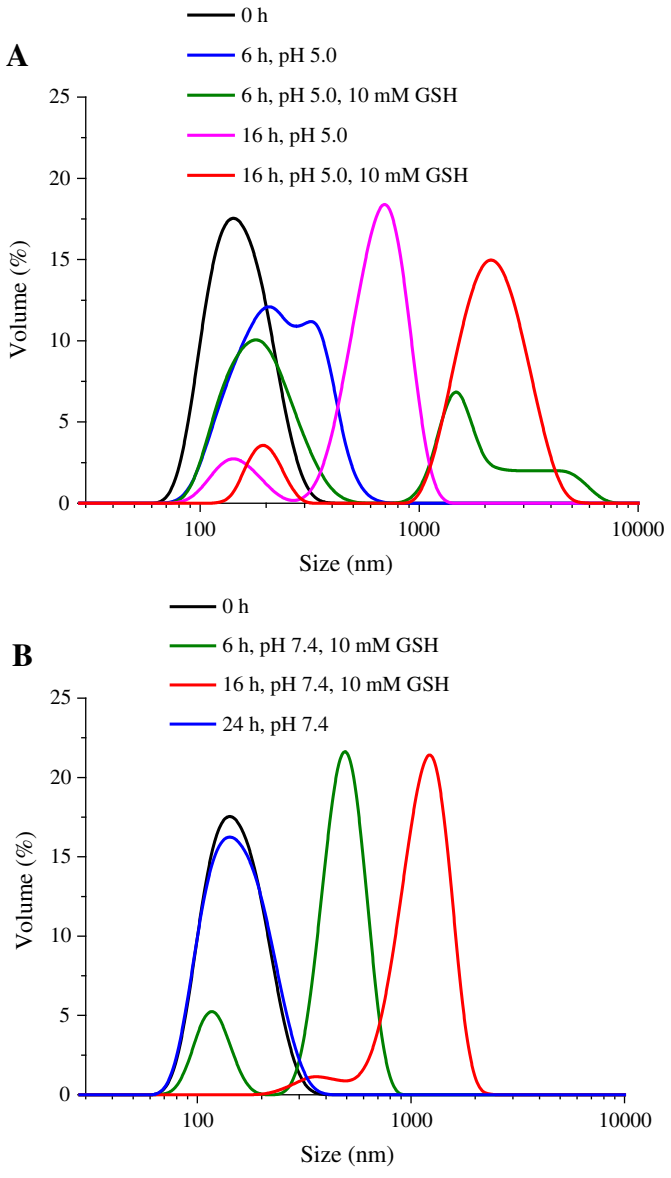


Fig. 4. pH and redox-induced size change of PEG-SS-PTMBPEC micelles in time followed by DLS. (A) pH 5.0; (B) pH 7.4.

3.4. Loading and in vitro release of DOX

DOX is a potent anticancer drug applied for the treatment of varying types of solid malignant tumors in the clinics [42]. DOX was loaded into PEG-SS-PTMBPEC and PEG-PTMBPEC micelles at theoretical drug loading contents (DLC) of 4.8, 9.1 and 16.7 wt.%. The results showed similar drug loading levels for PEG-SS-PTMBPEC and PEG-PTMBPEC micelles, with drug loading efficiencies (DLE) in the range of 58.4% to 66.6% (Table 2). The average diameters of PEG-SS-PTMBPEC micellar particles decreased from 140.2 to 130.3 nm following loading of 3.2 wt.% DOX, and then increased to 186.2 and 196.8 nm with further increasing DOX loading contents to 6.3 and 11.3 wt.%, respectively (Table 2). The analogous trends were also observed for DOX-loaded PEG-PTMBPEC micelles (Table 2).

The *in vitro* release of DOX from PEG-SS-PTMBPEC micelles was investigated at 37 °C under different conditions, *i.e.* (i) pH 7.4, (ii) pH 5.0, (iii) pH 7.4 and 10 mM GSH, (iv) pH 5.0 and 10 mM GSH, and (v) 2 or 4 h at pH 5.0 followed by pH 7.4 and 10 mM GSH (mimicking the intracellular trafficking pathway). The results showed that at physiological pH only ca. 24% DOX was released from DOX-loaded micelles in 21 h (Fig. 5A). The release of DOX was significantly accelerated at pH 5.0 with 62.8% of DOX released in 21 h under otherwise the same conditions, likely due to pH-induced acetal hydrolysis [23]. The release of DOX was also boosted under a reducing environment containing 10 mM GSH at pH 7.4, in which 74.3% of drug was released in 21 h. This redox-triggered drug release behavior has been reported for shell-sheddable PEG-SS-PCL and dextran-SS-PCL micelles [28,29]. Notably, the fastest and most complete drug release was observed at pH 5.0 in the presence of 10 mM GSH wherein 94.2% of DOX was released in 10 h (Fig. 5A). This is in line with the observation that PEG-SS-PTMBPEC micelles were rapidly destabilized under pH 5.0 and 10 mM GSH condition (Fig. 4A). In contrast, DOX release from reduction-insensitive PEG-PTMBPEC micelles was not influenced by presence of GSH both at pH 5.0 and pH 7.4 (Fig. S3). In order to simulate the intracellular trafficking process, drug release studies were performed at pH 5.0 (mimicking the acidic endosomal compartments) for 2 or 4 h and then at pH 7.4 and 10 mM GSH condition (mimicking the reductive environment of cytosols), which has interestingly resulted in faster and more thorough DOX release from PEG-SS-PTMBPEC micelles (ca. 91.5% of DOX released in a total time of 21 h) than at either pH 5.0 or pH 7.4 and 10 mM GSH condition (Fig. 5B). These results clearly indicate that redox and pH dual-sensitive degradable micelles present a synergistic effects in drug release. This is probably because partial hydrolysis of acetals in micellar core in response to acidic pH not only promotes partial DOX release but also improves the hydrophilicity of the micellar core that further facilitates ensuing reduction-triggered drug release.

Table 2
Characteristics of DOX-loaded PEG-SS-PTMBPEC and PEG-PTMBPEC micelles.

Copolymer	DLC (wt.%)		DLE (%)	Size (nm)	PDI
	theory	Determined[a]			
PEG-SS-PTMBPEC	4.8	3.2	66.2	130.3 ± 0.2	0.12
	9.1	6.3	66.6	186.2 ± 0.3	0.15
	16.7	11.3	64.2	196.8 ± 1.2	0.25
PEG-PTMBPEC	4.8	3.1	64.3	132.5 ± 0.7	0.21
	9.1	6.0	62.5	154.2 ± 0.6	0.28
	16.7	10.5	58.4	180.6 ± 1.6	0.30

[a] DOX loading content determined by fluorescence measurements.

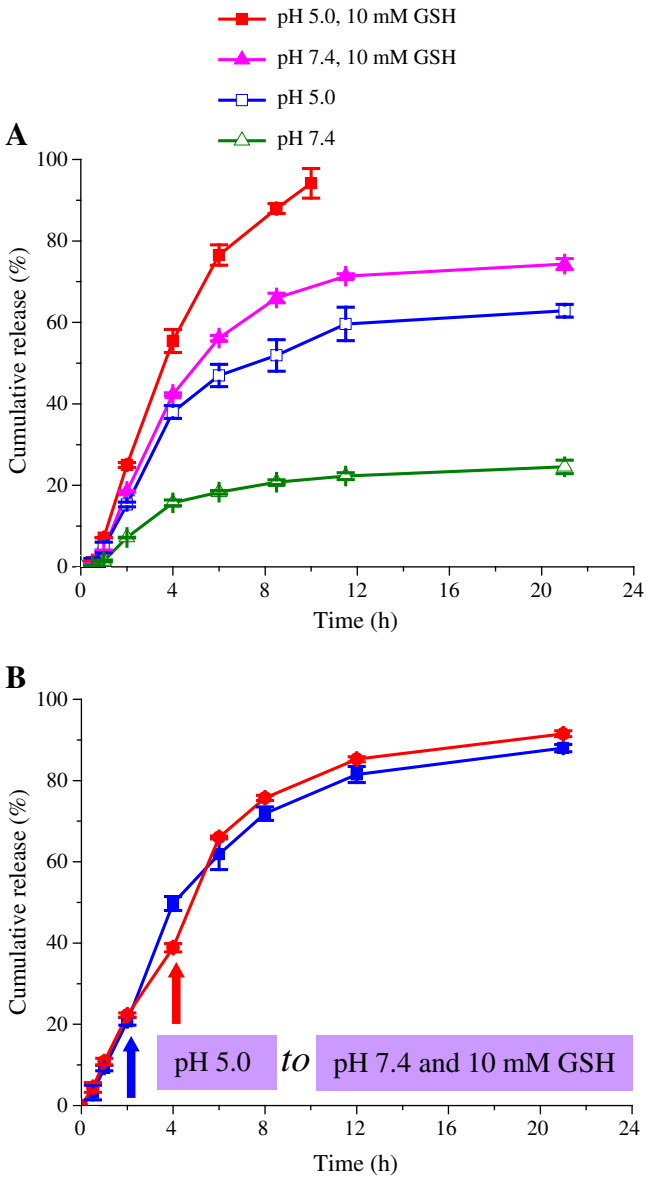


Fig. 5. pH and/or redox-triggered release of DOX from PEG-SS-PTMBPEC micelles at 37 °C. (A) DOX release at pH 7.4 or 5.0 in the presence or absence of 10 mM GSH; and (B) DOX release at pH 5.0 for 2 or 4 h followed by pH 7.4 and 10 mM GSH (mimicking the intracellular trafficking pathway).

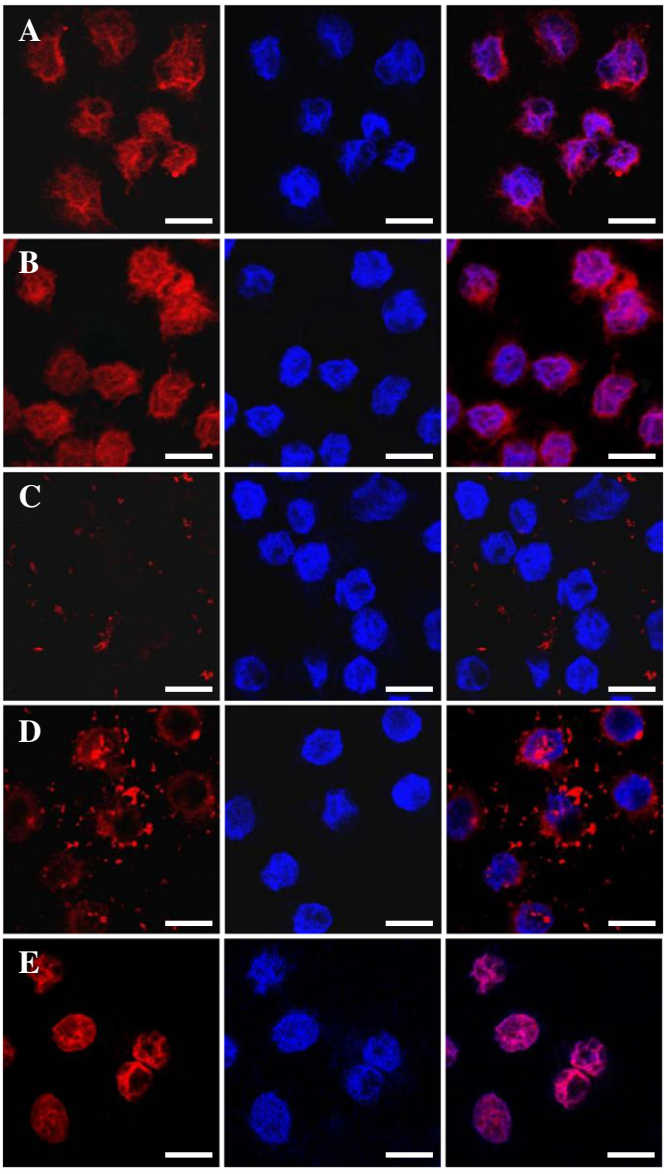


Fig. 6. CLSM images of HeLa cells following 4 or 8 h incubation with DOX-loaded micelles and free DOX (30 μg/mL). For each panel, the images from left to right showed DOX fluorescence in cells (red), cell nuclei stained by Hoechst 33342 (blue), and overlays of both images. The scale bars correspond to 20 μm in all the images. (A) DOX-loaded PEG-SS-PTMBPEC micelles, 4 h; (B) DOX-loaded PEG-SS-PTMBPEC micelles, 8 h; (C) DOX-loaded PEG-PTMBPEC micelles, 4 h; (D) DOX-loaded PEG-PTMBPEC micelles, 8 h; and (E) free DOX, 8 h.

3.5. Intracellular DOX release and antitumor activity

The cellular uptake and intracellular drug release profiles of DOX-loaded PEG-SS-PTMBPEC micelles were investigated in HeLa cells using CLSM. Notably, strong DOX fluorescence was observed in the cytoplasm and nuclei of HeLa cells following 4 h incubation with DOX-loaded PEG-SS-PTMBPEC micelles (Fig. 6A). The DOX fluorescence became even stronger in the cell nuclei at a longer incubation time of 8 h (Fig. 6B), which was notably similar to that observed for HeLa cells following 8 h incubation with free DOX (control experiments) (Fig. 6E). The uptake of DOX by the cell nuclei is crucial because DOX has to intercalate with DNA to induce cell death [43]. In contrast, little DOX fluorescence was observed for HeLa cells incubated for 4 h with DOX-loaded PEG-PTMBPEC micelles (reduction-insensitive control) under otherwise the same conditions (Fig. 6C). The DOX fluorescence though enhanced after 8 h incubation was found mainly in the perinuclei region of cells (Fig. 6D). This different intracellular drug release behaviors between PEG-SS-PTMBPEC and PEG-PTMBPEC micelles are most likely due to fast DOX release from PEG-SS-PTMBPEC micelles following escaping from endosomes as a result of reduction-triggered shell-shedding in the cytosol. In comparison, drug release from PEG-PTMBPEC micelles would be slow in the cytosol (neutral pH), as indicated in Fig. S3. These results have demonstrated that pH and reduction dual-responsive PEG-SS-PTMBPEC micelles mediate more efficient intracellular anticancer drug release than PEG-PTMBPEC micelles.

MTT assays in HeLa and RAW 264.7 cells revealed that PEG-SS-PTMBPEC and PEG-PTMBPEC micelles were practically non-toxic (cell viabilities ≥ 92%) up to a tested concentration of 1.0 mg/mL (Fig. 7), confirming that these degradable micellar particles have good biocompatibility. DOX-loaded PEG-SS-PTMBPEC micelles and DOX-loaded PEG-PTMBPEC micelles, however, displayed significant

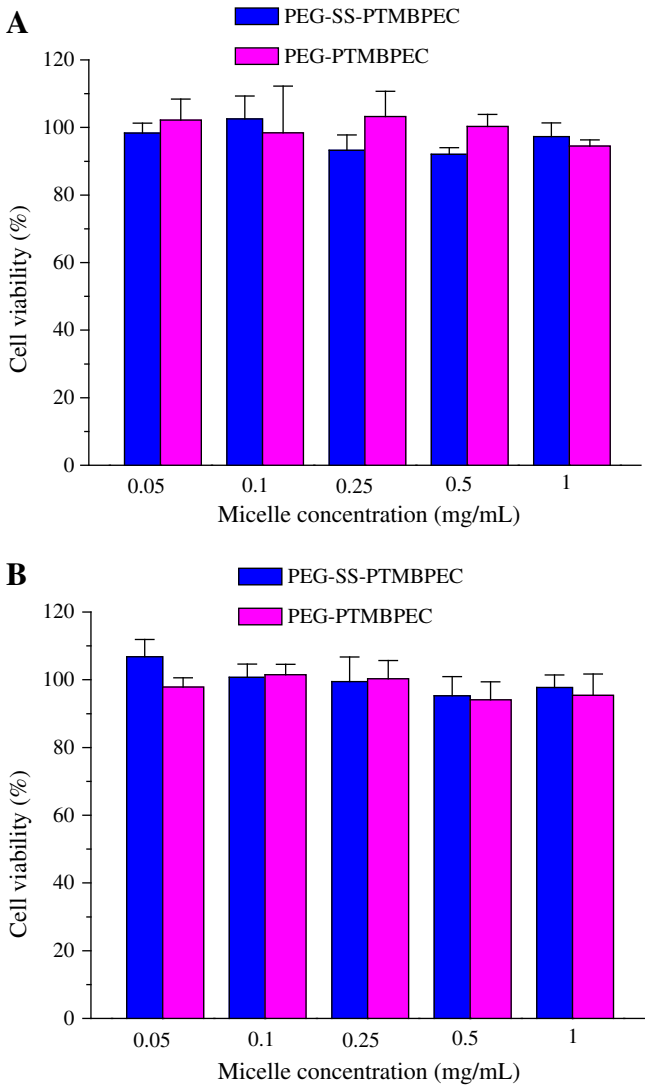


Fig. 7. Cytotoxicity of PEG-SS-PTMBPEC and PEG-PTMBPEC micelles. (A) HeLa cells; (B) RAW 264.7 cells. The cells were incubated with micelles for 48 h. Data are presented as the average ± standard deviation ($n = 4$).

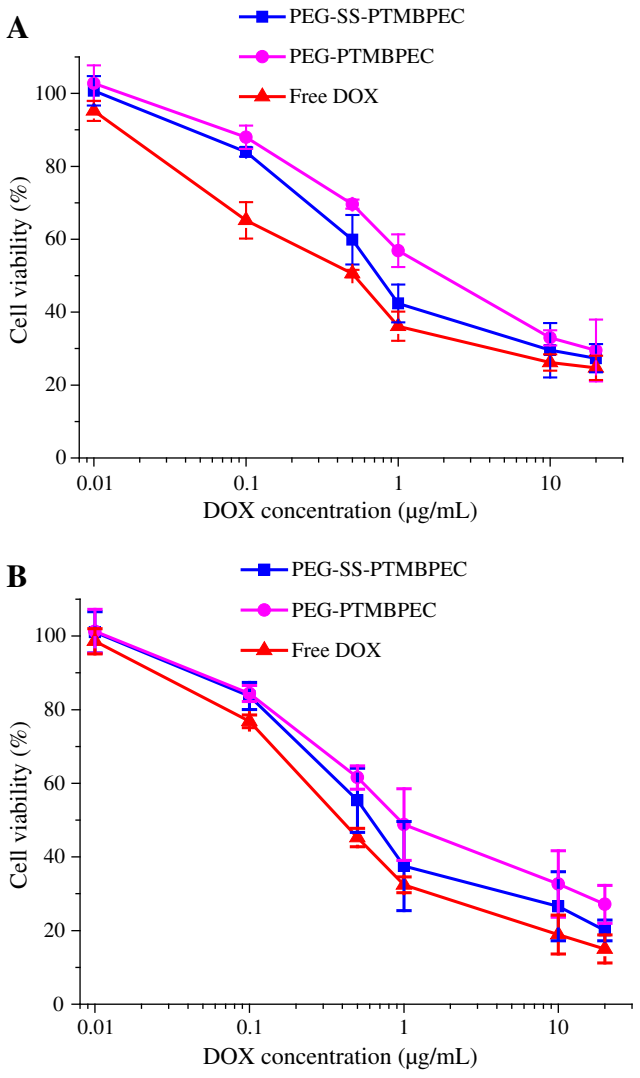


Fig. 8. Anti-tumor activity of DOX-loaded PEG-SS-PTMBPEC micelles, DOX-loaded PEG-PTMBPEC micelles and free DOX as a function of DOX dosages. (A) HeLa cells; (B) RAW 264.7 cells. The cells were incubated with DOX-loaded micelles or free DOX for 48 h. Data are presented as the average ± standard deviation ($n = 4$).

anti-tumor activity towards HeLa and RAW 264.7 cells following 48 h incubation (Fig. 8). It should be noted that DOX-loaded PEG-SS-PTMBPEC micelles had low IC_{50} (half inhibitory concentration) values of 0.75 and 0.60 μg DOX equiv./mL for HeLa and RAW 264.7 cells, respectively, which were lower than those obtained with the reduction-insensitive counterparts under otherwise the same conditions ($IC_{50} = 1.38$ and 0.94 μg DOX equiv./mL for HeLa and RAW 264.7 cells, respectively). This higher anti-tumor activity of PEG-SS-PTMBPEC micelles is in accordance with the confocal microscopy observations that PEG-SS-PTMBPEC micelles mediate fast intracellular drug release as compared to corresponding PEG-PTMBPEC micelles. Interestingly, the anti-tumor activity of DOX-loaded PEG-SS-PTMBPEC micelles, which was approaching to that of free DOX ($IC_{50} = 0.46$ and 0.42 μg DOX equiv./mL for HeLa and RAW 264.7 cells, respectively), was much higher than that reported for other DOX-loaded degradable block copolymer micelles (27 μg/mL [44], 15 μg/mL [45], 3.2 μg/mL [46]). It should further be noted that the anti-tumor activity of DOX-loaded PEG-SS-PTMBPEC micelles might be further enhanced by installing a targeting ligand such as folic acid, aptamer, peptide, and antibody fragment that facilitates efficient and specific cellular uptake of micelles. These redox and pH-sensitive degradable micelles with dually activated intracellular drug release property are highly promising for targeted cancer therapy.

4. Conclusions

We have demonstrated that reduction and pH dual-responsive degradable micelles based on PEG-SS-PTMBPEC copolymer efficiently deliver and release doxorubicin into cancer cells, resulting in superior anti-tumor activity. This represents a first design and study of degradable block copolymer micelles that respond to endosomal pH as well as cytoplasmic glutathione for enhanced intracellular anticancer drug release. These intelligent micelles have several interesting features: (i) they have low cytotoxicity and are degradable; (ii) they show decent drug loading levels; (iii) they are sufficiently stable with low drug release (only about 24% drug release in 21 h) under physiological conditions (pH 7.4, 37 °C); and (iv) they promote fast and maximum drug release inside the cancer cells in that drug release is activated during the whole intracellular trafficking process, i.e. not only in the mildly acidic endosomal compartments but also under the highly reducing cytoplasms and cell nuclei. These reduction and pH dual-responsive biodegradable micelles provide an interesting platform for targeted and controlled intracellular release of potent chemotherapeutics.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.01.001.

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