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Core-crosslinked pH-sensitive degradable micelles: A promising approach to resolve the extracellular stability versus intracellular drug release dilemma

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ABSTRACT

The extracellular stability versus intracellular drug release dilemma has been a long challenge for micellar drug delivery systems. Here, core-crosslinked pH-sensitive degradable micelles were developed based on poly(ethylene glycol)-*b*-poly(mono-2,4,6-trimethoxy benzylidene-pentaerythritol carbonate-*co*-acryloyl carbonate) (PEG-*b*-P(TMBPEC-*co*-AC)) diblock copolymer that contains acid-labile acetal and photo-crossslinkable acryloyl groups in the hydrophobic polycarbonate block for intracellular paclitaxel (PTX) release. The micelles following photo-crosslinking while displaying high stability at pH 7.4 were prone to rapid hydrolysis at mildly acidic pHs of 4.0 and 5.0, with half lives of ca. 12.5 and 38.5 h, respectively. Notably, these micelles showed high drug loading efficiencies of 76.0–93.2% at theoretical PTX loading contents of 5–15 wt.%. Depending on drug loading contents, PTX-loaded micelles had average sizes varying from 132.2 to 171.6 nm, which were decreased by 17–22 nm upon photo-crosslinking. The in vitro release studies showed that PTX release at pH 7.4 was greatly inhibited by crosslinking of micelles. Notably, rapid drug release was obtained under mildly acidic conditions, in which 90.0% and 78.1% PTX was released in 23 h at pH 4.0 and 5.0, respectively. MTT assays showed that PTX-loaded crosslinked micelles retained high anti-tumor activity with a cell viability of 9.2% observed for RAW 264.7 cells following 72 h incubation, which was comparable to PTX-loaded non-crosslinked counterparts (cell viability 7.5%) under otherwise the same conditions, supporting efficient drug release from PTX-loaded crosslinked micelles inside the tumor cells. These core-crosslinked pH-responsive biodegradable micelles with superior extracellular stability and rapid intracellular drug release provide a novel platform for tumor-targeting drug delivery.

1. Introduction

The past decade has witnessed an exploding advancement in biodegradable micelles for controlled release of hydrophobic anti-cancer drugs such as paclitaxel (PTX) and doxorubicin (DOX) [1–4]. A couple of micellar drugs (e.g. NK911® and Genexol-PM®) have been approved for different phases of clinical trials in Japan and South Korea [5,6]. The micelles have been shown to greatly improve drug water solubility as well as increase drug accumulation at the tumor sites via the enhanced permeability and retention (EPR) effect, resulting in better drug bioavailability and reduced side effects [7,8].

The self-assembled micellar carriers, however, often expose inadequate in vivo stability, which leads to premature drug release following i.v. injection [4,9]. The stability of micelles can be improved by crosslinking the micellar core or shell [10–12]. The work of Kissel [13], Hennink [14–16], Jing [17] and Zhong [18] groups on crosslinked biodegradable micelles showed that crosslinking of micelles have the benefits of high drug loading efficiency, superior stability against dilution, prolonged circulation time, and enhanced drug accumulation at the tumor site. We recently reported that ligand-decorated photo-crosslinked biodegradable micelles exhibit improved in vivo targetability, enhanced uptake by specific tumor cells, and greater anti-tumor effects as compared to the non-crosslinked counterparts [19,20]. It should be noted, nevertheless, that crosslinking of biodegradable micelles would also adversely influence drug release in the tumor tissues and cells, leading to compromised therapeutic outcomes.

The inferior drug release inside the tumor cells is one of the key reasons for low therapeutic efficacy of drug-loaded biodegradable micelles [21]. Gao and coworkers [22] reported that drug is released very slowly (over a few weeks) from biodegradable micelles via a diffusion-controlled mechanism. The intracellular drug release could be enhanced using designed bio-responsive (such as pH and

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reduction-sensitive) biodegradable micelles [23–25]. In particular, pH-responsive micelles have attracted great attention due to existence of mildly acidic pH in the tumor tissues as well as in the endo/lysosomal compartments of cells [26,27]. Over the past decade, different pH-responsive micelles have been developed based on acid-labile bonds such as ortho ester, hydrazone, cis-acotinyl, and acetal for enhanced intracellular drug release [28–36]. We recently reported that biodegradable micelles and polymersomes based on poly(ethylene glycol)-*b*-poly(mono-2,4,6-trimethoxy benzylidene-pentaerythritol carbonate) (PEG-*b*-PTMBPEC) block copolymers are prone to rapid hydrolysis thereby "actively" releasing drug under endo/lysosomal pH conditions [37,38].

For in vivo applications, the ideal micelles should be stable with minimal drug leakage during circulation while quickly releasing payloads after arriving at the tumor site [39]. In this paper, we report on novel design and preparation of core-crosslinked pH-sensitive degradable micelles based on poly(ethylene glycol)-*b*-poly(mono-2,4,6-trimethoxy benzylidene-pentaerythritol carbonate-*co*-acryloyl carbonate) (PEG-*b*-P(TMBPEC-*co*-AC)) diblock copolymer for targeted intracellular paclitaxel (PTX) release (Scheme 1). The acryloyl groups in the hydrophobic biodegradable polycarbonate block would facilitate photo-crosslinking resulting in steady micelles [19,20], while the pH-sensitive hydrolysis of acetal groups would result in fast intracellular drug release. In contrast to the previous work by Hennink and coworkers [14] on core-crosslinked biodegradable thermosensitive micelles that swell and release drug upon hydrolysis of oligolactate side chains, present crosslinked micelles are based on biodegradable polycarbonate backbone and acid-labile acetal side-chains that preferentially degrade at low pH. Here, the preparation of crosslinked pH-sensitive degradable micelles, loading and pH-responsive release of PTX, as well as anti-tumor activity of PTX-loaded crosslinked micelles were investigated.

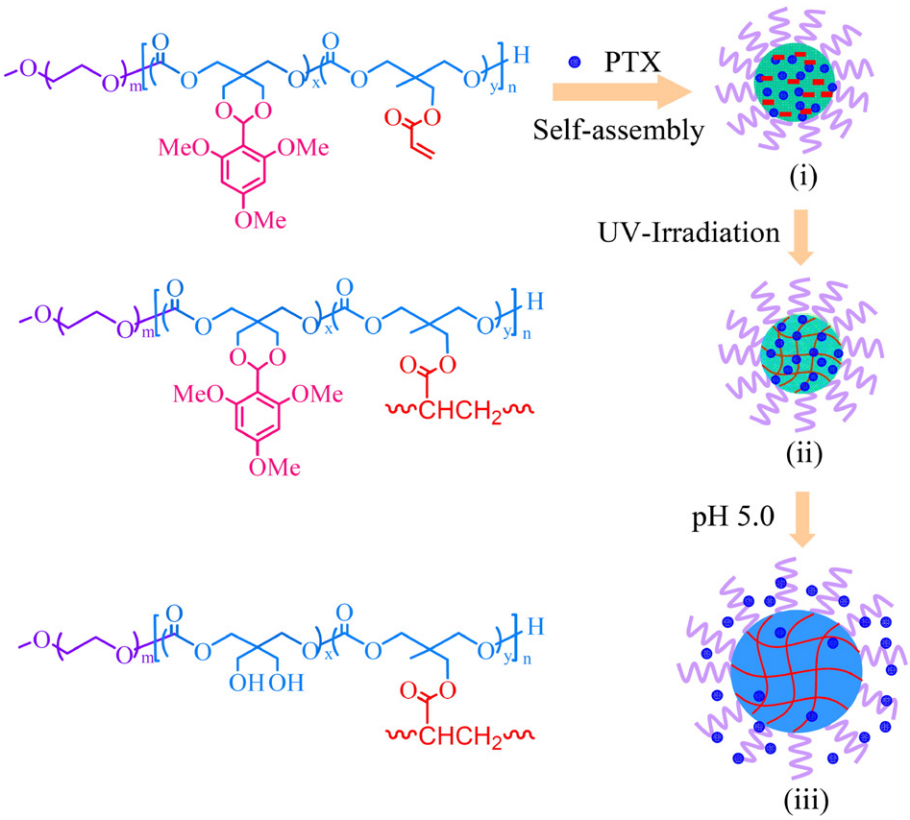
2. Materials and methods

2.1. Materials

Methoxy poly(ethylene glycol) (PEG, $M_n = 5.0$ kg/mol, PDI = 1.03, Fluka) was dried by azeotropic distillation from dry toluene. Dichloromethane (DCM) was dried by refluxing over CaH_2 and distilled prior to use. Zinc bis[bis(trimethylsilyl) amide] (97%, Aldrich), 1-[4-(2-hydroxy ethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone (Irgacure 2959 or I2959, 98%, Sigma), and paclitaxel (PTX, > 99%, Beijing Zhongshuo Pharmaceutical Technology Development) were used as received. Mono-2,4,6-trimethoxybenzylidene-pentaerythritol carbonate (TMBPEC) and acryloyl carbonate (AC) monomers were synthesized according to our previous reports [37,40]. The dialysis membranes (Spectra/Pore, MWCO 3500 and 12000–14000) were purchased from Spectrum Laboratories.

2.2. Synthesis of PEG-b-P(TMBPEC-co-AC) diblock copolymer

PEG-*b*-P(TMBPEC-*co*-AC) diblock copolymer was obtained by ring-opening polymerization of TMBPEC and AC using PEG (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. Briefly, in a glove-box under a nitrogen atmosphere, zinc bis[bis(trimethylsilyl)amide] (29 mg, 75 μmol) was quickly added to a stirred solution of PEG (0.50 g, 100 μmol), TMBPEC (0.40 g, 1.17 mmol) and AC (0.20 g, 1.0 mmol) in DCM (4.0 mL). The reaction vessel was sealed and placed into an oil-bath thermostated at 50 °C. The polymerization was allowed to proceed with magnetic stirring for 5 d. The resulting PEG-*b*-P(TMBPEC-*co*-AC) copolymer was isolated by twice precipitation from cold diethyl ether, filtration and



Scheme 1. Illustration of photo-crosslinkable pH-sensitive degradable micelles based on PEG-*b*-P(TMBPEC-*co*-AC) block copolymer. PTX-loaded crosslinked pH-sensitive degradable micelles exhibit superior extracellular stability while "actively" release PTX under a mildly acidic condition mimicking that of the endo/lysosomal compartments.

drying in vacuo at room temperature. Yield: 76.3%. ^{1}H NMR (400 MHz, $CDCl_3$): PEG: δ 3.38, 3.65; AC moieties: δ 6.47, 5.85, 4.14, 1.08; TMBPEC moieties: δ 6.08, 5.98, 4.74, 3.99, 3.82. M_n (^{1}H NMR) = 9.2 kg/mol, M_n (GPC) = 9.8 kg/mol, PDI = 1.06.

2.3. Characterization

The ^{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. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatography (GPC) instrument equipped with two linear PLgel columns 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 micelle size was determined using dynamic light scattering (DLS) at 25 °C using Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He–Ne laser using back-scattering detection. The amount of PTX was determined by HPLC (Waters 1525) with UV detection at 227 nm using a mixture of acetonitrile and water (v/v = 1/1) as a mobile phase. 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 PTX-loaded crosslinked micelle dispersion on the copper grid followed by staining with phosphotungstic acid.

2.4. Micelle formation and critical micelle concentration (CMC)

Micelles were prepared by the solvent exchange method. Typically, under stirring, 4.0 mL of phosphate buffer (PB, 10 mM, pH 7.4) was dropwise added to 1.0 mL of block copolymer solution in DMF (5.0 mg/mL) at room temperature. The resulting micelles were dialyzed against PB (10 mM, pH 7.4) for 12 h (Spectra/Pore, MWCO 3500).

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

2.5. Photo-crosslinking of micelles

The micelles were crosslinked in the presence of a biocompatible UV initiator, 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propanone (Irgacure 2959 or I2959). Typically, 50 μL of I2959 solution in acetone was introduced to PEG-*b*-P(TMBPEC-*co*-AC) micelle dispersion (1.0 mL, 0.67 mg/mL), resulting in a final I2959 concentration of 0.05 wt.%. The mixture was stirred at 37 °C for 3 h to evaporate acetone. Then, the micelle dispersion was irradiated under the UV light (Intelli-Ray 400, Uvitron) at an intensity of 100 mW/cm^2 for 10 min to give crosslinked micelles. The sizes and stability of crosslinked micelles were studied using DLS.

2.6. pH-dependent hydrolysis rate of acetals in non-crosslinked and crosslinked micelles

The acetal hydrolysis was followed by UV/vis spectroscopy by measuring the absorbance at 290 nm, according to our previous reports [37,38]. The non-crosslinked and crosslinked micelle dispersions prepared (1.76 mg/mL) as above were divided into three aliquots. Their pHs were adjusted to 4.0, 5.0, and 7.4 by adding 50 μL of 4.0 M pH 4.0 acetate buffer, pH 5.0 acetate buffer and pH 7.4 PB buffer, respectively. The dispersions were shaken at 37 °C. At the desired time intervals, 40 μL aliquot was removed and diluted with 1.75 mL of PB (0.1 M, pH 7.4). The absorbance at 290 nm for both micelles was measured. At the end, all the samples were completely hydrolyzed by the addition of two drops of concentrated HCl and were measured again to determine the absorbance at 100% hydrolysis, which was used to calculate extent of acetal hydrolysis.

2.7. Loading and pH-responsive release of PTX

PTX-loaded micelles were prepared by dropwise adding 4.0 mL of PB (10 mM, pH 7.4) to a mixture of 1.0 mL of block copolymer solution in DMF (5.0 mg/mL) and 100 or 200 μL of PTX solution in DMF (5.0 mg/mL), sonicating for 1 h, and dialyzing against PB (10 mM, pH 7.4) for 12 h at room temperature (MWCO 3500) with 5 times change of dialysis media to remove free PTX. The micelles were crosslinked as described above by UV irradiation. The release of PTX during photo-crosslinking process was negligible. To determine drug loading content (DLC) and drug loading efficiency (DLE), 50 μL of PTX-loaded non-crosslinked micelle dispersion was freeze-dried, the residue was dissolved in acetonitrile, and the amount of PTX was determined by HPLC (Waters 1525) with UV detection at 227 nm using a 1/1 (v/v) mixture of acetonitrile and water as a mobile phase. The DLC and DLE were determined according to the following formula:

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

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

The release profiles of PTX from micelles were studied at 37 °C in three different media (10 mM), i.e. (a) acetate buffer, pH 4.0; (b) acetate buffer, pH 5.0; and (c) PB, pH 7.4. The above prepared PTX-loaded micelle dispersions were divided into three aliquots. The pH of micelle dispersions was adjusted to pH 4.0 or 5.0 using acetate buffer or maintained at pH 7.4 using PB. 1.0 mL of PTX-loaded micelle dispersions (final concentration 32 μg/mL) was immediately transferred to a dialysis tube (MWCO 12000–14000). The dialysis tube was immersed into 20 mL of corresponding buffer (10 mM). The media was stirred at 37 °C. At desired time intervals, 7.0 mL of release media was taken out for HPLC measurement and replenished with an equal volume of fresh media. The amount of PTX was determined by HPLC (Waters 1525) with UV detection at 227 nm using a 1/1 (v/v) mixture of acetonitrile and water as a mobile phase. The release experiments were conducted in triplicate. The results presented are the average data.

2.8. MTT assays

The cytotoxicity of crosslinked PEG-*b*-P(TMBPEC-*co*-AC) micelles was studied by MTT assays using MCF-7 and RAW 264.7 cells. In brief, cells were plated in a 96-well plate (5×10^4 cells/well) in 90 μL of Dulbecco's Modified Eagle medium (DMEM) containing 10% FBS and 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL) for 12 h. 10 μL of micelle dispersion in PB (10 mM, pH 7.4) at varying micelle concentrations from 15 to 900 μg/mL was added. The cells were cultured at 37 °C under an atmosphere containing 5% CO_2 for another 72 h. Then, 10 μL of MTT solution in PBS (5.0 mg/mL) was added and incubated for another 4 h at 37 °C. The medium was aspirated, and the MTT-formazan generated by live cells was dissolved in 150 μL of DMSO. The absorbance at a wavelength of 490 nm of each well was measured using a microplate reader (Biorad, ELX808IU). The 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 anti-tumor activities of PTX-loaded non-crosslinked micelles, PTX-loaded crosslinked micelles and free PTX were also studied by MTT assays. MCF-7 and RAW 264.7 cells were plated in a 96-well

plate (5×10^4 cells/well) in 90 μL of DMEM containing 10% FBS and 1% L-glutamine, antibiotics penicillin (100 IU/mL), and streptomycin (100 μg/mL) for 12 h. The cells were incubated with PTX-loaded crosslinked micelles, PTX-loaded non-crosslinked micelles, and free PTX (drug dosage: 6 μg/mL), respectively, at 37 °C under an atmosphere containing 5% CO_2 for one or three days. Ethanol was used to solubilize PTX. Then, 10 μL of MTT solution in PBS (5.0 mg/mL) was added and incubated for another 4 h at 37 °C. The medium was aspirated, and the MTT-formazan generated by live cells was dissolved in 150 μL of DMSO, and the absorbance at a wavelength of 490 nm of each well was measured using a microplate reader (Biorad, ELX808 IU). The 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).

3. Results and discussion

3.1. Synthesis of PEG-b-P(TMBPEC-co-AC) diblock copolymer

The aim of this study was to develop robust pH-sensitive degradable micelles for efficient intracellular release of anti-cancer drugs, for which novel PEG-*b*-P(TMBPEC-*co*-AC) diblock copolymer containing photo-crosslinkable acryloyl groups and acid-labile acetal groups in the hydrophobic degradable polycarbonate block was designed. The crosslinking of micelles via UV irradiation was hypothesized to offer superior micellar stability preventing premature drug release and enhancing their accumulation at the tumor sites, whereas acid degradable acetals were instrumental to rapid intracellular drug release following taking up by tumor cells.

PEG-*b*-P(TMBPEC-*co*-AC) copolymer was prepared by ring-opening copolymerization of two cyclic carbonate monomers, mono-2,4,6-trimethoxybenzylidene-pentaerythritol carbonate (TMBPEC) and acryloyl carbonate (AC), using PEG (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 for 5 days (Scheme 2). The polymerization proceeded slowly due to steric hindering effect of large 2,4,6-trimethoxybenzylidene substituent [37,40]. ^{1}H NMR spectrum of PEG-*b*-P(TMBPEC-*co*-AC) copolymer showed besides signals attributable to PEG (δ 3.65, 3.38) also peaks due to AC moieties (δ 6.47, 5.85, 4.14, 1.08) and TMBPEC moieties (δ 6.08, 5.98, 4.74, 3.99, 3.82) (Fig. 1). The molecular weight of P(TMBPEC-co-AC) block was determined to be 4.2 kg/mol, among which are 3.6 kg/mol for TMBPEC and 0.6 kg/mol for AC units, by comparing the integrals of signals at δ 5.98 (acetal proton of TMBPEC moieties) and 5.85/6.47 (acryloyl protons of AC moieties) to 3.38 (methyl protons of PEG terminal), respectively. There was on average about three AC units per molecule. The molecular weight of PTMBPEC was close to the theoretical value of 4.0 kg/mol while that of PAC was lower than the designed (2.0 kg/mol). GPC revealed that resulting copolymer had a low polydispersity of 1.06 and an M_n of 9.8 kg/mol, close to that determined by ^{1}H NMR (9.2 kg/mol), confirming controlled synthesis of PEG-*b*-P(TMBPEC-*co*-AC) diblock copolymer.

3.2. Preparation and hydrolytic degradation of photo-crosslinked pH-sensitive degradable micelles

Micelles were prepared via solvent exchange method. Dynamic light scattering (DLS) showed that PEG-*b*-P(TMBPEC-*co*-AC) copolymer formed monodisperse micelles with an average diameter of approximately 94.3 nm and a low polydispersity (PDI) of about 0.08 (Table 1, Entry 1). The critical micelle concentrations (CMC) of PEG-*b*-P(TMBPEC-*co*-AC) copolymer determined using pyrene as a fluorescence probe were shown to be approximately 6.2 mg/L.

The micellar core was crosslinked by UV irradiation (100 mW/cm^2) in the presence of biocompatible photo-initiator I2959 (0.05 wt.%) in PB (pH 7.4, 10 mM). Notably, the average size of micelles following photo-irradiation was reduced by ca. 23 nm (Table 1, Entry 1), indicating that crosslinking of micelles has taken place. These crosslinked micelles displayed good colloidal stability with constant particle sizes and size distributions in PBS (pH 7.4) over a period of 4 weeks. Further stability studies showed that crosslinked micelles maintained similar size distribution even after 5000 times dilution (mimicking i.v. injection), while significant aggregation was observed for the non-crosslinked micelles (Fig. 2A). Moreover, unlike non-crosslinked micelles that were completely dissociated into unimers upon addition of 4-fold THF, crosslinked micelles just swelled (Fig. 2B). These results confirmed that micelles were successfully crosslinked by UV irradiation and crosslinked micelles had enhanced colloidal stability.

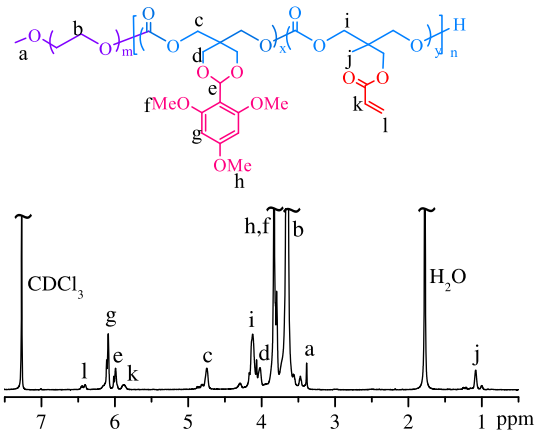
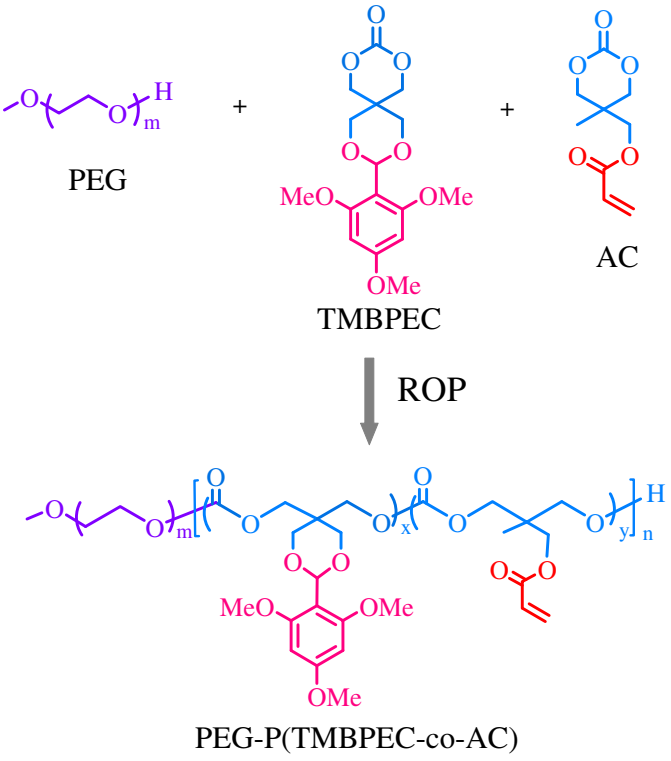


Fig. 1. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of PEG-*b*-P(TMBPEC-*co*-AC) copolymer.

The hydrolysis of acetals in P(TMBPEC-co-AC) within the micelle core was investigated at different pHs (i.e. pH 4.0, 5.0 and 7.4) at 37 °C. The extent of acetal hydrolysis was determined as reported by Frechet and



Scheme 2. Synthesis of PEG-*b*-P(TMBPEC-*co*-AC) diblock copolymer by ring-opening copolymerization of mono-2,4,6-trimethoxybenzylidene-pentaerythritol carbonate (TMBPEC) and acryloyl carbonate (AC) using PEG (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 for 5 days.

Table 1
PTX-loaded crosslinked pH-sensitive degradable micelles.

Entry	Theoretical drug loading content (wt.%)	PTX-NCLM		PTX-CLM		DLC (wt.%)[b]	DLE (%)[b]
		Size (nm)[a]	PDI[a]	Size (nm)[a]	PDI[a]		
1	0	94.3 ± 1.0	0.08	71.7 ± 0.3	0.053	–	–
2	5	131.2 ± 1.3	0.11	114.2 ± 0.5	0.081	4.45	93.2
3	10	152.3 ± 1.1	0.12	135.3 ± 0.5	0.079	8.36	91.2
4	15	171.6 ± 1.3	0.15	149.3 ± 0.7	0.087	10.2	76.0

[a] The average size and polydispersity index (PDI) of micelles determined by DLS measurements.
[b] The drug loading content (DLC) and drug loading efficiency (DLE) determined by HPLC analyses.

coworkers [31,32] using UV/vis spectroscopy by monitoring the absorbance at 290 nm, which are the characteristic absorbance of the hydrolysis product, 2,4,6-trimethoxybenzaldehyde. The results showed that the hydrolysis rates of acetals in both crosslinked and non-crosslinked micelles were highly pH dependent (Fig. 3). The crosslinked micelles exhibited in general somewhat slower acetal degradation than the non-crosslinked counterparts under otherwise the same conditions. The acetals in the non-crosslinked micelles had short half-lives of 5.0 and 28.0 h at pH 4.0 and 5.0, respectively, while crosslinked micelles showed acetal half-lives of 12.5 h and 38.5 h at pH 4.0 and 5.0, respectively. In contrast, only about 20.5% and 10.0% acetal hydrolysis was observed after 4 days at pH 7.4 for non-crosslinked and crosslinked micelles, respectively.

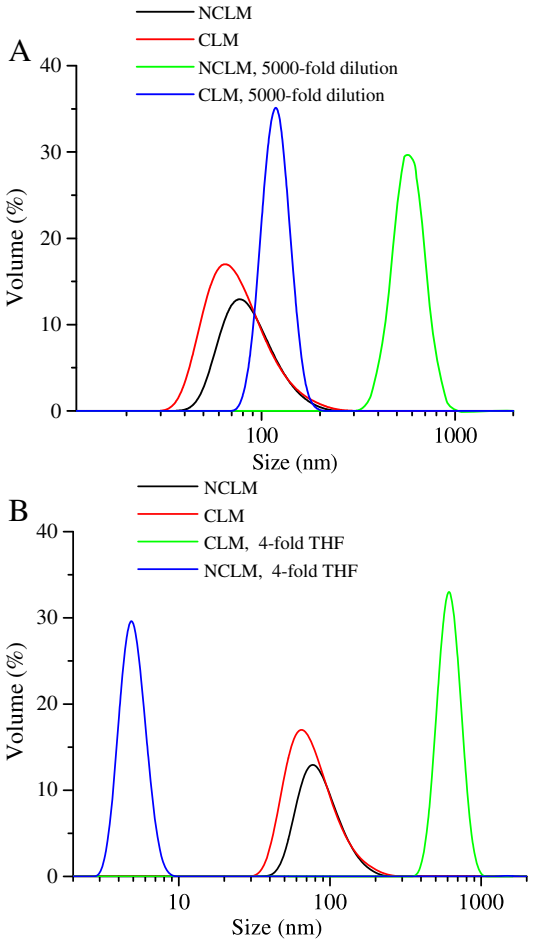


Fig. 2. Stability of non-crosslinked micelles (NCLM) and crosslinked micelles (CLM) against 5000-fold dilution (A) and addition of 4-fold THF (B), as measured by DLS. The initial micelle concentration was 0.75 mg/mL.

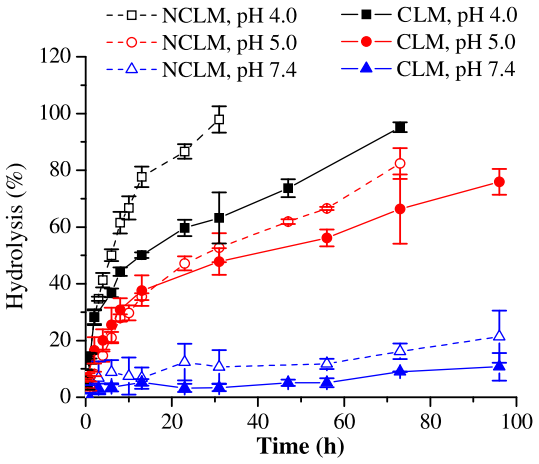


Fig. 3. pH-dependent hydrolysis of acetals in non-crosslinked and crosslinked micelles.

The size change of non-crosslinked and crosslinked micelles in response to acetal hydrolysis was monitored by DLS. Interestingly, non-crosslinked micelles swelled drastically, with micelle size increasing from ca. 94 nm to ca. 1000 and 1400 nm, following 23 and 71 h incubation, respectively, at pH 4.0 (0.1 M acetate buffer) (Fig. 4), due to acid-triggered acetal hydrolysis that leads to increased hydrophilicity of micellar core. It has been reported previously that hydroxy polycarbonate derived from pentaerythritol is highly hydrophilic but not soluble in water [41], which could explain the maintenance of micellar structures even after complete acetal hydrolysis. In contrast, small size increase from ca. 71 nm to ca. 82 nm was observed for crosslinked micelles in 23 h under otherwise the same conditions. At prolonged incubation time (e.g. 71 h), small portion of large-sized particles (ca. 1000 nm) was detected, likely due to significant degradation of micelle core. It should be noted that the polyacrylate formed after core-crosslinking is essentially non-biodegradable, which could be a subject for further improvement.

3.3. Preparation and pH-responsive drug release of PTX-loaded crosslinked micelles

In the following, loading and in vitro release of PTX were studied. The theoretical PTX loading contents were set at 5, 10 and 15 wt.%. The results showed high PTX loading efficiencies of 76.0–93.2% (Table 1, Entries 2–4). The sizes of PTX-loaded micelles increased from 131.2 to 171.6 nm with increasing PTX loading contents from 4.45 to 10.2 wt.% while PDI remained low (0.11–0.15). PTX-loaded micelles were readily crosslinked

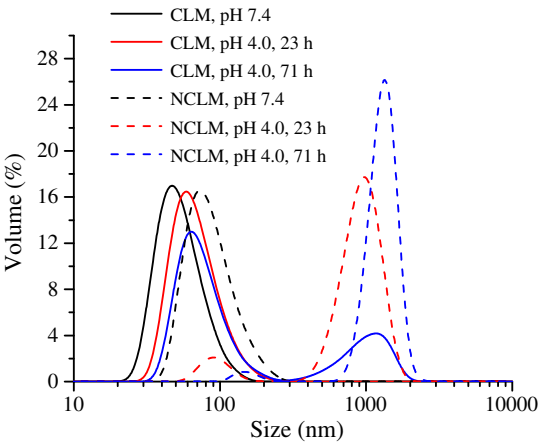


Fig. 4. Size change of non-crosslinked and crosslinked micelles at pH 4.0 (0.1 M acetate buffer) and 37 °C monitored by DLS.

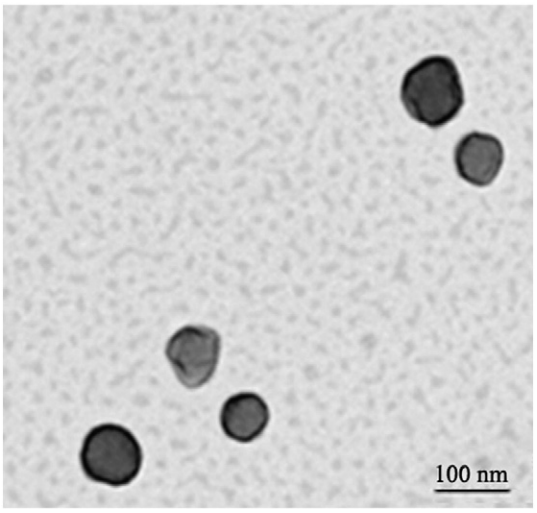


Fig. 5. TEM micrograph of PTX-loaded core-crosslinked pH-sensitive degradable micelles (Table 1, Entry 2).

by 5 min UV irradiation, which resulted in reduction of micelle sizes by 17–22 nm. Notably, drug leakage was not detected during crosslinking procedure as revealed by HPLC analysis. PTX-loaded crosslinked PEG-*b*-P(TMBPEC-*co*-AC) micelles were obtained with average sizes ranging from 114.2 to 149.3 nm depending on PTX loading contents (Table 1, Entries 2–4). TEM micrograph displayed that PTX-loaded crosslinked micelles had a uniform size distribution and spherical morphology (Fig. 5).

The in vitro release of PTX was carried out at three different pHs of 4.0, 5.0 and 7.4 using PTX-loaded non-crosslinked and crosslinked micelles (DLC = 10.2 wt.%) at a low micelle concentration of 0.032 mg/mL (corresponding to 3.27 μg equiv. PTX/mL). PTX was reported to have a water solubility of ca. 1.0 μg/mL [42]. In order to achieve sink conditions, 1.0 mL of PTX-loaded micelles was dialyzed against 20 mL of release media, and at different time intervals, 7.0 mL of release media was taken and replenished with an equal volume of fresh media. The results showed that PTX was quickly released from the PTX-loaded non-crosslinked micelles at pH 7.4 (ca. 73.1% release in 11 h), whereas drug release from PTX-loaded crosslinked micelles under otherwise the same conditions was largely inhibited (ca. 35.7% release in 50 h) (Fig. 6). It should further be noted that presence of 10% fetal bovine serum (FBS) had little influence on PTX release from crosslinked micelles. The enhanced stability and inhibited drug release behaviors have been observed for photo-crosslinked PEG-PCL micelles [13,19,20]. Interestingly, PTX-loaded crosslinked micelles were shown to rapidly release PTX under mildly acidic conditions, in which 90.0% and 78.1% of PTX was released in 23 h at pH 4.0 and 5.0, respectively (Fig. 6). The extents of drug release from PTX-loaded crosslinked micelles at pH 4.0 and 5.0 in 23 h were approaching to those for non-crosslinked counterparts under otherwise the same conditions. This pH-triggered drug release is most likely due to change of polarity in the micellar core as a result of acidic hydrolysis of acetals [21,37,38]. It appears, therefore, that core-crosslinked pH-sensitive degradable micelles on one hand possess superior stability at physiological pH due to covalent crosslinking and on the other hand are able to rapidly release drug at mildly acidic pH mimicking that of endo/lysosomal compartments owing to acetal hydrolysis.

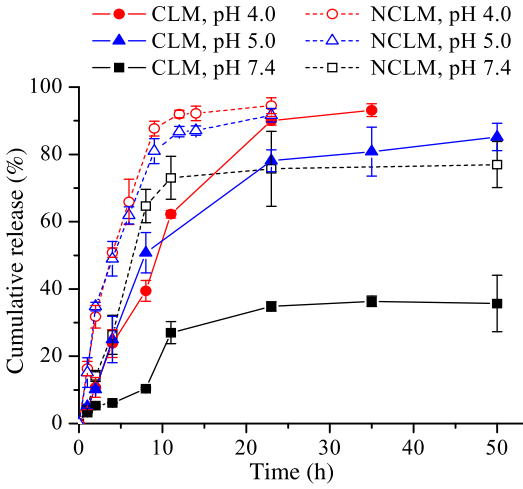


Fig. 6. pH-dependent drug release from PTX-loaded crosslinked micelles at 37 °C. The release profiles of PTX from non-crosslinked micelles were used as control. The initial micelle concentration was 32 μg/mL.

3.4. Cytotoxicity of empty and PTX-loaded micelles

The in vitro toxicity of non-crosslinked and crosslinked PEG-*b*-P(TMBPEC-*co*-AC) micelles was evaluated in MCF-7 and RAW-264.7 cells using MTT assays. The cells were incubated with micelles for 72 h at varying concentrations from 15 to 900 μg/mL. The results revealed that both non-crosslinked and crosslinked micelles had low cytotoxicities (cell viability > 80%) at all tested concentrations (Fig. 7).

The anti-tumor activities of PTX-loaded non-crosslinked and crosslinked PEG-*b*-P(TMBPEC-*co*-AC) micelles were also studied by MTT assays. MCF-7 and RAW 264.7 cells were treated for 24 or 72 h with free PTX or PTX-loaded micelles (PTX dosage: 6 μg/mL). Interestingly, the results showed that PTX-loaded crosslinked micelles caused significant cell death which was comparable to that of PTX-loaded non-crosslinked counterparts (Fig. 8). For example, cell viabilities of 26.7% and 9.2% were observed for RAW 264.7 cells following incubation with

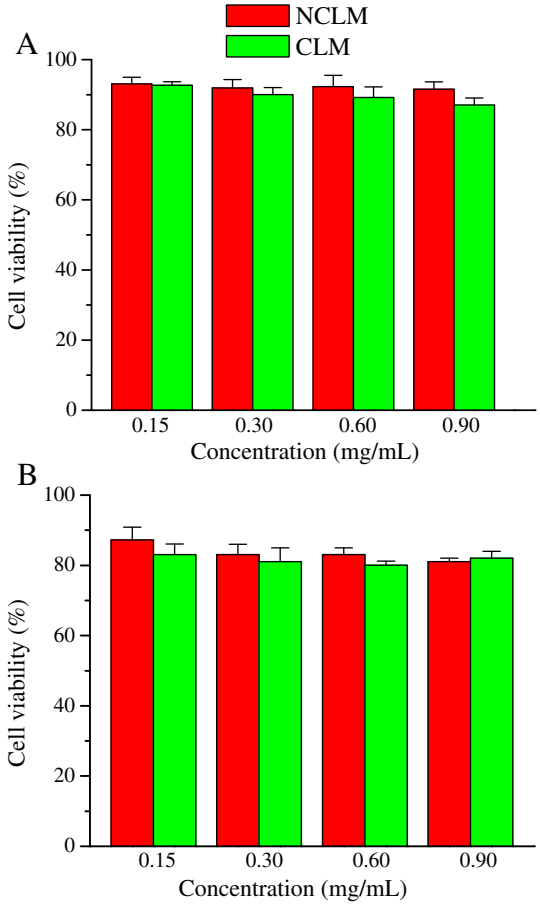


Fig. 7. Cytotoxicity of non-crosslinked and crosslinked PEG-*b*-P(TMBPEC-*co*-AC) micelles. (A) MCF-7 cells, (B) RAW 264.7 cells. The cells were incubated with micelles for 72 h. Data are presented as the average ± standard deviation (n = 4).

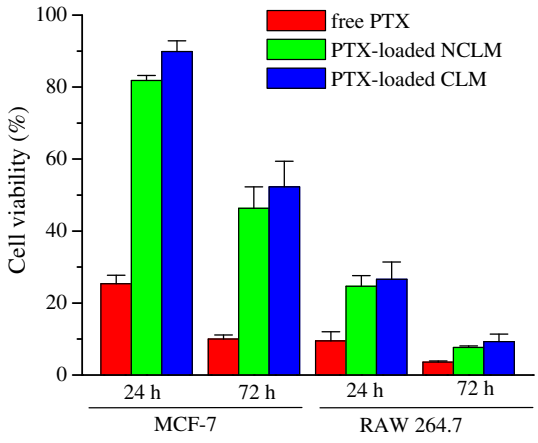


Fig. 8. The anti-tumor activity of PTX-loaded crosslinked micelles in MCF-7 and RAW 264.7 cells. PTX-loaded non-crosslinked micelles and free PTX were used as controls. PTX dosage was 6.0 μg/mL. The cells were incubated with micellar PTX or free PTX for 24 or 72 h. Data are presented as the average ± standard deviation (n = 4).

PTX-loaded crosslinked micelles for 24 and 72 h, respectively, which were analogous to those obtained for PTX-loaded non-crosslinked controls (cell viabilities of 24.5% and 7.5% for 24 and 72 h incubation, respectively). PTX-loaded micelles exhibited lower toxicity to MCF-7 cells than to RAW 264.7 cells, likely due to less efficient uptake of micelles by MCF-7 cells. However, comparable cell viabilities were observed for MCF-7 cells following incubation with PTX-loaded crosslinked and non-crosslinked micelles (cell viabilities 52.4% versus 46.5% in 72 h). These results indicate that crosslinking of PEG-*b*-P(TMBPEC-*co*-AC) micelles does not have adverse effects on the intracellular drug release and thereby anti-tumor activity. Similar results have also been reported for reduction-sensitive reversibly crosslinked nanoparticles that are automatically de-crosslinked and dissociated inside the tumor cells [43]. It should be noted that the anti-tumor activity of PTX-loaded crosslinked PEG-*b*-P(TMBPEC-*co*-AC) micelles may be further improved by attaching a targeting ligand that enhances specific cellular uptake. These core-crosslinked pH-responsive biodegradable micelles offer a novel and elegant approach to resolve the extracellular stability versus intracellular drug release dilemma of micellar drugs and provide a novel platform for tumor-targeting delivery of anti-cancer drugs.

4. Conclusions

We have demonstrated that core-crosslinked pH-sensitive degradable micelles while possessing superior stability at physiological pH are able to rapidly release loaded drugs under a mildly acidic condition mimicking that of the endo/lysosomal compartments. Notably, paclitaxel-loaded crosslinked pH-sensitive degradable micelles exhibit similar anti-tumor activity to the non-crosslinked counterparts, supporting that photo-crosslinking of micelle core has no significant adverse influence on intracellular paclitaxel release. These "smart" micelles provide an elegant approach to resolve the extracellular stability and intracellular drug release dilemma encountered by current nano drug delivery systems. It should further be noted that these core-crosslinked pH-sensitive degradable micelles can be readily prepared from designed amphiphilic degradable diblock copolymer based on functional polycarbonate hydrophobe containing photo-crosslinkable acryloyl groups and acid-labile acetal groups. These core-crosslinked pH-responsive degradable micelles are highly promising for targeted cancer therapy.

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