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pH-sensitive degradable chimaeric polymersomes for the intracellular release of doxorubicin hydrochloride

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ABSTRACT

pH-sensitive degradable chimaeric polymersomes were developed based on asymmetric poly(ethylene glycol)-*b*-poly(2,4,6-trimethoxybenzylidene-1,1,1-tris(hydroxymethyl)ethane methacrylate)-*b*-poly(-acrylic acid) (PEG-PTTMA-PAA) triblock copolymers for active loading as well as triggered intracellular release of hydrophilic doxorubicin hydrochloride (DOX·HCl). PEG-PTTMA-PAA copolymers were readily prepared with $M_{n\ PAA}$ ranging from 1.5, 2.1 to 2.7 kg/mol by sequential reversible addition-fragmentation chain transfer (RAFT) copolymerization of 2,4,6-trimethoxybenzylidene-1,1,1-tris(hydroxymethyl)ethane methacrylate (TTMA) and acrylic acid (AA) using PEG-CPADN ($M_{n\ PEG}$ = 5.0 kg/mol; CPADN: 4-cyanopentanoic acid dithionaphthalenoate) as a macro-RAFT agent. PEG-PTTMA-PAA copolymers formed mono-disperse polymersomes with average sizes of 63.9–112.1 nm, which decreased with increasing $M_{n\ PAA}$. The polymersomal structure was confirmed by transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM). Notably, the acetals in polymersomes while sufficiently stable at pH 7.4 were prone to rapid hydrolysis at mildly acidic pHs of 4.0 and 5.0, which resulted in swelling and eventually disassembly of polymersomes. These chimaeric polymersomes could actively load DOX·HCl resulting in remarkably high drug loading contents (up to 15.9 wt.%) and loading efficiencies (up to 88.8%). The *in vitro* release studies showed that DOX·HCl was released from chimaeric polymersomes in a controlled and pH-dependent manner. CLSM observations revealed that these chimaeric polymersomes could efficiently deliver and release DOX·HCl into the nuclei of HeLa cells. MTT assays in HeLa cells demonstrated that DOX·HCl-loaded PEG-PTTMA-PAA polymersomes exhibited high anti-tumor activity with IC_{50} (inhibitory concentration to produce 50% cell death) of 1.48–1.67 μg/mL, close to that of free DOX·HCl, while blank polymersomes were practically non-toxic up to a tested concentration of 2.0 mg/mL. These pH-sensitive degradable chimaeric polymersomes have appeared to be a promising alternative to liposomes for tumor-targeted delivery of DOX·HCl.

1. Introduction

The development in tumor-targeting nano delivery systems such as liposomes, micelles and nanoparticles has revived the therapeutic uses of numerous potent chemotherapeutics that are too toxic to be applied otherwise [1–3]. In the past decade, vastly different approaches have been designed for targeted delivery of hydrophobic drugs including paclitaxel (PTX) and doxorubicin (DOX) [4,5]. In comparison, few systems among which are macromolecular prodrugs [6], liposomes [7,8] and more recently polymersomes [9,10] have been developed for controlled release of hydrophilic small molecule anti-cancer drugs such as doxorubicin hydrochloride (DOX·HCl) and mitoxantrone hydrochloride. Liposomes and polymersomes that possess large aqueous interiors to accommodate water-soluble entities and hydrophobic membranes to control drug diffusion are the most ideal nano-carriers for hydrophilic small molecule anti-cancer agents. Unlike the macromolecular prodrug approach, drugs are physically encapsulated into liposomes and polymersomes (no chemical alternation of drug). It is interesting to note that liposomal doxorubicin (Doxil®, Caelyx® and Myocet®) is one of the very few nano drugs that are routinely used in the clinical settings for treating various forms of cancers including advanced ovarian cancer, breast cancer, multiple myeloma and Kaposi's sarcoma [11,12].

Polymersomes (also known as polymeric vesicles) have recently emerged as an improved alternative to liposomes [13,14]. As compared to liposomes, polymersomes usually exhibit better colloidal stability, higher mechanical strength, and lower chemical

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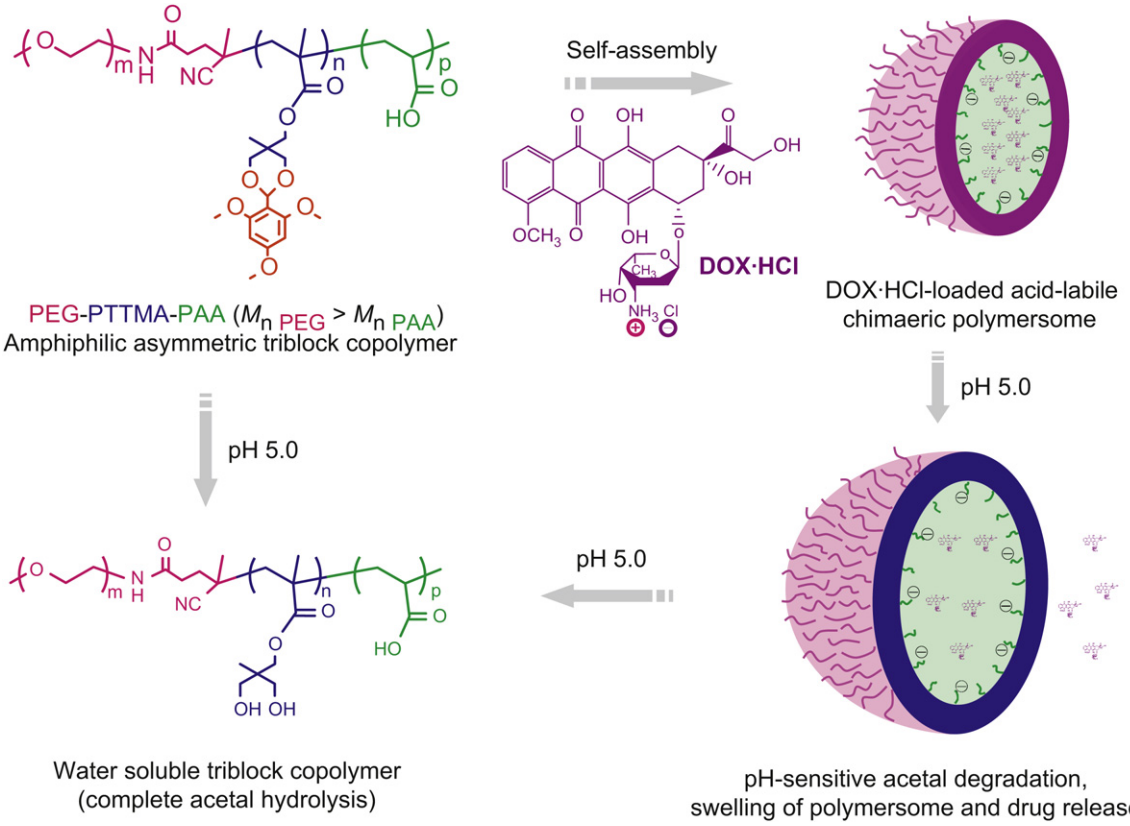
permeability (and lower drug leakage). Moreover, while liposomes have to be modified with poly(ethylene glycol) (PEG) to obtain "stealth liposomes" [15], polymersomes are intrinsically stealthed by non-fouling polymers such as PEG and dextran which typically form the hydrophilic part of macromolecular amphiphiles [16–18]. It should further be noted that polymersomes are much more versatile in structures and functions as compared to liposomes, which for example allows facile design of stimuli-responsive polymersomes for programmed drug release [19,20] and ligand-decorated polymersomes for tumor-targeting drug delivery [21,22]. In the past decade, polymersomes have been investigated for controlled release of various therapeutic agents including chemotherapeutics [23], proteins and siRNA [17,24]. However, despite presence of large aqueous reservoirs, polymersomes exhibit in general low loading levels and loading efficacies towards hydrophilic small molecule anti-cancer drugs [25]. In order to achieve enhanced loading of hydrophilic drugs like DOX·HCl, delicate encapsulation procedures like transmembrane pH-, sulfate-, citrate- or phosphate gradient loading [23,25–27] and nano-precipitation method [28] has been adopted.

In this paper, we report on endosomal pH-sensitive degradable chimaeric polymersomes based on asymmetric poly(ethylene glycol)-*b*-poly(trimethoxybenzylidene tris(hydroxymethyl)ethane methacrylate)-*b*-poly(acrylic acid) (PEG-PTTMA-PAA) triblock copolymers for active encapsulation and pH-triggered release of DOX·HCl (Scheme 1). Here, PEG block was designed to be longer than PAA block so that PEG would preferentially located at the outer surfaces endowing excellent biocompatibility and stealth effect, while PAA block would be placed inside the aqueous core offering efficient loading and stabilization of DOX·HCl via electrostatic interactions. The chimaeric PEG-PCL-PDEA polymersomes have shown to result in high encapsulation contents of exogenous proteins [29]. Moreover, the pH-sensitive degradation of acetals in PTTMA block would trigger DOX·HCl release in the endo/lysosomal compartments of cancer cells, resulting in high therapeutic effects. We reported previously that pH-sensitive degradable polymersomes containing trimethoxybenzylidene acetals released both hydrophilic and hydrophobic anti-cancer drugs in a pH-dependent manner [30]. In this study, synthesis of pH-sensitive degradable chimaeric polymersomes, loading and *in vitro* release of DOX·HCl, as well as anti-tumor activity of DOX·HCl-loaded PEG-PTTMA-PAA polymersomes were investigated.

2. Materials and methods

2.1. Materials

2,4,6-trimethoxybenzaldehyde (98%, Acros), methacryloyl chloride (97%, Alfa Aesar), triethylamine (99%, Alfa Aesar), 1,1,1-tris(hydroxymethyl)ethane (99%, Aldrich), nile red (99%, Sigma), pyrene (97%, Fluka), and doxorubicin hydrochloride (DOX·HCl, 90%, Beijing Zhongshuo Pharmaceutical Technology Development Co., Ltd.) were used as received. Tetrahydrofuran (THF), diethyl ether, ethyl acetate, dichloromethane (DCM), petroleum ether, anhydrous sodium sulfate, tris(hydroxymethyl) aminomethane (Tris), *p*-toluenesulfonic acid monohydrate and 5Å molecular sieves were purchased from Sinopharm Chemical Reagent Co., Ltd. (SCRC). Acrylic acid (AA, 99%, Alfa Aesar) was distilled under reduced pressure before used. Methoxy poly(ethylene glycol) (PEG, $M_n = 5.0$ kg/mol, PDI = 1.03) was purchased from Fluka and dried by azeotropic distillation from anhydrous toluene. Azobisisobutyronitrile (AIBN) was recrystallized twice from methanol. 2,4,6-trimethoxybenzylidene-1,1,1-tris(hydroxymethyl) ethane methacrylate (TTMA) was prepared with about 65% yield in two steps (Scheme S1), as previously report by Grinstaff [31]. The structure of TTMA was confirmed by ^{1}H NMR (Fig. S1). 4-cyanopentanoic acid dithionaphthalenoate (CPADN) was synthesized according to the described procedure for 4-cyanopentanoic acid dithiobenzoate [32]. PEG-CPADN macro-RAFT agent was obtained according to our previous report [33].



Scheme 1. Illustration of pH-sensitive degradable chimaeric polymersomes based on asymmetric PEG-PTTMA-PAA triblock copolymers (PEG block longer than PAA block) for active loading as well as endosomal pH-triggered release of doxorubicin hydrochloride.

2.2. Characterization

The ^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using deuterated chloroform ($CDCl_3$) or deuterated dimethylsulfoxide (DMSO-d_6) 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 chromatograph (GPC) instrument equipped with two linear PLgel columns (500Å and Mixed-C) following a guard column and a differential refractive-index detector (RI 2414). The measurements were performed using DMF containing 1 wt.% LiBr as an eluent at a flow rate of 0.8 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns. The size of polymersomes was determined using dynamic light scattering (DLS). Measurements were carried out at 25 °C using Zetasizer Nano ZS from Malvern Instruments equipped with a 633 nm He–Ne laser using back-scattering detection. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of 0.1 mg/mL polymersome dispersion on the copper grid.

2.3. Synthesis of PEG-PTTMA-PAA triblock copolymers

PEG-PTTMA-PAA triblock copolymers were synthesized by sequential RAFT copolymerization of TTMA and AA using PEG-CPADN (M_n = 5 kg/mol) as a macro-RAFT agent. In a typical example, under a nitrogen atmosphere, TTMA (0.16 g, 0.44 mmol), PEG-CPADN (50 mg, 10 μmol), AIBN (0.23 mg, 1.4 μmol) and DMF (2 mL) were added into a 10 mL Schlenk flask. The mixture was degassed with nitrogen for 30 min. The flask was sealed and placed into an oil bath thermostated at 65 °C. After 48 h polymerization, a sample was taken out to determine TTMA conversion. Then, second monomer AA (30 mg, 0.42 mmol) and additional AIBN (0.23 mg, 1.4 μmol) in DMF were added to the polymerization mixture. The polymerization proceeded for another 48 h at 65 °C. PEG-PTTMA-PAA triblock copolymer was isolated by precipitation in cold diethyl ether, filtration and drying in vacuo for 48 h at room temperature. Yield: 73.2%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.03 (aromatic protons), 5.83 (Ar–C*H*–), 4.17 (–COOC*H*$_2$C–), 3.89 (–OC*H*$_2$CC*H*$_2$O–), 3.75 (Ar–OC*H*$_3$), 3.65 (PEG), 2.23 (–C*H*COOH), 1.87 (–C*H*$_2$CHCOOH, –C*H*$_2$C–), 0.59–1.26 (C*H*$_3$CCOO–, C*H*$_3$C–).

2.4. Formation of polymersomes

PEG-PTTMA-PAA copolymer polymersomes were prepared by solvent exchange method. Briefly, to a stirred THF solution (200 μL) of PEG-PTTMA-PAA (2.1 mg/mL) was dropwise added 2.0 mL of phosphate buffer (10 mM, pH 7.4). The mixture was sonicated for 2 h and dialyzed against phosphate buffer (10 mM, pH 7.4) for 8 h (MWCO 7000) at room temperature. The size of the polymersomes was determined by DLS and TEM.

The critical aggregation concentration (CAC) was determined using pyrene as a fluorescence probe. The concentration of PEG-PTTMA-PAA copolymer was varied from 1.0×10^{-5} to 0.2 mg/mL and the concentration of pyrene was fixed at 1.0 μM. The fluorescence spectra were recorded using FLS 920 fluorescence spectrometer with the excitation wavelength of 330 nm. The emission fluorescence at 372 and 383 nm were monitored. The CAC was estimated as the cross-point when extrapolating the intensity ratio I_{372}/I_{383} at low and high concentration regions.

2.5. Confocal laser scanning microscopy (CLSM) observation of DOX·HCl and nile red-loaded polymersomes

DOX·HCl was firstly loaded into polymersomes by dropwise adding 40 μL of DOX·HCl aqueous solution (1.0 mg/mL) and 2.0 mL of phosphate buffer (10 mM, pH 7.4) to 0.2 mL of copolymer solution (2 mg/mL) in THF, sonicating for 2 h in the dark and dialysis against phosphate buffer (10 mM, pH 7.4, MWCO 7000). In order to load nile red, 10 μL of nile red solution in acetone (0.10 mM) was added into DOX·HCl-loaded polymersomes. The polymersome dispersions were stirred at 37 °C for 4 h to evaporate acetone. The resulting polymersomes were observed using CLSM with the emission fluorescence of DOX·HCl at 480 nm and nile red at 553 nm.

2.6. Determination of pH-dependent hydrolysis rate of the acetals in polymersomes

The acetal hydrolysis was followed by UV/vis spectroscopy by measuring the absorbance at 290 nm, according to the previous reports by Fréchet and coworkers [34,35]. The polymersome dispersions (1.0 mg/mL) prepared as above were divided into three aliquots. Their pH was adjusted to 4.0, 5.0 and 7.4, respectively, by addition of 400 μL of 0.1 M pH 4.0 and 5.0 acetate buffer or pH 7.4 phosphate buffer, while keeping the salt concentration the same. The solutions were shaken at 37 °C. At the desired time points, 40 μL aliquot was taken out and diluted with 3.5 mL phosphate buffer (0.1 M, pH 7.4). The absorbance at 290 nm was monitored. At the end, all samples were completely hydrolyzed by addition 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. Size change of polymersomes in response to acetal hydrolysis

The size change of polymersomes in response to acetal hydrolysis was followed by DLS measurements. Briefly, to 1.0 mL of polymersome dispersions (0.20 mg/mL) prepared as above at 37 °C was added 300 μL of acetate buffer (0.10 M, pH 5.0), which resulted in a final pH of 5.0. The change of polymersome sizes was monitored in time by DLS.

2.8. Loading of DOX·HCl into polymersomes

DOX·HCl-loaded polymersomes were readily prepared by solvent exchange method. In a typical example, 50 μL of DOX·HCl aqueous solution (2.0 mg/mL) and 2.0 mL of PB (10 mM, pH 7.4) were added to 0.20 mL of PEG-PTTMA-PAA copolymer solution in THF (5.0 mg/mL) (thus, theoretical DOX·HCl loading content was 10 wt.%). The mixture was sonicated for 2 h in the dark and dialyzed against phosphate buffer (10 mM, pH 7.4) for 8 h with at least 5 times change of media (MWCO 7000). The final polymersome concentration was approximately 0.50 mg/mL. For determination of drug loading content (DLC) and drug loading efficiency (DLE), DOX·HCl-loaded polymersomes were dissolved in DMF and analyzed with fluorescence spectroscopy (FLS 920, excitation at 480 nm), wherein calibration curve was obtained with DOX·HCl/DMF solutions with different DOX·HCl concentrations.

DLC and DLE were calculated according to the following formula:

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

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

2.9. In vitro release of DOX·HCl

The release profiles of DOX·HCl from PEG-PTTMA-PAA polymersomes were investigated at 37 °C in three different media, *i.e.* (a) acetate buffer, pH 4.0; (b) acetate buffer, pH 5.0; (c) phosphate buffer, pH 7.4. The concentrations of the release media were 10 mM. The above prepared DOX·HCl-loaded polymersomes were divided into three aliquots (each 0.5 mL). Their pH was adjusted to 4.0 or 5.0 using acetate buffer and to pH 7.4 using phosphate buffer, and immediately transferred to a dialysis tube with a MWCO of 12000–14000. The dialysis tube was immersed into 20 mL of corresponding buffer (0.1 M) at 37 °C. At desired time intervals, 5 mL of release media was taken out for fluorescence measurement (FLS920, excitation at 600 nm) and replenished with an equal volume of fresh media. To determine the amount of DOX·HCl released, calibration curves were run with DOX·HCl in corresponding buffer solutions at pH 4.0, 5.0 and 7.4, respectively. The emission spectra were recorded from 500 to 680 nm. The release experiments were conducted in triplicate. The results presented are the average data with standard deviations.

2.10. MTT assays

HeLa cells were seeded in 96-well plates (1×10^4 cells/well) in 100 μL of Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 24 h at 37 °C in a humidified 5% CO_2-containing atmosphere. The media was aspirated and replaced by 80 μL of fresh DMEM medium supplemented with 10% FBS. 20 μL of blank PEG-PTTMA-PAA polymersomes, DOX·HCl-loaded PEG-PTTMA-PAA polymersomes, or free DOX·HCl at various concentrations in phosphate buffer (10 mM, pH 7.4) was 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) solution (5 mg/mL) was added. The cells were incubated for 4 h. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 100 μL of DMSO, and the absorbance at a wavelength of 490 nm of each well was measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 490 nm with control wells containing only cell culture medium. Data are presented as average ± SD (n = 4).

2.11. Intracellular release of DOX·HCl

The cellular uptake and intracellular release behaviors of DOX·HCl-loaded polymersomes were followed with CLSM using HeLa cells. The cells were seeded on microscope slides in a 24-well plate (5×10^4 cells/well) using DMEM supplemented with 10% FBS for 24 h. The media were aspirated and replaced by 500 μL of fresh DMEM supplemented with 10% FBS. 50 μL of DOX·HCl-loaded polymersomes or free DOX·HCl (5.0 μg/mL) was added. The cells were incubated with DOX·HCl-loaded polymersomes or free DOX·HCl for 2 or 4 h at 37 °C in a humidified 5% CO_2-containing atmosphere. The culture media were removed and the cells were rinsed three times with PBS. The cells were fixed with 4% paraformaldehyde and the cell nuclei were stained with DAPI. CLSM images of cells were obtained using confocal laser scanning microscope (TCS SP2).

3. Results and discussion

3.1. Synthesis of asymmetric PEG-PTTMA-PAA triblock copolymers

PEG-PTTMA-PAA triblock copolymers were obtained by sequential radical addition-fragmentation chain transfer (RAFT) copolymerization of TTMA and AA monomers using PEG-CPADN (CPADN: 4-cyanopentanoic acid dithionaphthalenoate, $M_{n\ PEG}$ = 5.0 kg/mol) as a macro-RAFT agent at 65 °C (Scheme 2). CPADN is a robust and versatile RAFT agent, with which we have previously prepared a series of well-defined block copolymers including PEG-PCL-PDEA [29] and PEG–SS–PDEA [36]. In this study, three PEG-PTTMA-PAA copolymers with the same M_n of 16.0 kg/mol for hydrophobic PTTMA block and varying M_n of 1.0, 2.0–3.0 kg/mol for PAA block were designed. The results of synthesis are given in Table 1. ^{1}H NMR spectrum showed clearly signals characteristic of PEG (δ 3.65), PTTMA (δ 6.03, 5.83, 4.17, 3.89, 3.75, 1.87, 0.86, 0.52) and PAA (δ 2.23, 1.87) (Fig. S2). The M_n values of PTTMA and PAA blocks could be estimated by comparing the integrals of signals at δ 5.83 (acetal methine proton of PTTMA) and 2.23 (methine proton of PAA), respectively, with that of PEG methylene protons at δ 3.65. The results showed that all three PEG-PTTMA-PAA copolymers had similar M_n (13.4–15.2 kg/mol) for PTTMA block while M_n of PAA block varied from 1.5, 2.1, to 2.7 kg/mol, in line with the design (Table 1). In the following, corresponding copolymers were denoted as PEG-PTTMA-PAA(1.5k), PEG-PTTMA-PAA(2.1k) and PEG-PTTMA-PAA(2.7k), respectively. Notably, gel permeation chromatography (GPC) displayed that PEG-PTTMA-PAA copolymers had a unimodal distribution with low polydispersities of 1.22–1.34 and controlled M_n of 18.3–22.2 kg/mol that were close to those determined by ^{1}H NMR (Table 1). It is evident, therefore, that asymmetric PEG-PTTMA-PAA triblock copolymers were synthesized in a controlled manner via sequential RAFT copolymerization.

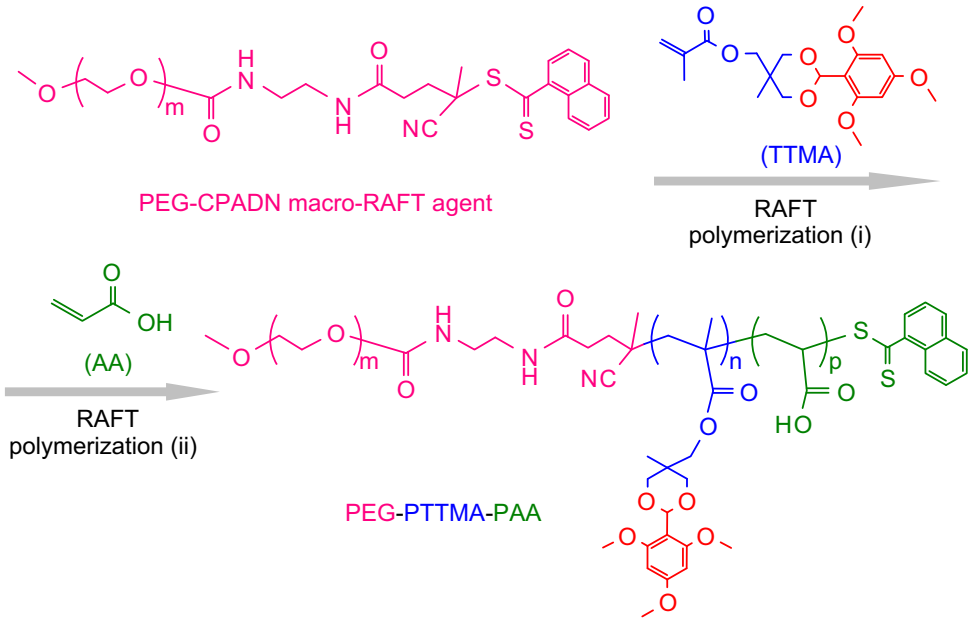
3.2. Preparation of chimaeric polymersomes

Polymersomes were prepared from PEG-PTTMA-PAA copolymers by solvent exchange method. DLS measurements showed that PEG-PTTMA-PAA copolymers formed mono-disperse nanoparticles with low polydispersities (PDI) of 0.15–0.21 (Fig. 1A). The average sizes of nanoparticles decreased from 112.1, 86.4, to 63.9 nm when increasing PAA block lengths from 1.5, 2.1 to 2.7 kg/mol (Table 2). These nanoparticles were sufficiently stable, with little size change in 24 h, at pH 7.4 (10 mM PB) and 37 °C. TEM micrograph displayed that these nanoparticles had a vesicular structure (Fig. 1B). In addition, confocal laser scanning microscopy (CLSM) observations of large polymersomes simultaneously encapsulated with DOX·HCl (hydrophilic) and nile red (hydrophobic) showed clearly co-localization of DOX·HCl and nile red, with fluorescence intensity profiles in accordance with location of DOX·HCl in the lumen and nile red in the membrane (Fig. S3), further confirming their polymersomal structure. The zeta potential measurements demonstrated that PEG-PTTMA-PAA polymersomes had modest negative surface charges (−12.7 ~ −17.5 mV). The critical aggregation concentration (CAC) measurements using pyrene as a fluorescence probe showed that these asymmetric PEG-PTTMA-PAA triblock copolymers had low CAC of 1.25–1.45 mg/L. In contrast, the parent PEG-PTTMA diblock copolymer with a similar PTTMA block length yielded relatively large polymersomes with an average size of about 300 nm that tended to precipitate during dialysis. These results indicate that these asymmetric triblock copolymers are thermodynamically favorable for self-assembling into nano-sized polymersomes with short PAA block located inside the polymersomes, in line with our previous report [29].

Table 1
Synthesis of asymmetric PEG-PTTMA-PAA triblock copolymers.

Entry	Copolymer	M_n (kg/mol)			M_w/M_n[b]
		Design	^{1}H NMR[a]	GPC[b]	
1	PEG-PTTMA-PAA(1.5k)	5-16-1	5-13.4-1.5	18.3	1.34
2	PEG-PTTMA-PAA(2.1k)	5-16-2	5-14.3-2.1	21.7	1.22
3	PEG-PTTMA-PAA(2.7k)	5-16-3	5-15.2-2.7	22.2	1.30

[a] Determined by ^{1}H NMR by comparing the integrals of signals at δ 5.83 (acetal methine proton of PTTMA) and 2.23 (methine proton of PAA), respectively, with that of PEG methylene protons at δ 3.65.
[b] Determined by GPC (eluent: DMF, flow rate: 0.8 mL/min, standards: polystyrene, 30 °C).



Scheme 2. Synthesis of PEG-PTTMA-PAA triblock copolymers by sequential RAFT copolymerization of TTMA and AA using PEG-CPADN (M_n = 5 kg/mol) as a macro-RAFT agent and AIBN as a radical source in DMF at 65 °C.

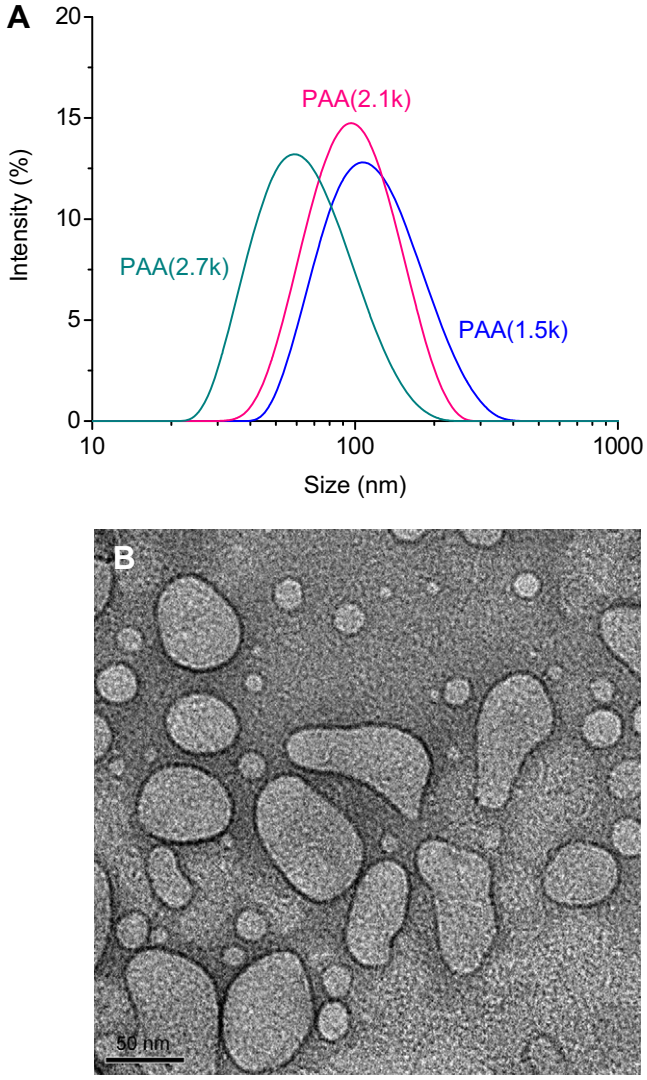


Fig. 1. Size distribution profiles of chimaeric polymersomes based on PEG-PTTMA-PAA triblock copolymers determined by DLS (A) and TEM image of PEG-PTTMA-PAA(2.1k) polymersomes (B).

3.3. pH-dependent hydrolysis of acetals in the polymersomes

The hydrolysis of acetals in PEG-PTTMA-PAA polymersomes was investigated at different pH values of 4.0, 5.0 and 7.4 at 37 °C. The extent of acetal hydrolysis was conveniently determined using UV/vis spectroscopy by monitoring the absorbance at 290 nm, which is the characteristic absorbance of the hydrolysis product, 2,4,6-trimethoxybenzaldehyde [34,35]. Notably, the results showed that hydrolysis rate of acetals in polymersomes was highly pH-dependent, in which significantly enhanced acetal hydrolysis was observed under mildly acidic conditions (Fig. 2), as our previous reports on acetal-containing micelles, polymersomes and polymer/DNA complexes [30,37,38]. For example, PEG-PTTMA-PAA(2.1k) polymersomes showed rapid acetal hydrolysis, with acetal half lives of approximately 3.0 and 11 h, at pH 4.0 and 5.0, respectively. In contrast, acetal hydrolysis rate was low (21.2% in 33 h) at pH 7.4 under otherwise the same conditions. The acetal hydrolysis was slightly faster for PEG-PTTMA-PAA polymersomes with longer PAA block length, likely due to occurrence of auto-catalytic degradation.

Table 2
Formation of chimaeric polymersomes from asymmetric PEG-PTTMA-PAA triblock copolymers.

Entry	Polymersome	Size (nm)[a]	PDI[a]	ζ[b] (mV)	CAC[c] (mg/L)
1	PEG-PTTMA-PAA(1.5k)	112.1 ± 10.2	0.20	−12.7	1.45
2	PEG-PTTMA-PAA(2.1k)	86.4 ± 4.6	0.15	−15.6	1.26
3	PEG-PTTMA-PAA(2.7k)	63.9 ± 7.3	0.21	−17.5	1.25

[a] Determined at 25 °C with a Zetasizer Nano ZS instrument (Malvern) equipped with a dynamic light scattering (DLS, 10 mW He–Ne laser, 633 nm wavelength) in PB buffer (pH 7.4, 10 mM).
[b] Determined at 25 °C with a Zetasizer Nano ZS instrument (Malvern) equipped with a standard capillary electrophoresis cell in PB buffer (pH 7.4, 10 mM).
[c] Critical aggregation concentration (CAC) determined in PB buffer (pH 7.4, 10 mM) using pyrene as a fluorescence probe.

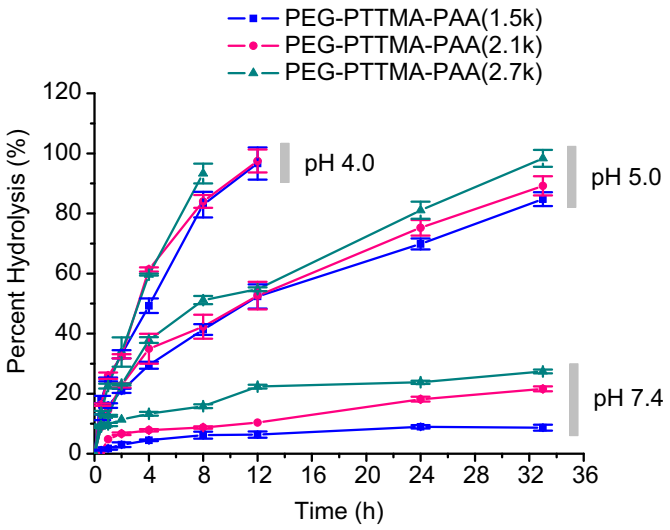


Fig. 2. pH-dependent hydrolysis of acetals in PEG-PTTMA-PAA polymersomes at 37 °C.

The size change of polymersomes in response to acetal hydrolysis at acidic pH was followed by DLS measurements. The results showed that PEG-PTTMA-PAA(2.1k) polymersomes swelled and/or aggregated at pH 5.0 (30 mM acetate buffer) with occurrence of a large population of sub-micrometer and micrometer-sized particles in 3 and 7 h (Fig. 3). Notably, only small-sized particles

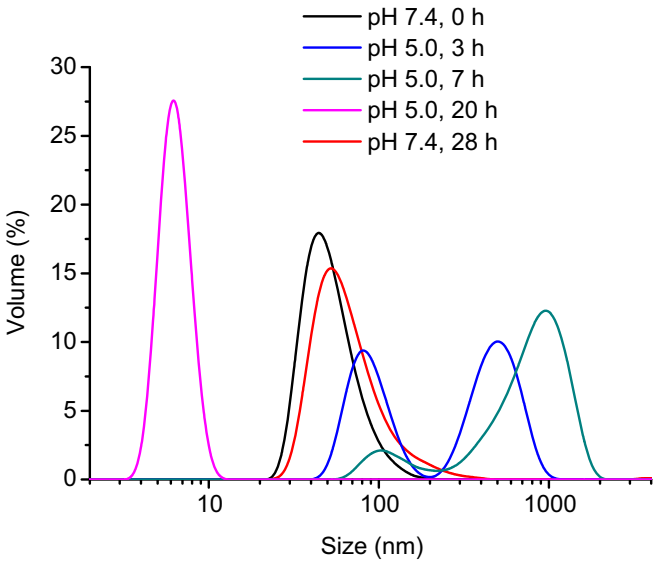


Fig. 3. Size change of PEG-PTTMA-PAA(2.1k) polymersomes in acetate buffer (pH 5.0, 30 mM) or phosphate buffer (pH 7.4, 30 mM) at 37 °C in time.

Table 3
Characterization of DOX·HCl-loaded PEG-PTTMA-PAA polymersomes in phosphate buffer (concentration 0.5 mg/mL).

Polymersome	Theoretical DLC (wt.%)	DLC (wt.%)	DLE (%)	Size[a] (nm)	PDI	ζ [b] (mV)
PEG-PTTMA-PAA(1.5k)	5	3.75	75.0	177.1 ± 5.2	0.15	−12.3
	10	7.23	72.3	184.5 ± 3.4	0.15	−11.0
	20	12.5	62.5	254.1 ± 5.8	0.15	−7.9
PEG-PTTMA-PAA(2.1k)	5	4.15	83.0	158.6 ± 7.4	0.17	−14.9
	10	8.22	82.2	159.1 ± 10.1	0.13	−12.2
	20	14.6	73.0	206.5 ± 9.2	0.23	−10.0
PEG-PTTMA-PAA(2.7k)	5	4.44	88.8	105.7 ± 5.9	0.19	−15.7
	10	8.53	85.3	143.3 ± 6.8	0.16	−13.6
	20	15.9	79.5	205.0 ± 6.3	0.28	−13.3

[a] Determined at 25 °C with a Zetasizer Nano ZS instrument (Malvern) equipped with a dynamic light scattering (DLS, 10 mW He–Ne laser, 633 nm wavelength) in PB buffer (pH 7.4, 10 mM).
[b] Determined at 25 °C with a Zetasizer Nano ZS instrument (Malvern) equipped with a standard capillary electrophoresis cell in PB buffer (pH 7.4, 10 mM).

(ca. 6 nm) were detected following 20 h incubation at pH 5.0, indicating that polymersomes were degraded into water-soluble unimers as a result of complete acetal hydrolysis. In contrast, little change of polymersome size was observed over 24 h at pH 7.4 under otherwise the same conditions (Fig. 3).

3.4. Encapsulation and in vitro release of DOX·HCl

The loading level of hydrophilic anti-cancer drugs such as DOX·HCl into polymersomes is usually very low. The transmembrane pH-, sulfate-, citrate-, or phosphate gradient loading method were reported to largely improve the loading of DOX·HCl into polymersomes [23,25–27]. However, these traditional "active loading" methods directly or indirectly involve low pH (*e.g.* pH 4.0 for pH gradient loading and pH <5.5 for sulfate-, citrate-, or phosphate gradient loading [39]) that would lead to undesired degradation of acetals in polymersomes. In this study, chimaeric polymersomes with PAA chains preferentially located in the aqueous core were designed to accomplish simple and "active" loading of DOX·HCl into the lumen of polymersomes. The theoretical drug loading contents (DLC) were set as 5, 10 and 20 wt.%. Interestingly, the results showed that all three chimaeric polymersomes achieved remarkably high loading efficiencies towards DOX·HCl (62.5–88.8%) (Table 3). Notably, drug loading efficiencies

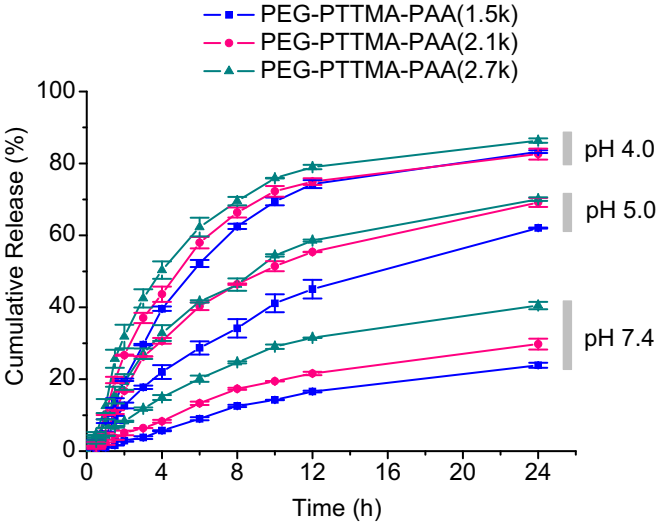


Fig. 4. pH-dependent release of DOX·HCl from PEG-PTTMA-PAA polymersomes at 37 °C.

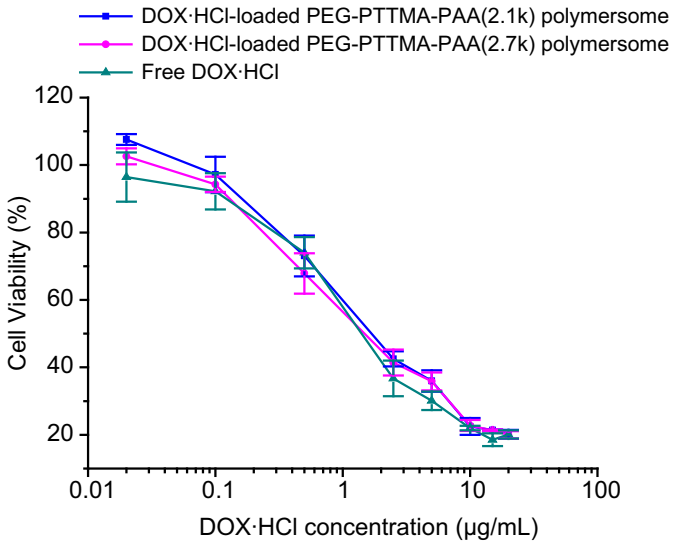


Fig. 5. Anti-tumor activity of DOX·HCl-loaded PEG-PTTMA-PAA polymersomes and free DOX·HCl as a function of DOX concentrations. HeLa cells were incubated with DOX·HCl-loaded PEG-PTTMA-PAA polymersomes or free DOX·HCl for 48 h. The cell viability was determined by MTT assays ($n = 4$).

(DLE) increased with increasing PAA block lengths, likely due to enhanced electrostatic interactions of polymersomal lumen with DOX·HCl. For example, at a theoretical DLC of 5 wt.%, DLE of 75.0%, 83.0% and 88.8% were obtained for polymersomes with $M_{n\ PAA}$ of 1.5k, 2.1k and 2.7k, respectively. It should further be noted that DLE decreased slightly with increasing theoretical DLC. For example, for PEG-PTTMA-PAA(2.7k) polymersomes, DLE decreased from 88.8% to 79.5% when increasing theoretical DLC from 5 wt.% to 20 wt.%. The sizes of DOX·HCl-loaded polymersomes increased with increasing DOX·HCl loading contents (Table 3). The polydispersity, however, remained typically low. It should further be noted that at similar drug loading levels, polymersome sizes decreased with increasing PAA block lengths from 1.5k to 2.7k (Table 3). The zeta potentials of DOX·HCl-loaded polymersomes were in the range of −7.9 ~ −15.7 mV, which increased with

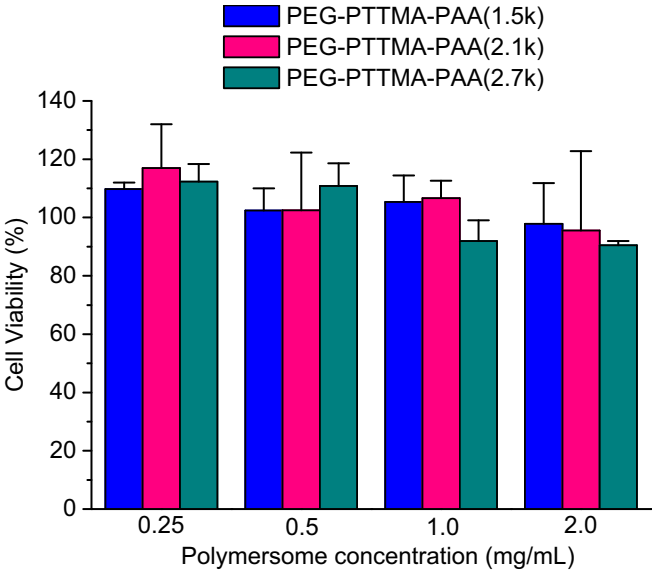


Fig. 6. Cytotoxicity of PEG-PTTMA-PAA polymersomes at varying concentrations of 0.25, 0.5, 1.0 and 2.0 mg/mL. HeLa cells were incubated with polymersomes for 48 h. The cell viability was determined by MTT assays ($n = 4$).

increasing DOX·HCl loading contents and decreasing PAA block lengths.

The *in vitro* release studies performed at 37 °C and different pHs demonstrated that DOX·HCl was released from chimaeric polymersomes much faster at acidic pHs than at physiological pH (Fig. 4). For instance, approximately 83.3% and 69.5% of DOX·HCl was released in 24 h from PEG-PTTMA-PAA(2.1k) polymersomes at pH 4.0 and 5.0, respectively. In contrast, drug release was low (about 29.8%) at pH 7.4 under otherwise the same conditions. In all cases, DOX·HCl was released in a controlled manner and initial burst drug release was not observed. Under the same pH condition, DOX·HCl-loaded PEG-PTTMA-PAA(2.7k) polymersomes displayed somewhat faster drug release than DOX·HCl-loaded PEG-PTTMA-PAA(1.5k) polymersomes (Fig. 4), which is most likely due to slightly faster acetal hydrolysis of PEG-PTTMA-PAA polymersomes with longer PAA lengths as shown above (Fig. 2). It is clear, therefore, that pH-sensitive degradable chimaeric polymersomes based on asymmetric PEG-PTTMA-PAA triblock copolymers are able to not only efficiently load DOX·HCl via "active loading" but also rapidly release encapsulated DOX·HCl in response to endo/lysosomal pH, which makes them unique and particularly appealing for intracellular release of DOX·HCl.

3.5. Anti-tumor activity and intracellular drug release of DOX·HCl-loaded PEG-PTTMA-PAA polymersomes

The anti-tumor activity of DOX·HCl-loaded polymersomes was investigated in HeLa cells by MTT assays. Interestingly, the results showed that DOX·HCl-loaded polymersomes retained high drug efficacy comparable to free DOX·HCl (Fig. 5). The viability of HeLa cells was reduced to about 42.0% following 48 h incubation with 2.5 μg DOX·HCl equiv./mL DOX·HCl-loaded polymersomes. The IC_{50} (inhibitory concentration to produce 50% cell death) of DOX·HCl-loaded PEG-PTTMA-PAA(2.1k) and PEG-PTTMA-PAA(2.7k) polymersomes was determined to be 1.67 and 1.48 μg DOX·HCl equiv/mL, respectively, which was close to that of free DOX·HCl (1.39 μg/mL) and lower than those reported for liposomal doxorubicin formulations (4.2 μg/mL [40], 8.2 μg/mL [41], and 150 μg/mL [42]). The high anti-tumor activity of DOX·HCl-loaded polymersomes indicates that DOX·HCl has been efficiently delivered and released into the nuclei of HeLa cells. It should be noted

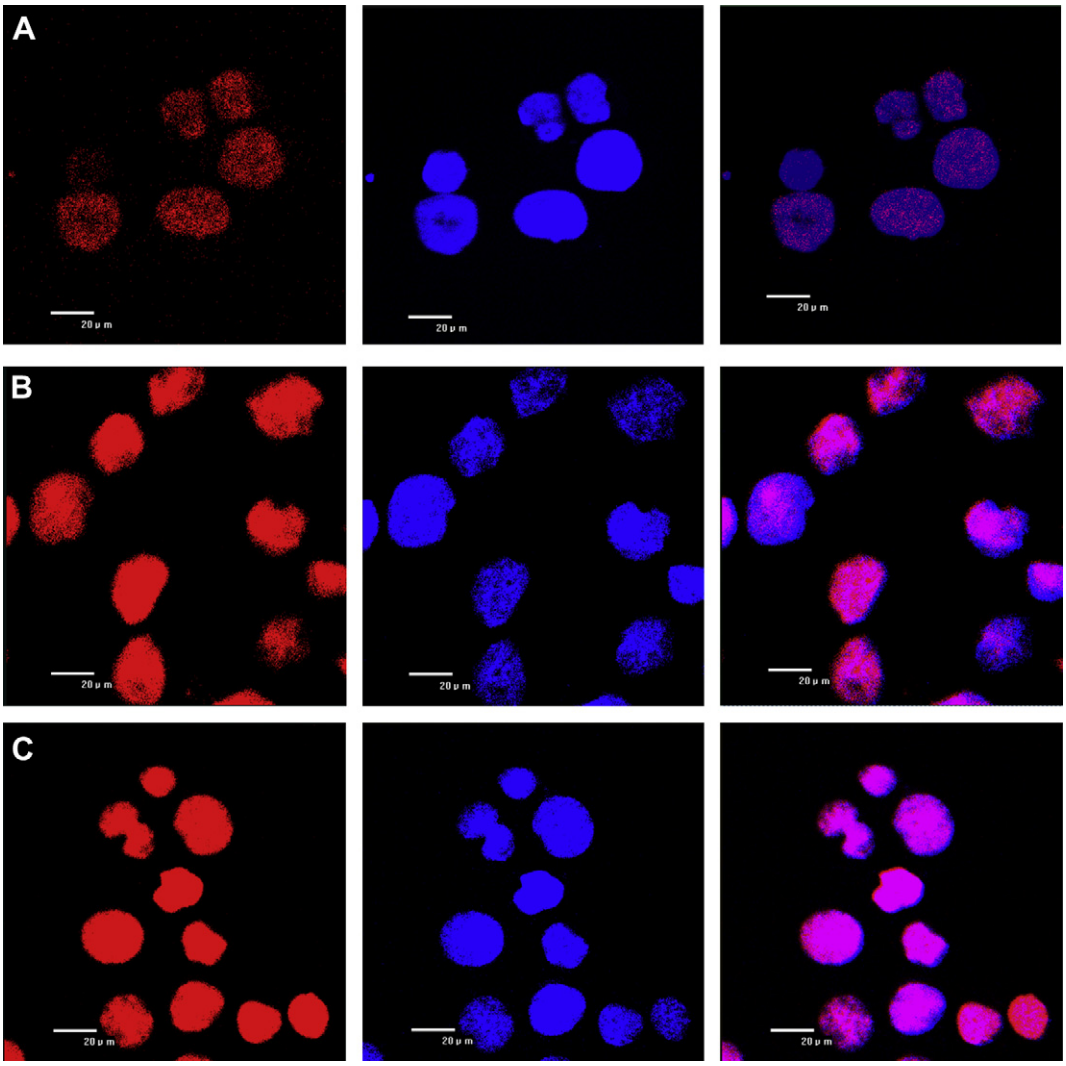


Fig. 7. CLSM images of HeLa cells incubated with DOX·HCl-loaded chimaeric polymersomes and free DOX·HCl (dosage 10 μg/mL). For each panel, images from left to right show DOX·HCl fluorescence in cells (red), cell nuclei stained by DAPI (blue), and overlays of two images. The scale bars correspond to 20 μm in all the images. (A) DOX·HCl-loaded PEG-PTTMA-PAA(2.1k) polymersomes, 2 h incubation; (B) DOX·HCl-loaded PEG-PTTMA-PAA(2.1k) polymersomes, 4 h incubation; and (C) free DOX·HCl, 4 h incubation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that the observed killing effect is partially due to free DOX·HCl leaked from polymersomes prior to their internalization by cells. While under otherwise the same conditions, blank PEG-PTTMA-PAA polymersomes were practically non-toxic (cell viability > 90%) up to a tested concentration of 2.0 mg/mL (Fig. 6), suggesting that PEG-PTTMA-PAA polymersomes possess good biocompatibility.

The cellular uptake and intracellular drug release behaviors of DOX·HCl-loaded PEG-PTTMA-PAA polymersomes were studied using confocal laser scanning microscopy (CLSM). The cell nuclei were stained with DAPI (blue). Remarkably, significant DOX·HCl fluorescence was observed in the nuclei of HeLa cells following 2 h incubation with DOX·HCl-loaded PEG-PTTMA-PAA(2.1k) polymersomes (Fig. 7A), indicating fast internalization of polymersomes and efficient release of DOX·HCl inside cells. The longer incubation time (*e.g.* 4 h) resulted in stronger DOX·HCl fluorescence in the nuclei of HeLa cells (Fig. 7B), which was comparable to the cells incubated with free under otherwise the same conditions (Fig. 7C).

These results confirm that pH-sensitive degradable chimaeric polymersomes can efficiently load and deliver DOX·HCl into tumor cells, achieving high drug efficacy. The anti-tumor activity of DOX·HCl-loaded PEG-PTTMA-PAA polymersomes might be further enhanced by installing a tumor-targeting ligand that facilitates specific cellular uptake [43]. These pH-sensitive degradable chimaeric polymersomes, therefore, possess several favorable properties including excellent biocompatibility, low CMC, small particle size, high drug loading, as well as pH-responsive drug release, which render them a highly promising alternative to liposomes for targeted cancer therapy.

4. Conclusions

We have demonstrated that pH-sensitive degradable chimaeric polymersomes are able to actively load hydrophilic anti-cancer drug DOX·HCl as well as efficiently deliver and release DOX·HCl into tumor cells, achieving high anti-tumor activity comparable to that of free DOX·HCl. These advanced polymersomes are potential alternatives to liposomes and have following unique features: (i) they are readily formed from designed asymmetric pH-sensitive degradable PEG-PTTMA-PAA triblock copolymers that can be conveniently prepared by controlled sequential RAFT copolymerization; (ii) they show remarkably high loading of DOX·HCl due to presence of electrostatic interactions between DOX·HCl and shorter PAA located in the aqueous core. This represents an emerging “active loading” method for small hydrophilic drugs into polymersomes. This alternative “active loading” approach is more straightforward, efficient, and widely applicable than traditional transmembrane pH-, sulfate-, citrate- or phosphate gradient loading method; (iii) they possess excellent biocompatibility and are intrinsically stealthed as longer PEG constitutes the outer surface of polymersomes; and (iv) they can be quickly destabilized at endosomal pH due to acid-triggered hydrolysis of acetals in the membrane of polymersomes, achieving triggered intracellular release of DOX·HCl and high anti-tumor activity. It should be noted that the drug efficacy of DOX·HCl-loaded pH-sensitive degradable chimaeric polymersomes might be further enhanced by attaching a specific targeting ligand such as folic acid and antibody. These pH-sensitive degradable chimaeric polymersomes are highly promising for tumor-targeting delivery of water-soluble anti-cancer drugs.

Acknowledgments

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2012.06.034.

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