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pH-responsive Polymersome Based on PMCP-*b*-PDPA as a Drug Delivery System to Enhance Cellular Internalization and Intracellular Drug Release*

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Abstract Choline phosphate (CP) as a novel zwitterion possesses specific and excellent properties compared with phosphorylcholine (PC), as well as its polymer, such as poly(2-(methacryloyloxy)ethyl choline phosphate) (PMCP), has been studied extensively due to its unique characteristics of rapid cellular internalization *via* the special quadrupole interactions with the cell membrane. Recently, we reported a novel PMCP-based drug delivery system to enhance the cellular internalization where the drug was conjugated to the polymer *via* reversible acylhydrazone bond. Herein, to make full use of this feature of PMCP, we synthesized the diblock copolymer poly(2-(methacryloyloxy)ethyl choline phosphate)-*b*-poly(2-(diisopropylamino)ethyl methacrylate) (PMCP-*b*-PDPA), which could self-assemble into polymersomes with hydrophilic PMCP corona and hydrophobic membrane wall in mild conditions when the pH value is ≥ 6.4. It has been found that these polymersomes can be successfully used to load anticancer drug Dox with the loading content of about 11.30 wt%. After the polymersome is rapidly internalized by the cell with the aid of PMCP, the loaded drug can be burst-released in endosomes since PDPA segment is protonated at low pH environment, which renders PDPA to transfer from hydrophobic to hydrophilic, and the subsequent polymersomes collapse thoroughly. Ultimately, the "proton sponge" effect of PDPA chain can further accelerate the Dox to escape from endosome to cytoplasm to exert cytostatic effects. Meanwhile, the cell viability assays showed that the Dox-loaded polymersomes exhibited significant inhibitory effect on tumor cells, indicating its great potential as a targeted intracellular delivery system with high efficiency.

Keywords Drug delivery; Choline phosphate; Polymersome; PDPA; Enhanced cellular internalization

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INTRODUCTION

Zwitterionic polymers composed of anionic and cationic on the corresponding monomer unit have been widely implemented as biomaterials due to their excellent biocompatibility, high hydrophilicity and nonfouling properties[1–7]. For example, polysulfobetaine, polycarboxybetaine and especially polyphosphorylcholine, which were inspired by the headgroup of cell membrane, have been exploited as drug delivery systems[1–4], surface coating to reduce nonspecific adsorption[5, 6] and resist bacteria[7]. Recently, polymers based on choline phosphate (CP), reversing from the phosphorylcholine (PC) structure, have attracted tremendous interest since they exhibited specific, tunable interactions with cell membrane and showed great potential in tissue

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engineering, drug delivery system and surface modification[8–20]. Hu and Emrick[8], Hu *et al.*[9] and Morozova *et al.*[10] reported that they synthesized the CP-polymers where alkyl can be *n*-Bu or other functional groups, which opened new opportunities in post-polymerization modification, bioconjugate chemistry of zwitterionic polymers, and expanded the application of CP functional materials in biology[8–10]. Meanwhile, Wang and Liu[11], Perttu[12] and Motion *et al.*[13] reported the CP liposomes as a novel kind of delivery system, which showed more excellent properties than PC liposomes[11–13]. In our previous works, we firstly found the CP groups in a multivalent fashion could strongly bind to a variety of cell membrane, and such strong adhesion was attributed to the unique quadrupole interaction between the PC and CP groups[14–18]. Recently we exploited the polyvalent CP as a drug delivery system, which showed excellent properties as a novel delivery system to enhance cell internalization efficiency in a short time[14, 15, 20, 21]. Although great progress on CP-based polymer has been made, there are still some challenges, such as how to control the cargo to be released rapidly and then transfer from endosome to cytoplasm effectively, especially for substances such as proteins and therapeutic nucleic acid, which are easily denatured in endosome and only exert cytostatic effects until they spread throughout cytoplasm.

Polymersomes can not only encapsulate hydrophilic substances in lumen, but also integrate hydrophobic molecules within the membrane wall[22]. In contrast to liposomes, polymersomes own larger aqueous lumen as well as thicker hydrophobic membrane wall, which makes it one of the most novel and fascinating delivery systems for the controlled release of antitumor drugs, proteins and therapeutic nucleic acids (siRNA, rRNA, pDNA, *etc.*)[23–29]. In addition, the controlled release of cargo molecules can be achieved by integrating stimuli-responsive (such as pH, temperature, voltage, reduction *etc.*) hydrophobic segments to the amphiphilic copolymer[30–34]. The 2-(diisopropylamino)ethyl methacrylate (DPA) based copolymers have been proven to be an effective delivery system capable of controlling the release of cargo in the desired pH region, such as PMPC-*b*-PDPA, which could assemble to various morphology, and has been developed as an effective delivery system to overcome endo/lysosome barrier[35–37]. The PDPA segment is hydrophilic below pH 6.2, while becomes hydrophobic when the pH of solvent is above 6.4 as a result of the deprotonation of PDPA[38–41]. In addition, the PDPA based amphiphilic copolymers can self-assemble into nanoparticles under extremely mild conditions without using organic solvents (*i.e.*, adjusting solution pH from 2.0 to 7.4 in water), which minimizes the possibility of the cargo molecules like protein, nucleic acid to be denatured and inactivated. Meanwhile, the diisopropyl-substituted tertiary amines in pendant display a prominent "proton sponge" effect, which facilitate the cargo molecules to escape from endosome to cytoplasm, and have been extensively used as genes delivery systems to overcome endosomal barrier[35–37].

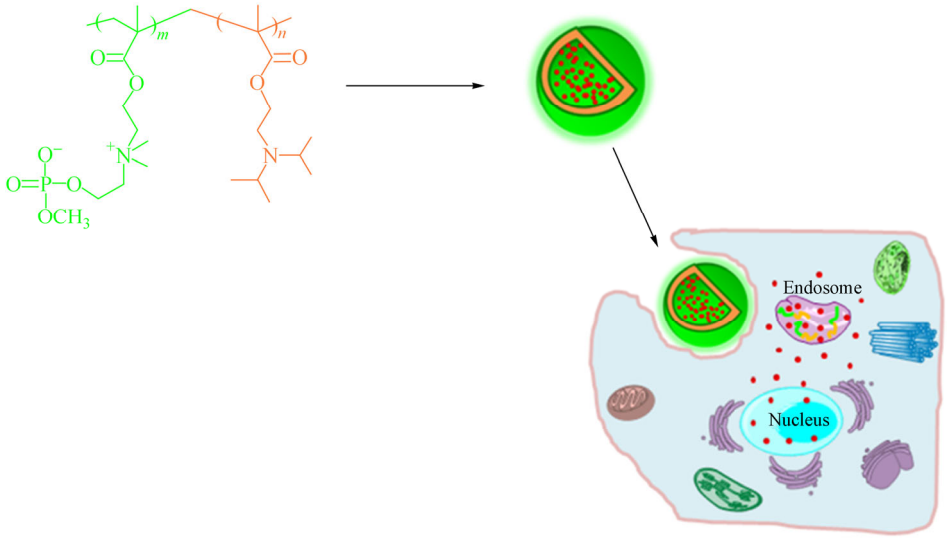
Herein, to overcome the challenges mentioned above, we designed and synthesized a novel diblock copolymer poly(2-(methacryloyloxy)ethyl choline phosphate)-*b*-poly(2-(diisopropylamino)ethyl methacrylate) (PMCP-*b*-PDPA) by atom transfer radical polymerization (ATRP). This diblock copolymer is soluble in dilute acidic solution, while becomes amphiphilic at pH > 6.4 since the PDPA segments become hydrophobic at pH > 6.4. We focused on the self-assemble behavior of PMCP-*b*-PDPA and found that it can self-assemble into polymersomes with PMCP to be coronas and hydrophobic PDPA to be membrane walls (Scheme 1) at pH = 6–7. It has been found that the drug-loaded PMCP-*b*-PDPA polymersomes can rapidly enter the tumor cell, since the PMCP coronas greatly enhance cellular internalization efficiency due to the strong quadrupole interaction between CP groups and PC groups of cell membrane[15, 16, 19, 20]. After the polymersomes enter the endo/lysosome (pH = 5.0), the cargo molecules Dox can be rapidly and comprehensively released from the delivery system due to the disassembly of the vesicle structure. Moreover, the "proton sponge" effect of PDPA chains further help the cargo molecules escape from endo/lysosome to exert cytostatic effects.

EXPERIMENTAL

Materials

The ATRP initiator of 3-azidopropyl 2-bromo-2-methylpropanoate and the monomer of 2-(methacryloyloxy) ethyl choline phosphate (MCP) were synthesized according to previous methods[18, 20]. Copper(I) bromide (CuBr,

99.999%), 2,2-bipyridine (bpy), 2-(diisopropylamino)ethyl methacrylate (DPA) and doxorubicin hydrochloride (Dox·HCl) were purchased from Sigma-Aldrich. The DPA monomer was purified by vacuum distillation prior to use. All chemicals were used without further purification unless otherwise mentioned.



Scheme 1 Illustration of pH-responsive polymersomes based on PMCP-*b*-PDPA diblock copolymer for Dox loading and triggered intercellular release of anticancer drug

Characterization

NMR spectra were recorded by Unity-400 NMR spectrometer at room temperature in D_2O/HCl (pH = 2.0). The size distribution of the PMCP-*b*-PDPA at different pHs was determined by dynamic light scattering (DLS) using a Zetasizer Nano-ZS from Malvern Instruments with He-Ne laser. The measurements were made with wavelength of 633 nm at 25 °C and angle detection at 173°. The morphology of diblock copolymers nanoparticles was visualized by transmission electron microscopy (TEM) using a JEOL JEM-1011 electron microscope. To prepare TEM samples, the polymersome solution (2 μL) was dropped onto a carbon-coated copper grid, and the water droplet was allowed to evaporate slowly under ambient conditions[29]. The Dox content loaded in the polymersome was determined by UV-Vis spectroscopy (NanoDrop 2000c from Thermo Scientific). The fluorescence intensity internalized into the cells at fixed time interval was measured by flow cytometry (FACS, Guava easyCyte 6-2L from Milipore), the excitation and emission were set at 480 nm and 560 nm. The cell viability was detected by synergy microplate reader using a Synergy H1 Microplate Reader from BioTek. The fluorescence images were obtained by confocal laser scanning microscopy (CLSM, LSM 700 Carl Zeiss Microscopy).

Synthesis of the Copolymer Poly(2-(methacryloyloxy)ethyl choline phosphate)-block-poly(2-(diisopropylamino)ethyl methacrylate) (PMCP-b-PDPA)

The typical ATRP procedure was adapted from previous methods[17, 20]. ATRP initiator of 3-azidopropyl 2-bromo-2-methylpropanoate (25 mg, 0.1 mmol), 2,2′-dipyridyl (bpy, 31.2 mg, 0.2 mmol), 2-(methacryloyloxy) ethyl choline phosphate (MCP, 0.738 g, 2.5 mmol) and 5 mL of methanol were added to a 25 mL Schlenk flask, and put through three cycles of freeze-pump-thaw with argon, then added CuBr (14.3 mg, 0.1 mmol). The reaction was processed at room temperature for 24 h. Then the reaction mixture was exposed to air and dialyzed against deionized water (MWCO 2000) for 24 h. After freeze-drying, the macro-initiator $PMCP_{25}$-Br was collected with a yield of 95.4%.

Then, $PMCP_{25}$-Br (0.381 g, 0.05 mmol), 2,2′-dipyridyl (bpy, 31.2 mg), 2-(diisopropylamino)ethyl methacrylate (DPA, 1.32 g, 6.2 mmol) and methanol (8 mL) mixture were added into the flask, then put through

three cycles of freeze-pump-thaw with argon. Finally, CuBr (14.3 mg, 0.1 mmol) was added and the reaction proceeded at room temperature for 48 h. The reaction was stopped by exposing the solution to air, and the catalyst was removed by dialysis bag with MWCO 2000 membrane. After freeze-drying for 24 h, $PMCP_{25}$-*b*-$PDPA_{120}$ was collected with yield 91.6%.

Preparation of PMCP-b-PDPA Polymersomes

The diblock copolymers $PMCP_{25}$-*b*-$PDPA_{120}$ (15 mg) was dissolved into dilute aqueous HCl (pH = 2, 15 mL, the initial copolymer concentration was 1 mg/mL) and stirred for 2 h. Then the pH was slowly adjusted over 30 min period from pH 2.0 to pH 5.0 using 0.10 mol/L aqueous NaOH. After equilibrium for 30 min at pH 5.0, the pH was adjusted to 6.4 using 0.01 mol/L aqueous NaOH over the same time scale and equilibrium for another 30 min. Finally, the pH was adjusted over 30 min from pH 6.4 to pH 7.4 using 0.005 mol/L aqueous NaOH and equilibrium for another 2 h to prepare TEM samples. The final copolymer concentration after pH adjustment was calculated by the final volume of solution (the final concentration was about 0.85 mg/mL). The average sizes and polydispersity at different pH were measured by DLS.

Loading Dox into Polymersomes

Dox-loaded polymersomes were prepared by adjusting the pH of $PMCP_{25}$-*b*-$PDPA_{120}$ and Dox·HCl aqueous solution from pH 2.0 to 7.4. Following the polymersome preparation method described above, $PMCP_{25}$-*b*-$PDPA_{120}$ (15 mg), Dox·HCl (8 mg) were dissolved in dilute aqueous HCl (pH = 2, 15 mL) and stirred for 2 h. The pH was gradually raised to pH 7.4, and the solution was allowed to stand overnight prior to dialysis. The Dox-polymersome mixture was then dialyzed against 500 mL phosphate buffered saline (PBS) at pH 7.4 for 12 h to remove excess free Dox from the solution[29, 34]. The final Dox concentration in the polymersome solution after dialysis was measured by UV-Vis spectrophotometry using the calibration curve (shown in Fig. S1 in the supporting information, SI). In addition, 5 mL of this solution was lyophilized to determine the drug loading content (DLC) and drug loading efficiency (DLE) using the Eqs. (1) and (2)[34].

$$\text{DLC (\%)} = \frac{\text{Weight of drug encapsulated in polymersomes}}{\text{Weight of polymer}} \times 100 \tag{1}$$

$$\text{DLE (\%)} = \frac{\text{Weight of drug encapsulated in polymersomes}}{\text{Weight of drug in feed}} \times 100 \tag{2}$$

In Vitro Drug Release

The drug release from the PMCP-*b*-PDPA polymersomes was examined using a dialysis method against PBS at pH 5.0 and pH 7.4 at 37 °C, respectively. Dox-loaded polymersomes solution (3 mL) was transferred to a dialysis bag (MWCO 3500) and immersed in a flask containing 17 mL PBS (pH 5.0, 7.4) with constant stirring at 37 °C. At fixed time intervals, solution outside the dialysis bag (0.5 mL) was taken out and subjected to UV-Vis measurements to determine the Dox concentration in solution, and the same volume of fresh PBS was replaced after every measurement. The concentration of released Dox at every time interval was measured *via* UV-Vis spectroscopy, using the previous standard calibration curve experimentally obtained, and the released amount was calculated by Eq. (3) according to our previous method[20].

$$m_{\text{act}} = \left(c_{\text{t}} + \frac{v}{V} \times \sum\nolimits_{0}^{\text{t}-1} c_{\text{t}} \right) \times V \tag{3}$$

where m_{act} is the actual mass of Dox released at time t, c_{t} is the Dox concentration in solution at time t measured *via* UV-Vis spectrometer, v is the sample volume taken at fixed time interval, V is the total volume of solution (20 mL). The experiment was performed in triplicate.

Cellular Uptake Assays

MCF-7 cells were seeded into 24-well plates at a density of 7×10^4 cells per well, and incubated in Dulbecco's modified eagle media (DMEM) containing fetal bovine serum (FBS), 100 units per mL of penicillin and

100 μg/mL of streptomycin at 37 °C for 24 h. The cells were treated with Dox-loaded polymersomes in serum-free DMEM for 1, 2 and 4 h respectively. The original concentration of Dox in medium was 2 μg/mL. After incubation at 37 °C for corresponding time intervals, the culture medium was removed, and the cells were washed three times with PBS. Then, the cells were fixed with 4% formaldehyde at room temperature for 30 min and washed three times with PBS. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 10 μg/mL) for 10 min and washed at least four times with cold PBS. Finally, the CLSM images were recorded using confocal microscope.

Flow Cytometry

MCF-7 cells were seeded into 12-well plates at a density of 2×10^5 cells per well, and incubated in DMEM containing fetal bovine serum (FBS), 100 units per mL of penicillin and 100 μg/mL of streptomycin at 37 °C for 24 h. After that, the cells were incubated with serum-free DMEM containing Dox-loaded polymersomes for 1, 2 and 4 h respectively. The original concentration of Dox in medium was 2 μg/mL. After incubation for fixed time, the cells were washed three times with PBS, then treated with trypsin and centrifuged. Finally, the cells were suspended in 0.3 mL PBS and determined with flow cytometer.

Cell Viability Assays

The cell viability was evaluated by Celltiter-Blue cell viability assay. MCF-7 cells were seeded into 96-well plates at a density of 7×10^3 cells per well, and incubated in DMEM containing fetal bovine serum (FBS), 100 units per mL of penicillin and 100 μg/mL of streptomycin at 37 °C for 24 h. After that the medium was replaced by serum-free DMEM containing different concentrations of Dox-loaded polymersomes. After incubation for 24 h, the medium was removed, then 0.1 mL of fresh serum-free DMEM containing 10 μL of Celltiter-Blue reagent was added to each well and the plates were incubated for another 4 h at 37 °C. Finally, the fluorescence signal was measured by microplate reader (λ_{ex} = 560 nm, λ_{em} = 590 nm). The cell viability was calculated based on the Eq. (4):

$$\text{Cell viability (\%)} = \frac{\text{Fluorescent intensity (sample)}}{\text{Fluorescent intensity (control)}} \times 100 \quad (4)$$

RESULTS AND DISCUSSION

Synthesis of the Copolymer PMCP-b-PDPA

The diblock copolymer PMCP-*b*-PDPA was synthesized by sequential ATRP in methanol at room temperature. To ensure the diblock copolymers PMCP-*b*-PDPA have the same polymerization degree of MCP, we firstly synthesized the PMCP-Br by ATRP as a macro-initiator. Then, the PMCP-*b*-PDPA was synthesized by ATRP in the presence of PMCP-Br macro-initiator at the room temperature. Herein, we synthesized a series of PMCP-*b*-PDPA copolymers with similar PMCP segment but different PDPA lengths. The compositions of these copolymers were determined by ^{1}H-NMR spectrum in D_2O (pH = 2) by comparing the integration of the PMCP characteristic peak at δ = 3.38 ppm and that of the PDPA characteristic peak at δ = 1.50 ppm. The ^{1}H-NMR and ^{31}P-NMR spectra of these diblock copolymers are shown in Fig. 1 and Fig. S2 (in SI).

Preparation and Characterization of pH-responsive Polymersomes

The PMCP-*b*-PDPA copolymers were soluble in water at pH 2.0–5.0 as the PDPA segments existed as a cationic polyelectrolyte. The PDPA is a weak polybase with the pK_a of 6.2 in water, and it is found that the PDPA chain lengths have no obvious influence on the transition pH from hydrophilic cationic polyelectrolyte to hydrophobic segments[32, 33, 35, 36]. The DLS measurements (Fig. 2a) showed that these copolymers existed as unimer below the pK_a, and the average sizes ranged from 4.3 nm to 8.3 nm at pH 5.0. However, upon adjusting the solution pH gradually over 6.4, the copolymer turned to be amphiphilic since the PDPA segments became hydrophobic as a result of the deprotonation, and then the amphiphilic copolymers could assemble into nanoparticles with various morphologies according to different molar ratios between PDPA to PMCP. These nanoparticles had average sizes varying from 37 nm to 55 nm, which were much larger than those of unimer, and proper size distributions

(Fig. 2b, PDI: 0.228–0.368) at pH 7.4.

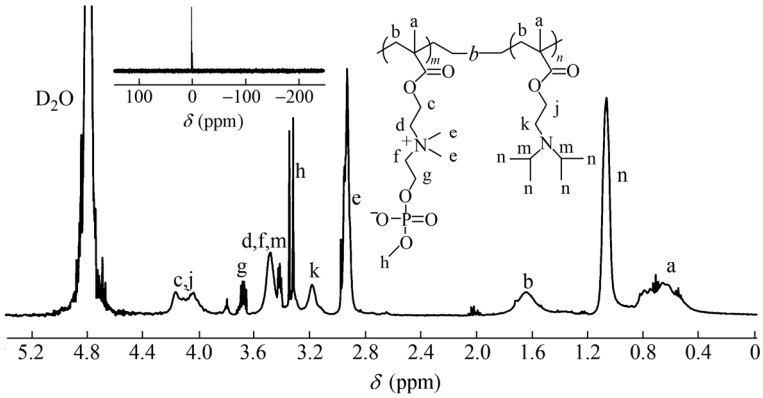


Fig. 1 ^{1}H-NMR and ^{31}P-NMR spectra (inset image) of PMCP$_{25}$-*b*-PDPA$_{30}$ in D_2O (pH = 2)

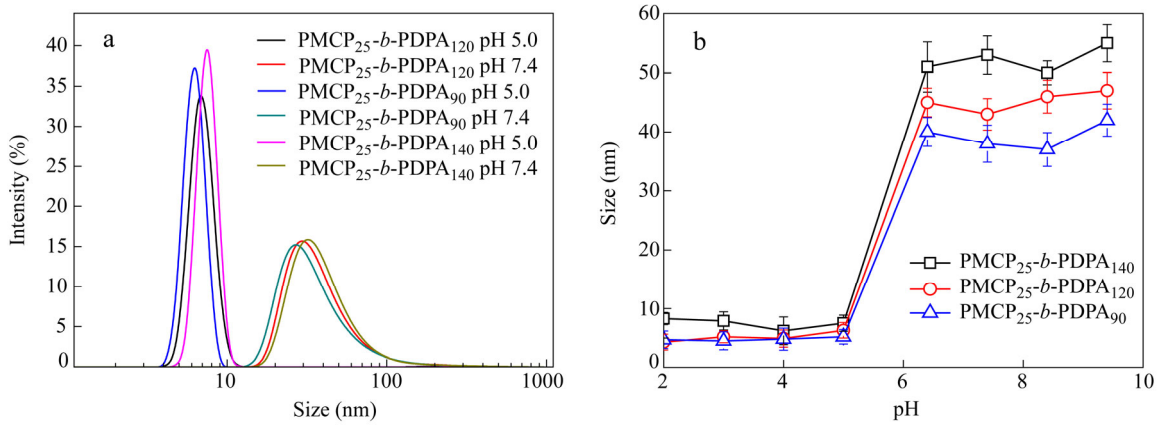


Fig. 2 The DLS measurement of PMCP-*b*-PDPA: (a) size distribution profiles of PMCP-*b*-PDPA copolymers at pH 5.0 (unimers) and 7.4 (nanoparticles), (b) PMCP-*b*-PDPA copolymer size distribution as a function of pH

According to the Eisenberg empirical rule, amphiphilic copolymers with more than 45% hydrophilic contents preferred to form spherical micelles, otherwise polymersomes or inverted nanostructures were formed when the hydrophilic contents were 35% or < 25%[23]. Therefore, it can be speculated that the PMCP$_{25}$-*b*-PDPA$_{90}$ tended to assemble into spherical micelles at pH 7.4 (Fig. S3, in SI). When increasing the PDPA polymerization degree to 120, the copolymer favored the formation of polymersomes (Fig. 3a) and the giant aggregates formed when the PDPA polymerization degree was increased to ≥ 140 (Fig. S4, in SI). The reason for this morphology change is that the molecular curvature will reduce greatly with the increase of PDPA volume fraction. The amphiphilic copolymers will preferentially form membrane at low curvature, and then the membrane will wrap up to form a hollow sphere known as polymersome[33]. However, it becomes difficult to form polymersome but to aggregate to giant aggregates when the contents of hydrophobic PDPA are too high.

The polymersomes formed by PMCP$_{25}$-*b*-PDPA$_{120}$ at pH 7.4 have a proper size distribution (PDI: 0.228–0.236). Meanwhile, the size statistics of TEM show that the average diameter of these polymersomes is about (66 ± 19) nm, and the thickness of membrane wall is about (15 ± 3) nm (Fig. 3b). The thick membrane wall suggested these polymersomes may be tougher than the conventional liposomes. It is notable that there are no obvious changes for the diameter and morphology of the polymersomes with the increase of pH after it is over 6.4 (Fig. 2b and Fig. S5 in SI). This may be because that almost all the DPA units have been deprotonated over pH 6.4, and the changes of pH have no influence on the size and morphology of the polymersomes.

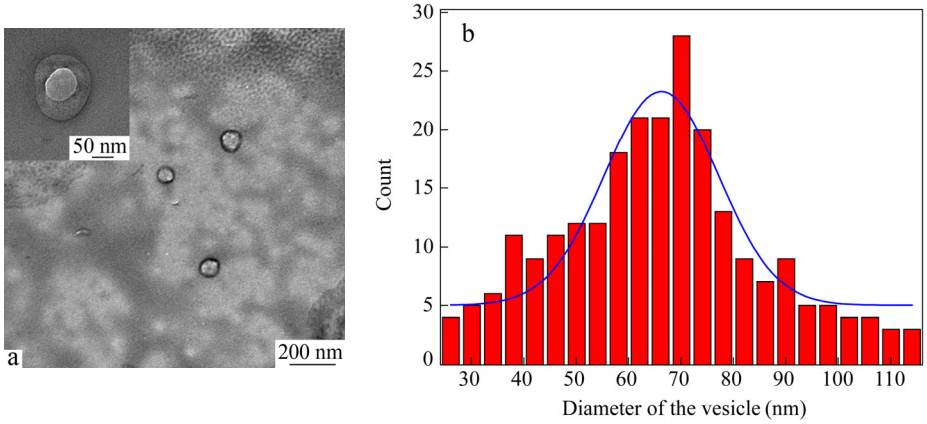


Fig. 3 pH-induced formation of polymersomes from $PMCP_{25}$-*b*-$PDPA_{120}$: (a) the TEM images of the polymersomes prepared at pH 7.4, (b) the diameter distribution of these polymersomes analyzed from TEM images

Loading and pH-responsive Release of Anticancer drug

$PMCP_{25}$-*b*-$PDPA_{120}$ was further studied to work as the drug delivery system. Dox-loaded polymersomes were readily prepared by adjusting the pH of the Dox and $PMCP_{25}$-*b*-$PDPA_{120}$ aqueous solution from 2.0 to 7.4 followed by extensive dialysis to remove free Dox. The drug loading content was calculated to be about 11.30 wt%, and the loading efficiency was approximately 32.49% according to the UV-Vis spectroscopy (Fig. S1 in SI, and Eqs. 1 and 2). In addition, there are no obvious changes between the sizes of polymersomes before and after Dox loaded (Fig. 4a), which further suggested that the Dox was mainly packaged into the lumen of polymersomes.

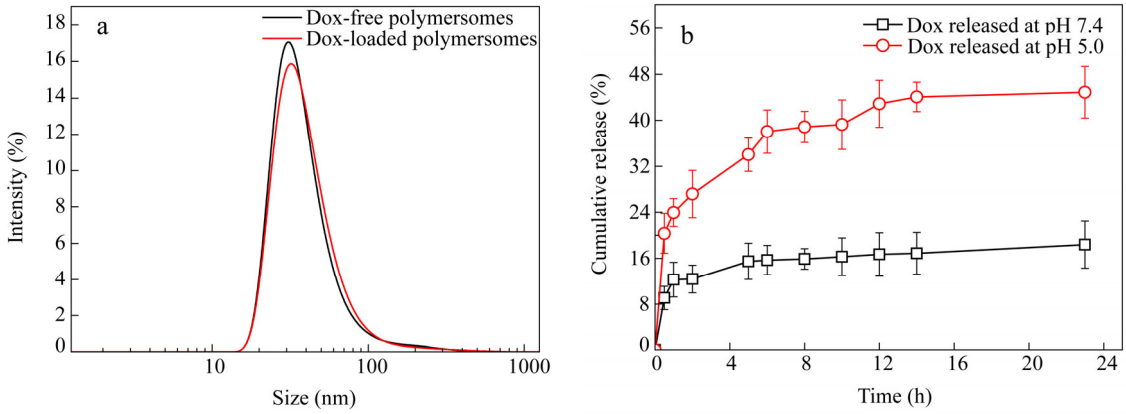


Fig. 4 (a) The size distribution of Dox-free polymersomes and Dox-loaded polymersomes from $PMCP_{25}$-*b*-$PDPA_{120}$; (b) *In vitro* Dox release from the Dox-loaded polymersomes from $PMCP_{25}$-*b*-$PDPA_{120}$ in PBS buffer under different pHs (pH 7.4 and pH 5.0)

The release profile of the cargo molecules Dox was investigated under conditions mimicking the normal body environment (pH 7.4) and acidic environment in endo/lysosome (pH 5.0) at 37 °C. It can be found from Fig. 4(b) that the release rate of Dox was increased to be 2.45 times at the acidic condition (PBS, pH 5.0) compared to that in the physiological condition (PBS, pH 7.4) within 23 h. This is because that the PDPA chains were protonated to form hydrophilic polycation at the acidic environment (pH = 5.0), which led to the disassembly of polymersome, and the Dox was rapidly released with the collapse of polymersome. Simultaneously this pH-responsive release minimized the premature release of anticancer drug.

Cellular Uptake Assays

We investigated the cellular uptake efficiency of Dox-loaded polymersomes *via* flow cytometry. As shown in Fig. 5, the relative fluorescence intensities of MCF-7 cell lines incubated with Dox-loaded polymersomes were increased when the incubation time was prolonged from 1 h to 4 h, indicating that the Dox-loaded polymersomes were rapidly internalized into the cells. The reason may be that the PMCP corona of the polymersomes enhanced their cellular internalization. Based on our previous studies, the multivalent CP groups could strongly bind to the cell membrane *via* special CP-PC quadrupole interaction, and accelerate the internalization of CP-based materials over a short time[15–20]. Herein, the PMCP corona outside of the polymersomes enhanced these nanoparticles to bind to cell membrane *via* multivalent CP-PC internalization at first, then internalized into the endosomes to further release the cargo molecules.

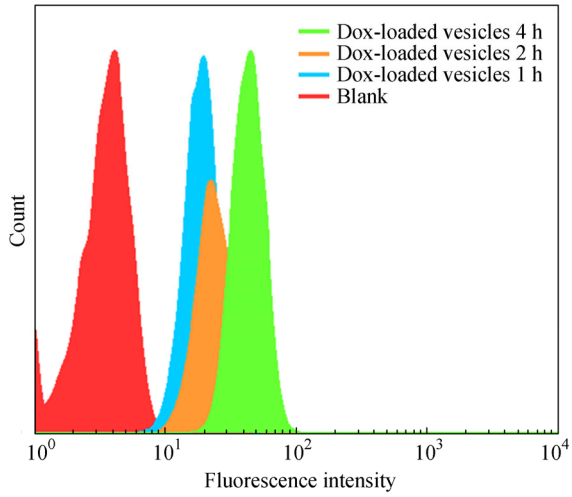


Fig. 5 Flow cytometry analysis of MCF-7 cells treated by Dox-loaded polymersomes from $PMCP_{25}$-*b*-$PDPA_{120}$

We further studied the cellular uptake of Dox-loaded polymersomes by using fluorescence microscopy. MCF-7 cells were incubated with the same concentration of Dox-loaded polymersomes for the same time. As shown in Fig. 6, the red fluorescence signal of Dox clearly appeared after incubated for 1 h, indicating that the Dox-loaded polymersomes had been successfully uptaken. Meanwhile, the red fluorescence was intensity distinctly enhanced with the prolongation of incubation time, and the red fluorescence signal mainly accumulated in the nucleus after incubation for 4 h, suggesting that the cargo had escaped from the endosomes. The reason is that the Dox-loaded polymersomes disassembled after these particles entered into the endosomes (pH = 5.0) due to the protonation of PDPA segment, promoting cargo's rapid release from the polymersomes. What was more, the "proton sponge" effect of PDPA chains further accelerated the Dox escaping from the endosomes to the nucleus to exert its cytostatic effect[35–37].

Cell Viability Assays

To investigate the anticancer efficacy of Dox-loaded polymersomes, we determined the cell viability using Celltiter-Blue assays. We incubated MCF-7 cell line with different concentrations of Dox-loaded polymersomes and blank polymersomes for 24 h. The blank polymersomes were nontoxic to the MCF-7 cells at a certain concentration scale as shown in Fig. S6 (in SI). However, the Dox-loaded polymersomes exhibited significant anticancer efficacy to MCF-7 cells. With increasing Dox concentration, the cell viability was further decreased (Fig. 7). Notably, the cell viability of free Dox was lower than that of Dox-loaded polymersomes. The reason may be that free Dox directly diffused into the nucleus to break the DNA double-strand and inhibit the replication and transcription[2], but the Dox-loaded vesicles were captured into the endo/lysosome initially, and then escaped from these compartments to diffuse into nucleus, which was time dependent.

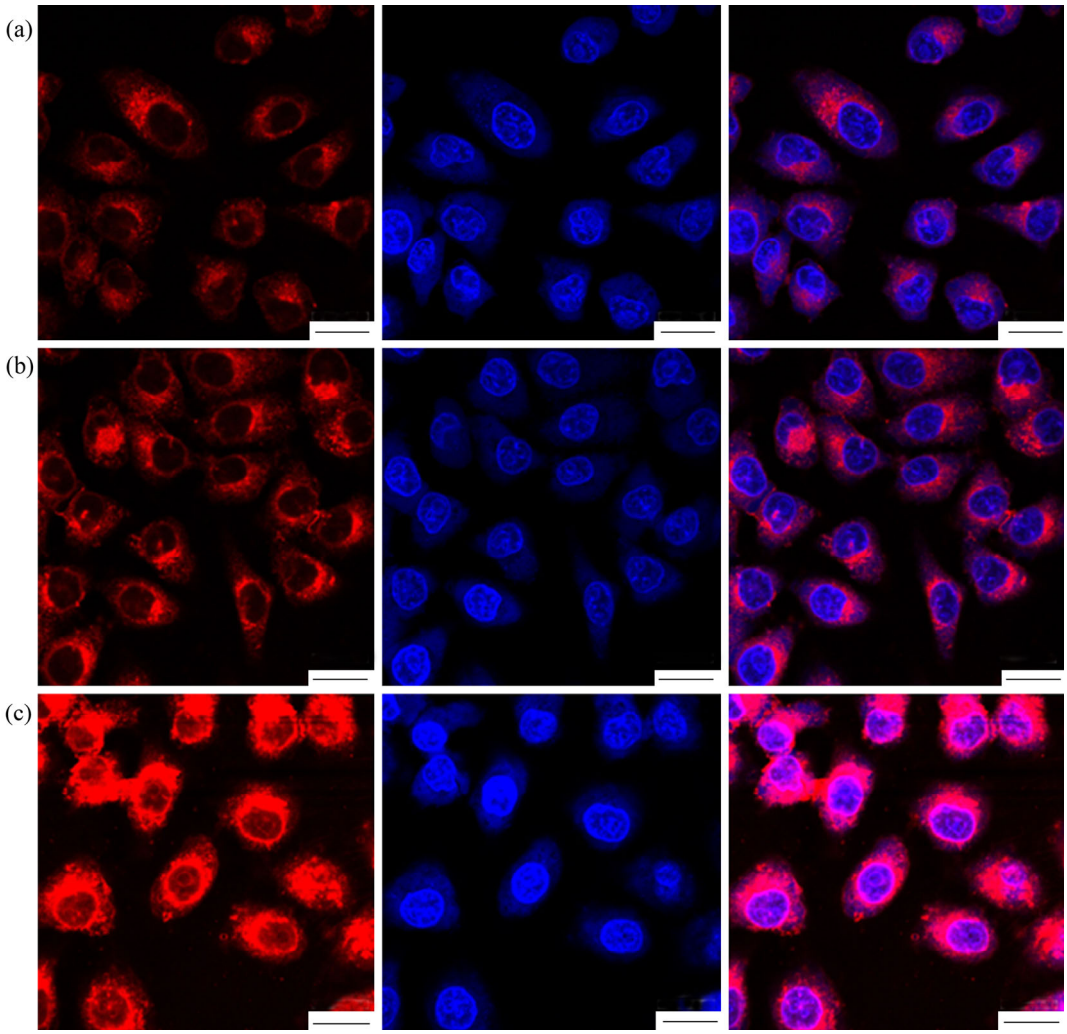


Fig. 6 CLSM images of MCF-7 incubated with Dox-loaded polymersomes (with Dox concentration of 2 μg/mL) from $PMCP_{25}$-*b*-$PDPA_{120}$: (a) incubated for 1 h, (b) incubated for 2 h, (c) incubated for 4 h (Scale bars: 20 μm)

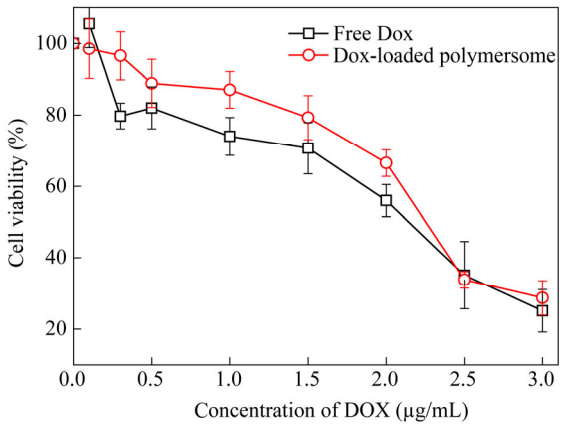


Fig. 7 Cell viability of MCF-7 cells incubated with various concentrations of Dox-loaded polymersomes from $PMCP_{25}$-*b*-$PDPA_{120}$ for 24 h

CONCLUSIONS

In this work, we synthesized a zwitterionic copolymer PMCP-*b*-PDPA, which could self-assemble into polymersomes, and the polymersome formation is pH-dependent. The polymersomes consist of a thick PDPA membrane wall and a corona of PMCP as evidenced by TEM and DLS, where the anticancer drug Dox is entrapped into the lumen of the polymersomes to minimize the premature release due to the protection of these thick membrane walls, and the PMCP corona can enhance the cellular internalization which has been demonstrated in our previous studies. These drug-loaded polymersomes existed steadily at body environment (pH 7.4), while collapsed inside the endosome (pH 5.0) due to the PDPA segments' changes from hydrophobic to hydrophilic, which contributed to the drug's rapid release. Moreover, the drug escaped more easily from the endosome to exert effects due to the "proton sponge" effect of PDPA chains. Finally, the *in vitro* cytotoxicity studies showed that the Dox-loaded polymersomes had significant inhibitory effect on tumor cells. Meanwhile, the polymersomes were prepared under mild conditions without using organic solvents, which minimized the damage to cargo molecules, especially for nucleic acid and protein, indicating that these polymersomes are highly promising as a targeted intracellular delivery system.

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