Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting Information

Dual-responsive polymersomes as anticancer drug carriers for co-delivery of doxorubicin and paclitaxel

Dongxu Zhou, Zhixiong Fei, Lunqiang Jin, Peng Zhou, Chenxi Li, Xiaoling Liu,*
Changsheng Zhao*

1. Experimental Section

1.1. Materials

N-isopropylacrylamide (NIPAM, 98%, Best Reagent) was recrystallized before being used. Methoxyl poly(ethylene glycol) (mPEG, M_w= 2000 g/mol, Best Reagent), 2,2'-azobis(2-methylpropionitrile) (AIBN, 98%, Best Reagent), 2-(diethylamino)ethyl methacrylate (DEAEMA, 99%, Aladdin), 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (DMA, 98%, TCI), 2-hydroxy-4-(methacryloyloxy)benzophenone (BMA, 99%, Alfa Aesar), N,N-dimethylformamide (DMF, anhydrous, Aladdin), 1,4-dioxane (safe dry, Adamas), dichloromethane (DCM, ultra dry, Aladdin), 4-(dimethylamino) pyridine (DMAP, 99%, Best Reagent), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 98% Aladdin), doxorubicin hydrochloride (DOX, 98%, Aladdin), paclitaxel (PTX, 99%, Aladdin), and coumarin 6 (98%, Aladdin) were used as received.

1.2. Instrumentation

^{1}H NMR spectra were recorded by Bruker AV III HD 400 spectrometer using $CDCl_3$ as solvent at room temperature. The molecular weight and distribution indexes of the polymers were measured at 40℃ by TOSOH EcoSEC HLC-8320GPC, 2×TSK gel Super AWM-H columns, DMF as the mobile phase (0.4 mL/min) and PMMA as the standard sample. Dynamic light scattering (DLS) analysis was performed by Malvern Nano-ZS to determine the sizes of polymersomes. The morphologies of polymersomes were determined via transmission electron microscope (TEM) images, which were collected on FEI Tecnai G2 F20 S-TWIN, using the sample of 1 mg/mL crosslinked polymersome solution negatively stained by phosphotungstic acid for 2 min. The UV-vis absorption spectra and absorbances were collected on Shimadzu UV-1750 spectrophotometer, where all samples were analyzed in quartz cuvettes. The microplate reader (Thermo Fisher Multiskan Sky) was used to determine the OD values in Cell Counting Kit-8 (CCK-8) tests. The cellular uptake behavior of polymersomes and free drugs towards HeLa and MCF-7 cells was observed by inverted fluorescence microscope (Leica DMi8).

1.3. Synthesis of mPEG macroinitiator

The synthesis process of mPEG macroinitiator was similar to general esterification. Herein, 0.063 g DMA (M = 364.63 g/mol) was dissolved in 15 mL DCM, followed by adding 6.4 mg DMAP (M = 122.17 g/mol) and 33.0 mg EDC (M = 191.70 g/mol). After complete dissolution, 0.173 mL triethylamine (TEA) was added dropwise at 0℃. The mixture was stirred at room temperature for 2 h to activate the carboxyl groups of DMA. Then 0.25 g mPEG (M = 2000 g/mol), dissolved in 5 mL DCM, was dropped into the reaction flask by a syringe at 0℃. The reaction was kept first at 0℃ for 1 h and then at room temperature for 48 h. Afterwards, most of the solvent was removed by rotary evaporation. The remains were precipitated in cold n-hexane, and the precipitates were dialyzed against DI water for 3 days, followed by freeze-dried. Then, yellow mPEG-macro flakes were collected.

1.4. Synthesis of block copolymers

The block copolymers mPEG-*b*-PNIPAM (PN), mPEG-*b*-PNIPAM-*b*-P(DEAEMA-co-BMA) (PNDB) were synthesized via RAFT. Briefly, in a 50mL Schlenk tube, 234.7 mg mPEG-macro (M = 2346.63 g/mol), 1.6 mg AIBN (M = 164.21 g/mol), 662 mg NIPAM (M = 113.16 g/mol), were added, then the flask was sealed by a rubber stopper. The tube was vacuumed and purged with N_2 for three cycles, followed by adding 3 mL anhydrous DMF deoxygenated for 30 min by a syringe. After the mixture was stirred at 60℃ for 12 h, it was quenched in cold water. The monomer/polymer mixture was precipitated in cold diethyl ether, then the precipitates were filtered and dried under

vacuum. White PN powder was collected.

Likewise, 39.5 mg PN (M = 7892 g/mol, calculated from ^{1}H NMR spectra), 0.8 mg AIBN, 24.4 mg BMA (M = 282.29 g/mol) were charged in a Schlenk tube wrapped by foil to avoid light, followed by being vacuumed and purged with N_2 for three cycles. 239.9 mg deoxygenated DEAEMA (M = 185.26 g/mol) and 0.6 mL deoxygenated 1,4-dioxane were transferred into the reaction flask by syringes. The reaction was kept at 60℃ for 24 h and cooled rapidly. Then, the mixture was precipitated in cold n-hexane, and the precipitates were dried under vacuum to obtain PNDB.

1.5. Preparation of polymersomes

The formation of polymersomes was carried out in dark. A certain amount of PNDB copolymer was dissolved in aqueous HCl solution (pH = 4~5) and then passed through a 0.22 μm nylon filter to remove any impurities. 1 M NaOH solution was added dropwise to perform the self-assembly process, until the pH increased to about 7.4. The final concentration of polymer solution was fixed at 1.0 mg/mL. Then, the solution was stirred for 3 days.

1.6. Crosslinking of polymersomes

The polymersome solution was first passed through 0.8 μm nylon filter to remove impurities, and placed into a UV chamber (UVACUBE 100, honle UV Technologies, Germany). A short time (30 min) of irradiation was enough. To protect polymersomes from overheating, the solution was irradiated for 6 times, 5 min each time.

1.7. Drug loading on polymersomes

DOX and PTX were post-loaded on polymersomes. At room temperature, 1 M HCl was used to adjust the pH of 10 mL crosslinked polymersome solution to 6.0, and 2 mg DOX was added, followed by stirring in dark for 2 days. Then the pH was adjusted to 7.4 using NaOH. The solution was later dialyzed against PBS (pH = 7.4, 25℃) to remove the excess DOX. To ensure it was removed completely, the absorption spectra of polymersome solution and PBS were scanned by UV-vis spectroscopy during the dialysis process.

Due to the poor solubility of PTX in water, 2 mg PTX was first dissolved in 100 μL methanol, then dripped into 10 mL polymersome solution (pH = 7.4) under ultrasonic. After being stirred in dark for 2 days at room temperature, it was dialyzed against PBS (pH = 7.4, 25℃) to remove methanol and excess PTX.

Similarly, to co-encapsulate DOX and PTX, PTX in methanol solution was added dropwise into DOX loaded polymersome solution (pH = 7.4, 25℃) and dialyzed against PBS (pH = 7.4, 25℃).

To determine the encapsulation efficiency and loading capacity of DOX and PTX, the absorbance of drug-loaded polymersome solution was measured by UV-vis at λ = 482 nm (for DOX) and λ = 231 nm (for PTX). Encapsulation efficiency (EE) and loading capacity (LC) were calculated using the following equations:

$$Encapsulation\ efficiency\ (EE)\ (\%) = \frac{Drug\ loaded\ on\ polymersomes\ (mg)}{Drug\ initially\ used\ (mg)} \times 100\%$$

$$Loading\ capacity\ (LC)\ (\%) = \frac{Drug\ loaded\ on\ polymersomes\ (mg)}{Amount\ of\ polymer\ (mg)} \times 100\%$$

In the process of determining the amounts of PTX, the blank polymersomes or DOX loaded polymersome were used as control samples.

The fluorescent polymersomes loading coumarin 6 used in cellular uptake tests were prepared by substituting hydrophobic coumarin 6 for PTX.

1.8. In vitro release of DOX and PTX

10 mL DOX loaded polymersome (DOX-PS), PTX loaded polymersome (PTX-PS) and DOX/PTX loaded polymersome (DOX/PTX-PS) solutions at pH 6.0 or 7.4 were poured into dialysis bags (MWCO = 3500 Da) and placed in beakers containing 1 L PBS (pH = 6.0 or 7.4, containing 0.2% w/v SDS to increase the solubility of PTX) preheat at 25℃, 37℃ and 45℃. At predetermined time intervals, 1 mL PBS was withdrawn for UV-vis measurement (λ = 482 nm or 231 nm) and quickly returned to the beaker after analysis. Finally, DOX and PTX release behavior of the drug-loaded polymersomes was obtained.

1.9. Cell culture

Mouse fibroblasts L929 cells, human cervical cancer cells (HeLa) and human breast cancer cells (MCF-7) were cultured in minimum Eagle's medium (MEM, HyClone), containing 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) antibiotics (penicillin-streptomycin, PS, 10000 U/mL penicillin and 10000 μg/mL streptomycin, HyClone), in a humified atmosphere containing 5% CO_2 at 37℃.

1.10. Cellular uptake study

The cellular uptake behavior and intracellular distribution of polymersomes and free drugs were determined by inverted fluorescence microscope. PTX was replaced by hydrophobic fluorescent probe coumarin 6. Firstly, coumarin 6 and DOX were encapsulated in polymersomes as described in the drug loading section, apart from the change of PTX to coumarin 6. HeLa and MCF-7 cells were seeded in 48-well plates at a density of 2000 cells per well and incubated for 24 h, and then treated with DOX-PS, DOX/coumarin 6-PS, and free DOX, DOX/coumarin 6 cocktail, where the concentrations of DOX and coumarin 6 were kept at 7.2 and 8.5 μg/mL, respectively (calculated according to the loading capacities of polymersomes). After being incubated for 2, 6, 24 h, the cells were washed with PBS and fixed by paraformaldehyde (biosharp Life sciences) at room temperature for 30 min. Then the cells were washed with PBS for three times, followed by treated with 0.25% Triton X-100 (BioFroxx) for 10 min to enhance the permeability of cell membranes. Afterwards, the cells were washed with PBS and stained by 2 μg/mL DAPI (Sigma-Aldrich) for 15 min in the dark. Then the cells were washed by PBS for three times and visualized using inverted fluorescence microscope at λ = 405 nm (blue channel), λ = 470 nm (green channel) and λ = 570 nm (red channel).

1.11. In vitro cytotoxicity assay

Cytotoxicity was determined using CCK-8 assay. L929 cells were seeded in 96-well plates at an initial density of 1000 cells per well in MEM medium containing 10% FBS and 1% PS. After the incubation at 37℃ for 12 h, the cells were treated with sterile polymersomes loaded or not loaded with drugs at different concentrations, where the final polymersome concentrations were 10, 50 and 100 μg/mL. After being incubated for 24, 48 and 96 h, 10 μL CCK-8 solution (Dojindo Laboratories) was added in each well and incubated at 37℃ for another 1 h. To compare the anticancer efficacies between single drug and dual drugs, and between free drugs and drugs encapsulated in polymersomes, HeLa and MCF-7 cells were applied to cytotoxicity tests. The initial seeding quantities of HeLa and MCF-7 cells were 2000 and 5000 per well, respectively; and their expanding time was 24 h. Then the cells were treated with blank polymersomes, DOX-PS, PTX-PS, DOX/PTX-PS, and free DOX, PTX, DOX/PTX cocktail at different concentrations (the final polymersome concentrations were 10, 20, 50, 100 μg/mL; the DOX concentrations of single drug were fixed at 1.44, 2.88, 7.2, 14.4 μg/mL, while for dual drugs they were 1.2, 2.4, 6, 12 μg/mL; the PTX concentrations of single drug were 1.7, 3.4, 8.5, 17 μg/mL, while for dual drugs they were 1.4, 2.8, 7, 14 μg/mL; the drug concentrations were calculated according to the loading capacities of

polymersomes). The incubation times of HeLa and MCF-7 after adding CCK-8 solution were 3h and 4h, respectively. Then the OD value of each well was recorded by a microplate reader at λ = 450 nm. The sample containing only MEM (with FBS and PS) was set as blank sample, and that containing MEM (with FBS and PS) and cells was as control. Each experiment was carried out in triplicate. The cell viability was calculated using the following equation:

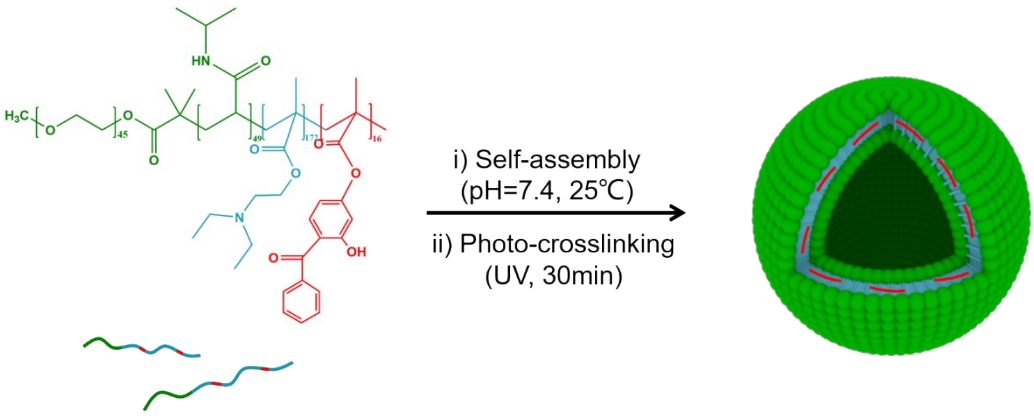
$$Cell\ viability\ (\%) = \frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100\%$$

To determine whether there was a synergistic effect when treating HeLa and MCF-7 cells with free DOX/PTX or DOX/PTX-PS, the combination index (CI) was analyzed based on the Chou-Talalay method[1] using the following equation:

$$CI_x = \frac{(D)_1}{(D_X)_1} + \frac{(D)_2}{(D_x)_2}$$

where $(D)_1$ and $(D)_2$ represent the concentrations of drug 1 and drug 2 in the combination system at the determined IC_x value, and $(D_x)_1$ and $(D_x)_2$ represent the concentrations of drug 1 and drug 2 alone. CI $<$ 1, = 1 and $>$ 1 suggest synergism, additive and antagonism, respectively. In our work, IC_{50} (inhibitory concentration of drugs to cause 50% cell death, calculated by GraphPad) was applied.

2. Supporting Figures



Scheme S1 The self-assembly and photo-crosslinking of polymersomes.

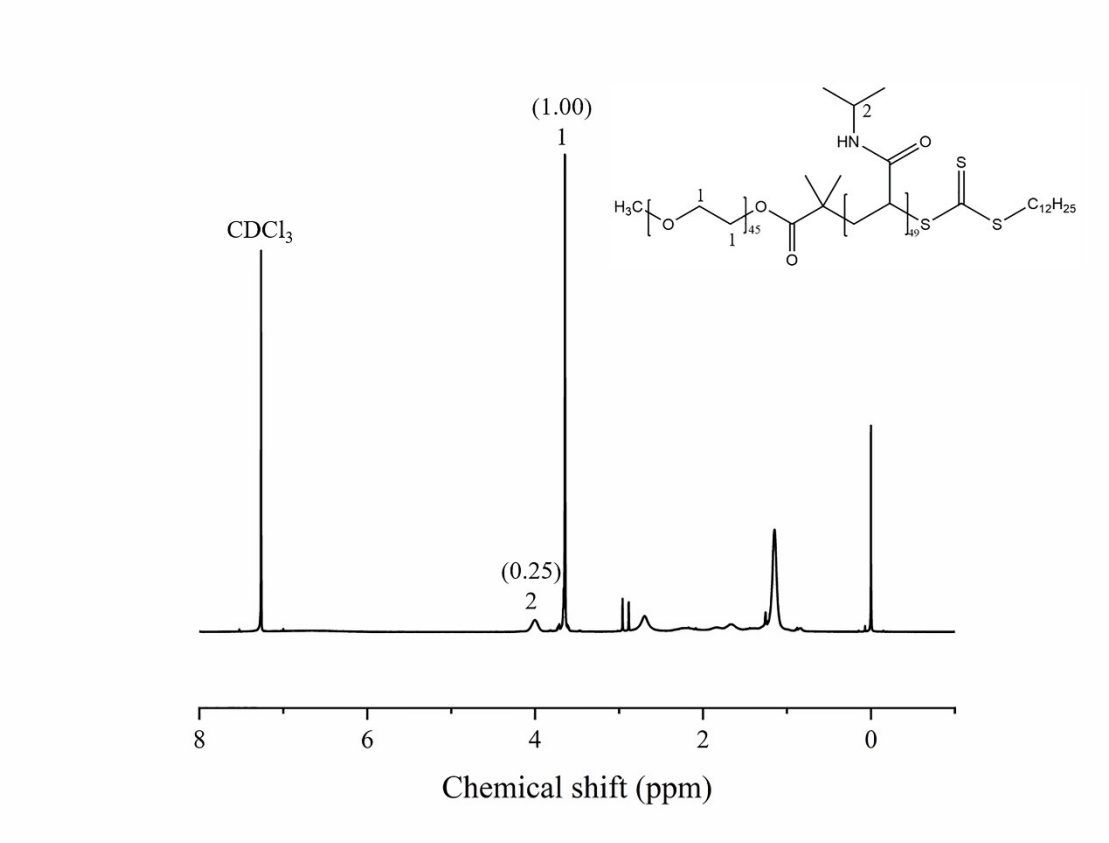


Figure S1 ^{1}H NMR spectrum of the copolymer mPEG-b-PNIPAM (PN). $CDCl_3$ was used as the solvent. The figures in the brackets indicate the normalized peak areas.

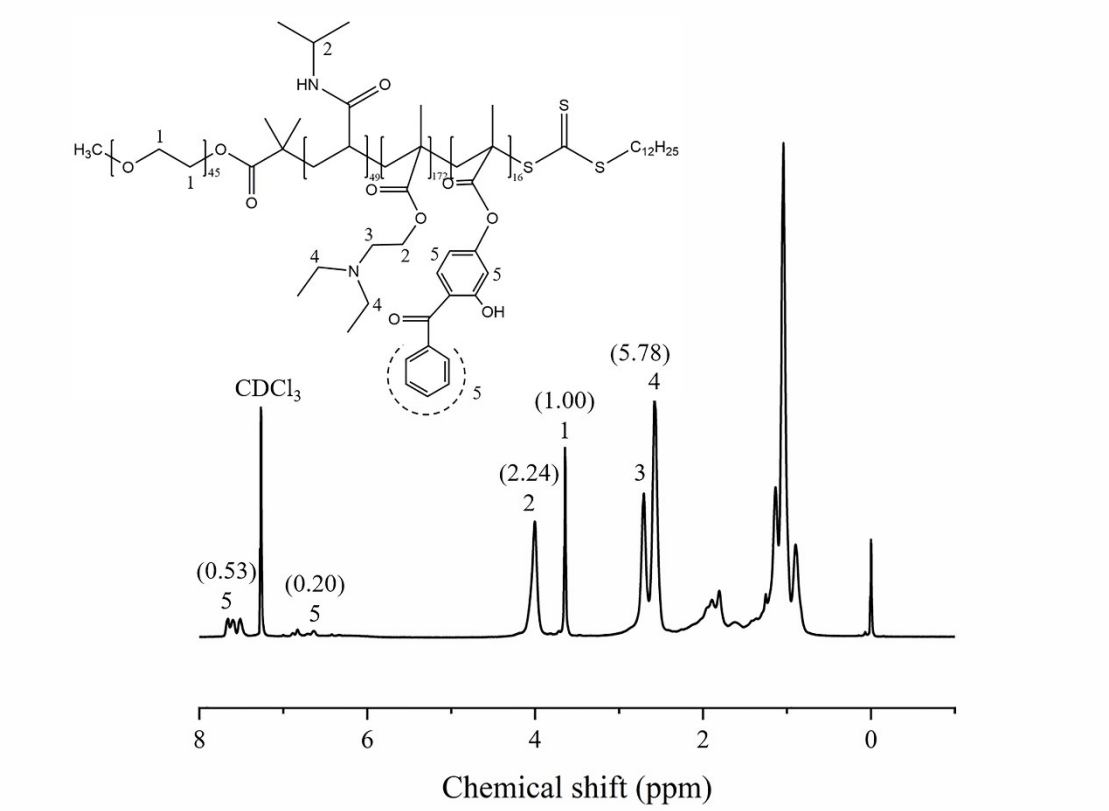


Figure S2 ^{1}H NMR spectrum of the triblock polymer mPEG-b-PNIPAM-b-P(DEAEMA-co-BMA) (PNDB). $CDCl_3$ was used as the solvent. The figures in the brackets indicate the normalized peak areas.

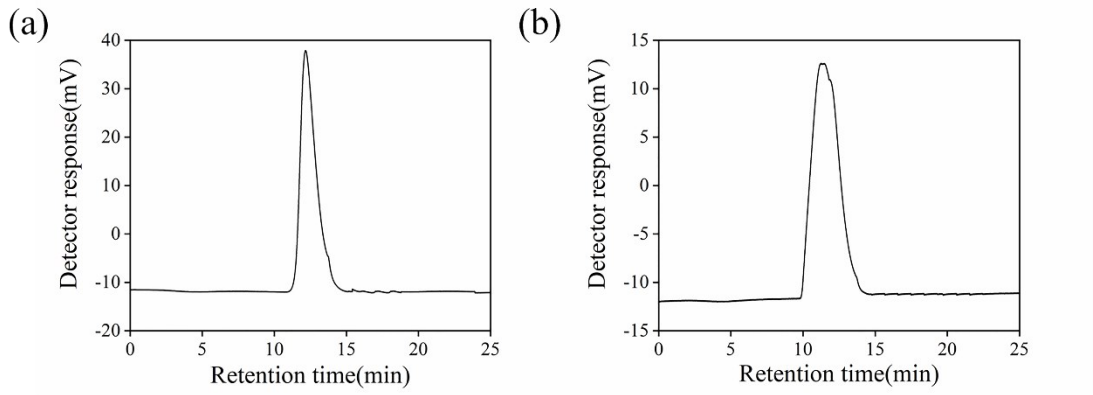


Figure S3 GPC elution curves of (a) mPEG-b-PNIPAM (PN) and (b) mPEG-b-PNIPAM-b-P(DEAEMA-co-BMA) (PNDB).

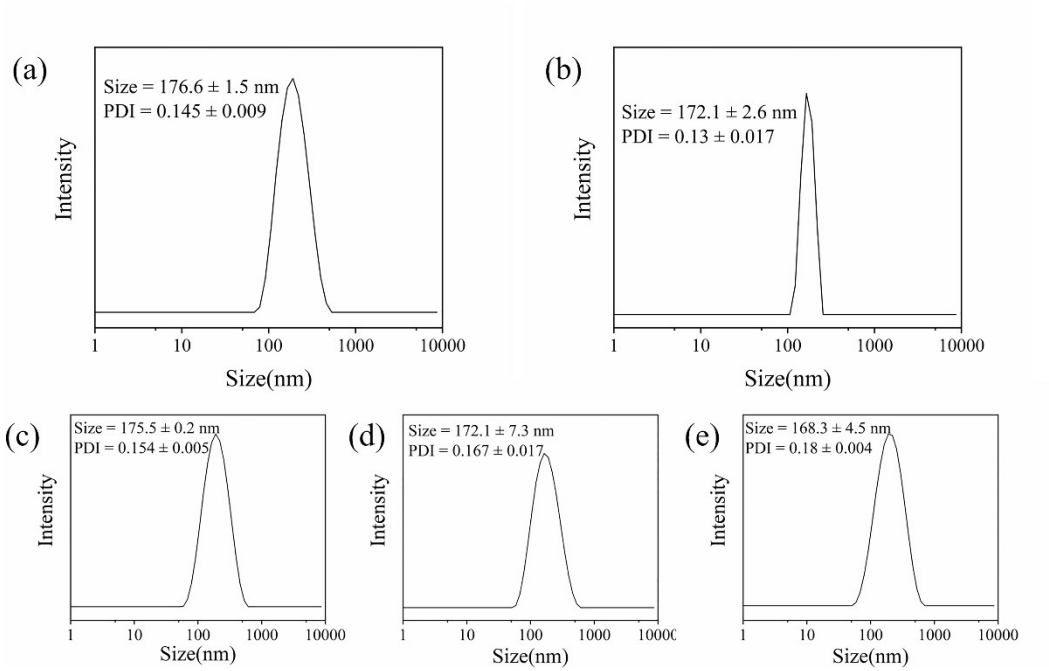


Figure S4 Sizes and distributions of (a) non-crosslinked polymersomes, (b) crosslinked polymersomes, (c) DOX-PS, (d) PTX-PS and (e) DOX/PTX-PS at 25℃, measured by DLS.

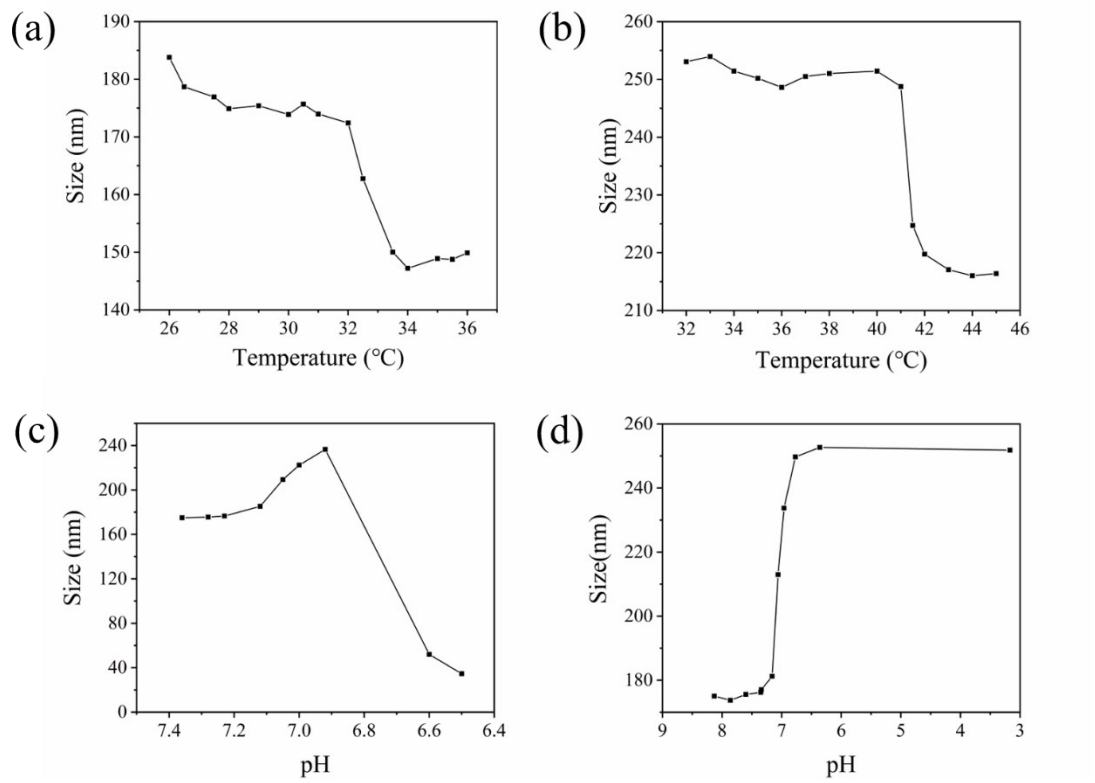


Figure S5 Dual-responsive behavior of crosslinked polymersomes. Temperature-dependent size change of polymersomes at (a) pH 7.4 and (b) pH 6.0; pH-dependent size change of (c) non-crosslinked and (d)crosslinked polymersomes at 25℃, measured by DLS. The maximum standard deviations of (a), (b), (c), (d) are ± 1.7%, ± 1.9%, ± 6.4%, ± 2.3%, respectively.

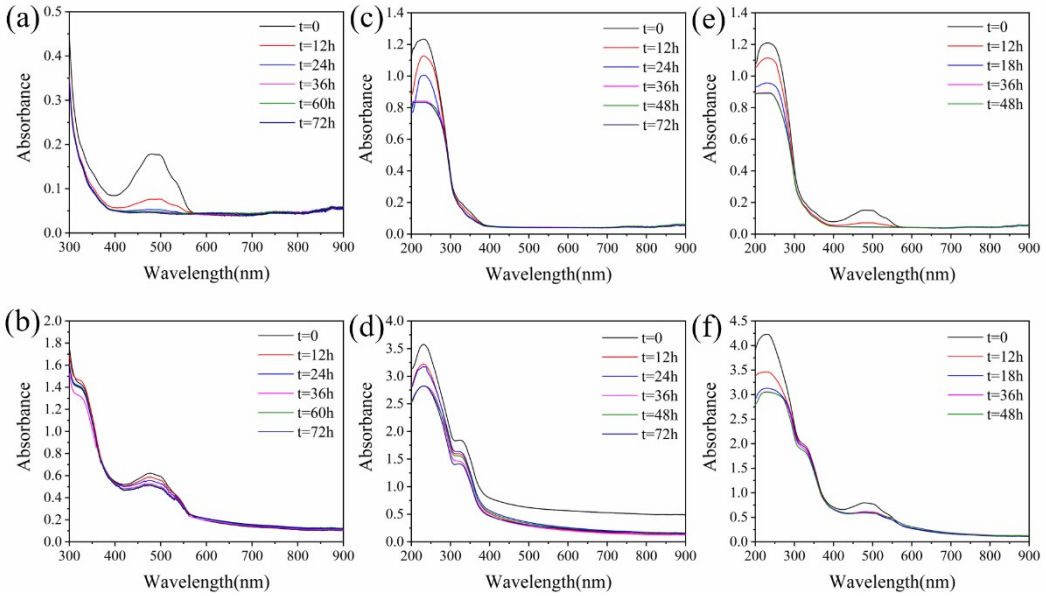


Figure S6 Monitoring the removal of excess reagents during dialysis process by scanning the UV-vis spectra of (a) DOX-PS, PBS; (b) DOX-PS solution; (c) PTX-PS, PBS; (d) PTX-PS solution; (e) DOX/PTX-PS, PBS; and (f) DOX/PTX-PS solution.

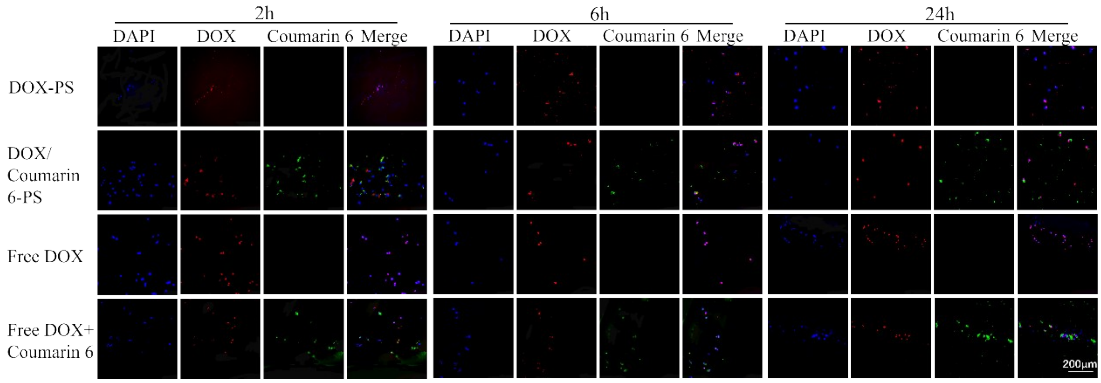


Figure S7 Fluorescence images of HeLa cells incubated with DOX-PS, DOX/coumarin 6-PS, free DOX and free DOX + coumarin 6 for 2, 6 and 24 h. Scale bar: 200 μm.

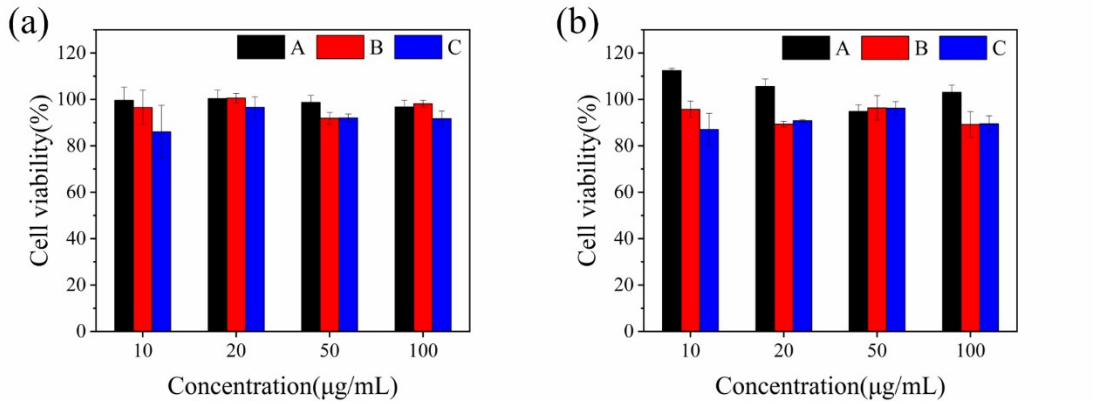


Figure S8 Cell viability of (a) MCF-7 and (b) HeLa cells after treated with blank polymersomes at different concentrations for (A) 24 h, (B) 48 h and (C) 96 h. Data are present as mean ± S.D. (n = 3).

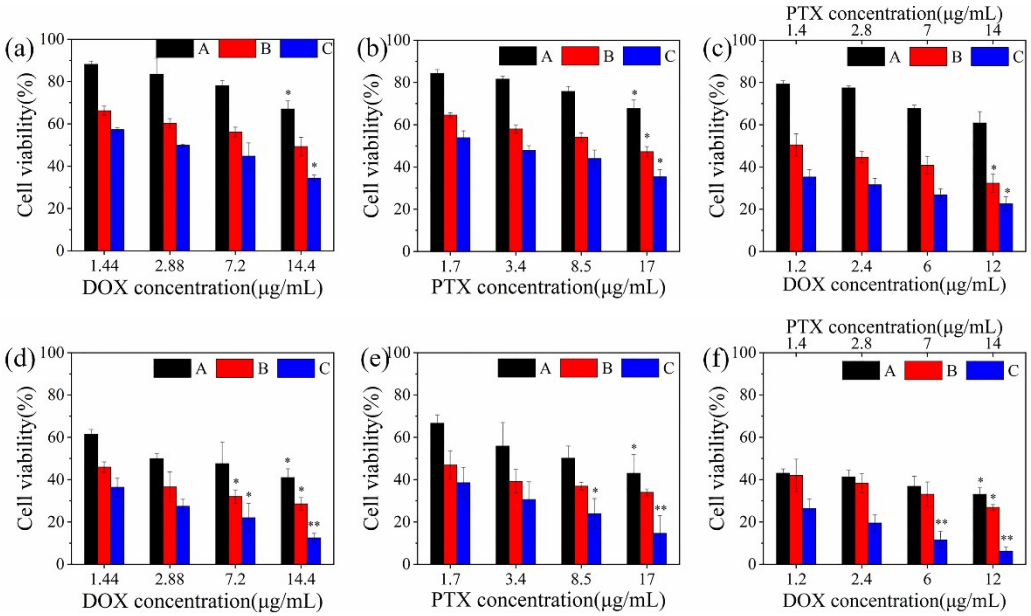


Figure S9 Cell viability of HeLa cells after incubated with (a) DOX-PS, (b) PTX-PS, (c) DOX/PTX-PS, (d) free DOX, (e) free PTX and (f) DOX/PTX cocktail at different drug concentrations for (A) 24 h, (B) 48 h and (C) 96 h. Data are present as mean ± S.D. (n = 3). *$p < 0.05$ and **$p < 0.01$ by two-tailed Student's t test.

Table S1 Molecular weight and distribution of the block polymers synthesized by RAFT.

Polymer	M_n[(a)] (g/mol)	M_n[(b)] (g/mol)	M_w[(c)] (g/mol)	Đ[(d)]
PN	7892	11785	16218	1.37
PNDB	44273	133408	187966	1.41

[(a)]Calculated by ^{1}H NMR spectra. [(b, c, d)]Obtained from GPC.

Table S2 The EE and LC of DOX and PTX encapsulated in polymersomes.

Drugs	DOX		PTX	
	in DOX-PS	in DOX/PTX-PS	in PTX-PS	in DOX/PTX-PS
EE	72.1%	59.9%	85.1%	70.2%
LC	14.4%	12.0%	17.0%	14.0%

Table S3 Combination index (CI_{50}) of free drugs and drug loaded polymersomes against MCF-7 cells after 48 h treatment.

Formulations	DOX IC_{50} (μg/mL)	PTX IC_{50} (μg/mL)	CI_{50}
DOX-PS	19.94	—	—
PTX-PS	—	20.87	—
DOX/PTX-PS	8.49	6.20	0.72
Free DOX	2.96	—	—
Free PTX	—	5.08	—
Free DOX/PTX	1.25	2.90	0.99

Table S4 Combination index (CI_{50}) of free drugs and drug loaded polymersomes against HeLa cells after 48 h treatment.

Formulations	DOX IC_{50} (μg/mL)	PTX IC_{50} (μg/mL)	CI_{50}
DOX-PS	15.25	—	—
PTX-PS	—	12.85	—
DOX/PTX-PS	5.39	5.98	0.82
Free DOX	5.97	—	—
Free PTX	—	6.51	—
Free DOX/PTX	1.27	3.45	0.74

References

1. T.-C. Chou, *Pharmacol. Rev.*, 2006, **58**, 621.