Supplementary Information for

High Drug Loading and Sub-Quantitative Loading Efficiency of Polymeric Micelles Driven by Donor-Receptor Coordination Interactions

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Materials and Methods

Materials

Poly(ethylene glycol) monomethyl ether (mPEG, M_n = 5000 Da), phenylboronic acid (PBA), and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received. mPEG-NH_2 (M_n = 5000 Da) was prepared according to our previous work.[1] γ-Benzyl-L-aspartate-*N*-carboxyanhydride (BLA-NCA) was synthesized according to the literatures.[2] Doxorubicin hydrochloride (DOX·HCl), epirubicin hydrochloride (EPI·HCl), camptothecin (CPT), and irinotecan hydrochloride (IR·HCl) were purchased from Beijing Huafeng United Technology Corporation (Beijing, China). 4-(Hydroxymethyl)phenylboronic acid pinacol ester, benzyl alcohol (Bn-OH), ethanolamine, carbonyldiimidazole (CDI), and 4-dimethylaminopyridine (DMAP) were purchased from Energy Chemical (Shanghai, China). *N*, *N*-Dimethylformamide (DMF) was stored over calcium hydride (CaH_2) and purified by vacuum distillation with CaH_2 before use. Other reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd. (Beijing, China) and used as received.

Characterization

The ^1H and ^{11}B nuclear magnetic resonance (NMR) spectra were recorded on Agilent 400 MHz. MestReNova 8.1.1 was used to analyze all spectra. For ^{11}B NMR, $BF_3{\cdot}OEt_2$ was used as the external reference to calibrate the spectra and the background absorption from the NMR tube was removed by whittaker smoother.

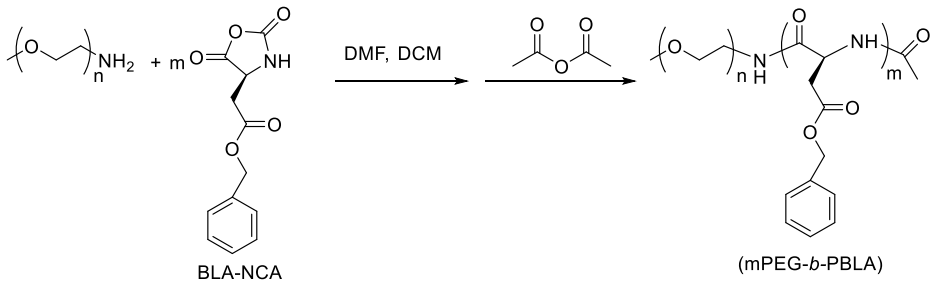
Molecular weight and polydispersity index (PDI = M_w/M_n) of the copolymers were determined by gel permeation chromatography (GPC). For mPEG-*b*-PBLA, the GPC analyses were performed on a Waters 1515 GPC instrument equipped with MZ-gel SDplus columns (500 Å, 10^3 Å, 10^4 Å) and a differential refractive-index detector (RI 2414). DMF with 0.05 M LiBr was used as the eluent at a flow rate of 0.8 mL/min at 60 °C, and polystyrene standards were used for the calibration of the columns. For mPEG-*b*-PHEA, the measurement was performed on a Waters 1515 HPLC and a 2410 refractive index detector, equipped with PL aquagel-OH MIXED-M columns. Aqueous acetate buffer (0.1 M, pH 2.8) was used as the eluent at a flow rate of 1.0 mL/min. The sample concentration was 2.0 mg/mL, and polyethylene glycol with different MWs (2 mg/mL) was used as the standard for determination of the calibration curve. Analysis of DOX in the PBA/DOX mixture was performed on a Shimadzu HPLC system (LC-20AT) connected with PDA detector (SPD-M20A). Shimadzu C18 column (3 μm, 50 mm × 4.6 mm) was used for analysis. Gradient method was adopted using 0.1% TFA-H_2O and acetonitrile as the mobile phase. HPLC analysis of free drug in the DOX-loaded P-PBA micelles was performed on Agilent 1220 LC system, with a mixture of acetonitrile and water (4:1, v/v) containing 0.1% TFA as the mobile phase. The column effluent was detected at 480 nm with a UV/Visible detector. The column type was Agilent eclipse plus C18 (3.5 μm, 4.6 mm × 100 mm). The size and zeta potential of particles in the aqueous solution were measured by dynamic light scattering (DLS) conducted on a Malvern Zetasizer Nano ZS90 with a He-Ne laser (633 nm) with 90° collecting optics. All the sizes were

presented in terms of intensity diameter unless otherwise specified.

Cell culture

NIH/3T3 (mouse embryo fibroblast), HeLa (human cervical carcinoma), HepG2 (human hepato-carcinoma), and B16F10 (mouse melanoma) cells were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 50 U/mL penicillin, and 50 U/mL streptomycin at 37 ºC in 5% CO_2 atmosphere.

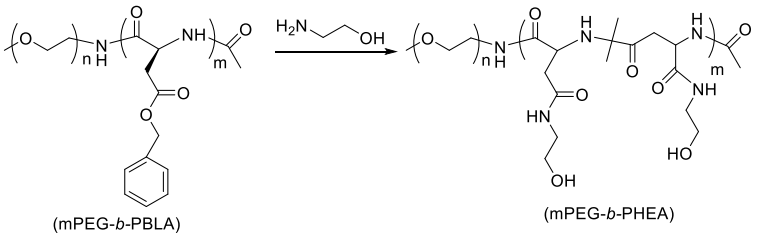
Synthesis of mPEG-*b*-PBLA copolymer



The mPEG-*b*-PBLA diblock copolymer (designed degree of polymerization (DP) = 22) was synthesized through the ROP of BLA-NCA monomer with mPEG-NH_2 as the macro-initiator. Briefly, BLA-NCA (5.58 g, 22.4 mmol) was dissolved in the mixture of dry DMF (5.58 mL) and DCM (50.2 mL). mPEG-NH_2 (5.09 g, 1.02 mmol) was dehydrated through an azeotropic process with toluene, and the remaining toluene was removed under vacuum. Afterwards, mPEG-NH_2 dissolved in dry DCM was added into the BLA-NCA solution *via* a syringe under argon. The reaction was maintained at 35 °C under gentle stirring for 3 days in a glove-box. Then excessive acetic anhydride was added to the solution to block the terminal amino group, and the

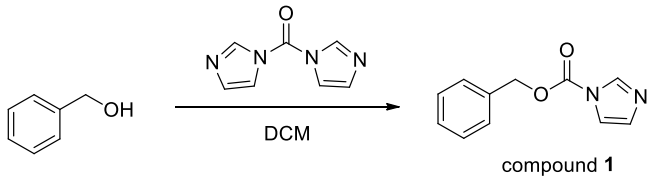
mixture was maintained at 35 °C for another 12 hours. The mixture was concentrated under vacuum at 60 °C. mPEG-*b*-PBLA copolymer was obtained via precipitation in excess amount of cold diethyl ether (yield: 86%). The ^{1}H NMR spectrum of mPEG-*b*-PBLA in CF_3COOD (TFA-d) was consistent with the previous report.[3] The M_n and PDI of the obtained polymer were 9.4 KDa and 1.09, respectively.

Synthesis of mPEG-*b*-PHEA



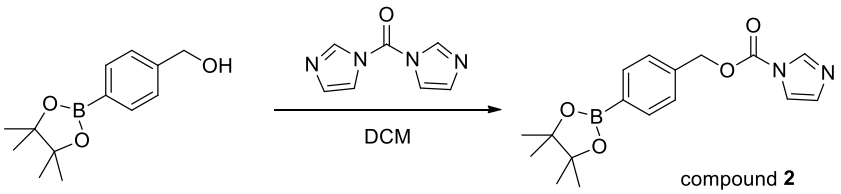
In brief, mPEG-*b*-PBLA (4.0 g) was dissolved in DMF (40 mL), and ethanolamine (3 equivalents to BLA unit) was added. The mixture was maintained at 35 °C for 12 h. The solution was precipitated with excess amount of cold diethyl ether to remove unreacted small molecules. The precipitation was washed 3 times by diethyl ether before removal of the residual solvent by vacuum. Then the precipitate was dissolved in DMF and dialyzed against distilled water. The purified product was obtained as a white solid after freeze-drying. The structures of the obtained mPEG-*b*-PHEA was determined by ^{1}H NMR in TFA-d/D_2O (v:v = 1:9), which was consistent with our previous report.[4] The M_n and PDI of the mPEG-*b*-PHEA were 8.3 KDa and 1.28, respectively.

Synthesis of benzyl carbonylimidazole (compound 1)



Briefly, benzyl alcohol (Bn-OH, 5.0 g, 46.2 mmol) was dissolved in anhydrous DCM (50 mL) in a flame-dried 100-mL flask. Carbonyldiimidazole (15 g, 92.5 mmol) was added, and the solution was stirred overnight. The mixture was diluted into ethyl acetate (200 mL) and washed with DI water (3 × 100 mL). The organic phase was washed with brine (3 × 50 mL), dried over $MgSO_4$, and concentrated in vacuum to obtain light-yellow liquid. The structure was determined by ^1H NMR using $CDCl_3$ as the solvent. The NMR peaks were consistent with the literature report.[5]

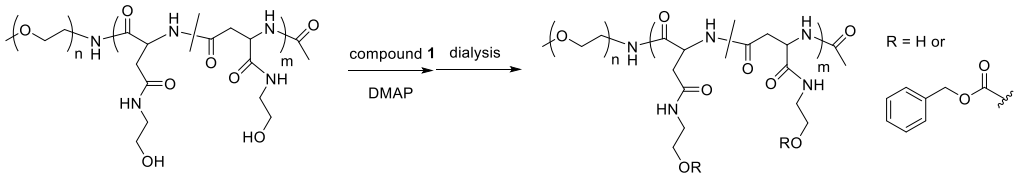
Synthesis of 4-(hydroxymethyl)phenylboronic acid pinacol carbonylimidazole (Compound 2)



Briefly, 4-(hydroxymethyl)phenylboronic acid pinacol ester (7.37 g, 31.5 mmol) was dissolved in anhydrous DCM (50 mL) in a flame-dried 100-mL flask. Carbonyldiimidazole (10.20 g, 62.9 mmol) was added, and the solution was stirred overnight. The mixture was diluted into ethyl acetate (200 mL) and washed with DI water (3 × 100 mL). The organic phase was washed with brine (3 x 50 mL), dried over $MgSO_4$, and concentrated in vacuum to obtain the white powder. The structure was determined by ^1H NMR using $CDCl_3$ as the solvent, which was consistent with

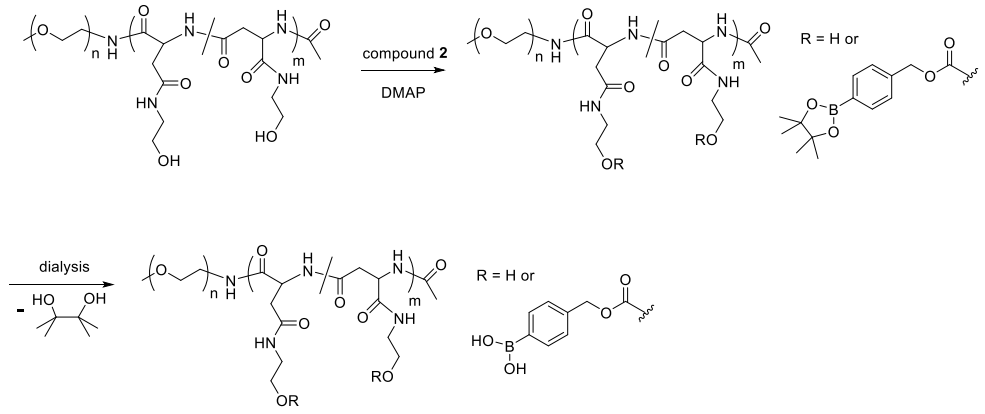
the literature report.[6]

Synthesis of polymer P-CBZ



In a flame-dried 100-mL flask, compound **1** (310 mg, 1.53 mmol), mPEG-*b*-PHEA (2.00 g, 0.12 mmol), and DMAP (190 mg, 1.56 mmol) were dissolved in anhydrous DMF (30 mL). The solution was stirred at 50 °C overnight, and was precipitated into excess amount of diethyl ether to remove unreacted small molecules. Then the crude product was dissolved in DMF and dialyzed against DI water. The final product P-CBZ was obtained as white solid after freeze-drying. The structure of P-CBZ was determined by ^{1}H NMR using TFA-d as the solvent, which was consistent with the previous report.[4] Modification efficiency of the terminal OH group was determined by ^{1}H NMR to be 47.6%.

Synthesis of polymer P-PBA



Into a flame-dried 100-mL flask, compound **2** (948 mg, 2.88 mmol),

mPEG-*b*-PHEA (2.00 g, 0.24 mmol), and DMAP (412 mg, 3.38 mmol) were dissolved in dry DMF (30 mL). The solution was stirred at 50 °C overnight, and was precipitated with excess amount of diethyl ether for twice to remove small molecules. The P-PBA precursor was obtained after removal of the residual solvent by vacuum. The structure was determined by ^{1}H NMR using DMSO-d_6 as the solvent. The P-PBA precursor was then dissolved in DMF and dialyzed against DI water to remove the pinacol. P-PBA was obtained as white solid after freeze-drying. The structure of P-PBA was determined by ^{1}H NMR using DMSO-d_6 as the solvent. Modification efficiency of the terminal OH group was determined to be 45.7% by ^{1}H NMR.

Preparation of DOX free base

For NMR study of DOX with PBA, DOX free base was prepared. In brief, DOX·HCl (100 mg) was dissolved in DI water (50 mL), and triethylamine (1.1 equiv to DOX) was added. The DOX free base was extracted three times by chloroform. Afterwards, chloroform and trace triethylamine were removed by vacuum, and the obtained DOX free base was kept at 4 °C in the dark before use.

Drug loading of P-CBZ and P-PBA micelles

The drug-loaded micelles were prepared by the typical nanoprecipitation method.[7] DOX·HCl was first neutralized by triethylamine (1.5 molar equivalents to DOX) in DMF. P-PBA or P-CBZ was dissolved in DMF and mixed with the DOX solution. The mixture was maintained at 35 °C for 6 h in the dark, and then added

dropwise into DI water under vigorous stirring. The mixture was stirred in the dark for another 6 h. DMF and other small molecules were removed by dialysis (MWCO 3500 Da) against DI water for 12 h. The drug aggregate was removed by filtration through 0.45-μm membrane. The drug-loaded micelles were obtained after lyophilization. To determine the DOX content in the micelles, they were dissolved in DMF and measured for the absorbance at 480 nm by the UV-Vis spectrometer. Loading of EPI, CPT and IR was similar processed and drug loading was quantified by UV absorption at 480, 367, 367 nm, respectively. Drug loading content (DLC, wt%) and drug loading efficiency (DLE, wt%) were calculated according to the following formula:

DLC = (amount of loaded drug / amount of drug-loaded NPs) × 100%

DLE = (amount of loaded drug / amount of feeding drug) × 100%

Aqueous solubility and colloidal stability of DOX-loaded P-PBA micelles

To test the lyophilization stability, the lyophilized DOX-loaded PB-DOX-2 micelles were resuspended with phosphate buffered saline (PBS, pH 7.4) before the particle size was monitored and the solution was imaged.

To explore the colloidal stability, PB-DOX-2 micelles were dissolved in PBS (pH 7.4) or PBS containing 10% FBS, and then the solutions were placed at 37 °C under gent shaking (100 rpm). At pre-determined time intervals (3, 6, 12, 24, and 48 h), an aliquot of the solution was withdrawn and measured by DLS.

H_2O_2-triggered degradation of P-PBA and disassembly of micelles

P-PBA (8 mg) was dissolved in D_2O (0.50 mL) with H_2O_2 (molar amount equal to the pendant phenylboronic acid) and incubated at 37 °C. At pre-determined time intervals, ^{1}H NMR measurement was performed to evaluate the degree of degradation of the polymer.

The disassembly of drug-loaded P-PBA micelles was also investigated in the presence of H_2O_2. PB-DOX-2 micelles were dissolved in PBS (pH 7.4) containing 100 μM H_2O_2, and the suspension was placed at 37 °C. At pre-determined time intervals, particle size of the micelles was measured by DLS.

***In vitro* drug release**

The drug release from micelles was studied using PBS (pH 7.4) containing 0.5% tween 80, PBS (pH 7.4) containing 0.5% tween 80 and 100 μM H_2O_2, and PBS (pH 5.5) containing 0.5% tween 80. Briefly, PB-DOX-2 or PC-DOX-1 micelles (5 mL, containing 334 μg DOX) were placed in a dialysis bag (MWCO 3500 Da), which was immersed in 45 mL of the release medium. The release study was performed at 37 °C under gently shaking (100 rpm). At pre-determined time intervals, 4 mL of the release medium was withdrawn and replaced with equal amount of fresh release medium. The DOX amount in the release medium was determined by spectrofluorimetry (λ_{ex} = 480 nm, λ_{em} = 590 nm).

***In vitro* anti-cancer efficacy**

The *in vitro* cytotoxicity of mPEG-*b*-PHEA and modified polymers (P-CBZ and P-PBA) was evaluated by the MTT assay. Briefly, NIH/3T3 cells were seeded in 96-well plates at 7,000 cells/well and incubated at 37 °C for 24 h. The cell culture medium was then replaced with fresh DMEM (200 μL) containing the polymer at the different concentrations. After 48-h incubation, the cell viability was measured by the MTT assay. Cells that did not receive treatment with polymers served as the control, and results were denoted as percentage viability of control cells.

To evaluate the anti-cancer efficacy of DOX-loaded micelles, B16F10, HeLa, and HepG2 cells were treated with DOX-loaded micelles in 96-well plates for 48 h at various DOX concentrations using the same method. NIN/3T3 (non-cancerous) cells were also used as the control.

Confocal laser scanning microscopy (CLSM) study

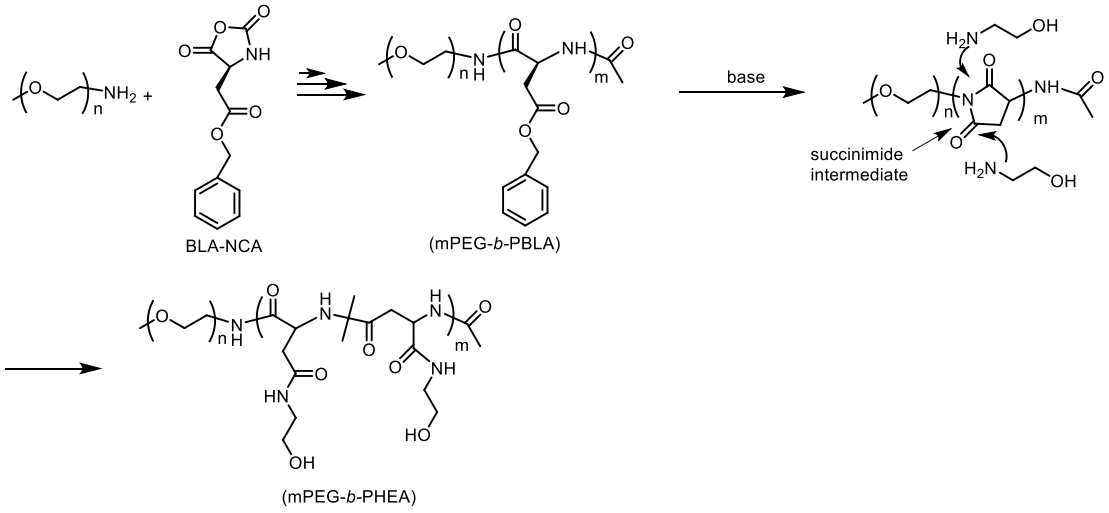
The cellular uptake of DOX-capsulated micelles in HeLa cells was investigated by CLSM. In brief, the cells were seeded on the coverslips in 6-well plates at 1×10^5 cells/well and incubated for 24 h. The medium was replaced with fresh DMEM (2 mL) containing free DOX or PB-DOX-2 micelles at the DOX concentration of 5 μg mL^{-1}. The medium was removed after 1- or 3-h incubation at 37 °C. The cells were washed with PBS and fixed with formaldehyde (4% in PBS) for 20 min at room temperature. Then the cell nuclei were stained with DAPI. The coverslips were placed onto the glass microscope slides. The subcellular localization of the PB-DOX-2 micelles was visualized by CLSM (Leica, TCS SP5, Germany).

Maximum tolerated dose (MTD) study

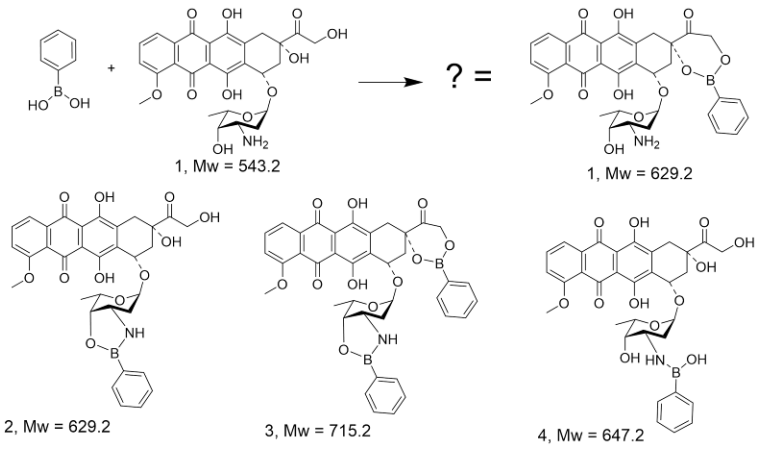
BALB/c male mice were divided into 10 groups (n = 5) and intravenously injected (once) with free DOX (5, 10, 15, 20, 25 mg/kg) or PB-DOX-2 micelles (20, 40, 60, 80, 100 mg DOX equiv./kg). Body weight and survival of mice were measured daily within the observation period of 14 days. The MTD was defined as the dose that causes neither mouse death due to the toxicity nor greater than 15% of body weight loss or other remarkable changes in the general appearance within the entire period of the experiments.

Statistical analysis

Experiments were performed at least three times and results were expressed as means ± SD. Statistical significances were analyzed using the Student's t-test, and differences between the test and control groups were judged to be significant at $*p < 0.05$ and very significant at $**p < 0.01$.



Scheme S1. Synthetic route of mPEG-*b*-PHEA.



Scheme S2. Possible products of DOX after interaction with PBA.

Table S1. Characterization of polymers.

Entry	Polymer	M_n (kDa)[a]	PDI [b]	Modification ratio (%)[a]
1	mPEG-*b*-PBLA	9.3	1.09	--
2	mPEG-*b*-PHEA	8.3	1.28	--
4	P-CBZ	--	--	45.7
3	P-PBA	--	--	47.6

[a] Determined by ^{1}H NMR.

[b] Determined by GPC.

Table S2. Sizes and zeta potentials of the DOX-loaded P-PBA micelles.

Entry	micelles	Diameter (nm)[a]	Diameter (nm)[b]	Zeta potential (mV)[a]
1	PB-DOX-1	22.0 ± 4.4	22.5 ± 4.7	-0.49 ± 1.07
2	PB-DOX-2	28.9 ± 5.5	29.7 ± 5.2	-0.76 ± 0.70
3	PB-DOX-3	33.8 ± 6.9	34.3 ± 6.5	0.21 ± 0.11
4	PB-DOX-4	46.9 ± 10.7	48.8 ± 10.9	-0.27 ± 0.28

[a] Measured before lyophilization.

[b] Measured after lyophilization.

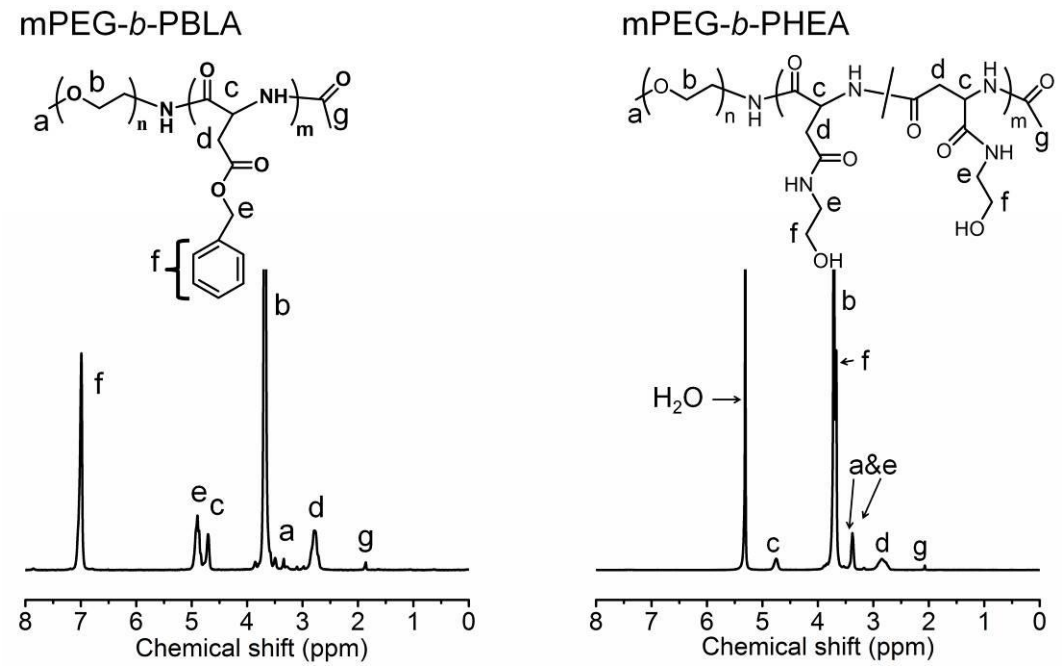


Figure S1. ^{1}H NMR spectra of mPEG-*b*-PBLA in TFA-d and mPEG-*b*-PHEA in TFA-d/D_2O (v:v = 1:9).

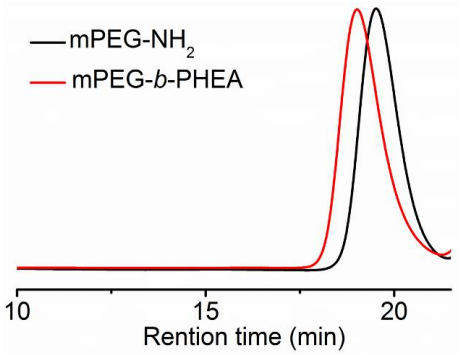


Figure S2. GPC traces of mPEG-NH_2 and mPEG-*b*-PHEA (mobile phase: 0.1 M acetate buffer).

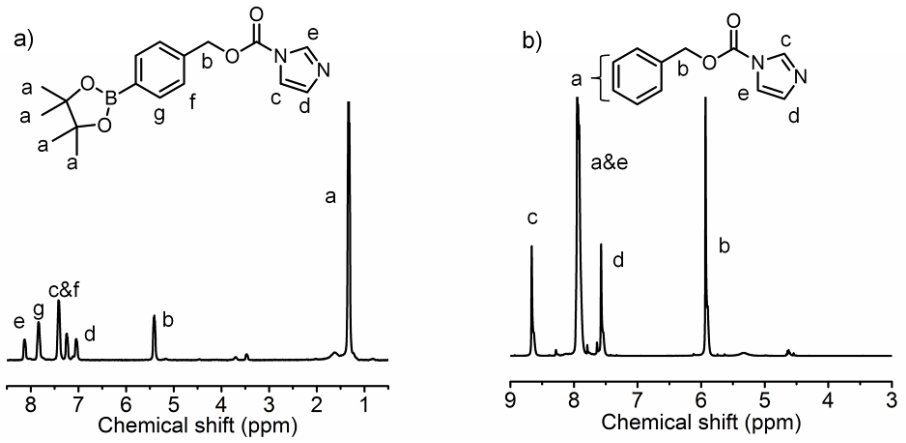


Figure S3. ^{1}H NMR spectra of compound **1** (a) and **2** (b) in $CDCl_3$.

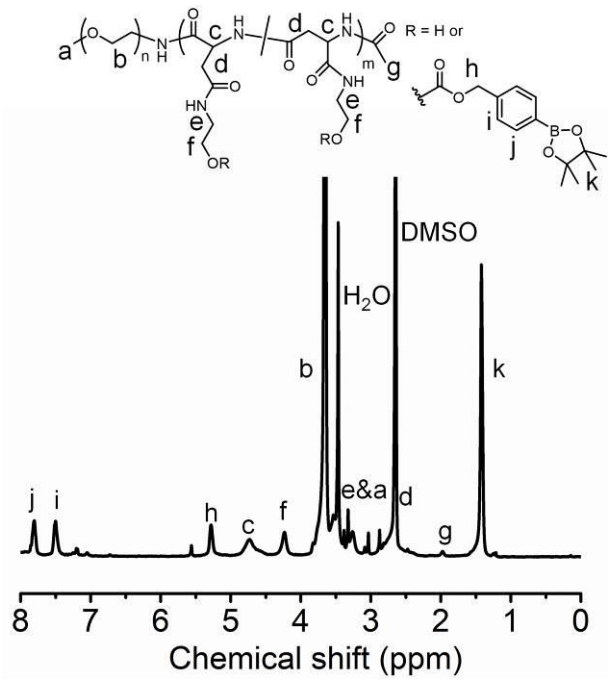


Figure S4. ^{1}H NMR spectrum of P-PBA precursor in DMSO-d_6.

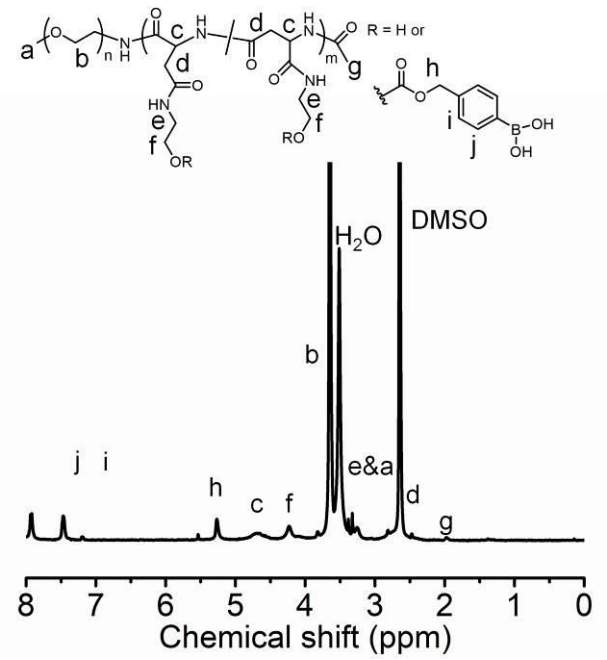


Figure S5. ^{1}H NMR spectrum of P-PBA in DMSO-d_6.

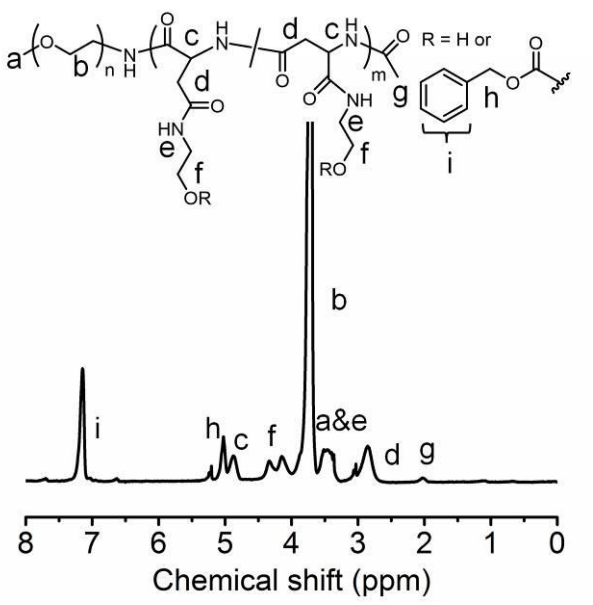


Figure S6. ^{1}H NMR spectrum of P-CBZ in TFA-d.

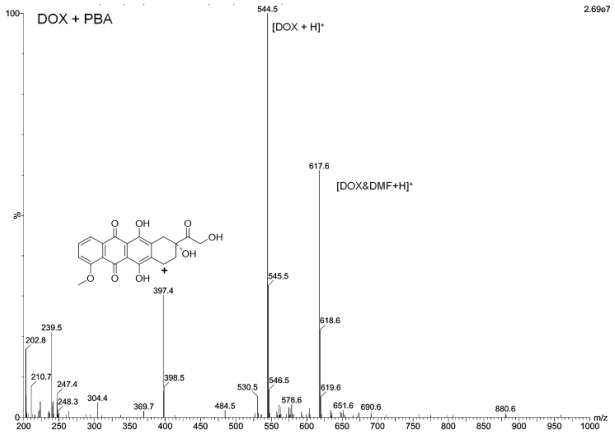


Figure S7. Mass spectrum of DOX after interaction with PBA in DMF.

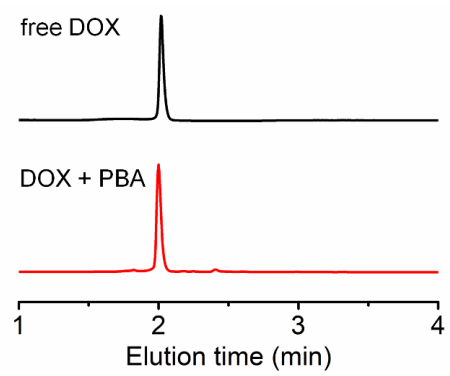


Figure S8. HPLC curves of free DOX and the mixture of DOX and PBA.

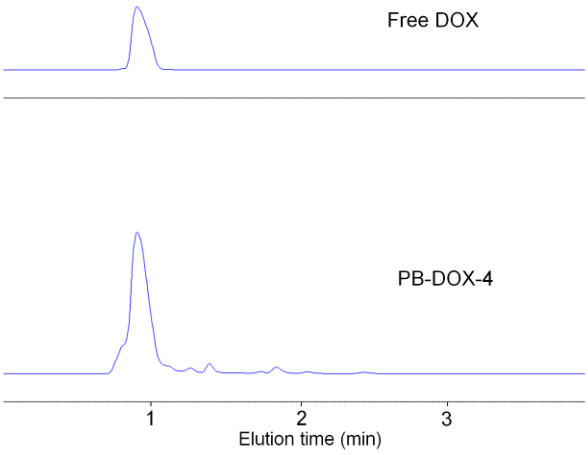


Figure S9. HPLC curves of free DOX and PB-DOX-4 micelles.

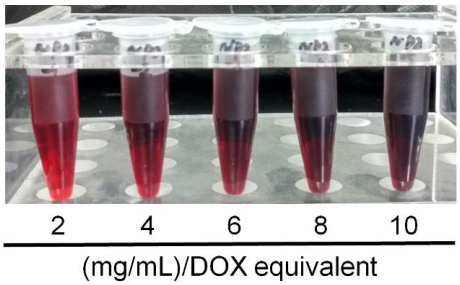


Figure S10. Aqueous solubility of lyophilized PB-DOX-2 micelles in PBS.

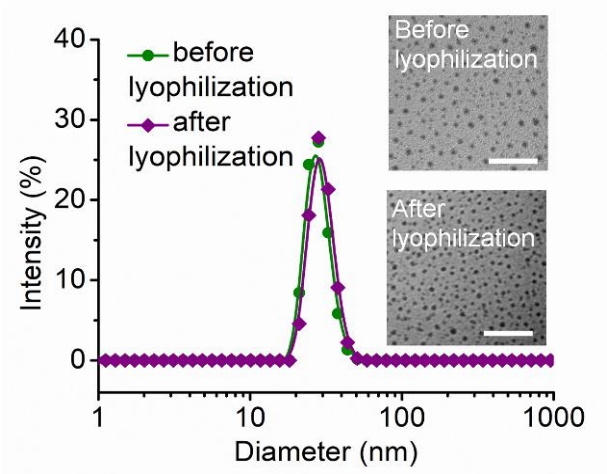


Figure S11. Size distributions and TEM images of PB-DOX-2 micelles before and after lyophilization. Scale bar = 200 nm.

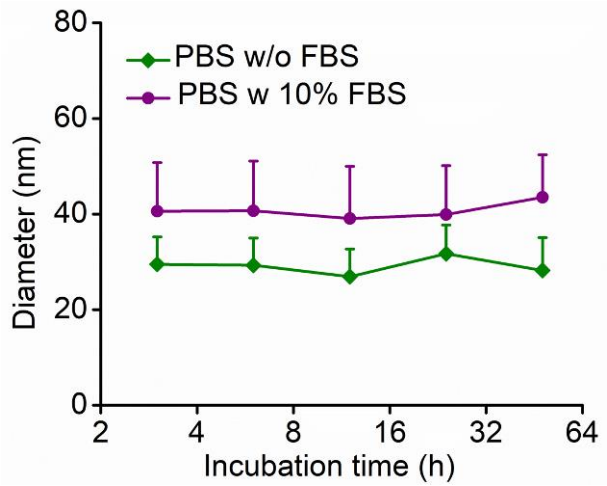


Figure S12. Stability of PB-DOX-2 micelles in PBS or PBS containing 10% FBS.

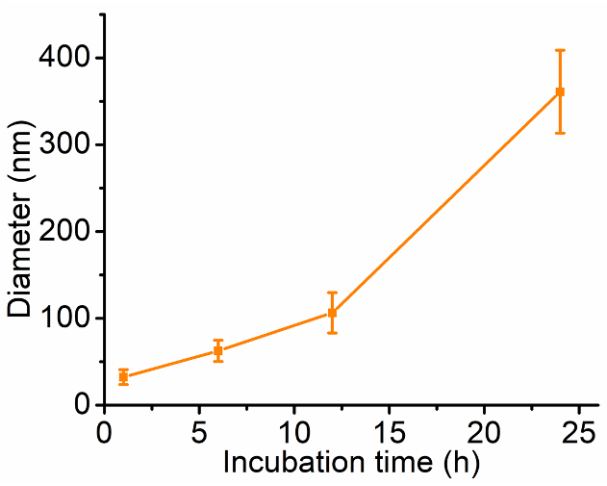


Figure S13. Size change of PB-DOX-2 micelles following incubation in PBS (pH = 7.4) containing 100 μM H_2O_2.

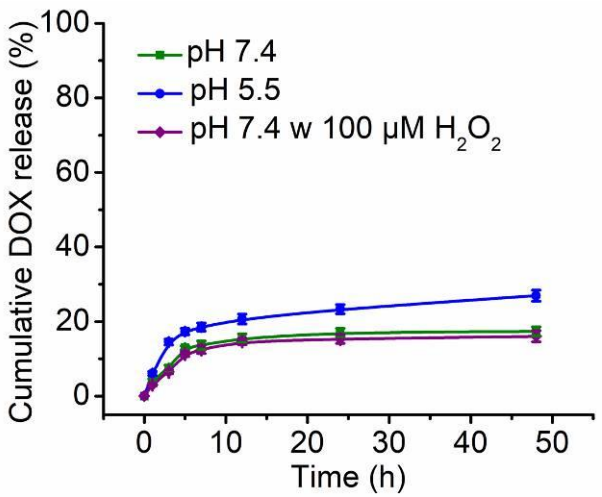


Figure S14. *In vitro* DOX release from PC-DOX-1 micelles following incubation for 48 h (n = 3).

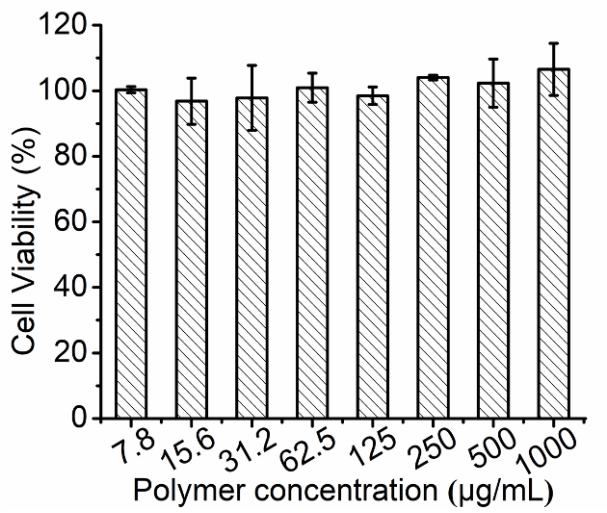


Figure S15. *In vitro* cytotoxicity of mPEG-*b*-PHEA in NIH/3T3 cells following incubation for 48 h (n = 3).

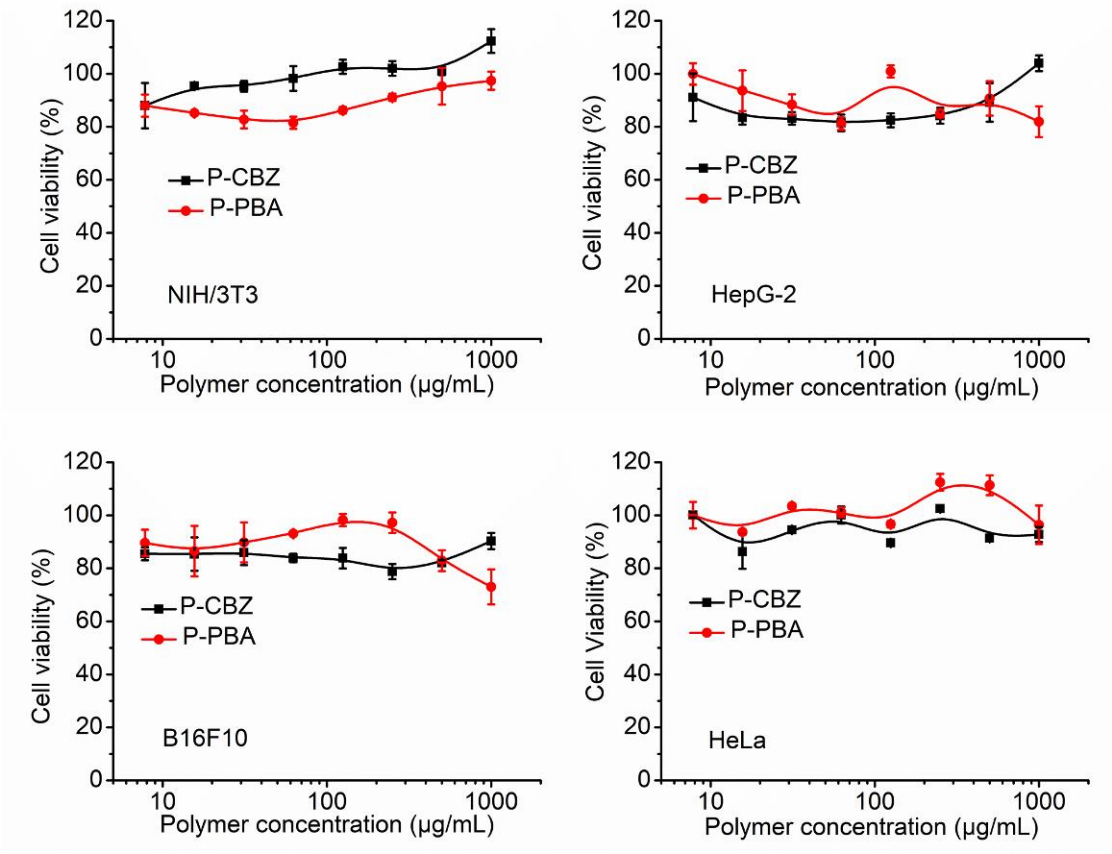


Figure S16. *In vitro* cytotoxicity of P-PBA and P-CBZ in different cell lines following incubation for 48 h (n = 3).

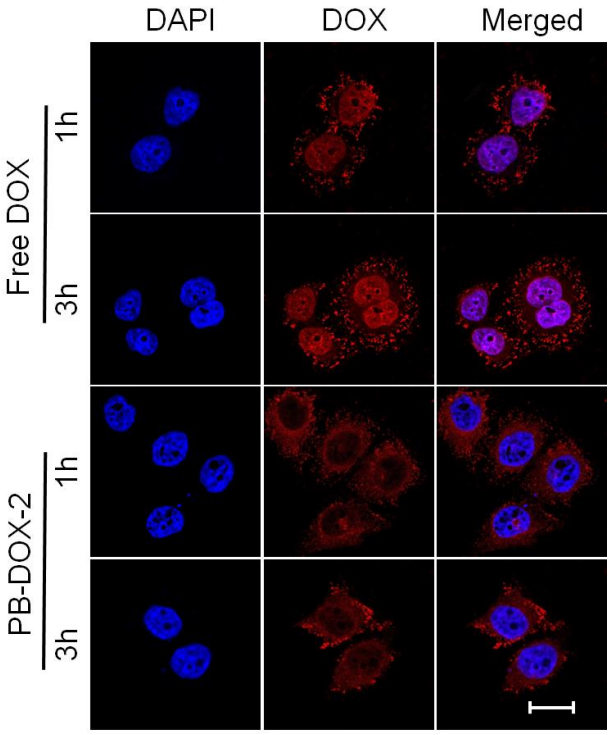


Figure S17. CLSM images of HeLa cells showing the effective cellular uptake of both free DOX or PB-DOX-2 micelles after incubation for 1 and 3 h (scale bar = 20 μm).

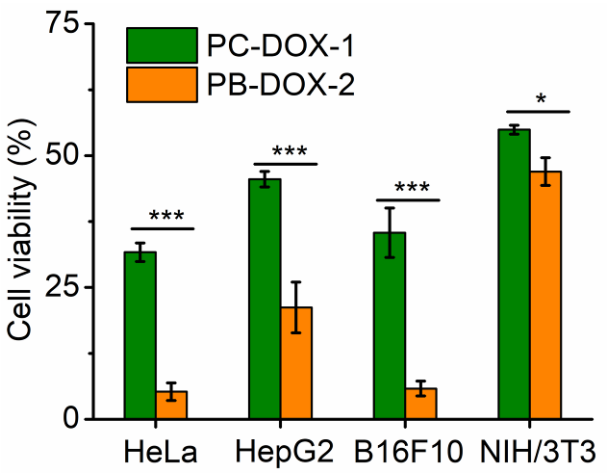


Figure S18. Cytotoxicity of DOX-loaded micelles toward various cell types at 2.5 μg/mL DOX equivalent after incubation for 48 h (n = 3).

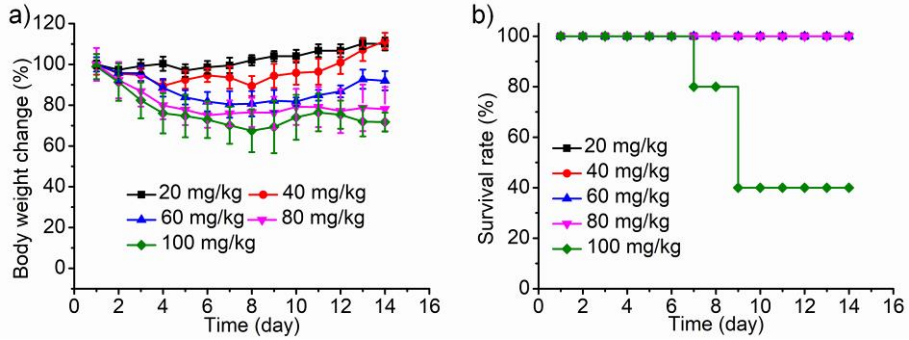


Figure S19. Body weight change (a) and survival rate (b) of mice after i.v. injection of PB-DOX-2 micelles at different DOX doses (n = 5).

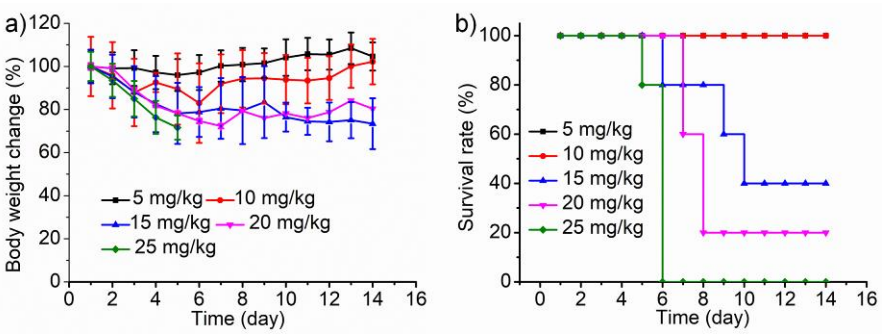


Figure S20. Body weight change (a) and survival rate (b) of mice after i.v. injection of free DOX at different doses (n = 5).

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