Coordinating bioorthogonal reactions with two tumor-microenvironment-responsive nanovehicles for spatiotemporally controlled prodrug activation

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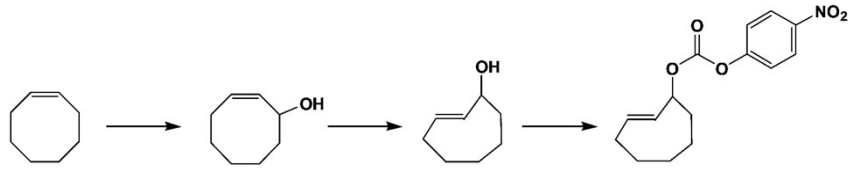
Experimental section

Materials

Diethyl ether, methyl benzoate, petroleum ether, aqueous ammonia, dichloromethane, ethyl acetate, acetone, methanol, 4-nitrophenyl chloroformate, $NaHCO_3$, Na_2SO_4, silica gel, $AgNO_3$, NaCl were purchased from Beijing Chemical Works. Dimethyl sulfoxide (DMSO) was purchased from AppliChem. Triethylamine and tetrahydrofuran (THF) were purchased from J&K Chemicals. N-bromosuccinimide (NBS), 4- (N, N dimethylamine) pyridine (DMAP), N, N-dimethyl formamide (DMF), N, N-Diisopropylethylamine (DIPEA) and azobisisobutyronitrile (AIBN) were purchased from Alfa Aesar Company. Pyrene was purchased from Aladdin Industrial Corporation. Dialysis membrane and doxorubicin hydrochloride were urchased from Solarbio. 4-(6-methyl-1,2,4,5-tetrazin-3 yl) phenyl] methanamine (Tz) was obtained from Chengdu Biocone Biological Technology Co., LTD. MMP-2 and MMP-2 inhibitor were purchased from Abcam. Trans-cyclooctene-PEG4-NHS ester (TCO-PEG4-NHS) was purchased from Click Chemistry Tools (USA). mPEG-$(CPLGLAGG)_2$ was purchased from Guoping Pharmaceutical Co., LTD. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies. Doxorubicin Hydrochloride Liposome Injection (Doxil) was purchased from Shijiazhuang Pharmaceutical Group co., LTD. (Z)-cyclooctene was purchased from Tokyo Chemical Industry Co., LTD.

Synthesis of (E)-cyclooct-2-en-1-yl (4-nitrophenyl)

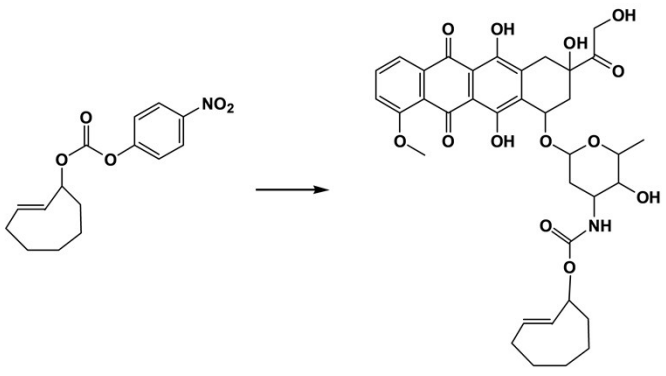


The compound (E)-cyclooct-2-enol was prepared according to the reference.[1-2] Briefly, the mixture of (Z)-cyclooctene (30 mL, 231 mmol), NBS (30 g, 168.6 mmol) and AIBN (23 mg, 0.14mmol) in carbon tetrachloride (120 mL) was purged with N_2 and stirred under reflux for 2 h. The reaction was cooled at 0 °C and the precipitate was removed by filtration. The solvent was rotary vaporized to give (Z)-3-bromocyclooctene as light yellow oil. The product was subsequently dissolved in a mixture of acetone (240 mL) and water (120 mL). Then $NaHCO_3$ (30 g, 360 mmol) was added to the solution and the mixture was stirred under reflux for 1 h. After which, the mixture was filtered and the filtrate was extracted three times with diethyl ether. Finally, the solvent in the ether layer was rotary vaporized to give (Z)-cyclooct-2-en-1-ol as light-yellow oil. 1H NMR (400 MHz, $CDCL_3$): δ= 5.70-5.54 (m, 1H), 5.54-5.38 (m, 1H), 4.62 (s, 1H), 2.24-1.96 (m, 1H), 1.95 – 1.73 (m, 2H), 1.64-1.29 (m, 8H).

Then (Z)-cyclooct-2-en-1-ol (8.5 g, 67 mmol) and methyl benzoate (9.2 g, 67 mmol) were dissolved in a mixture of diethyl ether (400 mL) and petroleum ether (800 mL). The solution was irradiated at 254 nm for 24 h while continuously pumped through a column in the dark containing $AgNO_3$ treated silica gel. After which, the silica was collected, stirred in a mixture of aqueous ammonia (500 mL) and dichloromethane (500 mL). The organic layer was separated, and the aqueous layer was extracted with dichloromethane (150 mL) for five times, and then the organic layer was combined, dried over Na_2SO_4, filtered and vaporized to give brown thick oil. The crude product was purified by silica gel column (petroleum ether: ethyl acetate =100:1 to 75:1) to give the pure product.

To prepare (E)-cyclooct-2-en-1-yl (4-nitrophenyl), (E)-cyclooct-2-enol (1 g, 7.9 mmol) was dissolved in dichloromethane (15 mL), and then DMAP (0.94 g, 12 mmol), 4-nitrophenyl chloroformate (1.92 g, 9.5 mmol) were added and stirred overnight in ice bath. After which, deionized water (50 mL) was added and the aqueous phase was extracted with ethyl acetate (30 mL) for three times. The extracted ethyl acetate was combined with dichloromethane, and then an appropriate amount of hydrochloric acid (0.01 M) was added to remove pyridine. After organic phase separation, saturated $NaHCO_3$ was added to remove excess hydrochloric acid. Then saturated NaCl solution was added to remove water. The organic phase was added to anhydrous sodium sulfate for drying and dehydrating. Finally, the solvent was removed by evaporation and purified by silica gel column (petroleum ether: ethyl acetate=200: 1). 1H NMR (400 MHz, Chloroform-d): δ= 8.30 (d, J = 8.9 Hz, 2H), 7.42 (d, J = 8.9 Hz, 2H), 6.06-5.96 (m, 1H), 5.60 (d, 1H,), 5.46 (s, 1H), 2.63-0.78 (m, 10H).

Synthesis of prodrugs

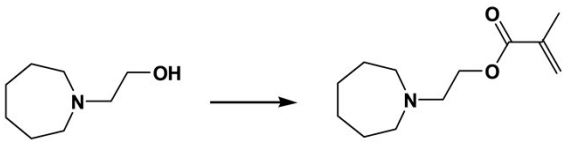


(E)-cyclooct-2-en-1-yl (4-nitrophenyl) (20 mg, 0.069 mmol) was dissolved in DMF (3 g), then DIPEA (80 mg, 0.62 mmol) and doxorubicin hydrochloride (45 mg, 0.0776mmol) were added, and then kept at 30 °C for 24 h. After which, deionized water (100 mL) was added and extracted with dichloromethane until there was no apparent

color in the water phase. Finally, Na_2SO_4 was used to remove water. The samples were purified by silica gel column (dichloromethane: methanol=200:1 to 50:1). 1H NMR (400 MHz, $CDCL_3$)=13.94 (s, 1H), 13.18 (s, 1H), 8.02 (d, J=10.0 Hz, 1H), 7.79 (dd, J_1=J_2= 6.0 Hz, 1H), 7.38 (d, J=11.6 Hz, 1H), 5.76 (m, 1H), 5.51 (m, 2H), 5.26 (d, J=6.8 Hz, 2H), 5.21 (d, J=7.6 Hz, 1H), 4.76 (s, 2H), 4.56 (s, 1H), 4.16 (q, J=4.8 Hz, 1H), 4.09 (s, 3H), 3.88 (m, 1H), 3.70 (s, 1H), 3.24 (d, J=16.0 Hz, 1H), 3.07 (s, 1H), 2.92 (s, 1H), 2.40 (m, 1H), 2.32 (d, J=11.6 Hz, 1H), 2.15 (d, J=10.0 Hz, 1H), 2.00-1.82 (m, 6H), 1.61 (m, 2H), 1.44 (m, 1H), 1.32 (d, J=4.8 Hz, 3H), 1.03 (m, 1H), 0.76 (m, 1H).

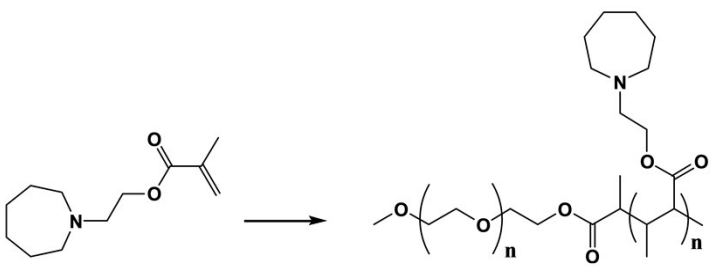
To synthesis of prodrug that cannot be activated (NDox-TCO), equatorial isomer of TCO-PEG_4-NHS (5 mg, 0.0097 mmol) and doxorubicin hydrochloride (5.6 mg, 1.43 mmol) were dissolved in dichloromethane (5 mL) and reacted at room temperature for 12 h. Then, the solvent was removed by evaporation to give NDox-TCO.

Synthesis of methacrylate monomers (C7A-MA)



The compound was prepared according to the reference.[3] Briefly, 2-(Hexamethyleneimino) ethanol (C7A) (14.3 g, 0.1 mol), triethylamine (10.1 g, 0.1 mol) and inhibitor hydroquinone (0.11 g, 1 mmol) were dissolved in THF (100 mL) and then methacryloyl chloride (10.4 g, 0.1 mol) was added dropwise. The solution was refluxed in THF for 2 h, and then filtered to remove the precipitated triethylamine-HCl salts, and the THF solvent was removed by rotary evaporator. The resulting residue was distilled in vacuo to give the C7A-MA. 1H NMR (400 MHz, $CDCL_3$): δ= 6.10 (s, 1H), 5.55 (s, 1H), 4.23 (t, 2H), 2.85 (t, J = 6.2 Hz, 2H), 2.73 (t, J = 5.5 Hz, 4H), 1.94 (s, 3H), 1.66-1.56 (m, 8H).

Synthesis of low pH-sensitive block copolymer (PEO-HMIE)



Firstly, C7A-MA (0.76 g, 5 mmol), PMDETA (21 μL, 0.1 mmol), and MeO-PEG_{114}-Br (0.5 g, 0.1 mmol) were charged into a polymerization tube. Then a mixture of 2-

propanol (2 mL) and DMF (2 mL) was added to dissolve the monomer and initiator. After three cycles of freeze-pump-thaw to remove oxygen, CuBr (14 mg, 0.1 mmol) was added into the reaction tube under nitrogen atmosphere, and the tube was sealed in vacuo. The polymerization was carried out at 40 °C for 8 h. After which, the reaction mixture was diluted with THF (10 mL), and passed through an Al_2O_3 column to remove the catalyst. The THF solvent was removed by rotary evaporator. The residue was dialyzed in distilled water and lyophilized to give white powder. 1H NMR (400 MHz, $CDCL_3$): δ=4.04 (s, 85H), 3.66 (s, 440H), 2.78-2.71 (m, 266H), 1.90-1.80 (s, 134H), 1.61(m, 378H), 1.04-0.88 (m, 95H).

Preparation of tetrazine loaded nanovehicles (MMP-2@Tz) and Dox-TCO loaded nanovehicles (pH@Dox-TCO)

3.5 mg MMP-2 sensitive diblock copolymer (mPEG-$(CPLGLAGG)_2$) and 0.5 mg tetrazine (Tz) were dissolved in 200 μL DMSO, and then the solution was added into 3.5 mL distilled water dropwise under sonication. Subsequently, it was transferred to a dialysis membrane (50kDa) and dialyzed against distilled water for 24 h to remove the unencapsulated Tz.

5 mg low pH-responsive block copolymer (PEO-HMIE) and 1 mg Dox-TCO were dissolved in 1 mL THF, and then the mixture was added into 4 mL distilled water dropwise under sonication. Subsequently, it was transferred to a dialysis bag (MWCO: 50kDa) and then dialyzed against distilled water for 24 h to remove the unencapsulated prodrug.

After micelle formation, the nanovehicles were characterized by transmission electron microscopy (TEM, HT7700, HITACHI) for micelle size and morphology, dynamic light scattering (DLS, Malvern) for hydrodynamic diameter. The Tz concentration in nanovehicles was determined by reading the absorbance at 540 nm using a microplate reader (BioTek) and Dox-TCO concentration in nanovehicles was determined by fluorescence spectrum (excitation wavelength was 488 nm and emission wavelength was 600 nm). The number of particles was measured by Nanoparticle Tracking Analysis (NTA, Particle Metrix). The encapsulation efficiency and loading content of Tz and prodrug in the particles were calculated according to the equations below:

$$\text{Encapsulation Efficiency \%} = \frac{weight\ of\ loaded\ Tz/Dox-TCO\ in\ nanoparticles}{weight\ of\ total\ Tz/Dox-TCO} \times 100\%$$

$$\text{Loading Content} = \frac{number\ of\ Tz/Dox-TCO\ molecules}{number\ of\ particles}$$

In vitro Tz and Dox-TCO release from nanovehicles

2 mL MMP-2@Tz solution was added into a dialysis bag (MWCO: 8000 Da) for dialysis at different conditions (with or without MMP-2). At different time intervals, 1 mL buffer solution was taken out, meanwhile, 1 mL fresh buffer solution was complemented. The released amounts of Tz were quantified by UHPLC-MS (LCMS 8045, Shimadzu). The MRM transitions was 202.20→116.05/185.05.89.10. Similarly, the released amounts of Dox-TCO were quantified by fluorescence spectrum.

UHPLC-MS analyses of Dox-TCO activation in different media.

The pH@Dox-TCO nanovehicles were added into distilled water (pH 7.4 or 6.5) at a final concentration of 15 ppb and the MMP-2@Tz nanovehicles were added into distilled water (with or without MMP-2) at a final concentration of 30 ppb, and then incubated at 37 °C for 48 h. After which, the two solutions were mixed at equal volume and then incubated for 5 min: (1) pH 6.5, without MMP-2, (2) pH 7.4, with 2 μg/mL MMP-2, (3) pH 6.5, with 2 μg/mL MMP-2. Finally, the prodrug activation was detected by UHPLC-MS.

UHPLC-MS was performed using a Triple Quadrupole Liquid Chromatograph Mass Spectrometer (LCMS 8045, Shimadzu). The mobile phase was as follows: solvent A consisted of 0.1% formic acid in water and B consisted of 0.1% formic acid in mass spectrometric grade acetonitrile. The mass spectrometer was interfaced with the liquid chromatograph using an electrospray ion source. The nitrogen nebulizing gas flow was set at 3 L/min and the drying gas flow at 10 mL/min. The interface voltage was 4kV. The temperature of the block heater was maintained at 400 °C and one of the desolvation line at 250 °C. The dwell time was set to 97 ms and the pause time was 3 ms. The MRM transitions were 544.10→361.00/130.05, 694.20→395.10/365.25, 965.30→569.20/460.85/417.30, 1116.40→282.05/185.05/89.10 respectively for Dox, Dox-TCO, NDox-TCO, NDox-TCO-Tz.

Fluorescence spectra of Drug-DNA complexes

The pH@Dox-TCO nanovehicles (20 μM) were added into PBS at pH 6.5, and the MMP-2@Tz nanovehicles (20 μM) were added into the buffer containing MMP-2 (2 μg/mL), and then incubated at 37 °C for 48 h. After that the two solutions were mixed at a molar ratio of 1: 2 and the final concentration of Dox-TCO was 1 μM. Subsequently, the mixture was added to equal volume DNA aqueous solution (1 mg/mL), and then the fluorescence spectrum of Dox was detected (excitation wavelength was 488 nm and emission wavelength was 550–650 nm). Similarly, the Dox or Dox-TCO was added into DNA aqueous solution, and then detected.

In vitro cytotoxicity assay

4T1 cells were seeded on the 96-well plates with 8×10^3 cells per well and incubated at 37 °C for 12 h. Then the cells were treated with different concentrations of pH@Dox-TCO nanovehicles and MMP-2@Tz nanovehicles at variant conditions: (1) pH 7.4, without MMP-2, (2) pH 6.5, without MMP-2, (3) pH 7.4, with 2 μg/mL MMP-2, (4) pH 6.5, with 2 μg/mL MMP-2, and all groups incubated at 37 °C for 48 h. After that, the media was removed and the cells were incubated with CCK-8 agent at 37°C for 2 h. Finally, the cell viabilities were measured by reading the absorbance of each well at 450 nm using a microplate reader.

Three-dimensional (3D) tumor sphere inhibition evaluation

Agarose was dissolved in serum-free 1640 medium (1.5%, w/v) by heating at 100 °C for 2 h, and then coated onto the 96-well plates. After which, the 4T1 cells were added to the wells (1000 cells/per well) and cultured at 37 °C for four days. Subsequently, the formed tumor spheres were individually treated with PBS or one of the following drug formulations: (1) Dox, (2) pH@Dox-TCO, (3) pH@Dox-TCO+MMP-2@Tz, (4) pH@Dox-TCO+MMP-2@Tz+inhibitor for 5 days, and then the sizes of the 3D tumor spheres were measured. The tumor change was calculated according to the equation: Tumor change=V_5/V_0

Animals experiments

BALB/c mice (6 weeks, female) were obtained from SPF (Beijing) biotechnology co., LTD. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals published in GB/T 35892-2018 and the experiments were approved by Institutional Animal Use and Care Committee of Peking University of China.

In vivo safety evaluation

The BALB/c mice were randomly divided into six groups (n = 6) and intravenously injected with (1) PBS, (2) Dox, (3) pH@Dox, (4) Doxil, (5) pH@Dox-TCO, (6) pH@Dox-TCO+MMP-2@Tz (10 mg/kg Dox equiv, the molar ratio of Dox-TCO to Tz was 1:2), respectively. For the group 6, the injection of MMP-2@Tz was followed 22 h after the injection of pH@Dox-TCO. After 7 days, the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and kidney function urea nitrogen (BUN) were analyzed using an automated analyzer (Hitachi-917, Hitachi), and the hematology levels of red blood cell (RBC), platelet (PLT), white blood cell (WBC), hematocrit (HCT) and hemoglobin(HGB) were analyzed using Celltac E (NIHON KOHDEN). Additionally, body-weight changes were recorded every two days.

UHPLC-MS analyses of activated Dox in blood samples and organs samples

The BALB/c mice were randomly divided into two groups (n = 6) and intravenously injected with Doxil or pH@Dox-TCO+MMP-2@Tz (10 mg/kg Dox equiv, the molar ratio of Dox-TCO to Tz was 1:2). For the second group, the injection of MMP-2@Tz was followed 22 h after the injection of pH@Dox-TCO. At different time intervals (0.5, 1, 2, 4, 8, 12 and 24 h), the blood and organs were collected. The blood samples were centrifuged at 10000 g for 10 min (4°C), and then the supernate was added into equal volume acetonitrile and centrifuged at 10000 g for 10 min to remove proteins. The organs were weighed, and then comminuted of 6 mL water. The comminuted organs were sonicated for 10 min and centrifuged at 10000 g for 10 min (4 °C), and then the supernate was added into equal volume acetonitrile and centrifuged at 10000 g for 10 min to remove proteins. Finally, the free Dox was analyzed by UHPLC-MS as mentioned above.

***In vivo* biodistribution and tumor-targeting capacity of the nanovehicles**

BALB/c mice were inoculated subcutaneously with 5×10^5 4T1 cells at the right leg. Then they were randomly divided into two groups (n = 6). When the tumor volume reached 500 mm^3, DiR-labeled pH@Dox-TCO or MMP-2@Tz nanovehicles were intravenously injected. At different time intervals (1, 2, 4, 8, 12, 24 and 48 h), the mice were imaged using an *in vivo* fluorescence imaging system (PerkinElmer), and the tumor tissues and major organs were collected for fluorescence analysis.

***In vivo* antitumor efficacy assay**

BALB/c mice were inoculated subcutaneously with 5×10^5 4T1 cells at the right leg. Then they were randomly divided into six groups (n = 8) when the tumor volume was greater than 80 mm^3, and intravenously injected with (1) PBS, (2) MMP-2@Tz, (3) pH@Dox-TCO, (4) Dox, (5) pH@Dox, (6) pH@Dox-TCO+ MMP-2@Tz (5 mg/kg Dox equiv, the molar ratio of Dox-TCO to Tz was 1:2) every 3 days for a total of three injections. For the group 6, the injection of MMP-2@Tz was followed 22 h after the injection of pH@Dox-TCO. The tumor volumes and body weight of each mouse was recorded every 2 days. The tumor volume and tumor control rate were calculated according to the equations below:

$V_{tumor}=1/2LW^2$ (L: tumor length, W: tumor width)

Tumor control rate % $=(1-V/V_{PBS}) \times 100\%$

Supplementary figures

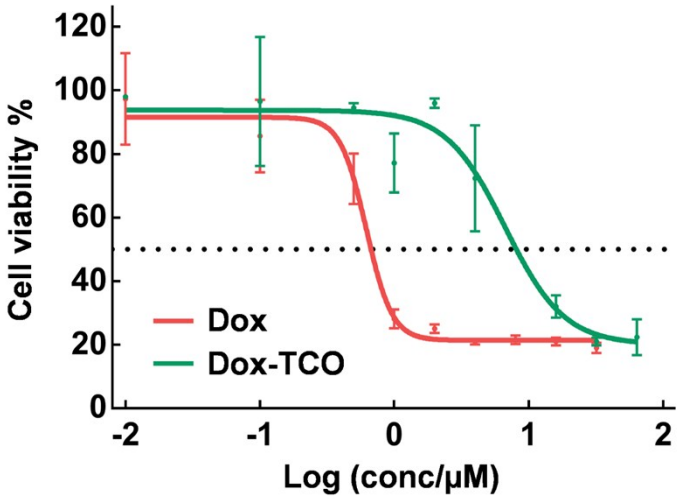


Figure S1. Comparison of the 24 h cytotoxicity of Dox-TCO and Dox. 4T1 Cells were incubated with different concentrations of Dox-TCO or Dox at 37°C for 24 h, and then the cell viability was determined by CCK-8 assay. Dox-TCO exhibited a 10-fold higher EC50 (6.682 μM) than that of native Dox (0.614 μM).

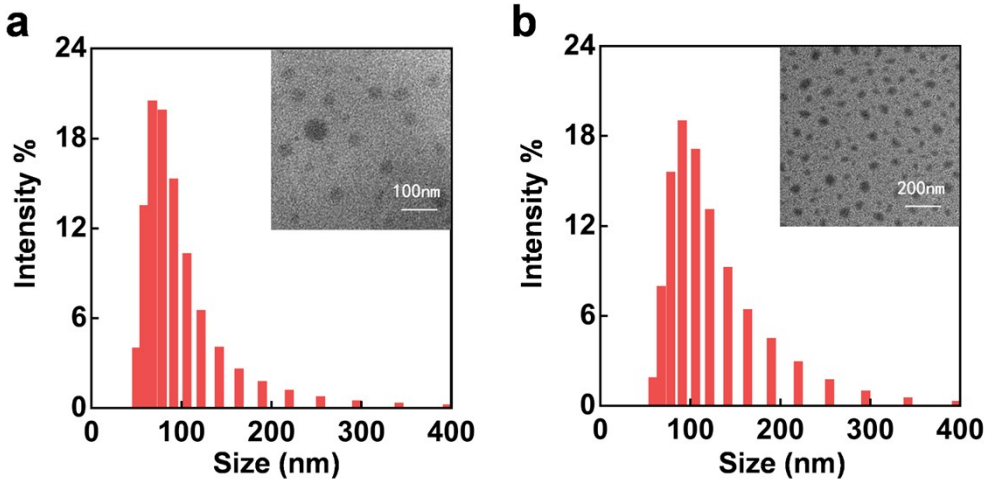


Figure S2. Dynamic light scattering (DLS) and transmission electron microscopy (TEM) imaging of two kinds of nanoparticles. The hydrodynamic size of pH responsive nanoparticles (a) and MMP-2 responsive nanoparticles (b) were about 91.6±18.5 nm and 117.1±22.3 nm, respectively.

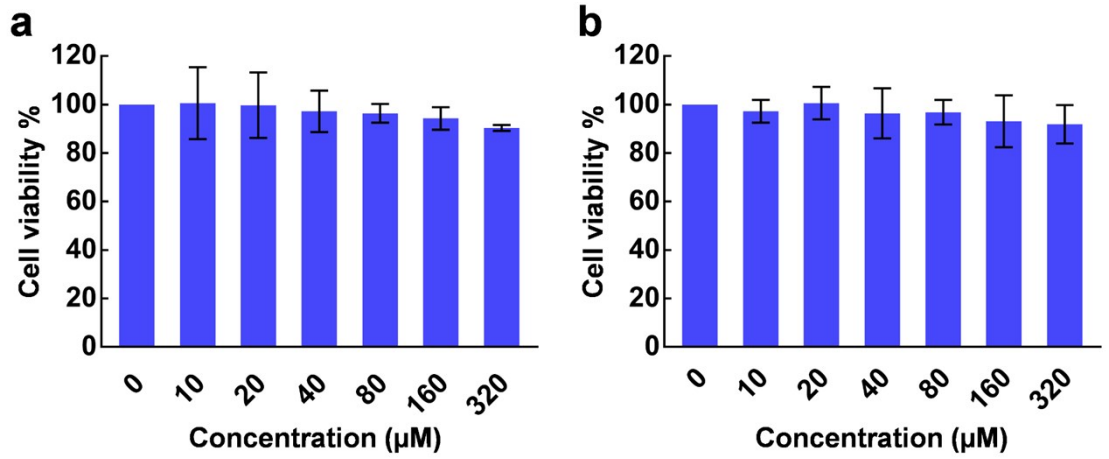


Figure S3. Relative cell viability of two kinds of nanoparticles with different concentrations. 4T1 cells were incubated with different concentrations of low pH responsive nanoparticles (a) or MMP-2 sensitive nanoparticles (b) at 37°C for 24 h. Both the low pH responsive nanoparticles and the MMP-2 sensitive nanoparticles had a satisfactory biocompatibility.

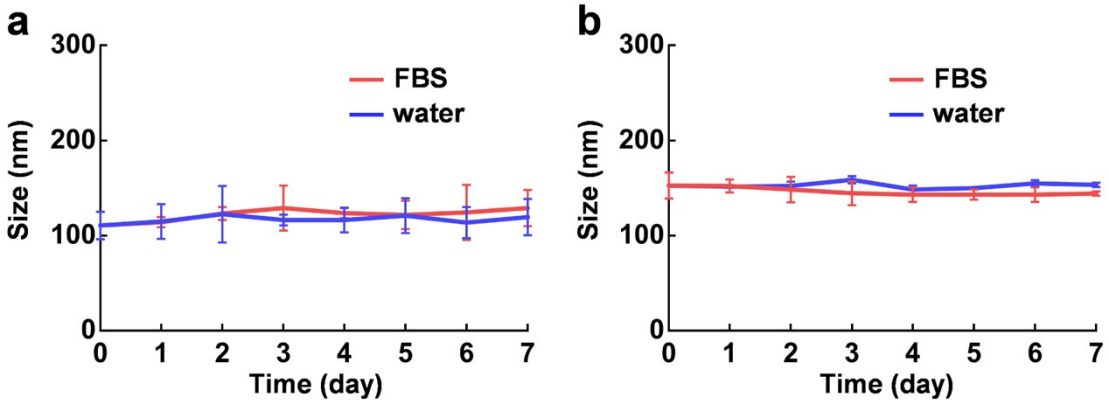


Figure S4. The stability of pH@Dox-TCO nanovehicles (a) and MMP-2@Tz nanovehicles (b) during 7 days incubation in FBS or water at 37°C. Both pH@Dox-TCO nanovehicles and MMP-2@Tz nanovehicles exhibited excellent stability. Bars represent the mean ± s.d. (n=6).

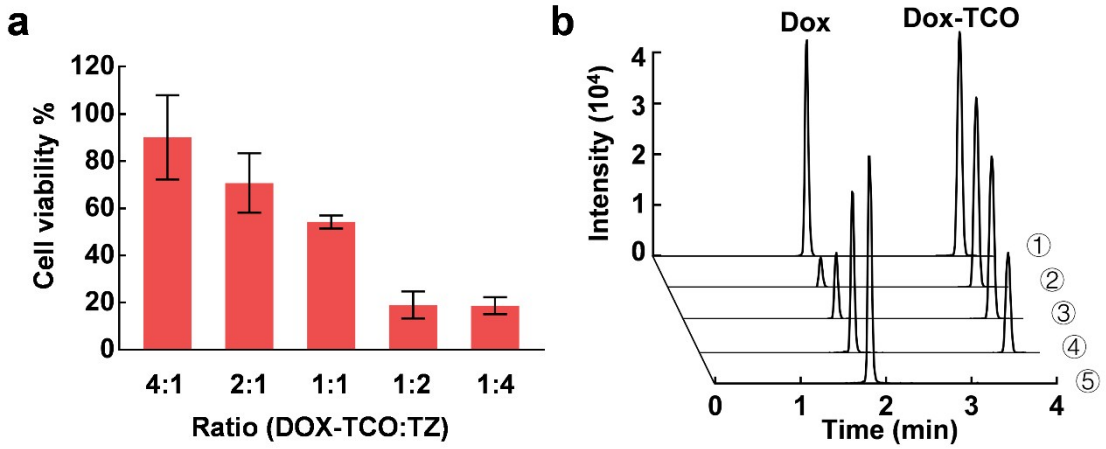


Figure S5. Measurement of the optimal ratio of Dox-TCO to Tz. (a) 5 μM Dox-TCO and different concentrations of Tz (1.25, 2.5, 5, 10, 20 μM) were added to 4T1 cells. After 24 h incubation at 37 °C, the cell viability was determined by CCK-8 assay. (b) UHPLC-MS analysis of activated Dox and Dox-TCO at different ratios of Dox-TCO to Tz. ①: references, ②: Dox-TCO: Tz=4: 1, ③: Dox-TCO: Tz=2: 1, ④: Dox-TCO: Tz=1: 1, ⑤: Dox-TCO: Tz=1: 2. When the molar ratio of Dox-TCO to Tz was 1:2 , Dox could be fully activated. Bars represent the mean ± s.d. (n=3).

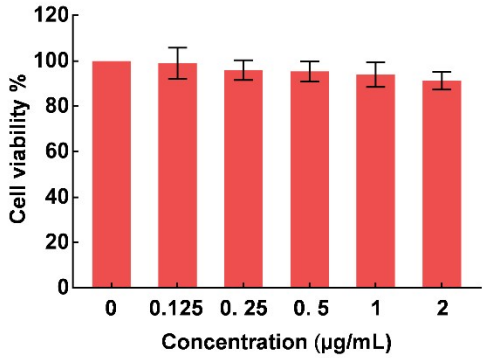


Figure S6. 4T1 cells were incubated with different concentrations of MMP-2 at 37 °C for 24 h. When the MMP-2 concentration was 2 μg/mL, the cell viability remained above 90%.

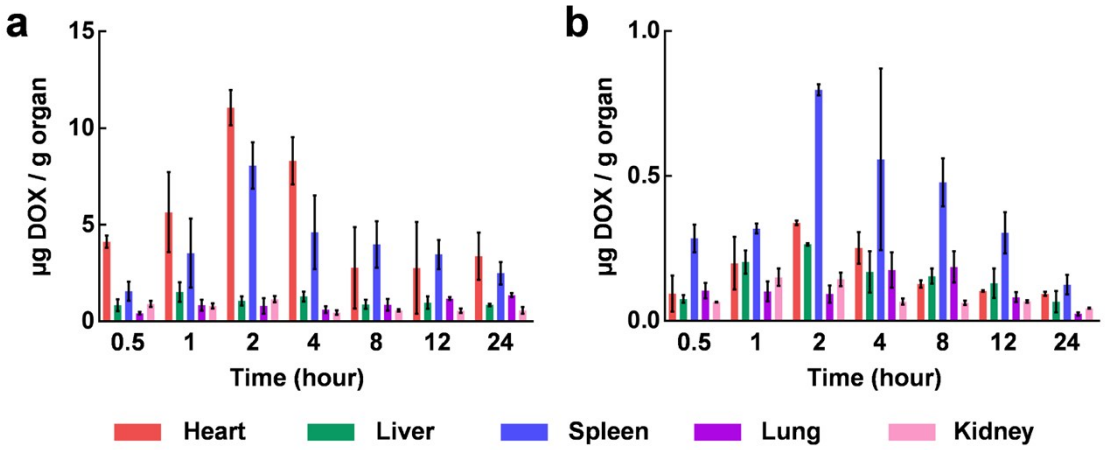


Figure S7. UHPLC-MS analysis of the Dox content in the main organs at different time points after *i.v.* injection of (a) Doxil and (b) pH@Dox-TCO+MMP-2@Tz (10 mg/kg Dox equiv). Concentrations of the Dox in the group treated with pH@Dox-TCO and MMP-2@Tz nanovehicles were always much lower than that of Doxil treated group in all organs, which confirmed the safety of our strategy. Bars represent the mean ± s.d. (n=6).

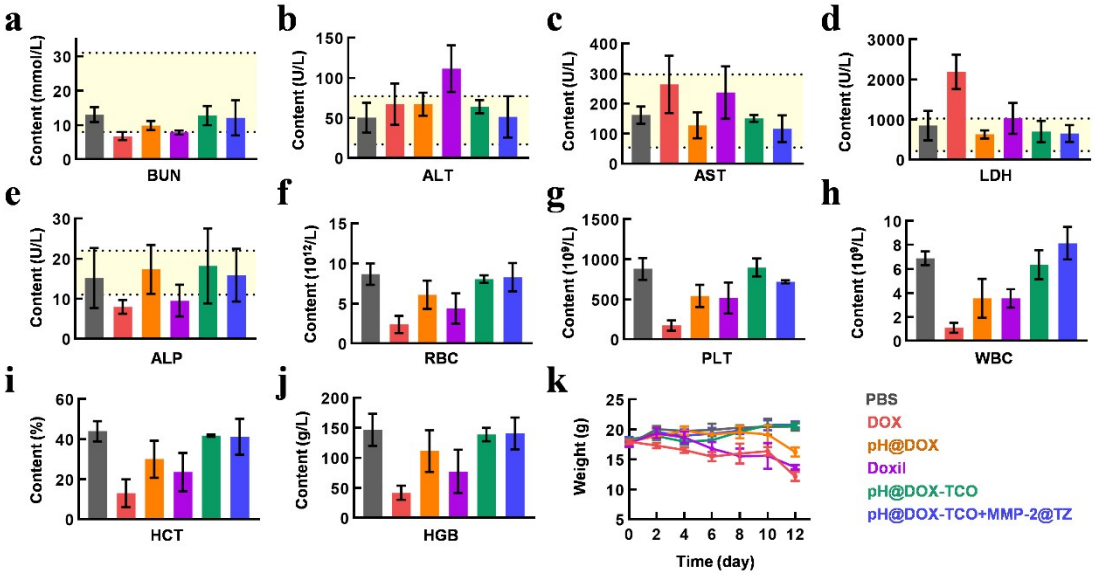


Figure S8. Biological safety evaluation. Blood biochemistry data and hematology data of the mice after *i.v.* injection 7 days of (1) control, (2) Dox, (3) pH@Dox, (4) Doxil, (5) pH@Dox-TCO, (6) pH@Dox-TCO+MMP-2@Tz. (a)-(j) blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactic dehydrogenase (LDH), and alkaline phosphatase (ALP), red blood cell (RBC), platelet (PLT), white blood cell (WBC), hematocrit (HCT), hemoglobin (HGB). (k) Body weight of mice in each group. All the markers were within normal ranges in group 6, which further confirmed the safety of our strategy. Bars represent the mean ± s.d. (n=6).

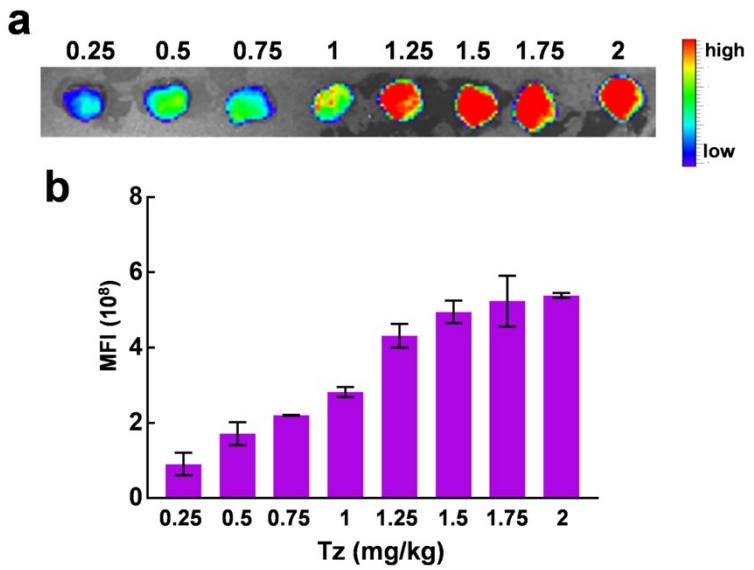


Figure S9. The saturated dose measurement of MMP-2@Tz nanovehicles. (a) *Ex vivo* fluorescence images of excised tumors after *i. v.* injection with different concentrations of MMP-2@Tz for 2 h. (b) Quantitative statistics of the MFI in (a). The accumulation of MMP-2@Tz nanovehicles saturated when intravenously injected with 1.5 mg/kg (Tz equiv) MMP-2@Tz nanovehicles after 2 h, when 5 mg/kg (Dox equiv) pH@Dox-TCO nanovehicles was used. Bars represent the mean ± s.d. (n=6).

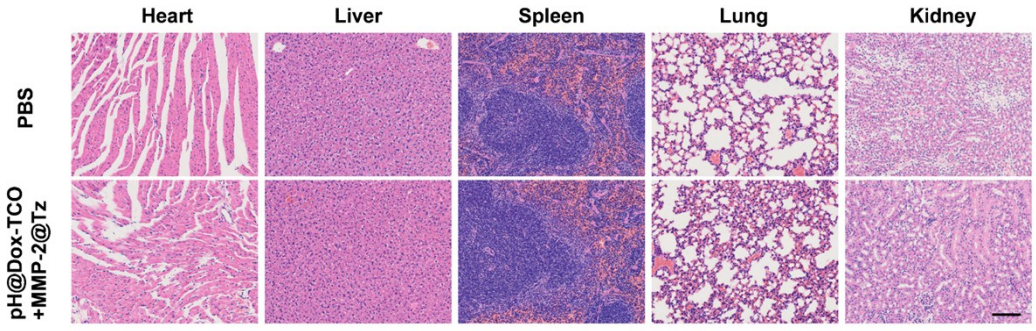


Figure S10. H&E staining of heart, liver, spleen, lung, kidney from PBS group and pH@Dox-TCO+ MMP-2@Tz group. Organs were harvested after treatment at day 14 and stained by hematoxylin eosin. There were almost no differences between the two groups in all organs. The scale bar is 100 μm.

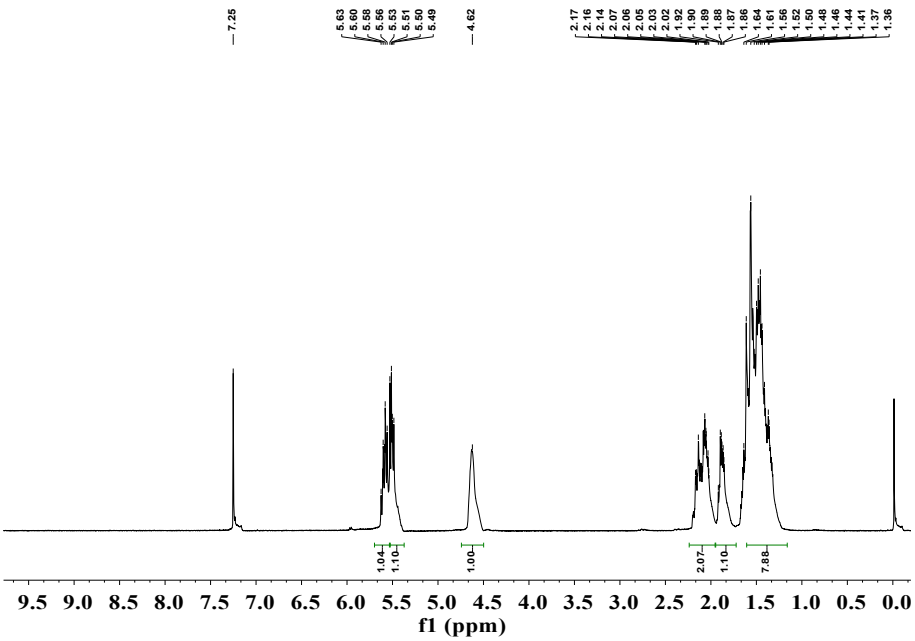


Figure S11. ^{1}H NMR spectrum of (Z)-cyclooct-2-en-1-ol.

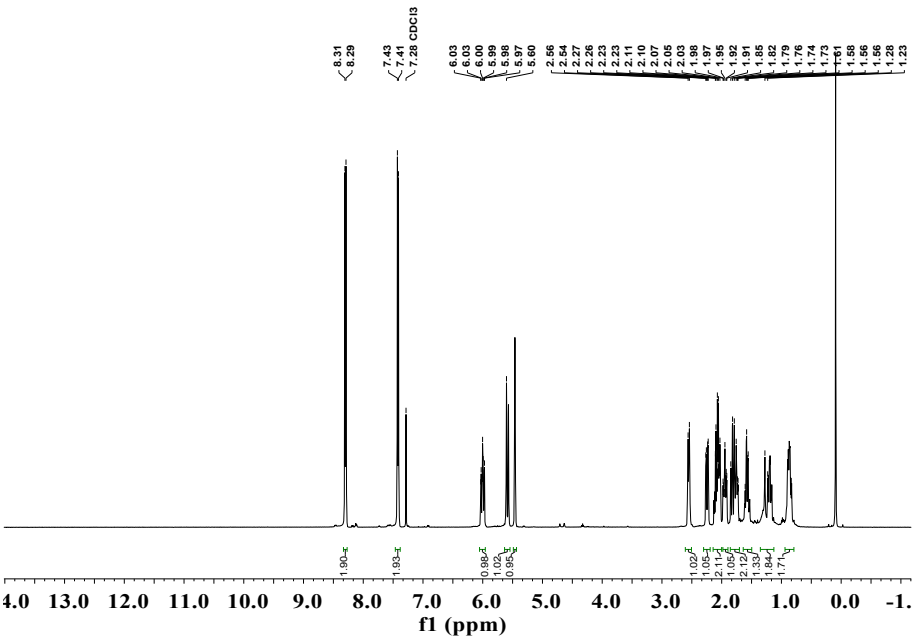


Figure S12. ^{1}H NMR spectrum of (E)-cyclooct-2-en-1-yl (4-nitrophenyl).

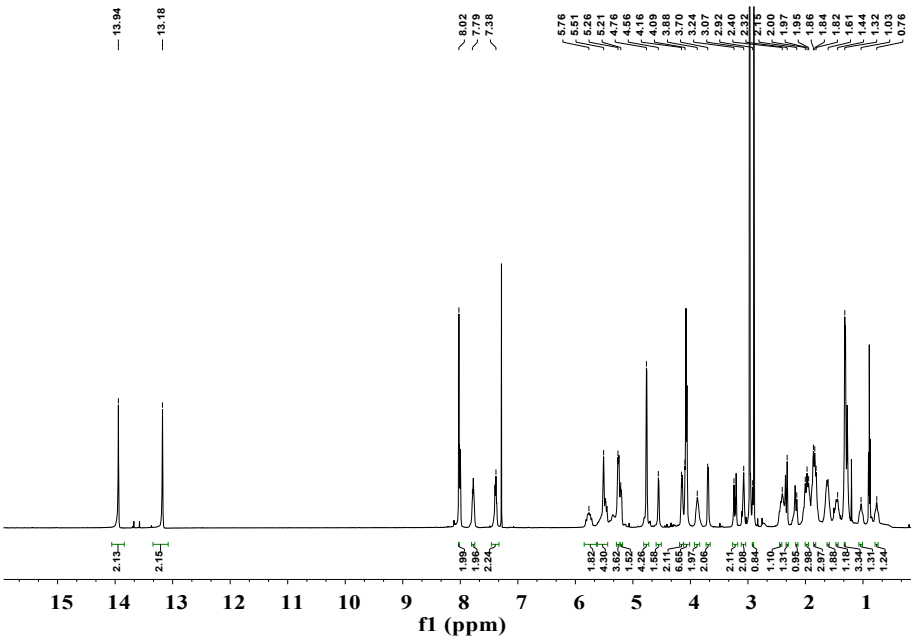


Figure S13. ^{1}H NMR spectrum of Dox-TCO.

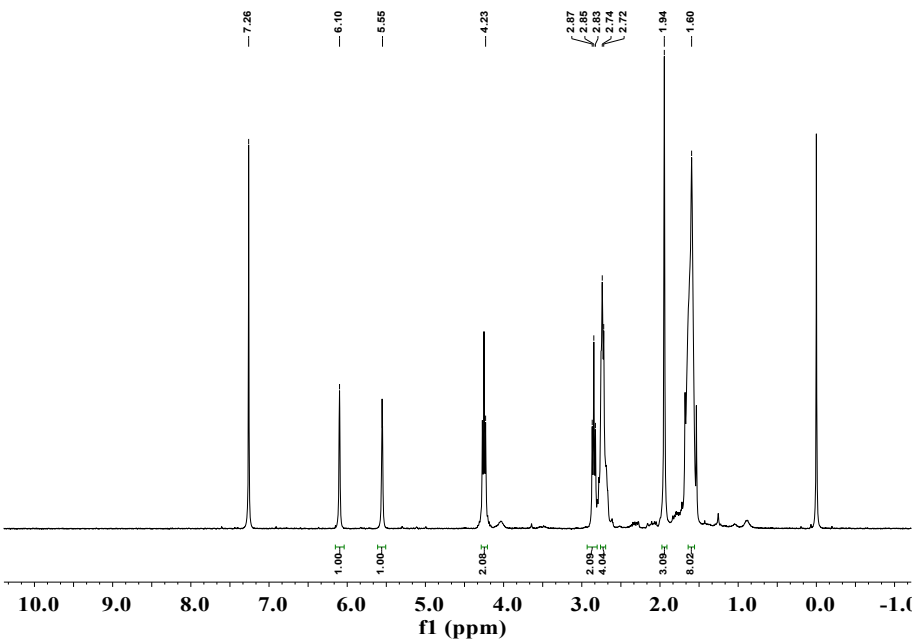


Figure S14. ^{1}H NMR spectrum of C7A-MA.

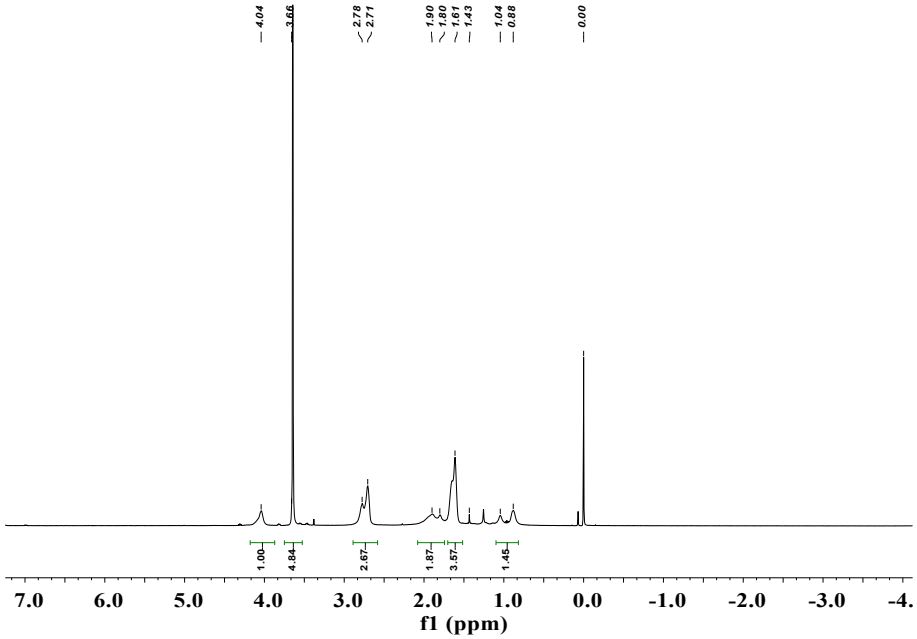


Figure S15. ^{1}H NMR spectrum of pH-sensitive block copolymer.

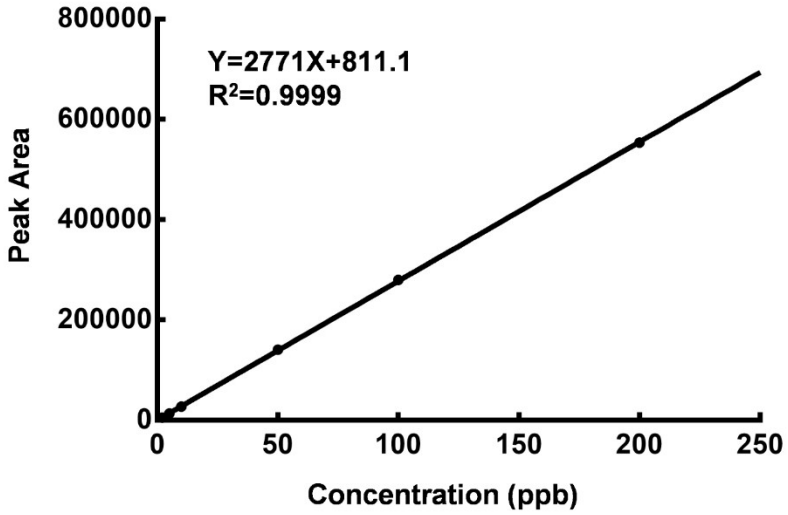


Figure S16. Calibration curve of Dox determined through fluorescence spectrum analysis.

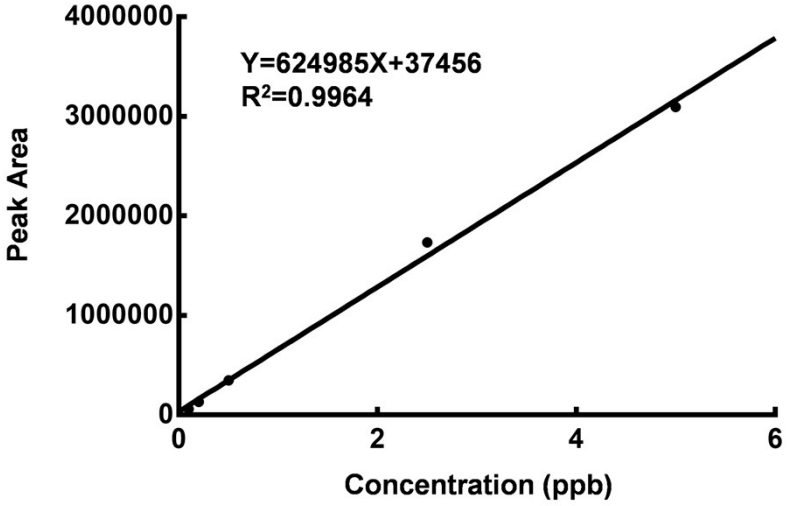


Figure S17. Calibration curve of Tz determined through UHPLC-MS analysis.

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