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Supporting Information

Reduction-Responsive Core-Crosslinked Hyaluronic Acid-*b*-Poly(Trimethylene Carbonate-co-Dithiolane Trimethylene Carbonate) Micelles: Synthesis and CD44-Mediated Potent Delivery of Docetaxel to Triple Negative Breast Tumor *In Vivo*

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Materials and methods

Materials

Trimethylene carbonate (TMC) and dichloromethane (DCM) were dried by refluxing over CaH_2 and distilled prior to use. Docetaxel (DTX, >99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd., Beijing, China), dimethyl sulfoxide (DMSO), anhydrous ether, and $CuSO_4 \cdot 5H_2O$ (99%) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Propargylamine (98%, J&K), sodium cyanoborohydride ($NaCNBH_3$, 95%, Alfar Aesar), 2-azidoethanol (96%, Adamas Reagent Co., Ltd), diphenylphosphate (DPP, 99%, TCI), sodium ascorbate (99%, J&K), dithiothreitol (DTT, 99%, Merck, Germany), glutathione (GSH, >98%, Amresco, USA), cyanine 5 (Cy5, 98%, Lumiprobe, USA), goat serum (Roche, Germany), DAPI (Invitrogen, USA), PE-Cy5 labeled CD44 antibody (eBioscience, San Diego, CA, USA), trypsin (Jinuo Biomedical Technology, Hangzhou, Zhejiang, China), rat monoclonal anti-mouse CD31 (BD Pharmingen, San Jose, California, USA), Alexa 594 conjugated donkey anti-rat secondary antibody (Molecular Probes, Eugene, OR, USA), cell culture dishes and 24 and 96-well plates (Thermo Fisher Scientific, USA) were used as received.

Cell culture and animal studies

The human breast cancer cell line MDA-MB-231 and murine fibroblast L929 cells were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). MDA-MB-231 and L929 cells were maintained in DMEM medium (HyClone, Logan, Utah, USA) supplemented with 1% (V/V) penicillin and streptomycin and 10% (V/V) fetal bovine serum. The cells were cultured as a monolayer in a humidified atmosphere containing 5% CO_2 at 37 °C.

Female Balb/c nude mice and Balb/c mice of 4-6 weeks age were purchased from the model animal research center of Nanjing University (Nanjing, China) and Shanghai SLAC laboratory animal Co., Ltd. (Shanghai, China), respectively. Mice were housed under normal specific pathogen-free (SPF) conditions. All animal procedures were performed following the protocol approved by the Animal Study Committee of Soochow University.

Synthesis of azido-P(TMC-*co*-DTC) copolymer

The azido-P(TMC-*co*-DTC) (N_3-P(TMC-*co*-DTC)) copolymers were synthesized by ring-opening polymerization (ROP) of dithiolane trimethylene carbonate (DTC) and trimethylene carbonate (TMC) using 2-azidoethanol as an initiator and diphenylphosphate (DPP) as a catalyst. In a typical example, under glove-box nitrogen atmosphere, DPP (250 mg, 1.0 mmol) was added into the stirred solution of 2-azidoethanol (8.7 mg, 0.1 mmol), TMC (450 mg, 4.41 mmol) and DTC (150 mg, 0.78 mmol) in DCM (1.2 mL). The reaction vessel was sealed and the polymerization was allowed to proceed with magnetic stirring at 40 °C for 24 h. The resulting polymer was isolated by precipitation in cold diethylether, filtered, and dried in vacuo at room temperature. Yield: 91%. ^{1}H NMR (400 MHz, $CDCl_3$, Fig. S2): TMC (δ 2.06, 4.32, 3.74), DTC (δ 3.02, 4.25, 3.32), and 3.51 (methylene, N_3CH_2-). M_n (^{1}H NMR) = 5.9 kg/mol, M_n (GPC) = 7.6 kg/mol, PDI (GPC) = 1.2.

Characterization

^{1}H NMR spectra were recorded on a Unity Inova 400 MHz spectrometer (Agilent, USA) or 600 MHz DirectDrive 2 (Agilent, USA) using deuterated chloroform ($CDCl_3$), deuterium oxide (D_2O, CIL, Andover, MA, USA), deuterated dimethylsulfoxide (DMSO-d_6, CIL, Andover, MA, USA) or a mixture of D_2O and DMSO-d_6 as a solvent. The molecular weights and polydispersities of the copolymers were determined using a gel permeation

chromatograph (GPC) instrument (Waters 1515, USA) equipped with two linear PL gel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector (RI 2414). The measurements were performed using DMF as the eluent at a flow rate of 1.0 mL/min at 30 °C and a series of narrow poly (methyl methacrylate) (PMMA) standards for the calibration of the columns. The size of the micelles was determined using dynamic light scattering (DLS). Measurements were carried out at 25 °C using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He-Ne laser using back-scattering detection. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. Fourier transform-infrared spectroscopy (FT-IR) spectra were recorded with a 6700 spectrometer (Nicolet, USA). Samples were studied as KBr pellets using 32 scans width (between 400 and 4000 cm^{-1}).

Micellar stability and GSH-triggered de-crosslinking of micelles

The stability of HA-CCMs was evaluated by determination of the size change against 100-fold dilution in PB buffer (10 mM, pH 7.4) or in the presence of 10% FBS. For the determination of the influence of serum on the stability of HA-CCMs, micelles in the presence of 10% FBS were maintained at 37 °C in a shaking bath at 200 rpm. After 24 h incubation, the size of the micelles was determined by DLS. The stability of DTX-loaded HA-CCMs in DMEM medium containing 10% FBS was evaluated over a period of 24 h in a similar way.

The destabilization of HA-CCMs in response to 10 mM GSH in PB buffer (10 mM, pH 7.4) was investigated by DLS measurements. Briefly, HA-CCMs dispersion and GSH solution (the pH was adjusted to 7.4 with 1 M NaOH) were gently bubbled with nitrogen gas for 10 min, respectively. Then the GSH solution was quickly added into the micelle dispersion (final GSH concentration: 10 mM) and the mixture was immediately placed in a shaking bath (200

rpm) at 37 °C. At different time intervals, the micelle size was measured using DLS. Samples without GSH were used as control groups.

Reduction-triggered drug release

The *in vitro* release of DTX from HA-CCMs was studied using a dialysis tube (Spectra/Pore, MWCO 14000) at 37 °C in PB (10 mM, pH 7.4) containing 0.1% (v/v) Tween 80 either in the presence or absence of 10 mM GSH. To acquire sink conditions, drug release studies were performed at a micelle concentration of 1.0 mg/mL (DTX concentration ~ 75 μg/mL) with 0.5 mL of micelle dispersion dialyzed against 25 mL of the same media. At desired time intervals, 5 mL of release medium was taken out and replenished with an equal volume of fresh medium. The amount of DTX released was determined by HPLC.

CD44 expression

The expression of CD44 receptors on MDA-MB-231 breast cancer cells was determined by fluorescence microscopy and flow cytometry. Murine L929 fibroblast cells were used as controls. Briefly, MDA-MB-231 and L929 cells were seeded at a density of 5×10^4 cells/well in 24-well plates and cultured at 37 °C for 24 h. Then CD44 antibody (5 μg/mL) was added. After 1 h incubation, the culture medium was removed. Cells were washed three times with PBS, fixed with 4% paraformaldehyde solution for 15 min and washed again with PBS. Finally cell nuclei were stained with DAPI for 8 min and washed with PBS for three times. The fluorescence images were obtained using a fluorescence microscope (Nikon ECLIPSE Ti-s, Nikon Inc., Japan).

For flow cytometry study, MDA-MB-231 and L929 cells were seeded in a 6-well plate at a density of 5×10^5 cells/well and cultured for 24 h. After 1 h incubation with CD44 antibody, cells were washed three times with PBS, detached with trypsin, centrifuged at 156.5 × g for 5 min and suspended in 0.5 mL of PBS. The cells were analyzed using flow cytometry (FACS

Calibur, BD Biosciences, USA). For each sample, 10,000 events were collected and cells cultured under normal conditions were used as the control.

Cellular uptake of micelles

The uptake behavior of HA-CCMs by MDA-MB-231 cells was studied using flow cytometry. Briefly, MDA-MB-231 cells were seeded at a density of 5×10^5 cells/well in 6-well plates and cultured for 24 h. Cy5-labled HA-CCMs were added at at a final micelle concentration of 200 μg/mL. For competition experiments, cells were pretreated with free HA (5 mg/mL) for 4 h. After an additional 4 h incubation with micelles, cells were treated as described above and then analyzed using flow cytometry (FACS Calibur, BD Biosciences, USA).

Live/dead assay

Briefly, MDA-MB-231 cells were seeded at a density of 8×10^3 cells/well in 96-well plates and cultured for 24 h. DTX-loaded HA-CCMs or free DTX (final DTX concentration: 1 μg/mL) in 10 μL of PBS were added. After 4 h incubation, the medium was removed and replaced with fresh medium, and the cells were incubated for another 44 h. A live/dead assay was performed to evaluate the cell viability of each group according to the standard procedure provided by Sigma Aldrich (04511 cell stain double staining kit) and finally the cells were observed within 1 h using fluorescence microscopy (Nikon ECLIPSE Ti-s, Nikon Inc., Japan).

Apoptosis assay

MDA-MB-231 cells were seeded at a density of 3×10^5 cells/well in 6-well plates and cultured for 24 h. DTX-loaded HA-CCMs micelles or free DTX (final DTX concentration: 1 μg/mL) were added. The cells were cultured for 4 h. Apoptotic cells were determined by dual staining

with an Annexin V and propidium iodide kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. In brief, the cells were detached with 0.25% trypsin (not containing EDTA) and centrifuged at 156.5 × g for 5 min. Then the cells were washed twice with PBS and resuspended in 300 μL binding buffer. Annexin V-FITC (5 μL) and propidium iodide (PI, 3 μL) were added followed by incubation at room temperature for 15 min in the dark. The cells were analyzed by flow cytometry within 1 h.

MTT assay

The antitumor activity of DTX-loaded HA-CCMs and free DTX were determined by the MTT assay. Briefly, MDA-MB-231 cells were seeded at a density of 8×10^3 cells/well in 96-well plates and cultured for 24 h. DTX-loaded HA-CCMs or free DTX (final DTX concentration: 0.5 or 1 μg/mL) in 10 μL of PBS were added. The cells were incubated for 4 h, the medium was removed and replaced with fresh medium, and the cells were incubated for another 44 h. Subsequently, 20 μL of MTT stock solution (5 mg/mL) was added to each well, and the plates were further incubated for 4 h at 37 ºC in the dark. The medium was discarded and 150 μL of DMSO was added to dissolve the blue formazan crystals. Cell viability was assessed by the absorbance at 492 nm of the DMSO solution measured on a microplate reader (Multiskan FC, Thermo). The data were expressed as the percentages of viable cells compared to the survival of a control group (untreated cells).

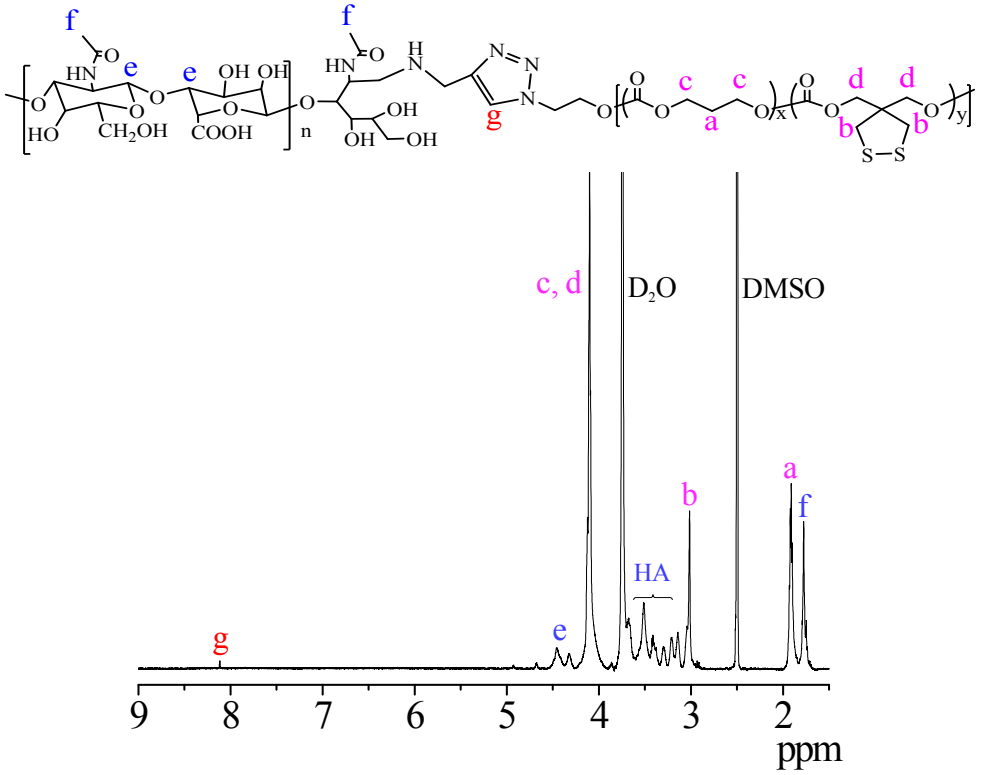


Fig. S1. ^{1}H NMR spectrum (600 MHz, D_2O/DMSO-d_6 = 1/9, v/v) of HA-P(TMC-DTC).

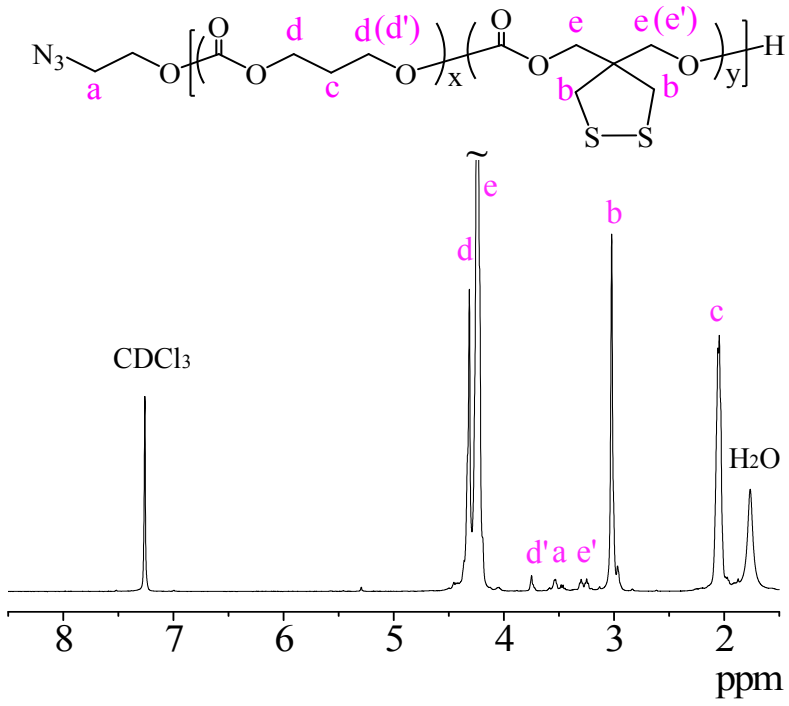


Fig. S2. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of azido-P(TMC-DTC).

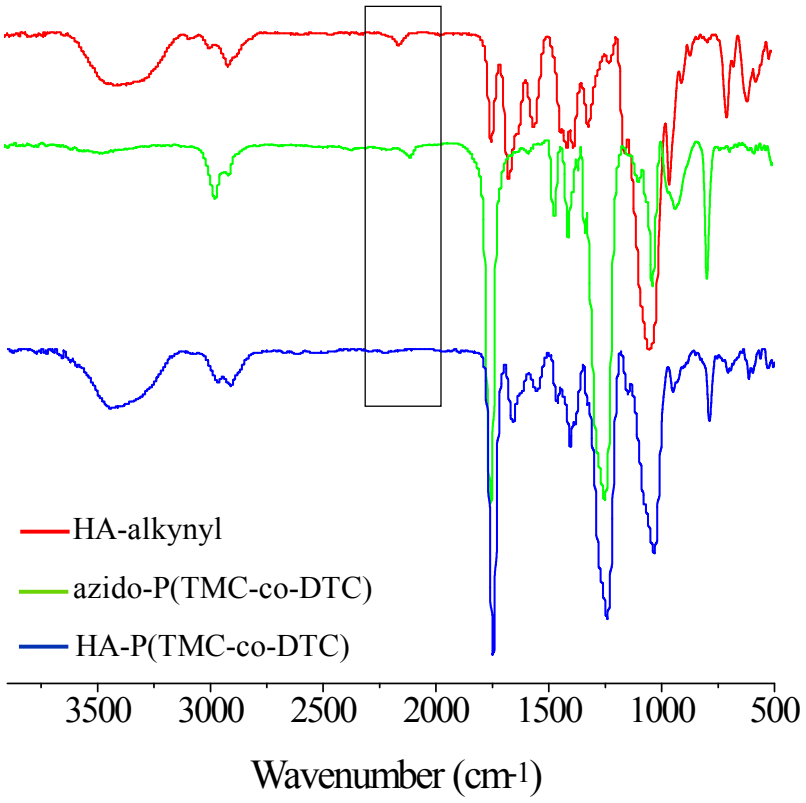
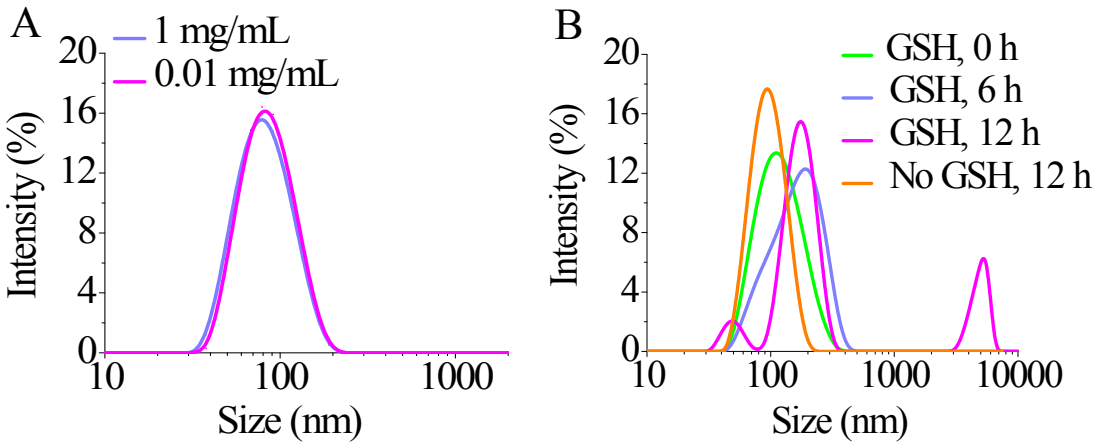


Fig. S3 Infrared spectrum of HA-alkynyl, azido-P(TMC-DTC) and HA-P(TMC-DTC).



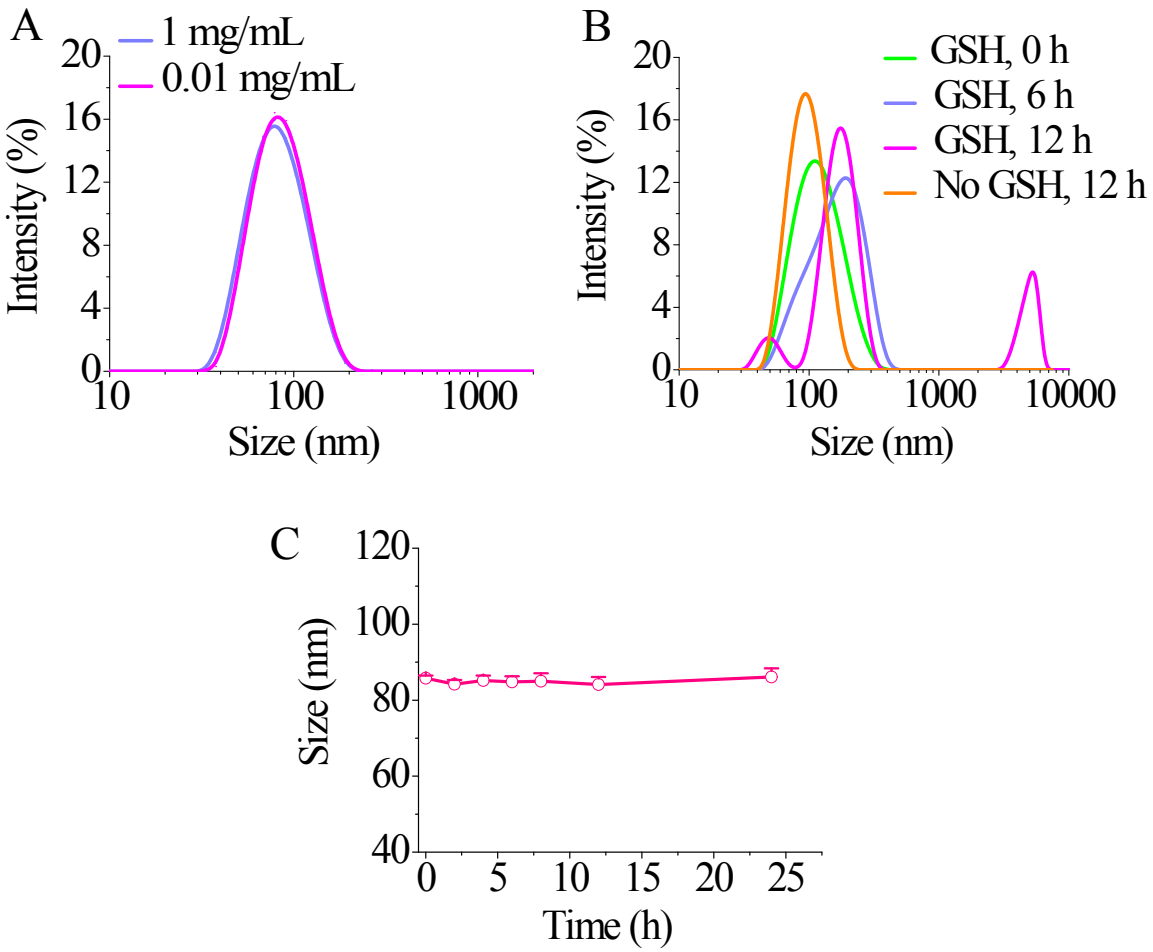


Fig. S4 (A) Stability of HA-CCMs against 100-fold dilution in PB buffer (10 mM, pH 7.4). (B) GSH-triggered destabilization (against 10 mM GSH) of HA-CCMs. (C) Stability of DTX-loaded HA-CCMs in DMEM medium containing 10% FBS.

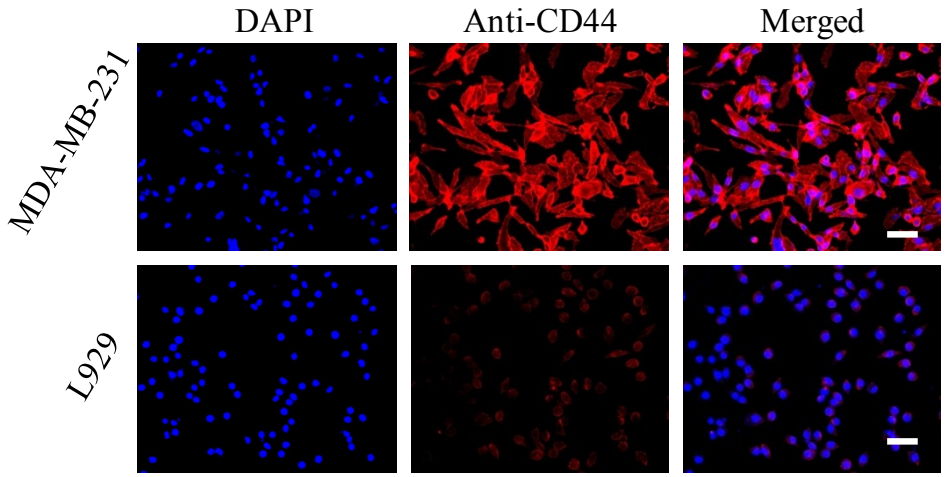


Fig. S5 CD44 receptor expression on MDA-MB-231 breast cancer cells determined by confocal microscopy. Normal L929 fibroblast cells were used as control. Bar: 40 μm.

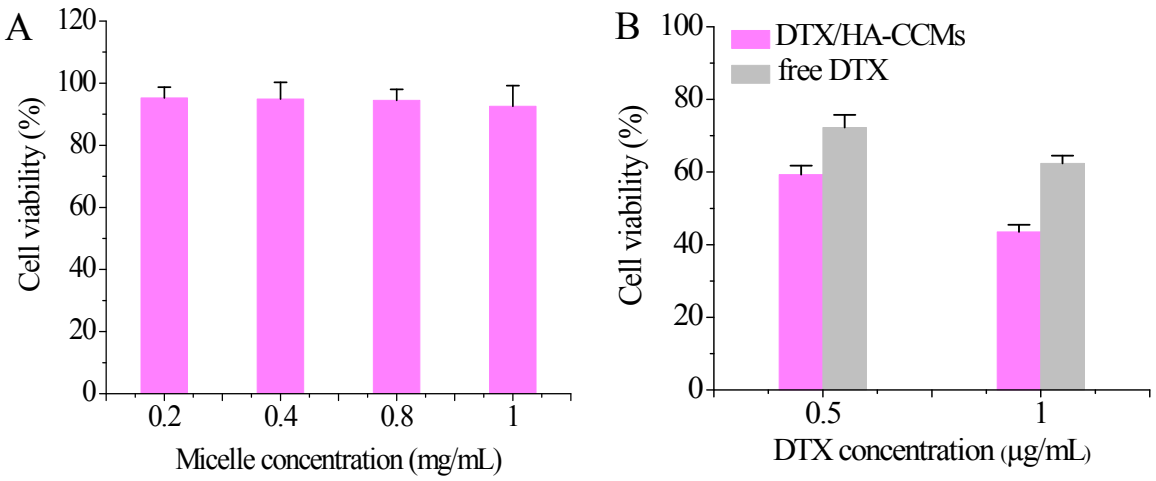


Fig. S6 (A) Cell viability of blank HA-CCMs in MDA-MB-231 breast cancer cells (n = 4). (B) MTT assay of MDA-MB-231 cells after 4 h incubation with either DTX-loaded HA-CCMs or free DTX (0.5 or 1 μg DTX equiv./mL) and another 44 h culture in fresh medium (n = 4).

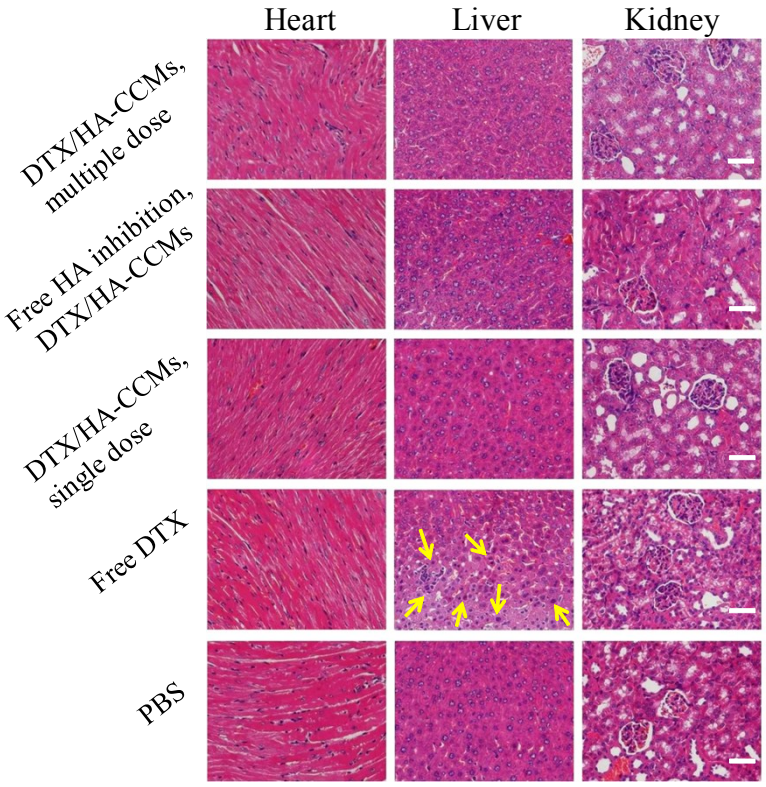


Fig. S7 H&E staining of heart, liver and kidney excised from subcutaneous MDA-MB-231 breast

tumor bearing nude mice following 21 d treatment with different formulations. Yellow arrows indicate hepatic necrosis. The images were obtained by a Leica microscope at 400× magnification. Bar: 50 μm.