

Supporting Information

Expansile crosslinked polymersome for pH sensitive delivery of doxorubicin

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1. Experimental

1.1. Materials

2,4,6-trimethoxybenzaldehyde, 1,1,1-tris(hydroxymethyl) ethane were purchased from Fisher (Pittsburgh, PA). Polyethylene glycol, fumaryl chloride, Tween 80, ammonium persulfate (APS), and tetramethylethylenediamine (TEMED) were purchased from Sigma Aldrich Co. (Milwaukee, WI) and used as received. Other chemicals or reagents were purchased from Fisher or Sigma unless noted otherwise.

1.2. Characterizations

The chemical structures of synthesized polymers were confirmed using ¹H NMR spectroscopy (300 MHz Varian NMR). Molecular weights of the obtained polymers were determined by Viscotek GPCMax/VE 2001 gel permeation chromatography (GPC) machine (Malvern Instruments, Inc.) with tetrahydrofuran as eluent.

1.3. Synthesis of 2,4,6-trimethoxybenzylidene-1,1,1-tris(hydroxymethyl)ethane (TT)

TT monomer was synthesized according to previous reports.^{1,2} Briefly, 6.7 g of 1,1,1-tris(hydroxymethyl)ethane (55.8 mmol) and 4.0 g of 2,4,6-trimethoxybenzaldehyde (20.5 mmol) was dissolved in 200 mL of tetrahydrofuran (THF) by vigorous stirring at room temperature. 50 g of 4 Å molecular sieves was then added as a desiccant and 0.473 g of *p*-toluenesulfonic acid (2.49 mmol) was added as catalyst. The reaction mixture was stirred for 24 hours at ambient temperature. At the completion of reaction, 8.0 mL of trimethylamine (TEA) was added to neutralize the acid. Afterwards, 200

mL of CH_2Cl_2 was added, and the mixture was filtered to remove the molecular sieves. Filtrate was collected and solvents were removed by rotary evaporation. Solid residue was re-dissolved in 200 mL of CH_2Cl_2 and washed with Tris buffer (100 mM, pH 8.0) three times to remove unreacted monomers, then dried over anhydrous magnesium sulfate. The CH_2Cl_2 solvent was then removed by rotary evaporation and the residue was washed with 200 mL of diethyl ether. The final product, 2,4,6-trimethoxybenzylidene-1,1,1-tris(hydroxymethyl) ethane, was dried in vacuum to give a light yellow solid. Yield: 4.9 g (80.3 %).

1.4. Synthesis of PEG-Fu-DiTT

Excess fumaryl chloride (FuCl, 1 mL, density 1.4 g mL^{-1} , 9 mmol) was added to 10 mL of anhydrous CH_2Cl_2 in a 50 mL flask with 2 g potassium carbonate (K_2CO_3) as proton scavenger. PEG600 (0.2 g, 0.33 mmol) dissolved in 5 mL of anhydrous CH_2Cl_2 was then added dropwise to the flask while stirring. The reaction mixture was further stirred overnight at room temperature and filtered to remove K_2CO_3 solids. The filtrate was proceeded for rotary evaporation under reduced pressure to remove CH_2Cl_2 solvent. Unreacted fumaryl chloride was then fully removed under vacuum to obtain FuCl-PEG-FuCl. To synthesis PEG-Fu-DiTT, 10 mL of THF was added to dissolve FuCl-PEG-FuCl and the mixture was then transferred to another flask containing 0.2 g TT, 50 mL THF and 2 g K_2CO_3 . This FuCl-PEG-FuCl/TT/ K_2CO_3 mixture was further stirred for 12 hours at room temperature and then filtered to remove K_2CO_3 solids. The filtrate was concentrated by rotary evaporation and the residue was precipitated in petroleum ether to obtain light brown soft solid product.

1.5. Self-assembly polymersomes preparation

10 mg PEG-Fu-DiTT polymer was dissolved in 1 mL of THF under stirring and then 10 mL of 0.3% poly(vinyl alcohol) (PVA) solution was added slowly. The mixture was allowed to stir for 5 min and residual THF was removed under reduced pressure. Final micelle concentration was 1.0 mg mL^{-1} . Crosslinked polymersomes were prepared by adding of 40 μL of 0.3 M ammonium persulfate and 40 μL of 0.3 M tetramethylethylenediamine to the above polymersome solution. Dynamic light scattering (DLS) was performed to determine the hydrodynamic sizes of formed micelles on a Zetasizer Nano ZS (Malvern Instruments). The size and morphology of micelles were further confirmed by Transmission Electronic Microscopy (TEM, JEOL 1400, Japan). Stabilities of self-assembled crosslinked and uncrosslinked PEG-Fu-DiTT polymersomes after exposure to proteins, surfactants and salt ions were evaluated by DLS in 10% FBS, 5 mM SDS and 0.9% NaCl solution, respectively. To investigate the size changes of these uncrosslinked and crosslinked PEG-Fu-DiTT polymersomes, the solution pH was adjusted to 7.4 or acidic 5.0 using phosphate buffer or acetate buffer, respectively. The size changes of these polymersomes under varied pH conditions were then tracked using DLS measurement at different time points.

1.6. Loading of DOX into polymersomes

DOX-loaded polymersomes were prepared by sequentially adding 400 μL DOX•HCl aqueous solution (7.5 mg/mL) and 4 mL of 0.3% PVA solution to 1 mL of PEG-Fu-DiTT copolymer dissolved in THF solvent (20 mg/mL) under stirring. Residual THF was removed by rotary evaporation. Crosslinked polymersomes were prepared by adding of 40 μL of 0.3 M AP and 40 μL of 0.3 M TEMED to the polymersomes solution. The mixture was transferred to a dialysis tubing (Pierce, MWCO 2000) and dialyzed against phosphate buffer (10 mM, pH 7.4) for 12 h in the dark with at least 3 times change of media. The dialysis media was collected and the concentration of DOX in the media was quantified using a UV-vis absorbance microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA). The detection wavelength was set at 490 nm and calibration curve was obtained with a series of DOX•HCl solutions of known concentrations. The drug loading content (DLC) and drug loading efficiency (DLE) of DOX encapsulated in polymersomes were calculated according to the following formula, as previously described.²

$\text{DLC (\%)} = [\text{weight of drug loaded} / \text{weight of drug-loaded polymersomes}] \times 100\%$

$\text{DLE (\%)} = [\text{weight of drug loaded} / \text{weight of drug in feed}] \times 100\%$

1.7. *In vitro* release profile of DOX

The release profiles of DOX from PEG-Fu-DiTT polymersomes were investigated in pH 5.0 and pH 7.4 at 37 °C. DOX-loaded polymersomes were prepared according to the above formula with final polymer concentration of 5 mg/mL and then divided into two aliquots of 4 mL volume for each sample. The pH was adjusted to pH 7.4 using phosphate buffer or to 5.0 using acid-buffered solution then transferred to dialysis tubes with molecular weight cut-off (MWCO) of 2000. Dialysis tubes were then immersed in corresponding pH 5.0 or pH 7.4 buffer (15 mL, 0.1 M) with constant shaking at 37 °C. At certain time points, 1 mL of release medium was taken out for DOX concentration determination on a UV-vis absorbance microplate reader (490 nm) using calibration curves established by corresponding buffer solutions (5.0 or 7.4) of known DOX•HCl concentrations. The release media was then replenished with equal volume of fresh buffer immediately. Each experiments were conducted in triplicates and average values were calculated with standard deviations.

1.8. Cell viability

HeLa cell was used as a model cancer cell for cytotoxicity evaluation. Trypsinized HeLa cells were seeded onto 48-well tissue culture polystyrene (TCPS) plates at 10,000 cells cm^{-2} . Cells were then cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin, 2 mM L-glutamine, and 0.1 mg ml^{-1} streptomycin for 24 h to allow adhesion. To evaluate the cytotoxicity of PEG-Fu-DiTT polymersomes, empty samples without loading drugs were prepared, dialyzed against PBS for 2 days, and lyophilized for 3 days. The dried polymersomes were then incubated with HeLa cells at concentrations of 0.1, 0.5, 1.0 and 2.0 mg mL^{-1} for 3 days. Wells seeded with the same HeLa cell density but without added polymersomes were used as positive controls. The relative cell densities in each group were determined by MTS assay (CellTiter 96 Aqueous

One Solution, Promega, Madison, WI) by comparing the OD value to that of positive controls (set as 100%). To evaluate the cancer killing effect, similar cell culture procedures were used by adding DOX-loaded polymersomes or free drugs at concentrations of 0.01, 0.1, 0.5, 1, 5, 10 and 50 $\mu\text{g mL}^{-1}$.

1.9. Cellular uptake of polymersomes

HeLa cells were co-cultured with DOX-loaded polymersomes in DMEM at a drug concentration of 10 $\mu\text{g mL}^{-1}$, similar to the above mentioned cancer killing studies. At time points of 1, 2, 4 hours, culture medium was removed and cells were washed with PBS for three times then fixed in 4% paraformaldehyde. After 10 min, paraformaldehyde was removed and cells were washed three times with PBS then stained with DAPI (4',6-diamidino-2-phenylindole) for 2 min. DOX fluorescence (red) from polymersomes and DAPI fluorescence (blue) from cellular nuclei were visualized and photographed by Axiovert 25 Zeiss light microscope (Carl Zeiss, Germany).

2. Results

2.1. Polymer characterization

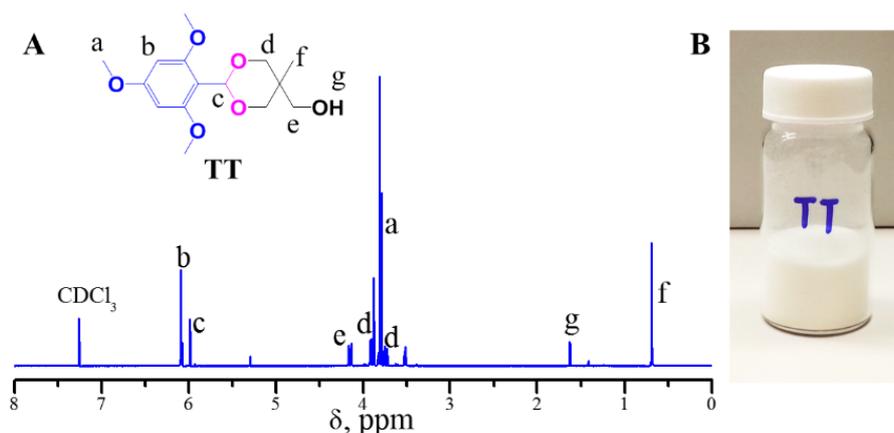


Fig. S1 (A) ^1H NMR spectrum of TT monomer (in CDCl_3). (B) Image of TT product obtained after purification.

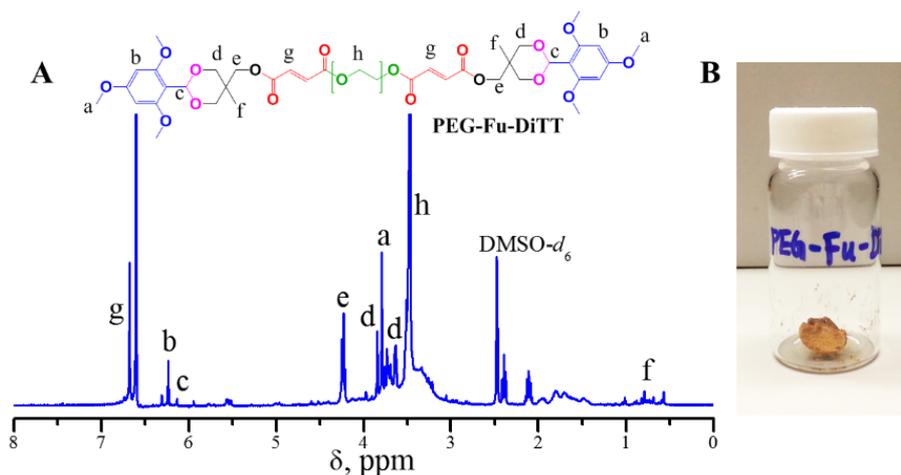


Fig. S2 (A) ^1H NMR spectrum of PEG-Fu-DiTT polymer (in $\text{DMSO-}d_6$). (B) Image of synthesized PEG-Fu-DiTT polymer product.

Table S1 Polymer properties including number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (PDI).

Polymers	M_n (g mol^{-1})	M_w (g mol^{-1})	PDI
PEG	540	560	1.04
PEG-Fu-DiTT	1380	1710	1.24

2.2. Self-assembled PEG-Fu-DiTT polymersome properties

Table S2 Polymersome properties before and after drug loading.

	Empty micelle			DOX-loaded micelle		
	Size (nm)	PDI	Zeta (mV)	Size (nm)	PDI	Zeta (mV)
Uncrosslinked	101.6 ± 3.6	0.46 ± 0.03	-2.4 ± 1.5	113.9 ± 4.6	0.44 ± 0.01	-1.3 ± 0.5
Crosslinked	96.3 ± 2.4	0.42 ± 0.03	-2.8 ± 1.1	103.5 ± 5.3	0.45 ± 0.06	-1.3 ± 0.3

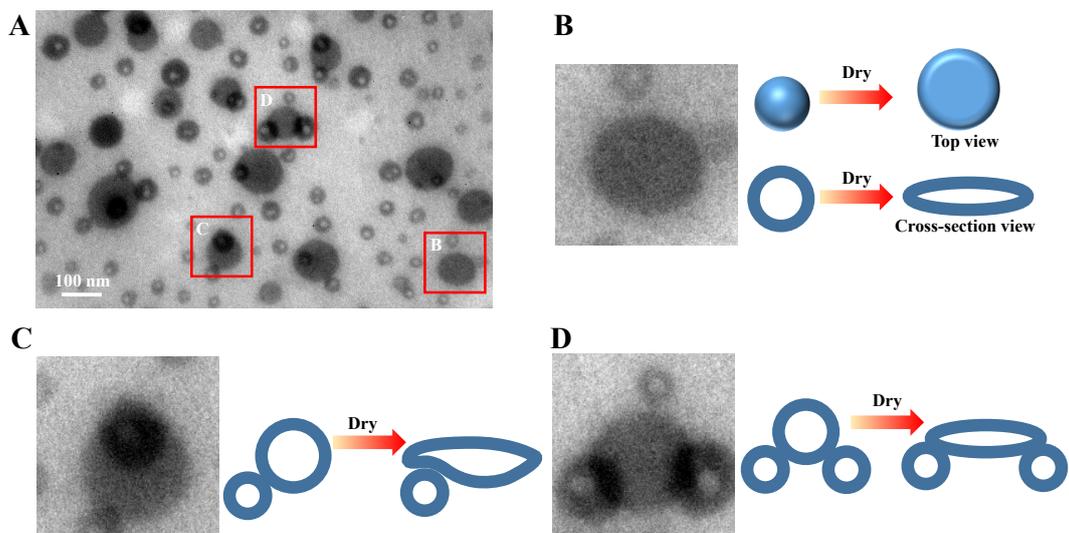


Fig. S3 (A) TEM images of polymersomes and schematic demonstration of different conformations of polymersomes created by the drying process including (B) void core collapse, (C) overlap and (D) edge overlap of multiple polymersomes.

2.3. HeLa cancer cell cytotoxicity

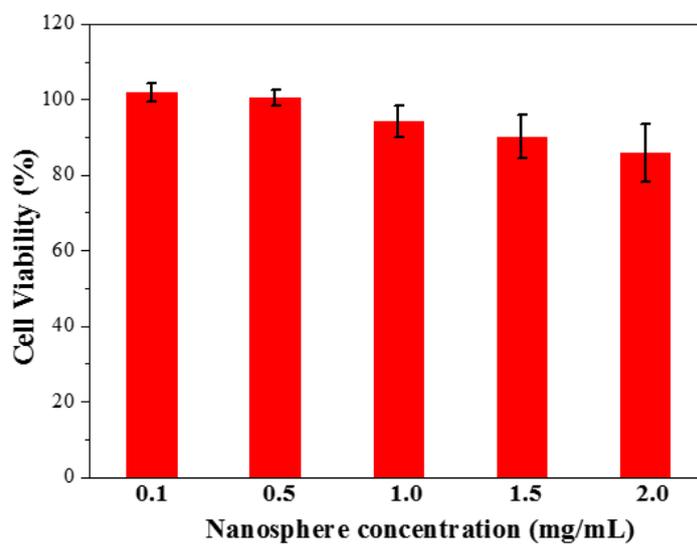


Fig. S4 HeLa cell viability after co-cultured with empty crosslinked polymersomes at varied concentrations of 0.1, 0.5, 1.0, 1.5 and 2.0 mg mL⁻¹ for 3 days.

2.4. HeLa cancer cell uptake of DOX loaded PEG-Fu-DiTT polymersome and free DOX•HCl drugs

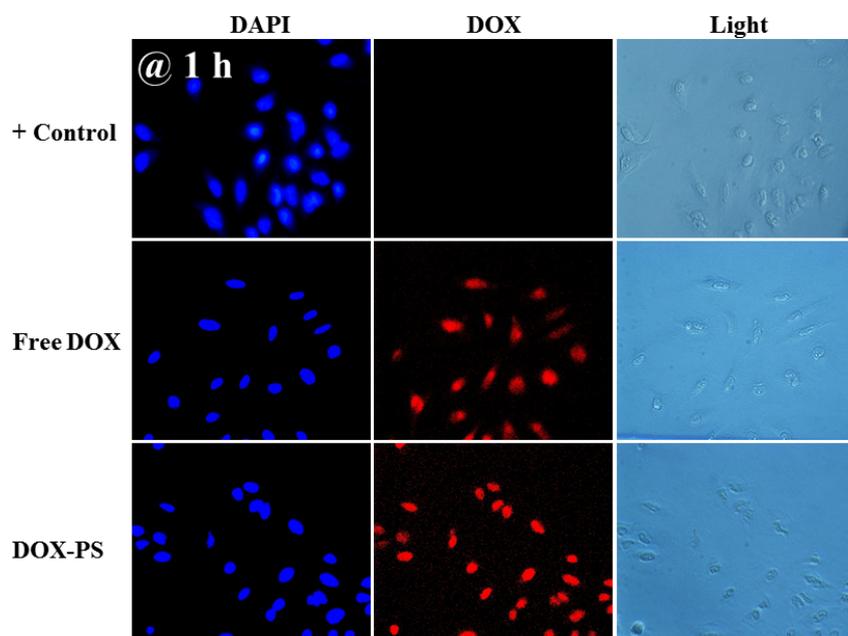


Fig. S5 DAPI (blue) and DOX (red) fluorescence photographs and light images of HeLa cells co-cultured for 1 h in the presence of free DOX or DOX loaded polymersome (DOX-PS). Cells without DOX administration were used as positive control.

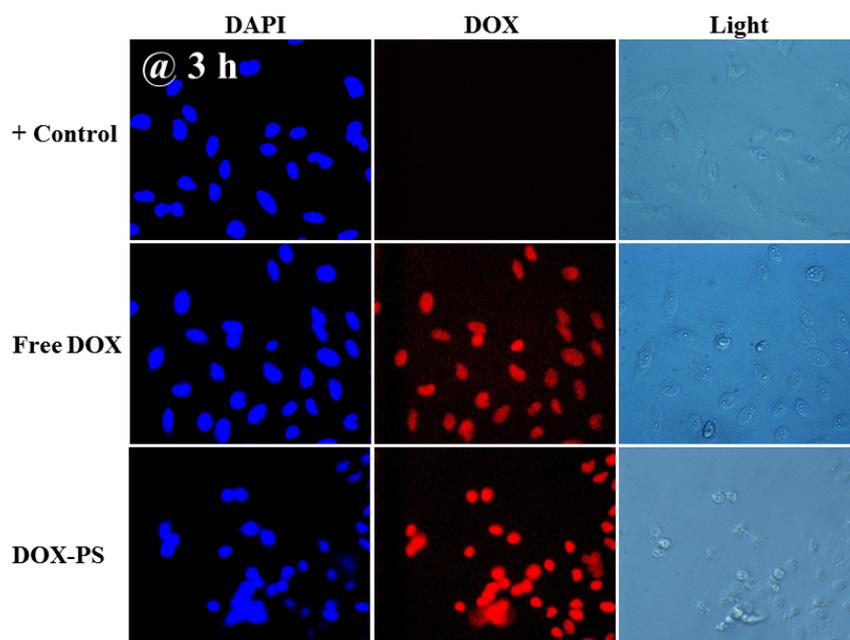


Fig. S6 HeLa cell imaging after 3 h co-culture without DOX, or with free DOX or DOX-PS.

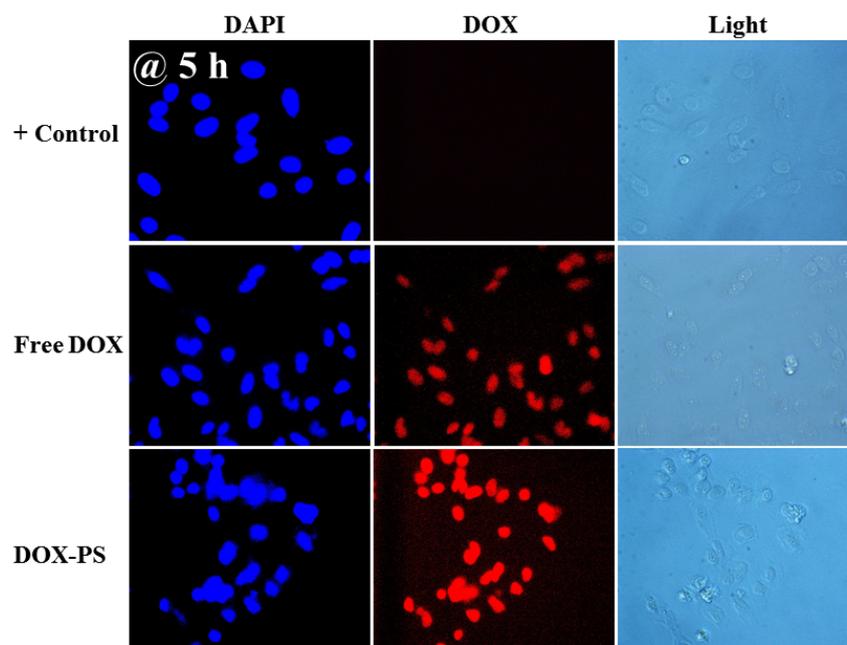


Fig. S7 HeLa cell imaging after 5 h co-culture without DOX, or with free DOX or DOX-PS.

References

1. A. P. Griset, J. Walpole, R. Liu, A. Gaffey, Y. L. Colson and M. W. Grinstaff, *J. Am. Chem. Soc.*, 2009, **131**, 2469-2471.
2. Y. Du, W. Chen, M. Zheng, F. Meng and Z. Zhong, *Biomaterials*, 2012, **33**, 7291-7299.