Electronic Supplementary Information (ESI)

"And" Logic Gate Regulated pH and Reduction Dual Responsive Prodrug Nanoparticles for Efficient Intracellular Anticancer Drug Delivery

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Experimental Section

1. Materials

Doxorubicin hydrochloride (DOX·HCl, HPLC purity ≥ 98%) was supplied by Zhongshuo Pharmaceutical Technology Development Co. Ltd (Beijing, China). Methoxypolyethylene glycol (MPEG, M_n 2000) was purchased from Sigma-Aldrich (Shanghai, China). 1-Ethyl-3-(3 – dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) and 3, 3'-dithiodipropionic acid (DTDP) were obtained from Aladdin Chemistry Co. Ltd (Shanghai, China). 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and glutathione (GSH) were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Other chemicals purchased from Kelong Chemical Industries Reagent Co. Ltd (Chengdu, China) were used without further purification.

2. Measurements

^{1}H NMR spectra were recorded on a Bruker Advance 400 spectrometer (Bruker, Germany) by using tetramethyl silane (TMS) as the internal reference. FTIR spectra were obtained on a Nicolet 6700 Fourier Transform Infrared spectrophotometer (Thermo Scientific Co., USA). The averaged hydrodynamic diameter and its distribution were determined by dynamic light scattering (DLS) on a Brookhaven model BI-200SM spectrometer (Brookhaven Instruments CO., USA) equipped with a 9000AT correlator using an Innova 304 He-Ne laser (1 W, λ=532 nm). UV-Vis absorption spectra were recorded on a Varian Cary® 50 UV-Vis Spectrophotometer (Varian Co., USA). Fluorescence spectra were observed on a Varian Cary Eclipse Fluorescence Spectrometer (Varian Co., USA). Transmission electron microscopy (TEM) was carried out on a Tecnai G2 F20 S-TWIN Transmission Electron Microscope (FEI Co., USA) operated at an acceleration voltage of 200 kV. Prior to test, sample dispersions were dropped onto carbon-coated copper grids, followed by staining with 0.5% phosphotungstic acid solution (pH =7.4). High resolution electrospray ionization time-of-flight mass spectrometry (HR ESI-TOF MS) was performed on a Waters Q-Tof Premier mass spectrometer operating in positive ion electrospray mode. The capillary and sampling cone voltages were set at 2.8 kV and 40 V for positive electrospray mode. The desolvation temperature was set at 150 °C and the source temperature at 90 °C.

3. Preparation of PEG-SS-DOX prodrug

3.1 Synthesis of PEG-NHS

A mixture of DTDP (0.632 g, 3 mmol), EDC (1.265 g, 6.6 mmol) and NHS (0.760 mg, 6.6 mmol) was dispersed into dry dichloromethane (DCM, 30 mL). The solution of MPEG (2.0 g, mmol, M_n

2000) in DCM (20 mL) was then added and reacted with DTDP for 24h under nitrogen atmosphere. The resulting mixture was concentrated under reduced pressure and dried in vacuum. The crude solid product was then dissolved in water and filtrated to remove precipitates, followed by dialyzing against water for 2 days (molecular weight cut-off 2000 Da). The resultant PEG-NHS (yield: 71%) was obtained after lyophilization.

3.2 Synthesis of PEG-SS-DOX

DOX·HCl (0.058 g, 0.1 mmol) was treated with triethylamine (0.04 ml, 0.3 mmol) in dimethyl sulfoxide (DMSO) and then reacted with PEG-SS-NHS (0.229 g, 0.1 mmol) at room temperature for 48 h. The resulting solution was dialyzed using a dialysis bag (molecular weight cut-off 2000 Da) against a phosphate buffer solution (PBS, pH = 8.0) for 3 days and lyophilized to obtain PEG-SS-DOX (yield: 56%). ^{1}H NMR (DMSO-d_6, ppm): δ 1.24~1.36 (s, 3H, -CH_3 of DOX), 3.20~3.24 (s, 3H, -OCH_3 of PEG), 3.25~3.80 (t, 180H, CH_2CH_2O of PEG), 3.93~4.05 (s, 3H, -OCH_3 of DOX), 4.54~4.66 (s, 2H, -CH_2CO), 7.63~7.69, 7.87~8.02 (s, 3H, CHCHCH of DOX). HR ESI-TOF MS: m/z 2713.6423 [$(M+H)^+$, $C_{124}H_{218}O_{58}NS_2$].

4. Preparation of PEG-SS-DOX and PEG-SS-DOX-Cu nanoparticles

For the preparation of PEG-SS-DOX nanoparticles, 100 mg of PEG-SS-DOX was dissolved in 10 mL DMSO, and the solution was dialyzed against water for 5 days. The preparation of PEG-SS-DOX-Cu nanoparticles was conducted in the same process except that a certain amount of $Cu(NO_3)_2$ was added prior to the dialysis treatment.

5. **Determination of critical micelle concentration** (CMC)

The CMC of PEG-SS-DOX and PEG-SS-DOX-Cu at pH 7.4 was determined by UV-Vis spectroscopy using pyrene as an extrinsic probe according to the previous report.[1] The absorbance of pyrene at different prodrug concentrations was observed at λ of 315 nm, and the CMC values were determined from the inflection point of the fitted sigmoid curve.

6. In vitro drug release

To investigate the release rate of DOX for the prodrug systems under different conditions (pH: 5.0 and 7.4; concentration of GSH: 2 μM, 2 mM, 5 mM), non-cross-linked and core-cross-linked prodrug nanoparticles dispersed in 1 mL of pH 7.4 PBS (4 mg mL^{-1}) were placed into dialysis bags, and the bags were incubated in 20 mL of release medium at 37 ^{o}C with continuous shaking at 100 rpm. At predetermined intervals, 4 mL of incubated solution was taken out and an equal volume of fresh release medium was replenished. The amount of released DOX was measured by fluorescence spectroscopy (excitation at 495 nm). The release experiments were performed in triplicate.

7. Cell culture

The human cervical cancer cells (HeLa) were obtained from American Type Culture Collection and cultured in a 10% fetal bovine serum (FBS)-containing Dulbecco's modified eagle medium (DMEM) supplemented with 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cells were grown in a humidified incubator at 37 ^{o}C under 5% CO_2 atmosphere. The cells were harvested with 0.02% EDTA and 0.025% trypsin and rinsed. The resultant cell suspension was used in the

following experiments.

8. Cell viability assays

The relative cytotoxicities of PEG-SS-DOX against HeLa cells were evaluated in vitro by MTT assay. The cells were seeded in 96-well plates at a density of 7000 cells per well in 200 μL complete DMEM containing 10% FBS supplemented with 50 U mL^{-1} penicillin and 50 U mL^{-1} streptomycin, and incubated at 37°C in the 5% CO_2 atmosphere for 24 h. Culture media were then removed, followed by addition of PEG-SS-DOX-Cu and free DOX solutions at different DOX concentrations (0~16 μg L^{-1}, DOX). After 48 h of incubation, 10 μL of MTT (5 mg mL^{-1}) solution was added to each well and incubation was continued for another 4 h. The medium was removed and 200 μL of DMSO were added into each well to dissolve the formazan by pipetting up and down for several times. The absorbance of each well was measured using a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., USA) at a test wavelength of 570 nm and a reference wavelength of 630 nm. The cell viability (%) was calculated based on the following equation:

$$\text{Cell viability}(\%) = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100\% \qquad (1)$$

where I_{sample} and $I_{control}$ represent the intensity determined for cells treated with different samples and for control cells (untreated), respectively.

9. Cellular uptake

The cellular uptake behavior of PEG-SS-DOX and PEG-SS-DOX-Cu nanoparticles was observed by inverted fluorescence microscope using HeLa cell lines. The cells were seeded in 6-well plates

at a density of 20000 cells per well in 2 mL of DMEM containing 10% FBS supplemented with 50 U mL^{-1} penicillin and 50 U mL^{-1} streptomycin, and incubated at 37°C in the 5% CO_2 atmosphere for 24 h. The cells for the reduction-response test group were then pretreated with GSH (used for cell culture) for 2 h. After being washed with PBS, the cells were incubated with PEG-SS-DOX, PEG-SS-DOX-Cu and free DOX solutions at a final DOX concentration of 8μg L^{-1} in DMEM at 37°C for an additional 4 h or 8 h. Cells without GSH pretreatment were evaluated for comparison. Then, the culture medium was removed and cells were washed with PBS thrice. After that, the cells were fixed with 4% formaldehyde for 30 min at room temperature, and the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue) for 20 min. Fluorescence images of cells were obtained by using a Leica DM2500 inverted fluorescence microscope (Leica Microsystems Ltd., Germany).

Supplementary Figures

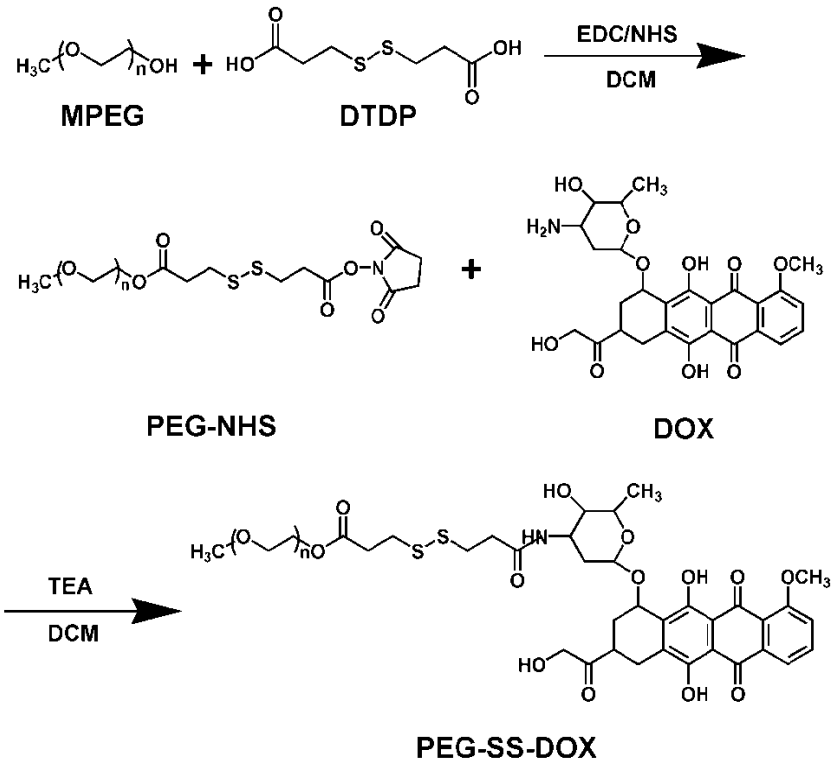

Figure S1. Synthetic route to PEG-SS-DOX

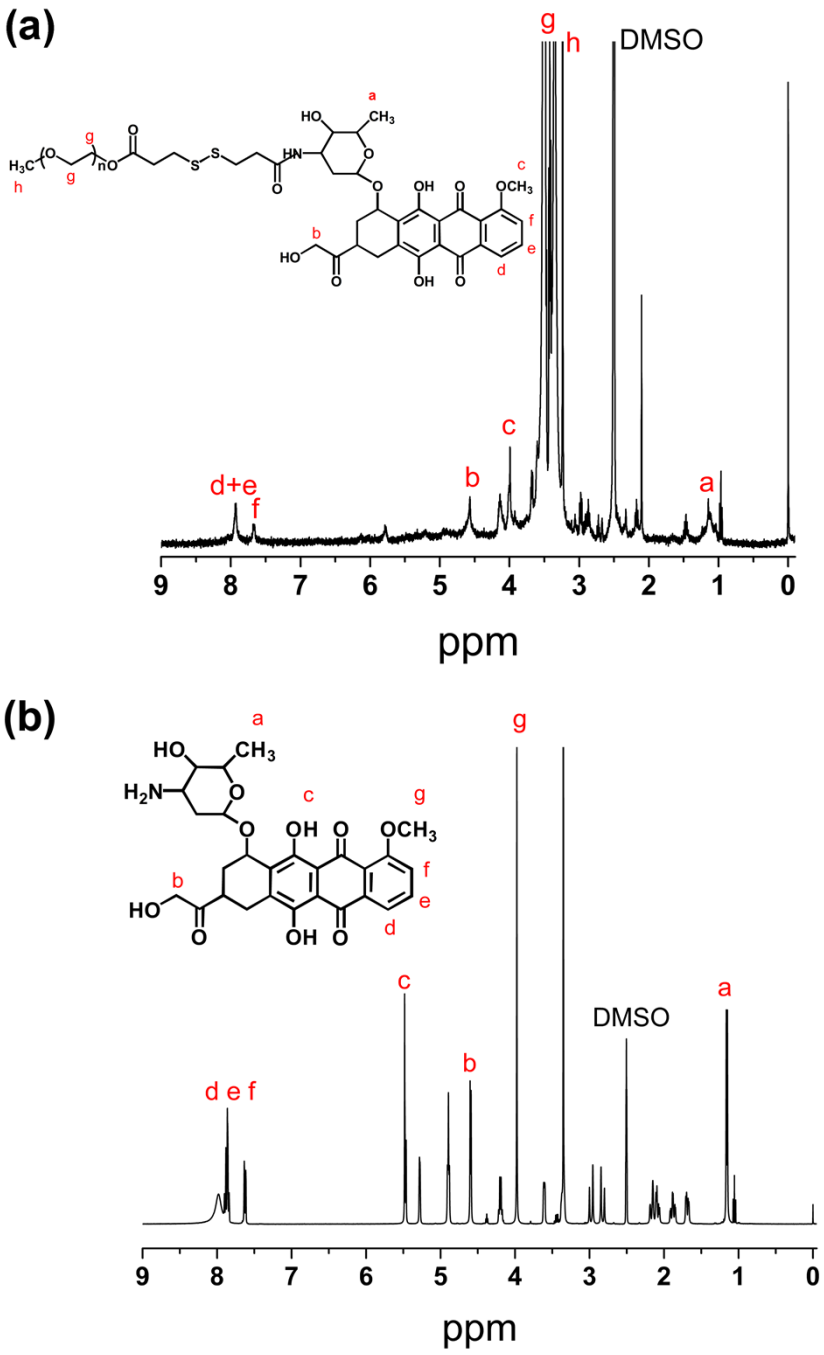

Figure S2. [1]H-NMR spectra of (a) PEG-SS-DOX prodrug and (b) DOX·HCl

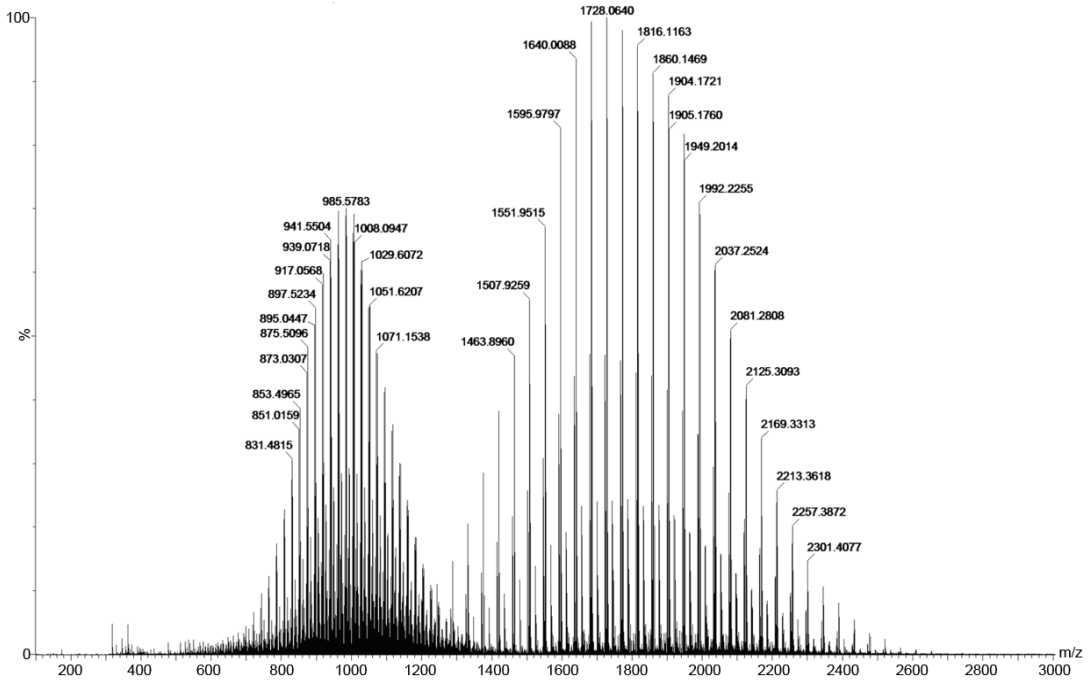

Figure S3. HR ESI-TOF MS spectra of PEG-SS-DOX prodrug

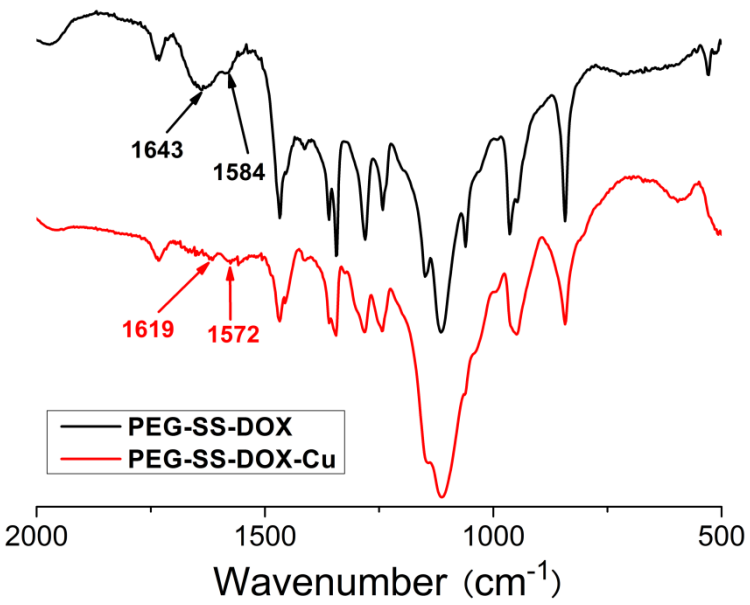

Figure S4. FT-IR spectra of PEG-SS-DOX and PEG-SS-DOX-Cu

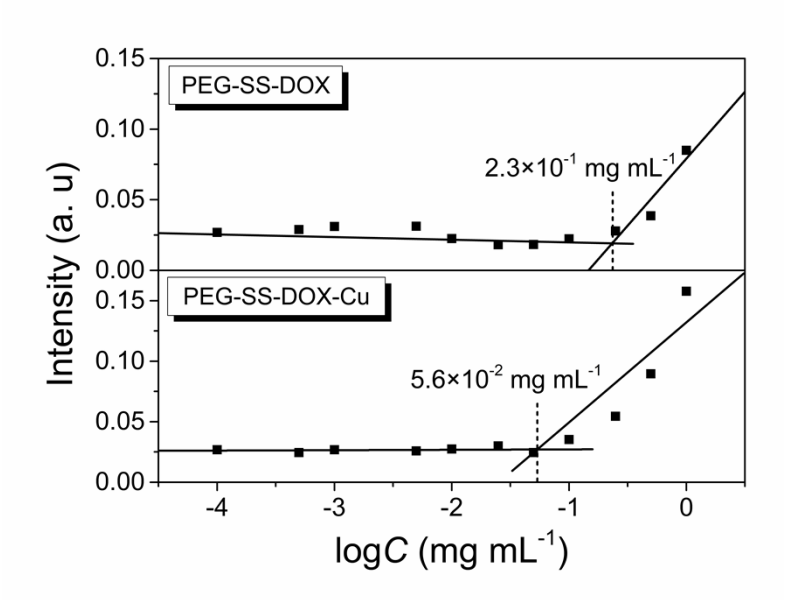

Figure S5. Critical micelle concentrations of (a) PEG-SS-DOX and (b) PEG-SS-DOX-Cu in aqueous solution.

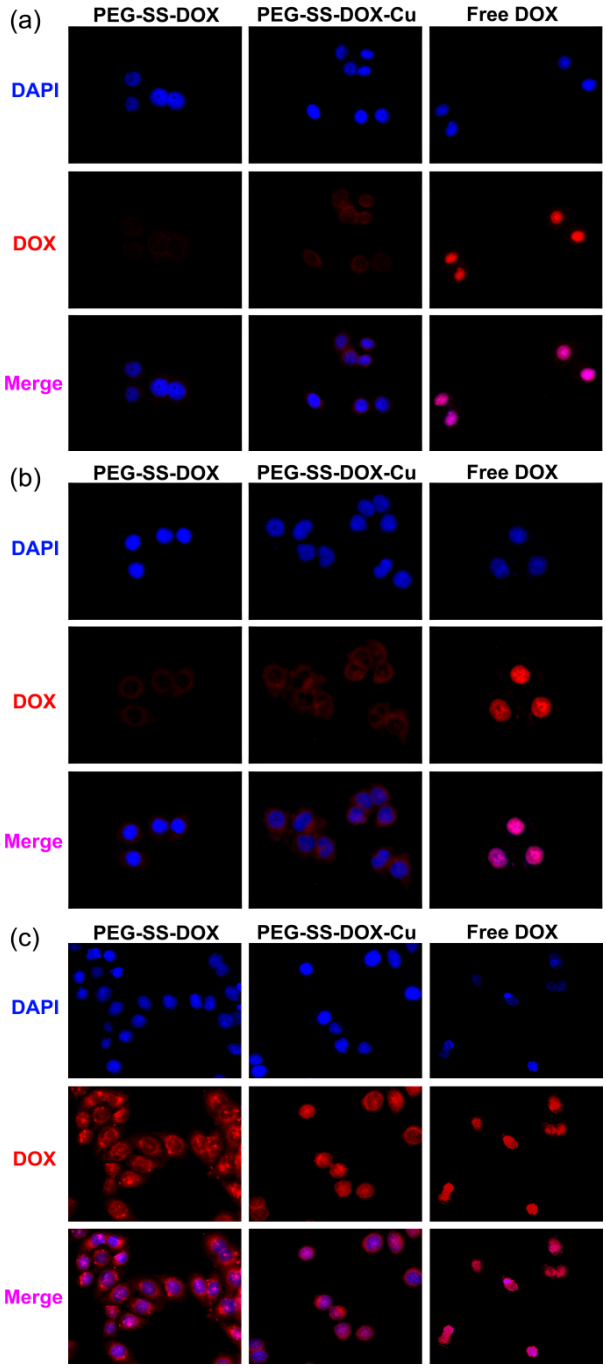

Figure S6. Fluorescence microscopy images of intracellular tracking of free DOX, PEG-SS-DOX and PEG-SS-DOX-Cu nanoparticles after (a) 4 h, (b) 8 h and (c) 24 h incubation. Nuclei were labeled with DAPI. Images were taken from DAPI channel (blue), the DOX channel (red), and their overlapped images.

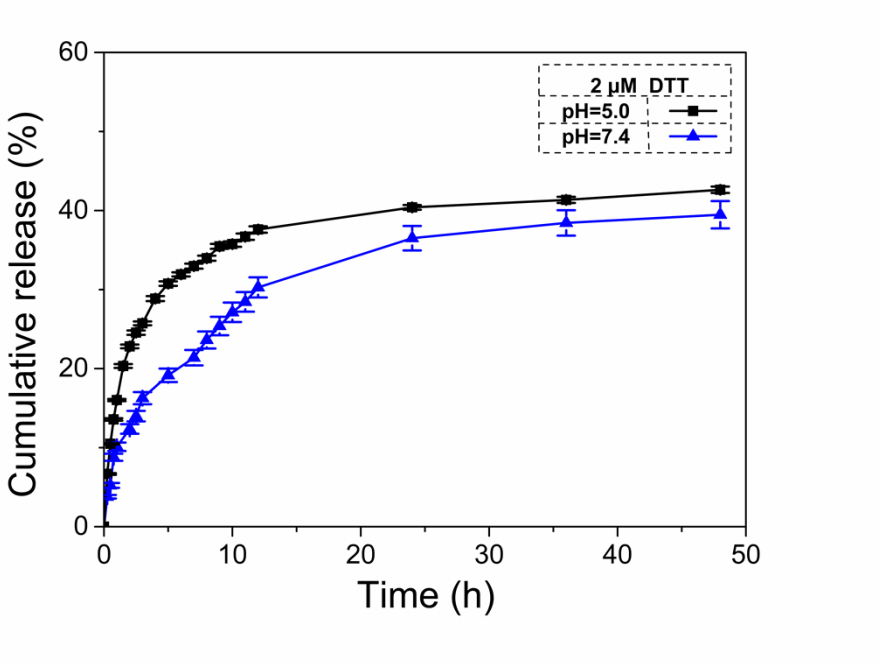

Figure S7. DOX release profiles of PEG-SS-DOX nanoparticles (mean ± standard deviation (SD), $n = 3$).

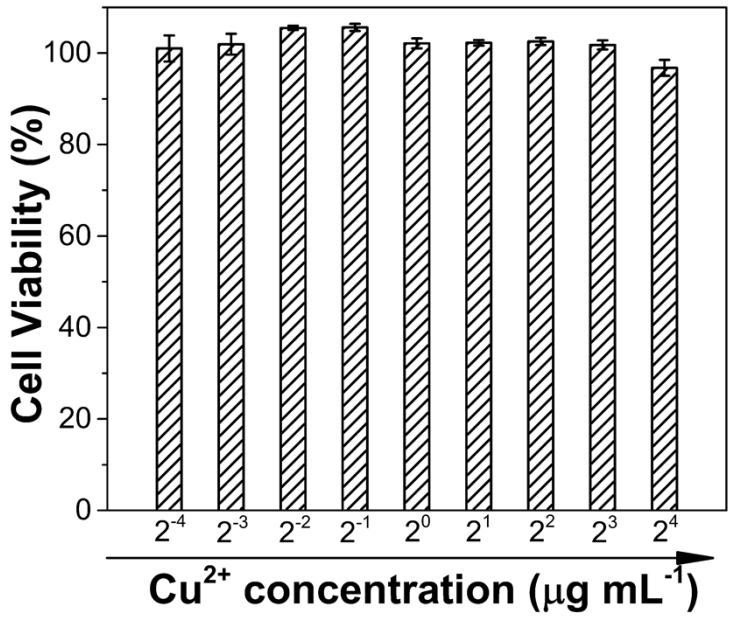

Figure S8. Viability of HeLa cells after incubation with Cu^{2+} for 72 h.

Reference

1 (*a*)B. Tanhaei, N. Saghatoleslami, M. Chenar, A. Ayati, M. Hesampour, M. Mänttäri, *J Surfact Deterg*, 2013, **16**, 357; (*b*)Y. Kim, M. Pourgholami, D. Morris, M. Stenzel, *Biomacromolecules*, 2012, **13**, 814.