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One-pot synthesis of glutathione-responsive amphiphilic drug self-delivery micelle of doxorubicin-disulfide-methoxy polyethylene glycol for tumor therapy

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EXPERIMENTAL

Materials and measurements

Doxorubicin (DOX), 2-hydroxyethyl disulfide and acryloyl chloride were purchased from TCI Shanghai (Shanghai, China). Amino-polyethylene glycol monomethyl ether (M_W=5000) and hexamethylene diacrylate (HDDA) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Glutathione (GSH) was purchased from Sigma Aldrich. Muse Annexin V & Dead Cell Kit (Merck-Millipore) was purchased from Shanghai Haoranbio Company, Inc. CCK-8 and Hoechst Staining Kit were purchased from Beyotime Company. Fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biotechnology Co., Ltd. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Sigma-Aldrich. N, N-Dimethyl formamide (DMF) and tetrahydrofuran (THF) were purchased from J&K Scientific Ltd. (Beijing, China). All other chemicals and reagents were analytically pure and used directly without further purification.

Nuclear Magnetic Resonance (NMR) measurement was carried out on a BrukerAvance 400 spectrometer (BrukerBioSpin, Switzerland) to collect the ^{1}H spectra in $CDCl_3$ or DMSO-d_6. TEM images were obtained with a Tecnai transmission electron microscope (Hillsboro, USA). The average hydrodynamic radius of micelles was measured using a Zetasizer ZEN 3500 dynamic light scattering system (DLS) (Malvern instrument, UK). All DLS measurements were performed with an angle detection of 173° at 25℃. AUV-2450 UV–Vis spectrophotometer (Shimadzu, Japan) was used to determine the drug loading and DOX release rate of DOX-DSDA-PEG and DOX-HDDA-PEG micelles. The molecular weight was measured by Gel Permeation Chromatograph (GPC, Waters 1515, USA) in DMF with flow rate of 1 mL min^{-1}, and the standard was Polystyrene (PS). Cell viability

was detected by M200 Pro NanoQuant (TECAN). The cell uptake experiment was conducted by inverted fluorescence microscope (Olympus IX73). Cell apoptosis was evaluated by Muse Cell Analyzer (Merck & Millipore, Germany). All data were averaged over three measurements. All samples were filtered through 0.45 μm filters to remove dust prior to use.

Synthesis of disulfide-based diacrylate (DSDA)

2,2'-Dithiobis-ethanol (1 g, 6.5 mmol) was dissolved in THF, and acryloyl chloride (1.4 g, 15.6 mmol) was added to the reaction flask drop by drop in an ice-water bath. Then, triethylamine (1.58 g, 15.6 mmol) was added dropwise into the above mixture and the reaction was carried out for six hours. The reaction solution was filtered by Buchner funnel and concentrated to 2 ml. The disulfide-based diacrylate product was purified by silica gel column chromatography to obtain pure monomer. ^{1}H NMR ppm: 3.0 (t, 2H), 4.45 (t, 2H), 5.87, 5.89 (d, 1H), 6.12-6.19 (m, 1H), 6.43, 6.48 (d, 1H).

Preparation of micelles of DOX-DSDA-PEG and DOX-HDDA-PEG

Disulfide-based diacrylate (DSDA, 100 mg, 0.48 mmol) or hexamethylene diacrylate (HDDA, 108.5 mg, 0.48 mmol) and DOX·HCl (267 mg, 0.46 mmol) were added to anhydrous DMF in a flask and allowed to react for 24 h at 80℃. Then, amino-methoxy polyethylene glycol (100 mg) was added to the flask and the reaction was continued for another 24 h at room temperature. The reaction solution was placed into a dialysis bag and dialyzed in DMF solvent for three days (DMF was changed three times), then the dialysis bag was placed in pure water for three days (and pure water was changed three times). The micelle solutions of DOX-DSDA-PEG and DOX-HDDA-PEG

were re-assembled by centrifugation at 12000 rpm, then the supernatants were freeze-dried to yield red powders.

Measurement of critical micelle concentration

The critical micelle concentration (CMC) was determined by fluorescence measurement (excitation wavelength was 335 nm). A calculated volume of pyrene solution in acetone was added to a series of volumetric flasks, and then acetone was removed under reduced pressure. Then, polymer solutions at different concentrations were added to the volumetric flasks, and the pyrene concentration was fixed at 6×10^{-6} mol L^{-1}. All the samples were allowed to stand for one day before fluorescence measurement.

Drug loading and drug release triggered by glutathione

The freeze-dried red powders of DOX-HDDA-PEG and DOX-DSDA-PEG were dissolved in PBS and detected by UV-Vis. According to the standard curve of DOX at 490 nm, the drug loading amounts of DOX-HDDA-PEG and DOX-DSDA-PEG were 22% and 25.5%.

The micelle solutions of DOX-HDDA-PEG and DOX-DSDA-PEG were divided into six separate parts. The six parts of 3 ml micelle solution with pH 7.4 PBS were placed into a dialysis bag (MW=1000), then the dialysis bags were placed into flasks with pH 7.4 PBS & GSH=1 μg mL^{-1} and pH 7.4 PBS & GSH=1 mg mL^{-1}. Then, 3 ml of the dialysis solution outside dialysis bag was removed at fixed intervals, and 3 ml of fresh PBS or PBS with GSH was added to the dialysis solution. The six 3 ml aliquots removed at eight points-in-time were prepared to be measured. The dialysis bags with solution were cut off at 72 h and the six separate solutions were measured by UV-Vis. (the

experiments were carried out twice in parallel).

Cell viability

1) The cell viabilities of DOX, DOX-HDDA-PEG and DOX-DSDA-PEG were evaluated using the Cell Counting Kit (CCK-8) assay. The concentrations of DOX in micelles of DOX-HDDA-PEG and DOX-DSDA-PEG were 1 μg mL^{-1}, 5 μg mL^{-1}, 10 μg mL^{-1}, and 15 μg mL^{-1}, respectively.

The Lung A549 cells were seeded in a 96 well culture plate at a density of 10^4 cells per well and cultured in DMEM medium supplemented with 10% fetal bovine serum at 37℃ in a humidified environment of 5% CO_2 for one day. Thereafter, the cells were incubated with DOX-HDDA-PEG and DOX-DSDA-PEG for 24 h and 48 h, respectively. Then, 10 μL of CCK-8 solution was added to each well and incubated for further 1 h at 37℃. The cell viability was obtained by scanning with a microplate reader at 430 nm. The relative cell viability (%) was expressed as a percentage of the cell viability of pure cell control culture. The experiments were carried out six times in parallel. The results presented are the average data.

2) The cell viabilities of DOX, DOX-HDDA-PEG and DOX-DSDA-PEG were evaluated using the Muse Cell Analyzer. The concentration of DOX in micelles of DOX-HDDA-PEG and DOX-DSDA-PEG was 15 μg mL^{-1}. The cells were incubated with DOX, DOX-HDDA-PEG and DOX-DSDA-PEG for 24 h and 48 h, respectively. The FBS dissolved with DOX, DOX-HDDA-PEG and DOX-DSDA-PEG was removed from 96 well culture plate, then the cells were digested by Trypsin (0.25%)-EDTA (0.02%) solution. The cell suspension was prepared with a mixed solution of digested cells solution and removed FBS. About 100 μL of cells in suspension and Muse™ annexin V & Dead Cell Reagent were added to a tube and incubated for 15 min at room temperature. Then, the incubated mixed solution was

detected by Muse Cell Analyzer.

Cellular uptake assay

Lung A549 cells were seeded with a density of 5×10^4 per dish in 35 mm plastic microscopy dishes and incubated overnight at 37 °C. Then, the A549 cells were treated with DOX, DOX-HDDA-PEG and DOX-DSDA-PEG (15 μg mL^{-1} DOX in micelles) for 4 h and 8 h, respectively. Then, the cells were fixed and stained by Hoechst Staining Kit for 10 min, and then gently rinsed with PBS three times and observed under fluorescence microscope.

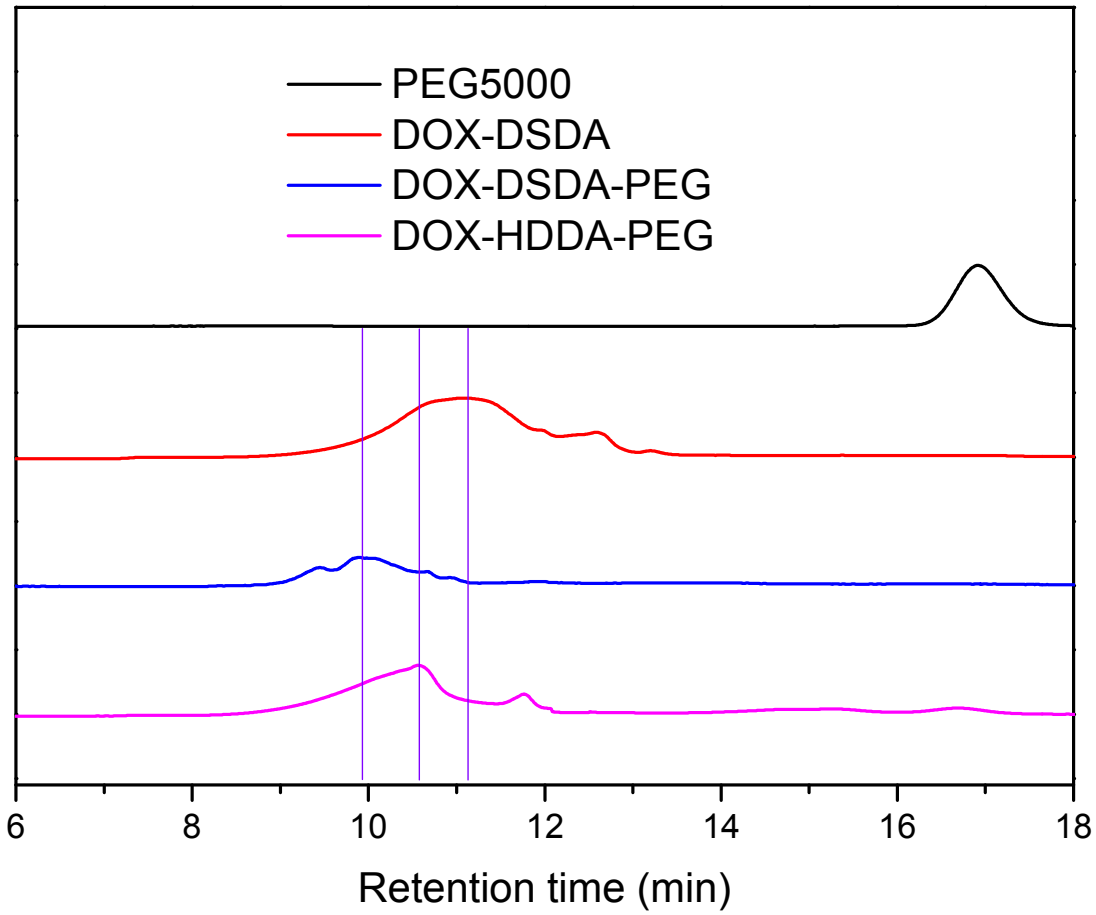

Figure S1. The retention times of PEG, DOX-DSDA, DOX-DSDA-PEG and DOX-HDDA-PEG measured by GPC in DMF (standard reagent: polystyrene).

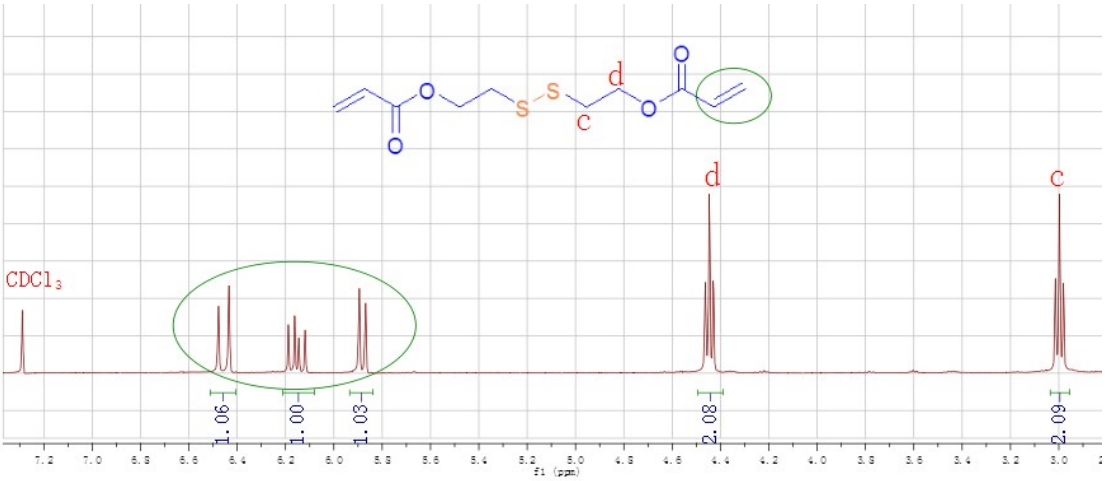

Figure S2. The ^{1}H NMR spectrum of DSDA in $CDCl_3$.

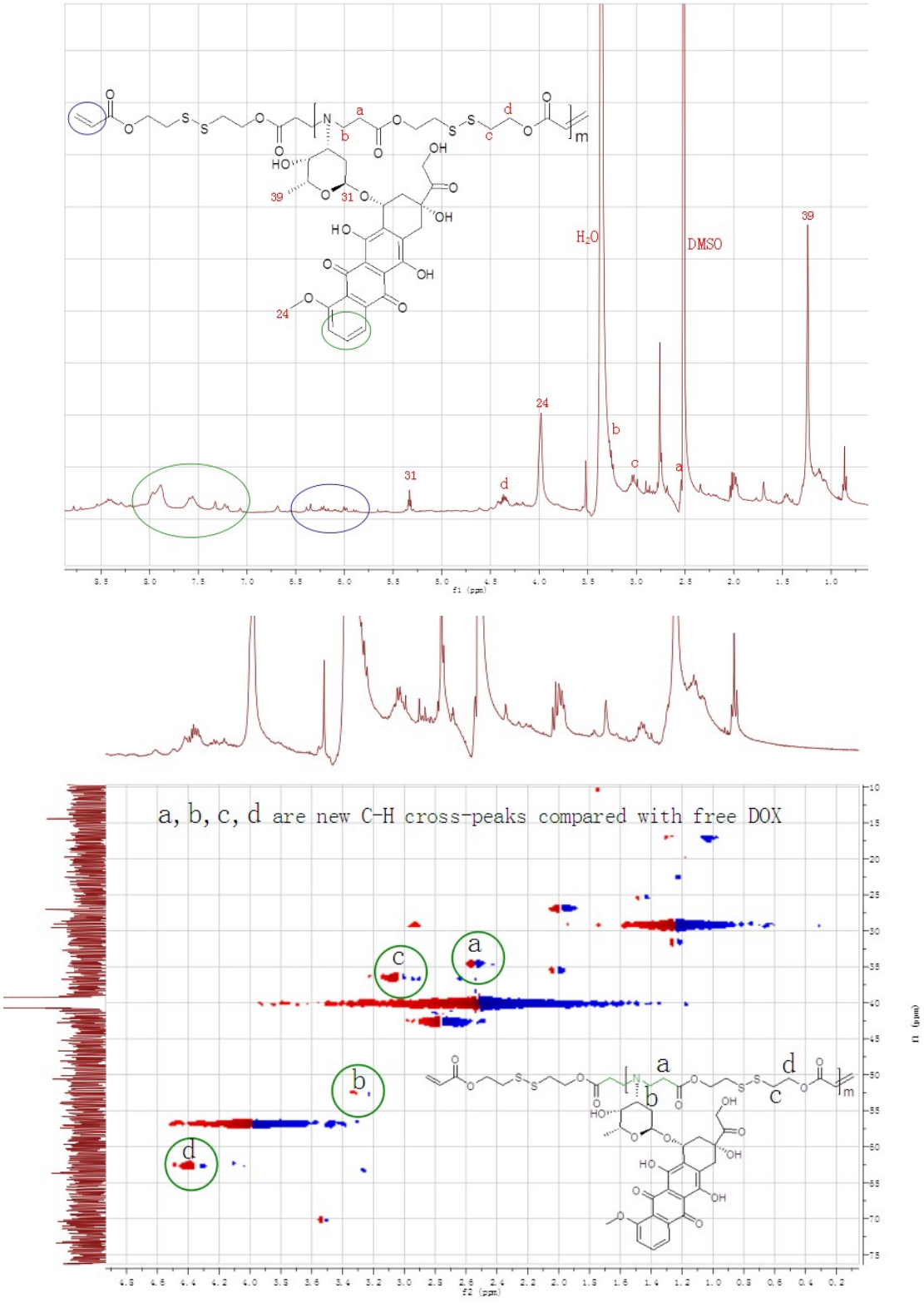

Figure S3. The ^{1}H NMR and C-H COSY spectra of DOX-DSDA in DMSO-d_6.

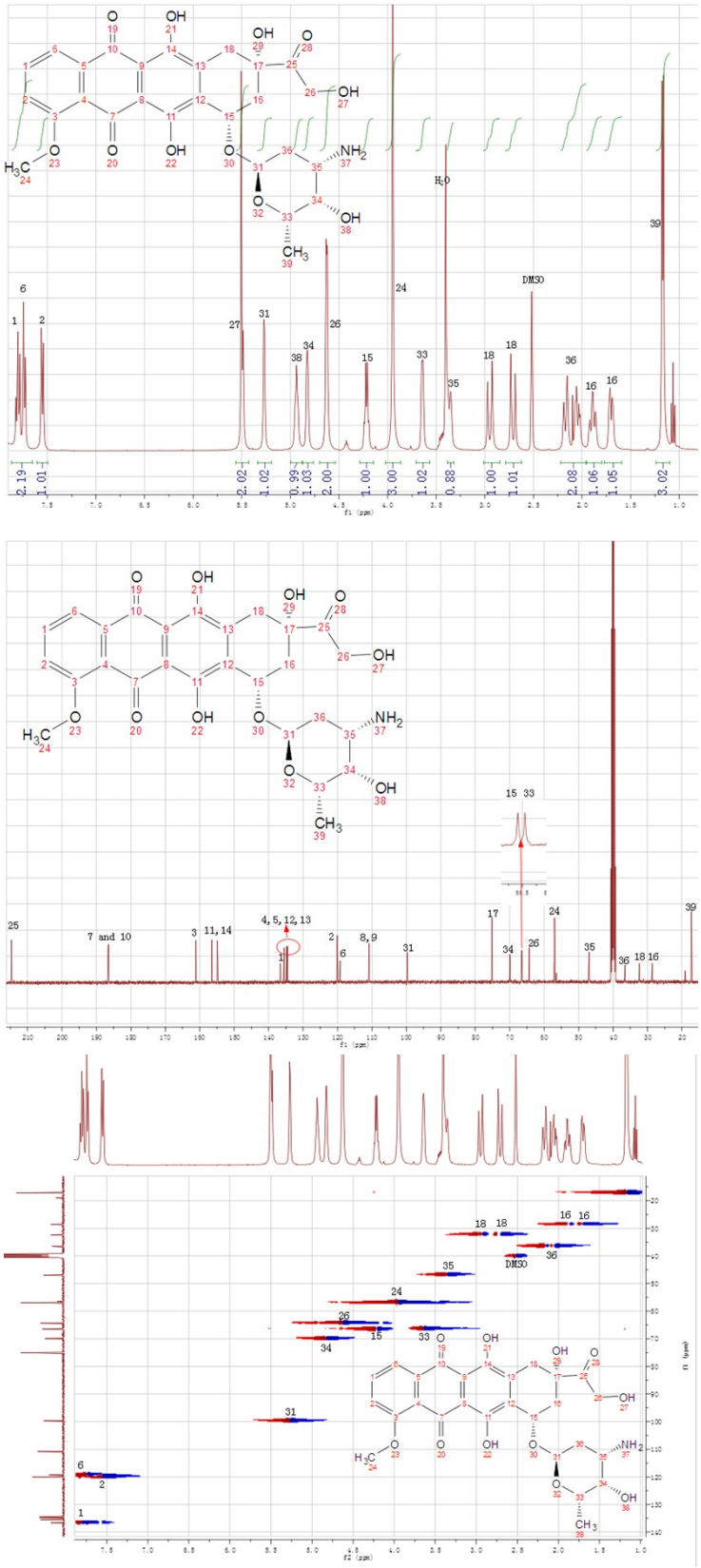

Figure S4. The ^{1}H NMR, ^{13}C NMR and C-H COSY spectra of DOX in DMSO-d_6.

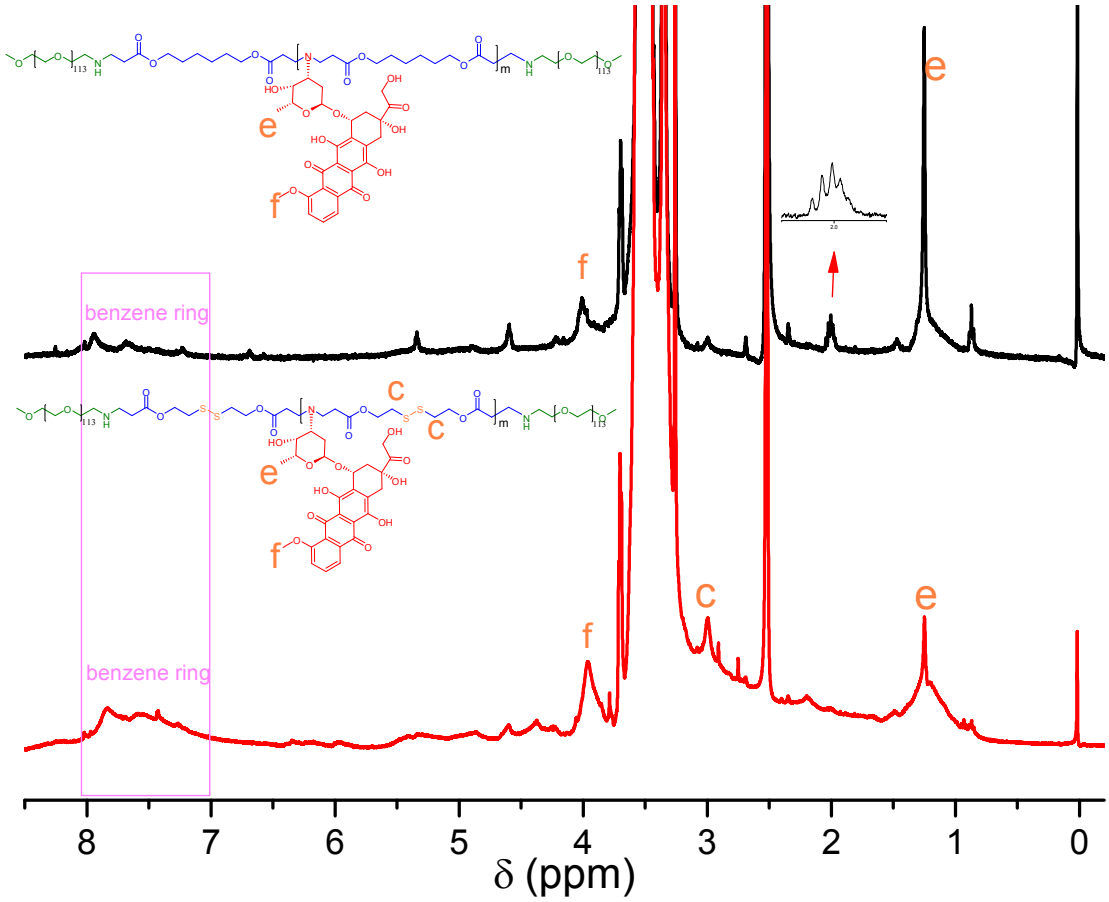

Figure S5. The ^{1}H NMR spectra of DOX-DSDA-PEG (Red) and DOX-HDDA-PEG (Black) in DMSO-d_6.

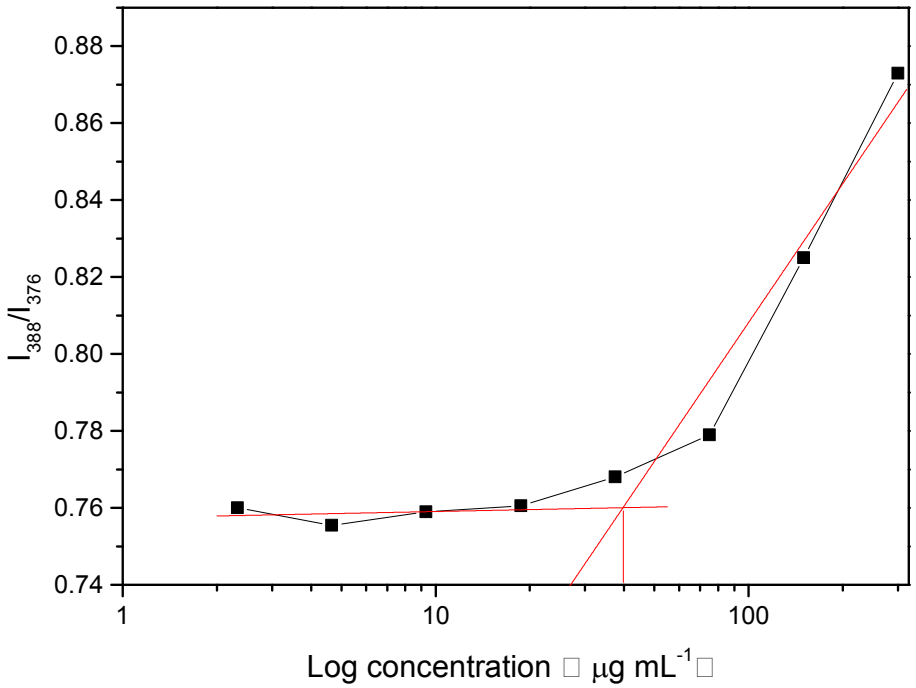

Figure S6. The critical micelle concentration of DOX-DSDA-PEG in water.