A pH-sensitive prodrug micelle self-assembled from multi-doxorubicin-tailed polyethylene glycol for cancer therapy

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Experimental Section

Materials

α-Methoxy-ε-hydroxy-poly(ethylene glycol) (mPEG-OH, M_n = 2 kDa) was converted into α-methoxy-ε-amino poly(ethylene glycol) (mPEG-NH_2) as previously described [see: Nanoscale 2014, 6, 1732.]. The following reagents were of analytic grade and used as received: BOC-Lys(BOC)-OH (Sigma), 4-formylbenzoic acid (Sigma), dicyclohexylcarbodiimide (DCC, Sigma), N-hydroxysuccinimide (NHS, Sigma), 4-(Dimethylamino)pyridine (DMAP, Sigma), trifluoroacetic acid (TFA, J&K Chemical), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, Sigma).

Dialysis bag (MWCO: 1.0 kDa) was purchased from Shanghai Green Bird Technology Development Co., Ltd., China. Doxorubicin hydrochloride (DOX·HCl) was purchased from Dalian Meilun Biotechnology Co., Ltd., China. Triethylamine (TEA) was pre-dried with potassium hydrate and distilled. Chloroform ($CHCl_3$), tetramethylene oxide (THF), and dichloromethane (DCM, CH_2Cl_2) were dried over CaH_2 and then distilled under ambient pressure. All other reagents were of analytical grade and used as received. C6 and MCF7 cells were obtained from the Experimental Animal Center of Zhongshan School of Medicine. MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were purchased from SigmaeAldrich (St Louis, MO, USA).

Synthesis of prodrug polymers (PELD)

Poly(ethylene glycol)-$(Lysine)_2$-$(formylbenzoic\ acid\text{-}doxorubicin)_4$ (PELD) was synthesized according to the literature [see: Macromol. Rapid Commun. 2013, 34, 548] and as shown in Scheme 1.

PEG-LL-BOC_2: NHS (0.69 g, 6 mmol), EDC·HCl (1.146 g, 6 mmol) and mPEG (10 g, 5 mmol) were added to a solution of BOC-Lys(BOC)-OH (2.076 g, 6 mmol) in chloroform (100 mL) . After being stirred for 24 h, the solution was filtered， concentrated, precipitated into a large amount of cool diethyl ether. The obtained solid was filtered, washed with diethyl ether, and dried in vacuum until a constant weight. PEG-LL-BOC_2 was obtained as yellow solid. ^1H NMR ($CDCl_3$): δ (ppm) 4.06 (OC-C***H***-NH_2-), 3.88 (-OC***H₂***CH_2-), 3.65 (-OC***H₂***CH_2-), 3.55 (C***H₃***-O-, -OCH_2C***H₂***-NH- and -CH_2C***H₂***-NH_2), 1.70 (-CH_2C***H₂***CH_2-), 1.41 (BOC).

PEG-LL: 2.3284 g (1.0 mmol) PEG-LL-BOC_2 was treated with 6 mL TFA/DCM

mixture (v/v = 2:1) at room temperature for 2 h. After adjusting to alkaline with TEA, the sultion was precipitated into a large amount of cool diethyl ether, filtered, washed with diethyl ether, and dried in vacuum until a constant weight. PEG-LL was obtained as yellow solid. ^{1}H NMR ($CDCl_3$): δ (ppm) 4.06 (OC-C***H***-NH_2-), 3.88 (-O**C*****H*****$_2$**CH_2-), 3.65 (-O**C*****H*****$_2$**CH_2-), 3.55 (C***H*****$_3$**-O-, -OCH_2C***H*****$_2$**-NH- and -CH_2C***H*****$_2$**-NH_2), 1.70 (-CH_2C***H*****$_2$**CH_2-).

PEG-LL$_2$-BOC$_4$: NHS (0.2762 g, 2.4 mmol), EDC·HCl (0.4584 g, 2.4 mmol) and PEG-LL (2.128 g, 1.0 mmol) were added to a solution of BOC-Lys(BOC)-OH (0.83 g, 2.4 mmol) in chloroform (50 mL). After being stirred for 24 h, the solution was filtered. The filtrate was concentrated, precipitated into a large amount of cool diethyl ether, filtered, washed with diethyl ether, and dried in vacuum for 24 h until a constant weight. PEG-LL$_2$-BOC$_4$ was obtained as yellow solid. ^{1}H NMR ($CDCl_3$): δ (ppm) 4.06 (OC-C***H***-NH_2-), 3.88 (-O**C*****H*****$_2$**CH_2-), 3.65 (-O**C*****H*****$_2$**CH_2-), 3.55 (C***H*****$_3$**-O-, -OCH_2C***H*****$_2$**-NH- and -CH_2C***H*****$_2$**-NH_2), 1.70 (-CH_2C***H*****$_2$**CH_2-), 1.41 (BOC).

PEG-LL$_2$: 2.784 g (1.0 mmol) PEG-LL-BOC_2 was treated with 6 mL TFA/DCM mixture (v/v = 2:1) at room temperature for 2 h. After adjusting to alkaline wit TEA, the sultion was precipitated into a large amount of cool diethyl ether, filtered, washed with diethyl ether, and dried in vacuum for 24 h until a constant weight. PEG-LL$_2$ was obtained as yellow solid. ^{1}H NMR ($CDCl_3$): δ (ppm) 4.06 (OC-C***H***-NH_2-), 3.88 (-O**C*****H*****$_2$**CH_2-), 3.65 (-O**C*****H*****$_2$**CH_2-), 3.55 (C***H*****$_3$**-O-, -OCH_2C***H*****$_2$**-NH- and -CH_2C***H*****$_2$**-NH_2), 1.70 (-CH_2C***H*****$_2$**CH_2-).

PEG-LL$_2$-(CHO)$_4$: DCC (1.0315 g, 5.0 mmol), DMAP (0.0244 g, 0.2 mmol) and 4-

formylbenzoic acid (1.5 g, 10.0 mmol) were added to a solution of PEG-LL_2 (2.384 g, 1.0 mmol) in DCM (30 mL) and DMSO (2 mL). After being stirred for 24 h, the solution was filtered. The filtrate was concentrated, precipitated into a large amount of cool diethyl ether, filtered, washed with diethyl ether, and dried in vacuum until a constant weight. PEG-LL_2-$(CHO)_4$ was obtained as yellow solid. ^{1}H NMR ($CDCl_3$): δ (ppm) 10.10 (-Ph-C***H***O), 8.31 (-CO-***Ph***-), 8.03 (-CO-***Ph***-), 4.06 (OC-C***H***-NH_2-), 3.88 (-OC***H₂***CH_2-), 3.65 (-OC***H₂***CH_2-), 3.55 (C***H₃***-O-, -OCH_2C***H₂***-NH- and -CH_2C***H₂***-NH_2), 1.70 (-CH_2C***H₂***CH_2-).

PEG-LL_2-$(CHO\text{-}DOX)_4$: conjugation of DOX to PEG-LL_2-$(CHO)_4$ was carried out through Schiff's reaction between the amino group of DOX and the benzaldehyde end group of PEG-LL_2-$(CHO)_4$ [see: Langmuir, 2012, 28, 11988-11996]. PEG-LL_2-$(CHO)_4$ (0.2856 g, 0.1 mmol) and an excess amount of DOX (0.2901 g, 0.5 mmol) were dissolved in a mixed solvent of methanol (10 mL) and DMSO (1 mL), and then TEA (70 μL) was added. The solution was protected from light and stirred at 40 °C for 8 h. The resulted dark orange solution was dialyzed (MWCO: 1 kDa) against $NaHCO_3$ solution at weak basic pH for 72 h, and then freeze-dried to achieve PEG-LL_2-$(CHO\text{-}DOX)_4$ as dark red powder. ^{1}H NMR (DMSO-d_6): δ (ppm) 8.28 (-Ph-C***H***=N-), 8.15 (-CO-***Ph***-), 8.00 (-***Ph***-CH-, -***Ph***-), 7.64 (-***Ph***-), 5.40 (-O-C***H***(CH_2)-O-), 4.96 (-O-C***H***(CH_2)-CH-), 4.56 (-OC-C***H₂***-OH), 4.03 (-O-C***H***(CH)-CH_3-), 3.98 (-N-C***H***(CH)-CH_2-), 3.74 (-OC***H₂***CH_2-), 3.65 (-OC***H₂***CH_2-), 3.05 (C***H₃***-O-, -CH-C***H***(CH)-OH-, C***H₃***-O- and -CH_2C***H₂***-NH-), 2.97 (-C-C***H₂***-C-), 2.14 (-CH-C***H₂***-C-), 1.14(-CH-C***H₂***-CH-, CH-C***H₃***).

Characterization

^{1}H NMR spectra were obtained using a Varian Unity 300 MHz spectrometer or a Bruker 400 MHz spectrometer. $CDCl_3$ or DMSO-d_6 was used as the solvent depending on polymer solubility. FTIR spectral studies were carried out using a Nicolet/Nexus 670 FTIR spectrometer in the range between 4000 and 500 cm^{-1} at a resolution of 2 cm^{-1}. All powder samples were compressed into KBr pellets in the FTIR measurements. Transmission electron microscopy (TEM) was performed using a Hitachi model H-7650 TEM operated at 80 kV. The samples were prepared by drying a drop (10 μL, 1 mg/mL) of the sample solution on a copper grid that was coated with amorphous carbon. For the negative staining of samples, a small drop of uranyl acetate solution (1 wt% in water) was added to the copper grid, which was then blotted with a filter paper after 1 min. The grid was finally dried overnight in a desiccator before TEM observation. The hydrodynamic sizes were determined *via* dynamic light scattering (DLS). Measurements were performed at 25 °C using a 90 Plus/BI-MAS equipment (Brookhaven Instruments Corporation, USA). The data was collected on an auto-correlator with a detection angle of scattered light at 90°. For each sample, the data from three measurements were averaged for the mean ± standard deviation (SD).

Preparation of micelle

30.0 mg of PEG-LL_2-$(CHO\text{-}DOX)_4$ was dissolved in 5 mL chloroform, and then the solution was added dropwise to PBS at pH 7.4 (30 mL) under ultrasonic agitation using a Type 60 Sonic Dismem-brator (Fisher Scientiffic) at a power level of 50%.

After chloroform was removed by rotary evaporation, the solution was adjusted to pH 7.4. Afterwards, the solution was filtered with a syringe filter (pore size: 0.45 μm) to eliminate aggregates, concentrated, and washed three times using a MILLIPORE Centrifugal Filter Device (MWCO: 100 000 Da).

Determination of critical micellization concentration (CMC)

The critical micellization concentration (CMC) of PEG-LL_2-$(CHO\text{-}DOX)_4$ micelles in phosphate-citrate buffer (pH 6.5) or PBS (pH 7.4) was measured at 25 °C on a Shimadzu RF-5301PC fluorescence spectrometer (Kyoto, Japan), using pyrene as a fluorescent probe as reported [see: Adv. Mater. 2012, 24, 115]. The excitation wavelength was set to 335 nm, and the fluorescence intensity was detected at 373 and 384 nm. CMC was measured from the onset of a decrease in the intensity ratio of peak at 373 nm to peak at 384 nm plotted versus the logarithm of polymer concentration.

Fluorescence spectra of prodrug micelle at different pH values

The micelle solutions (DOX: 20 μg/mL; 1 mL each) were adjusted to different pH values, and then diluted to 5 mL with an aqueous solution at the same pH. The fluorescence spectra of DOX at different pH values were measured using a PerkinElmer PE-LS55 fluorescence spectrometer (Waltham, MA, USA). An excitation wavelength of 485 nm was used, and the emission spectra were recorded from 500 to 700 nm with a bandwidth of 10 nm.

***In vitro* DOX release from micelle**

Micelle solutions at the same concentration in PBS at pH 5.0 and 7.4 were transferred into a dialysis bag (Mw cut-off: 14,000 Da). The bag was placed into the same buffered solution (50 mL), and the release study was performed at 37 °C in an incubator shaker (ZHWY-200B, Shanghai Zhicheng, China). At certain time intervals, 1 mL of solution outside the dialysis bag was replaced with the same volume of fresh buffer solution for UV-vis analysis. DOX concentration was calculated based on the absorbance intensity of DOX at 485 nm. In the assessment of drug release, the cumulative amount of released drug was calculated, and the percentages of drug released from micelles were plotted against time.

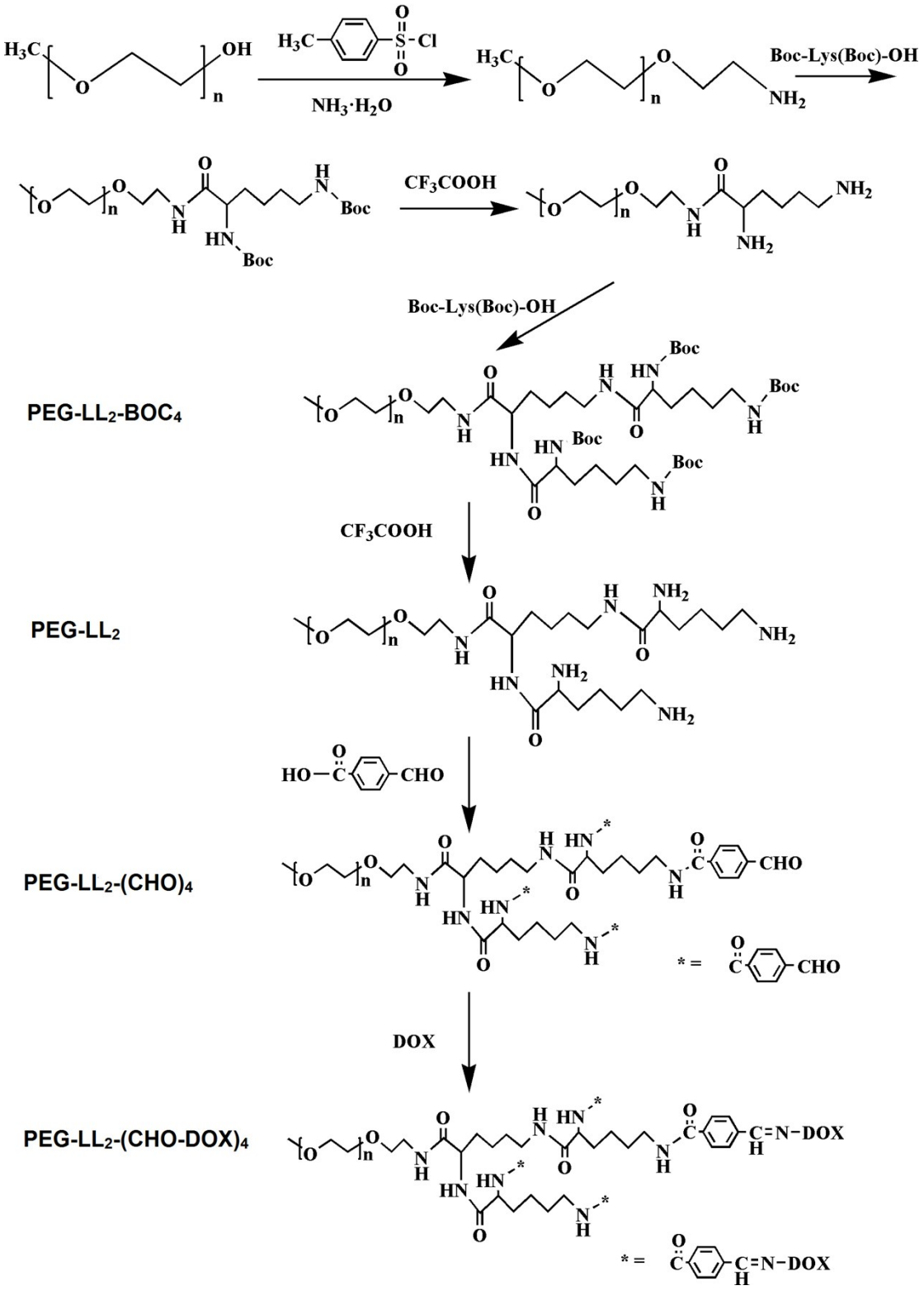
Confocal laser scanning microscopy (CLSM)

The cellular uptake of PELD micelle in C6 and MCF-7 cells were observed on CarlZeiss LSM 710 confocal laser scanning microscope (Oberkochen, Germany). C6 or MCF-7 cells were seeded in 40 mm glass bottom Petri dishes at a density of 1×10^3 cells per dish and incubated overnight at 37 °C in 1 mL of DMEM medium containing 10% fetal bovine serum (FBS) and then incubated with micelle (DOX concentration:10 μg/mL) for different time. The cells were washed twice with PBS, and then stained with DAPI solution (1 μg/mL) for 15 min for CLSM observation. The excitation and emission wavelengths of DOX and DAPI are 488 nm and 590 nm, 352 nm and 455 nm, respectively.

MTT assay

C6 glioma cells and MCF-7 breast cancer cells were cultured in DMEM medium containing 10% FBS and 1% Pen-Strep under a humidified atmosphere of 5% CO_2 at

37 °C. The cells were isolated with 0.25% trypsin-EDTA at the cell confluence of 80% or above and seeded into 96-well plates at a density of 1×10^4 cells per well with fresh medium, then cultured overnight at 37 °C. The cells were incubated with PELD micelle at various concentrations for 48 h before being measured with MTT assay. After the medium in each well was replaced with 100 μL fresh medium containing 10 μL MTT solutions (5 mg/mL in PBS), the cells were incubated for additional 4 h at 37 °C. The medium in each well was then replaced with 100 μL DMSO to dissolve the substrate, and the absorbance at 570 nm was detected using a Tecan Infinite F200 (Tecan, Crailsheim, Germany). The cell viabilities were calculated compared to the absorbance of negative control. All experiments were conducted in triplicate.



Scheme S1. Synthesis of PEG-LL_2-$(CHO\text{-}DOX)_4$.

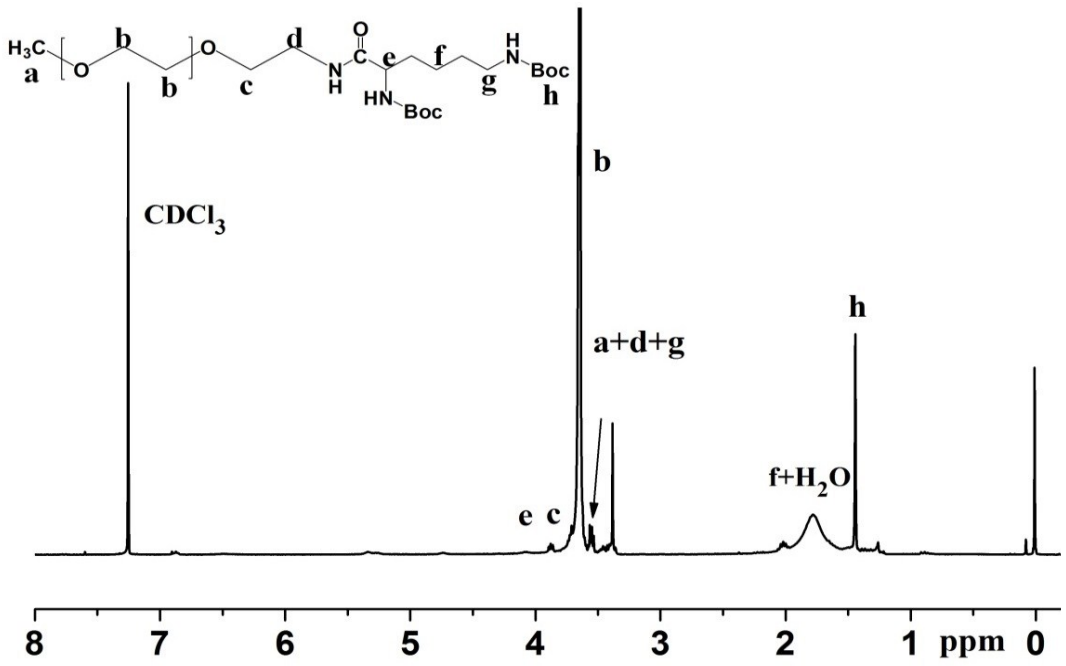


Figure S1. ^{1}H NMR spectrum of PEG-LL-BOC$_2$ (CDCl$_3$, 300MHz).

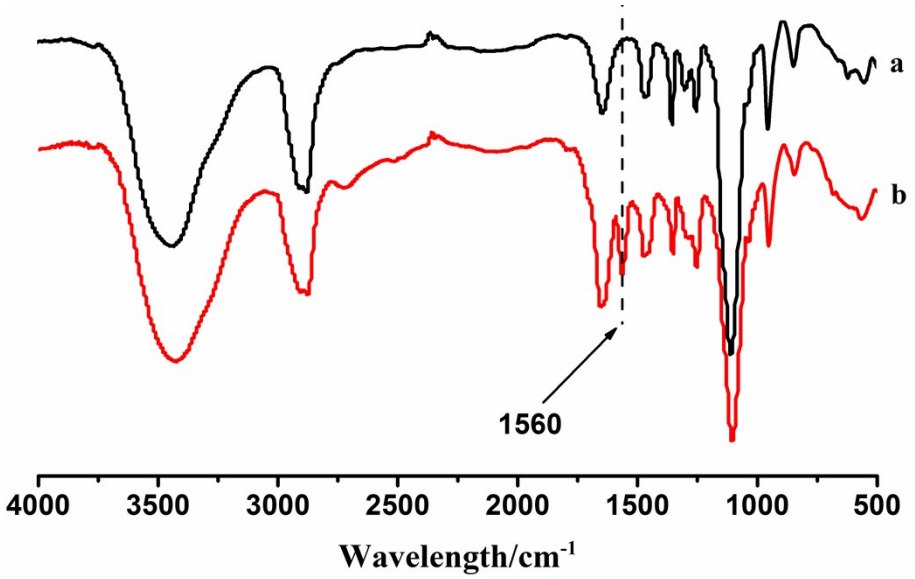


Figure S2. FT-IR spectra of a) PEG-NH$_2$; b) PEG-LL-BOC$_2$.

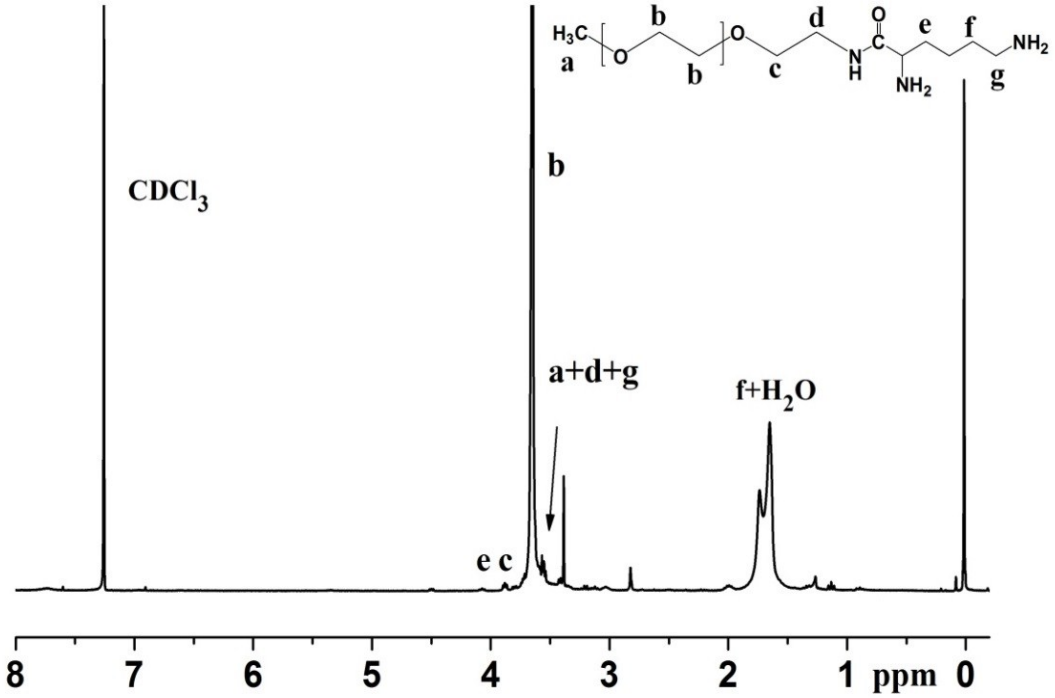


Figure S3. ^{1}H NMR spectrum of PEG-LL ($CDCl_3$, 300MHz).

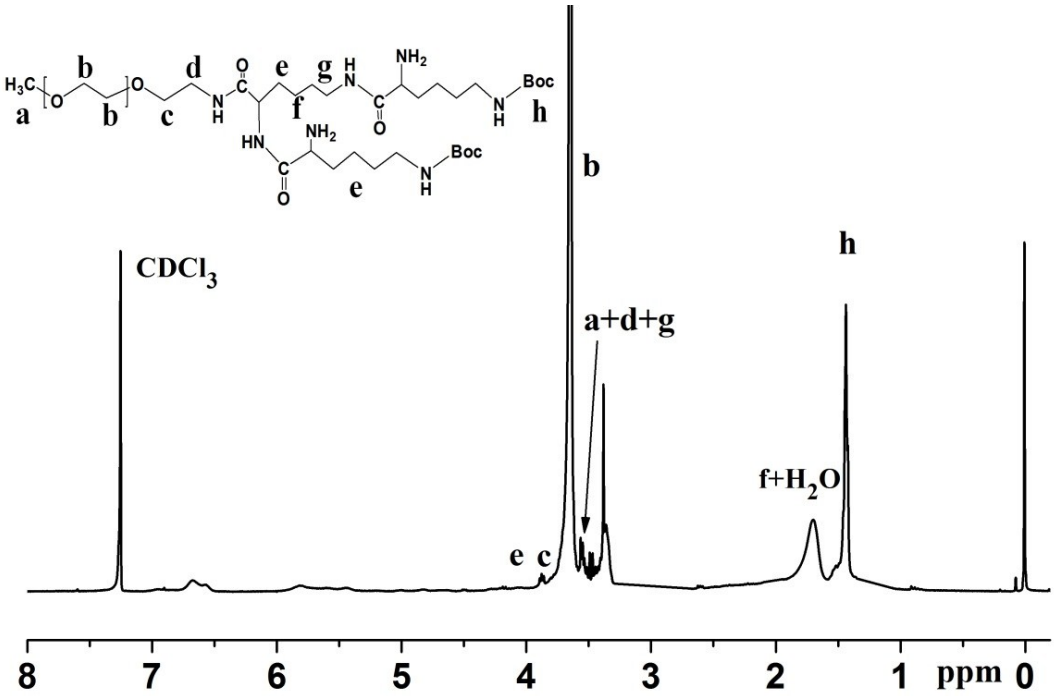


Figure S4. ^{1}H NMR spectrum of PEG-LL_2-BOC_4 ($CDCl_3$, 300MHz).

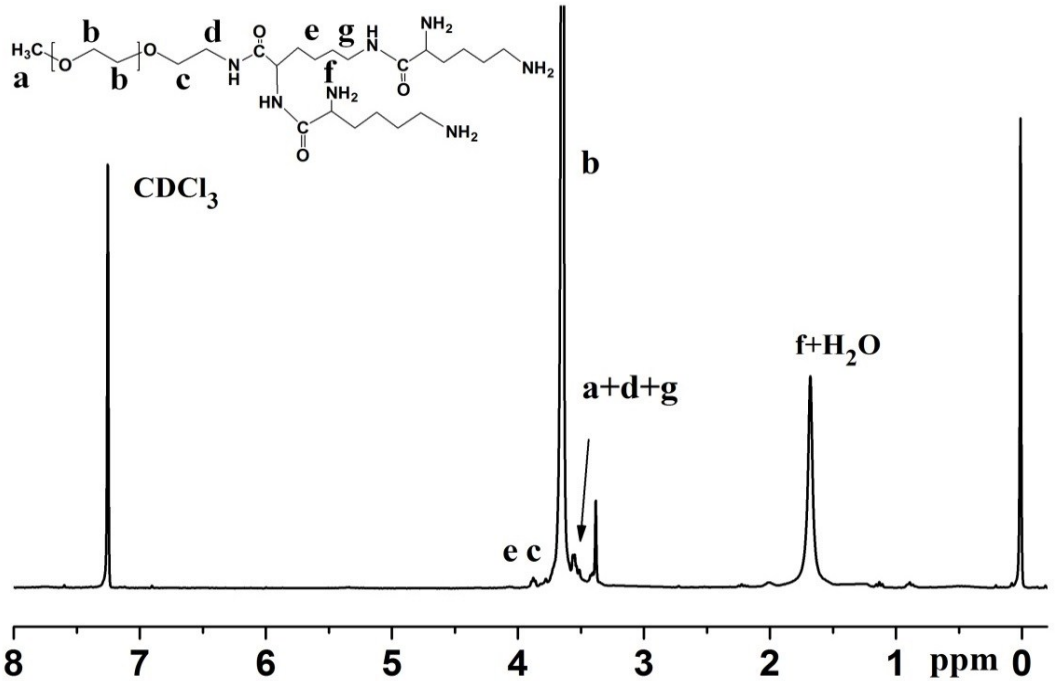


Figure S5. ^{1}H NMR spectrum of PEG-LL_2 ($CDCl_3$, 300MHz).

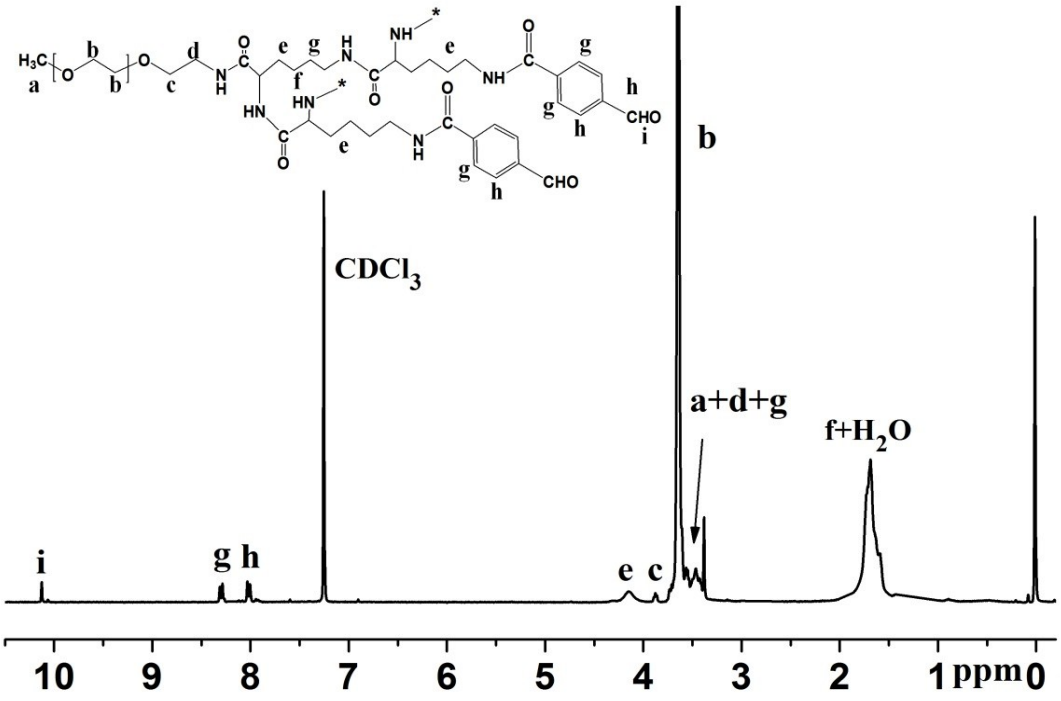


Figure S6. ^{1}H NMR spectrum of PEG-LL_2-CHO_4 ($CDCl_3$, 300MHz).

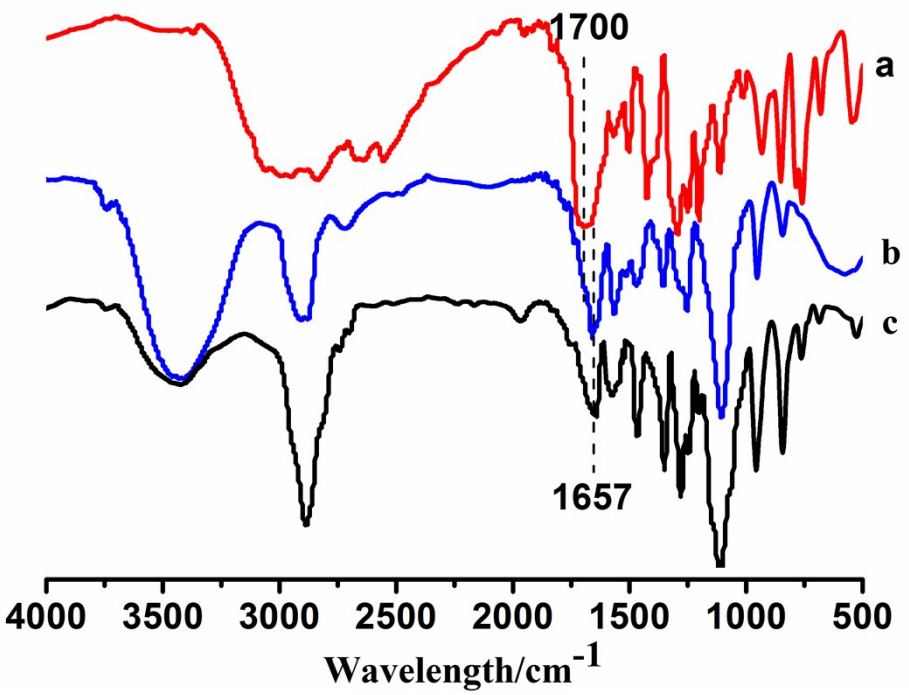


Figure S7. FT-IR spectra of a) 4-Formylbenzoic acid; b) PEG-LL_2; c) PEG-LL_2-CHO_4.

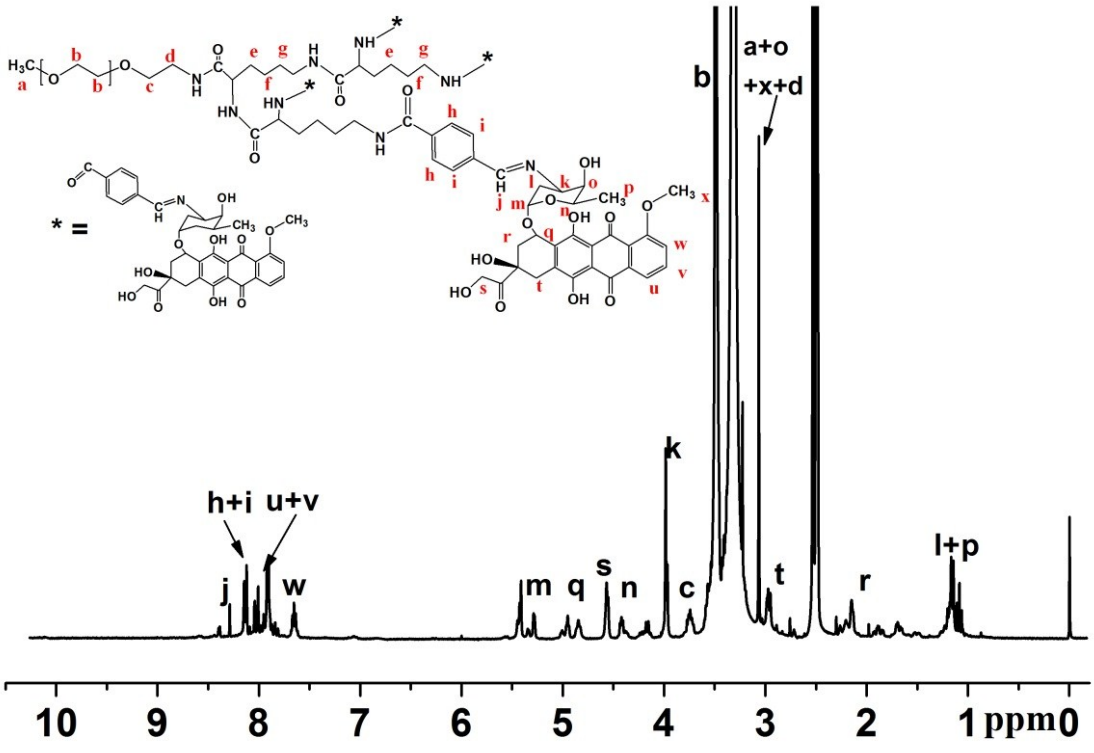


Figure S8. ^{1}H NMR spectrum of PEG-LL_2-(CHO-DOX)$_4$ (DMSO-d_6).

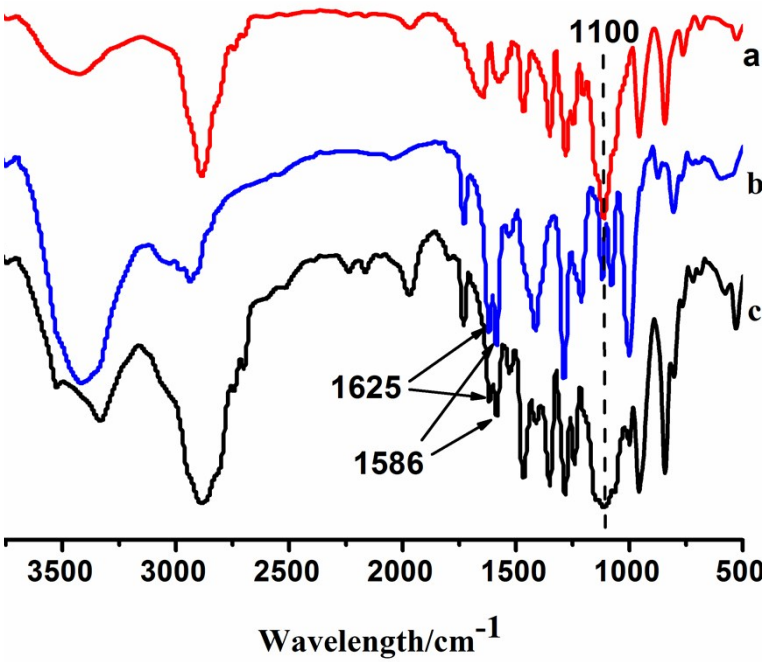


Figure S9. FT-IR spectra of a) PEG-LL_2-$(CHO)_4$; b) DOX; c) PEG-LL_2-$(CHO\text{-}DOX)_4$.

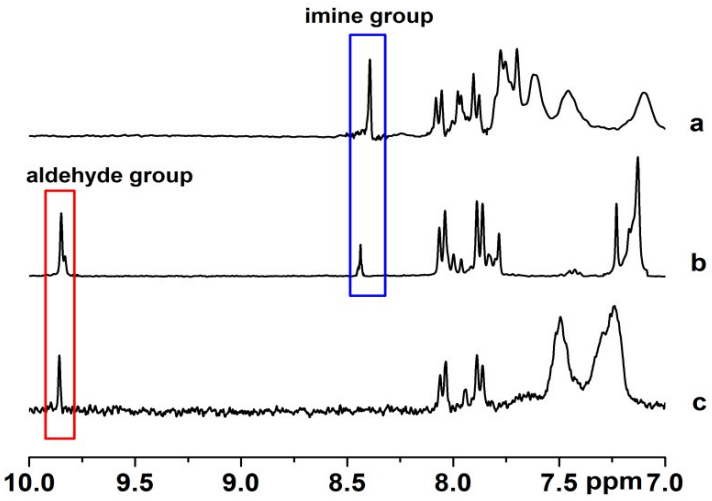


Figure S10. Magnified ^1H NMR spectra of PEG-LL_2-$(CHO\text{-}DOX)_4$ in D_2O with NaOD at pH 7.4 (a), with DCl at pH 6.5 (b) and in D_2O with DCl at pH 5.0 (c).

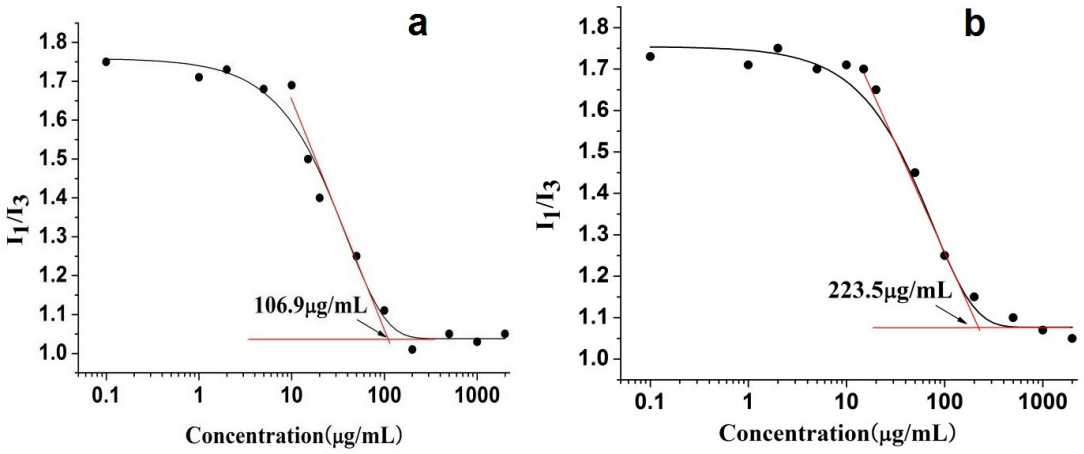


Figure S11. Determination of CMC values of PELD at pH 7.4 and 6.5, respectively.