Supporting Information

PDEAEMA-based pH-responsive copolymers mixed micelles for targeting anticancer drug control release

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1. Materials

Poly(ethylene glycol) methyl ether (mPEG, M_n=5000 g/mol, Sigma-Aldrich). The inhibitor in the methyl methacrylate (MMA, Sigma-Aldrich, 99%), 2-(diethylamino) ethyl methacrylate (DEAEMA, Sigma-Aldrich, 99%) and poly(ethylene glycol) methyl ether methacrylate (PEGMA, M_n = 475 Da, Sigma-Aldrich, 99%) were removed by passing through a neutral alumina filled column, and then distilled and stored at -20 ^oC. Triethylamin (TEA), tetrahydrofuran (THF), dichloromethane (DCM) and toluene were dried with calcium hydride (CaH_2), and then distilled before use. Hexane, pyrene (Sigma-Aldrich, 99%), 2-bromoisobutyryl bromide (98%, Alfa Aesar), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA, Sigma-Aldrich,

99%), $CuBr_2$, stannous octoate ($Sn(Oct)_2$), dimethyl sulfoxide (DMSO), acetone, magnesium sulfate, ethyl 2-bromobutyrate (Sigma-Aldrich, 99%), 3-(4,5-Dimethyltlliazol-2-yl)-2,5-diphenyltetrazoxium bromide (MTT, Sigma-Aldrich), doxorubicin hydrochloride (DOX·HCl, Wuhan Yuancheng Gongchuang Technology Co., Ltd.) and all other reagents were used directly. fetal Bovine serum (FBS), dulbecco's modified eagle medium (DMEM), penicillin and streptomycin were supplied by Invitrogen. HepG2 cells were achieved from the American Type Culture Collection (ATCC) and cultured according to the supplier's recommended conditions.

2. Instruments and test methods

^1H NMR spectra were conducted on a Bruker AVANCE III 400 and $CDCl_3$ was used as solvent. The M_n and M_w/M_n were carried out on GPC, tetrahydrofuran (THF) was used as the eluent with a flow rate 1.0 mL/min, monodispersed polystyrene standards were selected to obtain the calibration curve. The dynamic light scattering (DLS，Malvern Zetasizer Nano S) was used to obtain size, zeta potential and polydispersity index data, the micelle solutions were filtered through a 0.45-μm cellulose membrane before analysis. Transmission electron microscopy (TEM) observation was conducted on a JEOL JEM-2100F Transmission Electron Microscope. Confocal Laser Scanning Microscopy (CLSM，Leica, TCS SP8) was used to obtain the cellular uptake images. Fluorescence spectra were measured by a fluorescence spectrophotometer (F-4500, Hitachi).

3. Synthesis of the copolymers

(1) Synthesis of triblock copolymers. The triblock copolymer mPEG-*b*-PDEAEMA-*b*-PMMA were synthesized by brominated mPEG as initiator,

followed by continuous activators regenerated by ARGET ATRP of DEAEMA and MMA, the synthesis routes are shown in **Scheme S1** and **Scheme S2**, and the typical procedures were as follows: mPEG and DMAP added to a dry 250 mL round bottom flask with a magnetic stir bar inside was placed in an oil bath at 80^{o}C under argon, maintained for 2 h and then allowed to cool to room temperature, DCM was added to dissolve the polymer and then an excess amount of TEA was added. Then the mixture was cooled and maintained at 0 ^{o}C in the ice bath, followed by the addition of 2-bromoisobutyryl bromide in a dropwise manner in 1 h. After completion of the addition, the reaction mixture was allowed to warm up to room temperature and continuous stirring was applied for 24 h. After all the residue of quaternary ammonium salt was removed by filtering and then the solution was concentrated, following by precipitation in cold ether to obtain the macroinitiator mPEG-Br, which was dried under vacuum at room temperature for 24 h. To a flamed-dried 50mL Schlenk flask with a magnetic stirring bar, $CuBr_2$ and the macroinitiator mPEG-Br were added, and the flask was evacuated and flushed with argon for thrice. Anhydrous toluene, DEAEMA and ligand HMTETA were introduced into the flask with degassed syringes in turn. The mixture was stirred for 10 min and a required amount of $Sn(Oct)_2$ solution in toluene was added into the flask by syringe. The flask was placed in a preheated oil bath maintained at 70 ^{o}C for 7 h, then the second monomer MMA was added by syringe to continue the polymerization for another 8 h. Then the flask was removed from oil bath and cooled to room temperature. The mixture was solved in THF and was purified by passing through a neutral alumina column to remove the catalyst. After rotary evaporation, the copolymers were recovered by being precipitated into 10-fold excess of *n*-hexane, filtered, and dried under vacuum for 24 h.

(2) Synthesis of diblock copolymers. The diblock copolymer PDEAEMA-*b*-PMMA and PPEGMA-*b*-PDEAEMA were synthesized with ethyl 2-bromobutyrate as initiator and ARGET ATRP of DEAEMA and MMA or PEGMA, the synthesis routes are shown in **Scheme S3** and **Scheme S4**. Typically, $CuBr_2$ were placed into a dry 1 schlenk flask with a magnetic stirring bar. And the flask was then evacuated and flushed with argon for thrice. Anhydrous toluene, DEAEMA and ligand HMTETA and initiator ethyl 2-bromobutyrate were added into the flask using degassed syringes in turn. The mixture was stirred for 10 min and a required amount of $Sn(Oct)_2$ solution in toluene was added into the flask by syringe. The flask was placed in a preheated oil bath maintained at 70 ^{o}C. After 7 h, the second monomer MMA or PEGMA was then introduced by syringe to continue the polymerization for another 8 h. Then the flask was removed from the oil bath and cooled to room temperature. THF (50 mL) was added into the flask and the mixture was then passed through a neutral alumina column to remove the catalyst. After removing the catalyst, the product was recovered by being precipitated into 10-fold excess of *n*-hexane, filtered, and finally dried under vacuum for 24 h.

4. Acid-base titration

In briefly, polymer A, polymer A/B and polymer A/C were dissolved in deionized water at concentration of 1 mg/mL, and the pH was adjusted to about 3 by HCl (0.1mol/L). The solution was titrated by NaOH (0.1 mol/L) aqueous solution at an increment of 100 μL. It was finished when the pH exceeded 11. The pH increase of the solution was monitored with an automatic titration titrator (Hanon T-860, Jinan,

China) at room temperature. The pK_b region of the polymers was calculated from the derivative values of the titration curves, responding to the inflection region.

5. Determination of LC and EE

For the determination of the drug loading content (LC) and entrapment efficiency (EE), a calibration curve (**Fig. S3**) was obtained with DOX-DMSO solutions with different DOX concentrations by UV-VIS at 480 nm. Firstly. Then, 1 mg DOX-loaded micelles were dissolved in 10 mL of DMSO under vigorous vortex and were analyzed by UV-VIS spectrophotometer (UV-2450, Shimadzu, Japan) at 480 nm. Finally, the LC and EE were calculated using the following Equation (1) and (2), respectively. All samples were analyzed in triplicates.

$$\mathrm{LC}(\%) = \frac{\text{Weight of loaded drug}}{\text{Weight of drug loaded micelle}} \times 100\% \quad (1)$$

$$\mathrm{EE}(\%) = \frac{\text{Weight of loaded drug}}{\text{Weight of drug in feed}} \times 100\% \quad (2)$$

6. *In vitro* DOX release

Specifically, 5 mL of micelle formulations were transferred to dialysis tubing (MWCO:3.5 kDa) and subjected to dialysis against 35 mL of PBS. The sink conditions were maintained under gentle shaking at 120 rpm and 37 ºC. At predetermined intervals, 4 mL aliquot of the test solution was withdrawn periodically and replaced with an equal volume of the fresh release medium. The drug concentration in the removed solution was detected by measuring the absorbance at 480 nm in a UV-vis spectrometer. All the drug release tests were performed in triplicate. The cumulative drug release percent (E_r) was calculated as following:

$$E_r(\%) = \frac{V_e \sum_{1}^{n-1} C_i + V_0 C_n}{m_{DOX}} \times 100\% \quad (3)$$

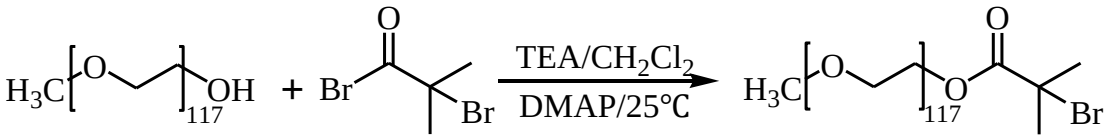
Where m_{DOX} represents the amount of DOX in the micelle, V_0 is the volume of the solution in the beaker (V_0 = 44 mL), and C_i represents the concentration of DOX in the i_{th} sample.

7. MTT assay

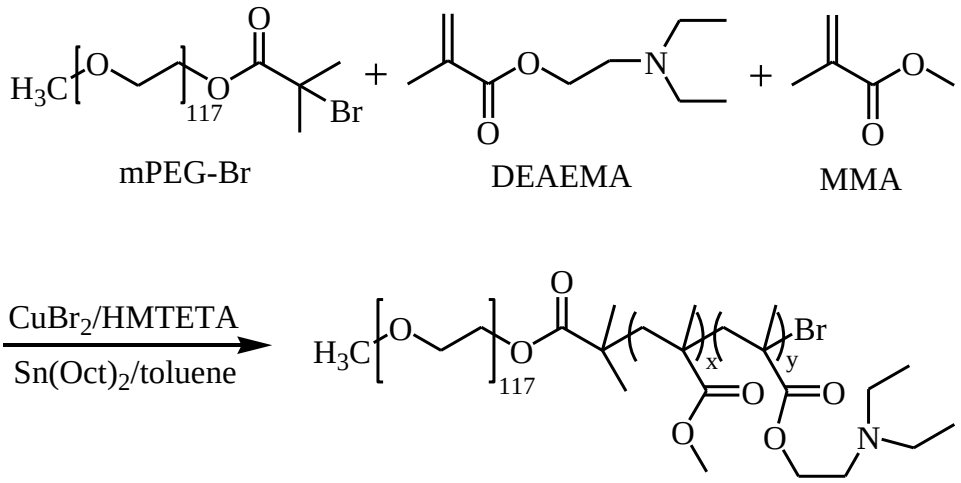
The HepG2 cells were incubated in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 µg/mL) without shaking at 37 ºC for 3 days in a CO_2 (5%) incubator for the attachment of the cells. After that, the cells were incubated with free DOX and micelle DMEM solutions with various concentrations as culture medium. An equivalent volume DMEM only medium cultured cells were treated as control. All the cytotoxicity assays were performed in sextuplicate. The cells were then incubated for 24 h and 48 h before MTT solution (20 µL 5 mg/mL in sterile-filtered PBS and 180 µL DMEM). After incubation for another 4 h, the medium was aspirated and the precipitated formazan was extracted with 200 µL of DMSO. The optical density (OD) value of the solution was measured by using microplate reader (Multiskan Spectrum, Thermo Scientific, Finland) at 570 nm. The relative cell viability compared to the control cell culture was calculated using Equation (4)

$$\text{Cell viability} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100\% \quad (4)$$

where $A_{control}$ and A_{sample} are the absorbance at 570 nm in the absence and in the presence of free DOX and the micelles, respectively. A_{blank} is the absorbance at 570 nm without both cells and samples.

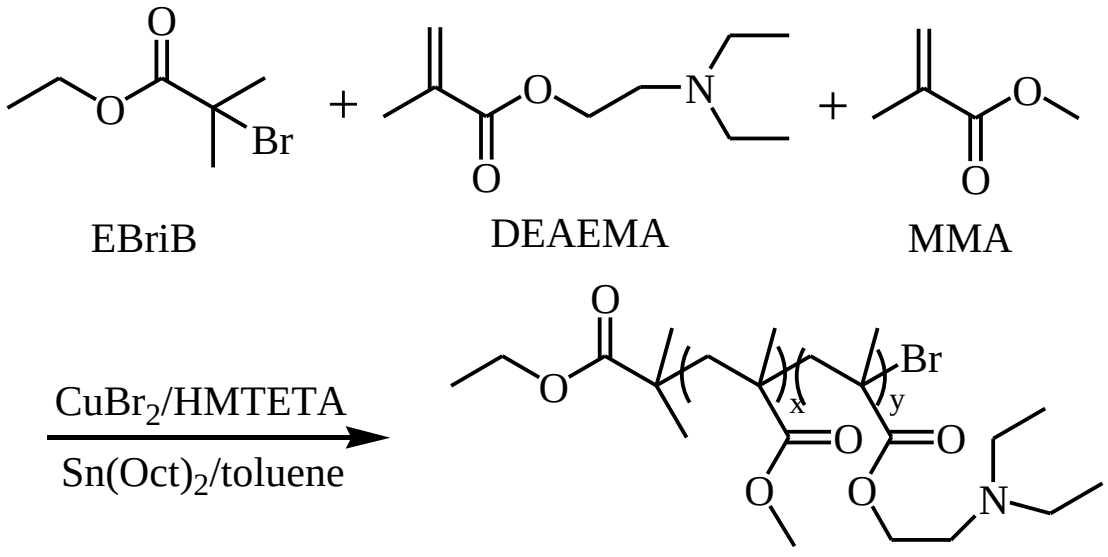


Scheme S1. Synthesis route of mPEG-Br



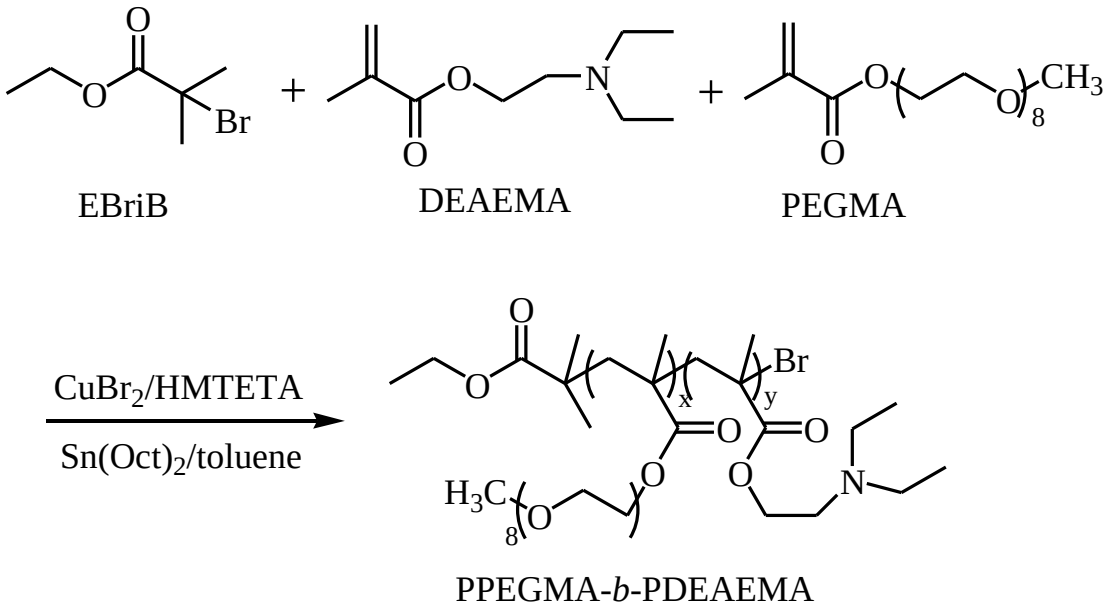
mPEG-*b*-PDEAEMA-*b*-PMMA

Scheme S2. Synthesis route of mPEG-*b*-PDEAEMA-*b*-PMMA



PDEAEMA-*b*-PMMA

Scheme S3. Synthesis route of PDEAEMA-*b*-PMMA



Scheme S4. Synthesis route of PPEGMA-*b*-PDEAEMA

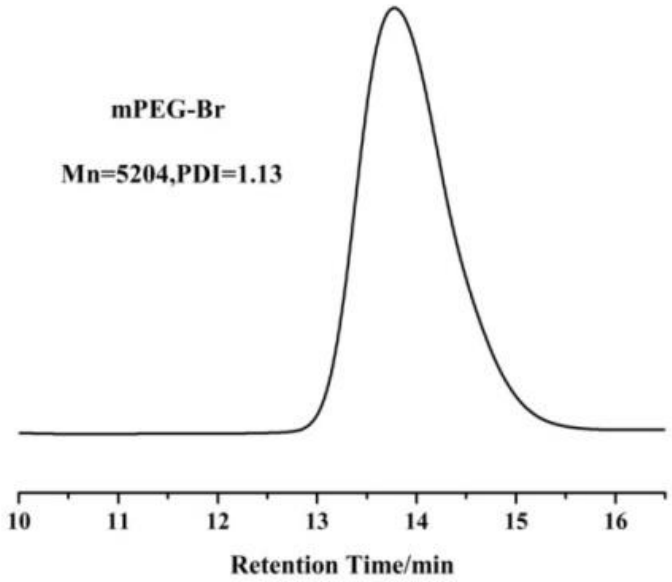


Fig. S1. GPC trace of mPEG-Br

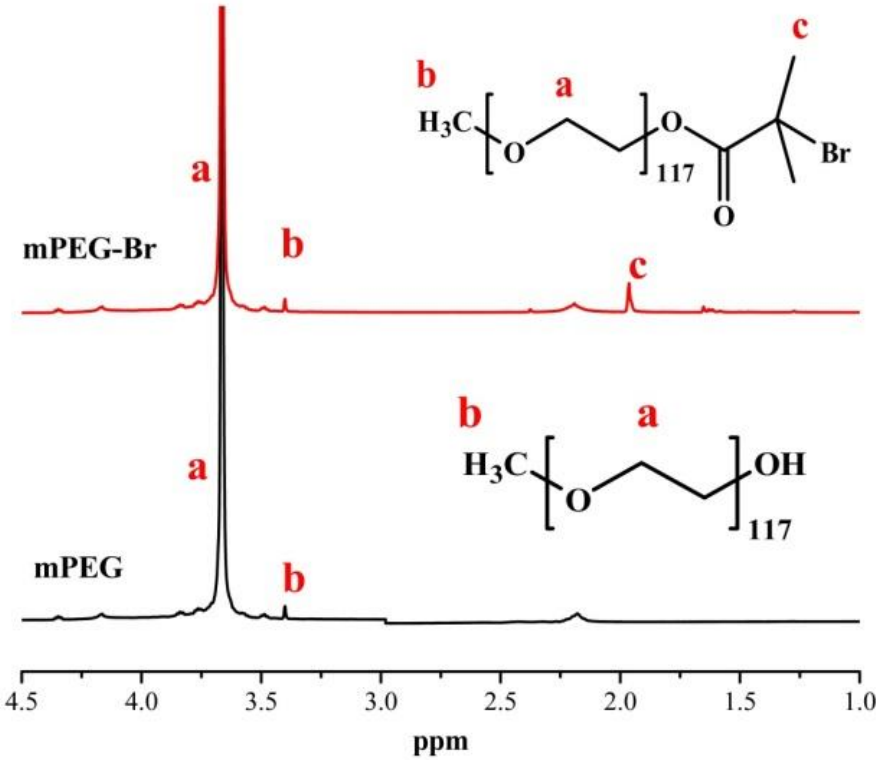


Fig. S2. ^{1}H NMR spectra of the mPEG and mPEG-Br

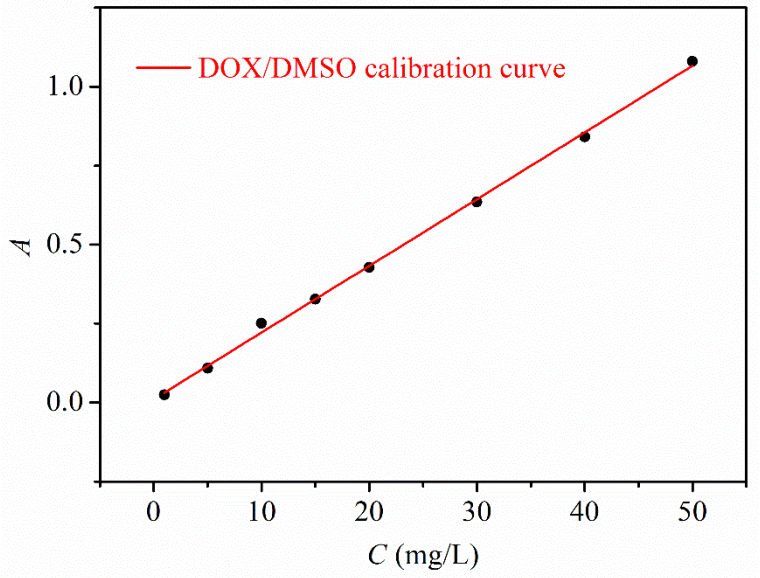


Fig. S3. DOX/DMSO calibration curve

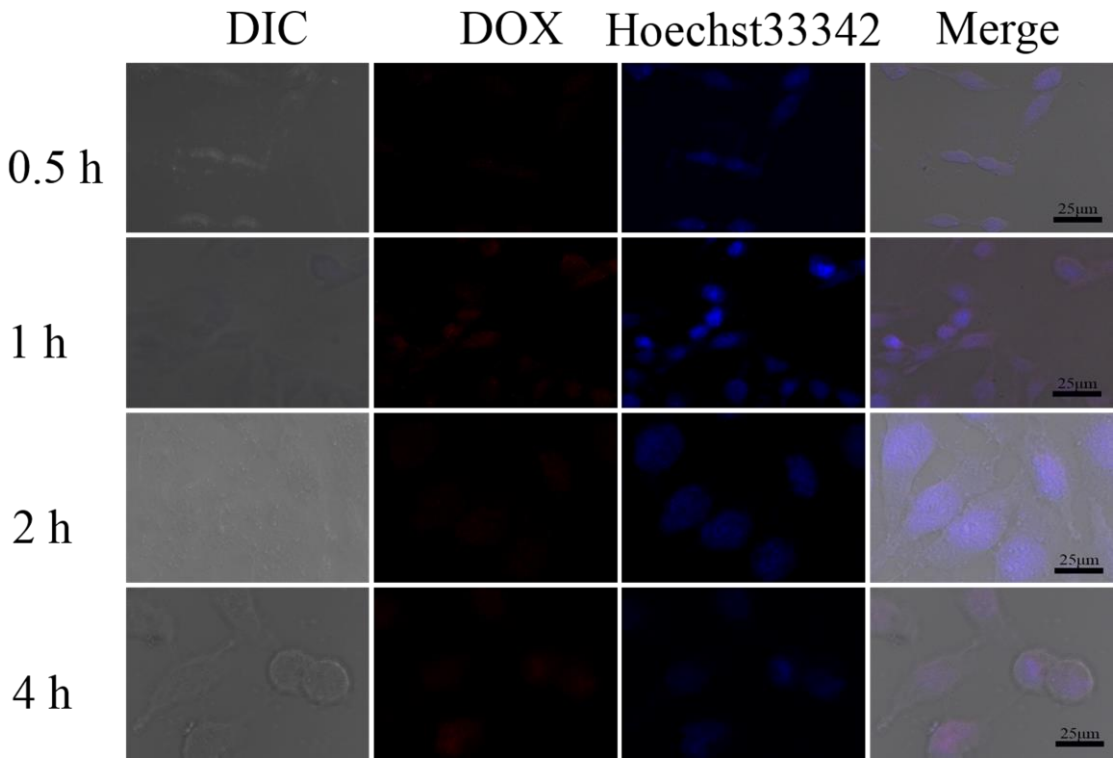


Fig. S4. CLSM images of HepG2 cells incubated with free DOX