Electronic Supplementary Information

of

MMP-2 Responsive Polymeric Micelles for Cancer-Targeted Intracellular Drug Delivery

Wei-Hai Chen, Guo-Feng Luo, Qi Lei, Hui-Zhen Jia, Sheng Hong, Qing-Rong Wang*, Ren-Xi Zhuo and Xian-Zheng Zhang*

Key Laboratory of Biomedical Polymers of Ministry of Education & Department of Chemistry, Wuhan University, Wuhan 430072, P. R. China

*Corresponding author. Tel./fax: +86-27-68754509.

E-mail addresses: wangqr@whu.edu.cn (W.Q.R.), xz-zhang@whu.edu.cn (Z.X.Z.).

1. Materials

Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA) and diisopropylethylamine (DIEA) were purchased from GL Biochem. Ltd. (Shanghai, China) and used as received. N,N-Dimethylformamide (DMF), methanol, dimethyl sulfoxide (DMSO), tetrahydrofuran (THF) and dichloromethane (DCM) were provided by Shanghai Chemical Co. (China) and distilled prior to use.

Doxorubicin hydrochloride (DOX·HCl) was purchased from Zhejiang Hisun Pharmaceutical Co. (China). The MMP-2 protease sensitive peptide substrate Ac-Cys-Pro-Leu-Gly-Leu-Ala-Gly-Gly-DOX (Ac-CPLGLAGG-DOX: peptide-DOX) was prepared according to our previous report.[S1] The Purity (95.8%) of the peptide-DOX (Fig. S1) was confirmed by high-pressure liquid chromatography (HPLC). 6-Maleimidocaproic acid was also synthesized according to the previous report.[S2] The ^{1}H NMR spectrum (Fig. S2) demonstrated that 6-maleimidocaproic acid was synthesized successfully. Biotin-poly(ethylene glycol)-NH_2-3400 (Biotin-PEG-NH_2) was purchased from Laysan Bio Inc. 1,10-Phenanthroline monohydrate and Brij35 were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). Matrix metalloproteinase (MMP-2) was purchased from RD-SYSTEMS. N(ε)-benzyloxycarebonyl-L-lysine (H-Lys(Z)-OH) and TIMP-2 were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zoliumbromide (MTT), trypsin, penicillin streptomycin, molecular probe (Hoechst 33258) and Dulbecco's phosphate buffered saline (PBS) were purchased from Invitrogen. All other reagents and solvents were of analytical grade and used directly.

2. Synthesis of Biotin-poly(ethylene glycol)-blocked-poly(L-lysine) (Biotin-PEG-

b-PLL)

Brifly, H-Lys(Z)-OH (4.0 g) was suspended in the distilled THF (40 mL) and the mixture was stirred at 50 ^{o}C under N_2 atmosphere. Then a solution of triphosgene (3.0 g) in THF (15 mL) was added dropwise to the suspension. And when the suspension became a clear solution, the reaction was stopped and then poured into excess dried n-hexane to obtain crude Z-Lys-NCA. The crude product was further recrystallized from dried THF/n-hexane twice and dried under vacuum. The polymerization of Z-Lys-NCA (4.5 g) by Biotin-PEG-NH_2 (0.1 g) was carried out in 20 mL anhydrous N,N-dimethylformamide (DMF) at 50 ^{o}C under N_2 atmosphere for 72 h. Then the solution was precipitated in an excess of diethyl ether three times to obtain Biotin-PEG-b-PLL(Z). Subsequently, 15 mL TFA was added to dissolve Biotin-PEG-b-PLL(Z) (600 mg) in an ice bath. After stirring for 15 min, HBr (5 mL of a 33 wt% solution in acetic acid) was added dropwise and stirred for another 1 h. Then the solution was precipitated in diethy ether. After collecting and drying, the product was resuspended in 10 mL DMF and the solution was dialyzed (MWCO: 3500 Da) against distilled water and finally lyophilized to obtain Biotin-PEG-b-PLL.

3. Synthesis of the 6-maleimidocaproic acid modified Biotin-PEG-b-PLL (Biotin-PEG-b-PLL(Mal))

Brifly, the 6-maleimidocaproic acid (48 mg, 1 eq. to amino groups of Biotin-PEG-b-PLL), DIEA (0.45 mL), PyBOP (177.4 mg) and HOBt (45.7 mg) were dissolved in distilled DMSO (5 mL) and stirred at room temperature. After 30 min, a solution of Biotin-PEG-b-PLL (50 mg) in DMSO (10 mL) was added and stirred for another 48 h. Then the mixture solution was dialyzing against distilled water (MWCO: 3500 Da). Biotin-PEG-b-PLL(Mal) was obtained after lyophilized.

4. Preparation of the MMP-2 protease sensitive Biotin-PEG-b-PLL(Mal)-

peptide-DOX

The Biotin-PEG-b-PLL(Mal)-peptide-DOX was synthesized via Michael addition according to a previous reference.[S3] Briefly, a solution of the previous prepared Biotin-PEG-b-PLL(Mal) (30 mg) containing the substrate Ac-CPLGLAGG-DOX (18.8 mg) in 15 mL DMSO was stirred at room temperature for 24 h. Then the mixture solution was dialyzing against DMSO/water (1:1, V/V) (MWCO: 3500 Da) for 24 h. Finally, the Biotin-PEG-b-PLL(Mal)-peptide-DOX was obtained after lyophilized as red solid.

5. Fabrication of the multifunctional polymeric micelles

The multifunctional polymeric micelles were prepared by directly dissolving the Biotin-PEG-b-PLL(Mal)-peptide-DOX (0.5 mg/mL) in the PBS (0.01 M, pH 7.4) and subsequently standing in the dark. The inherent amphiphilicity property of the Biotin-PEG-b-PLL(Mal)-peptide-DOX provided the opportunity to self-assemble into micelles. The hydrophobic peptide-DOX composed the core and the hydrophilic PEG formed the shell.

6. Characterizations

^{1}H NMR spectra were recorded on a Varian Unity 300 MHz spectrometer by using dimethyl sulfoxide-d_6 (DMSO-d_6) or D_2O as the solvent. The molecular weight and polydispersity index (PDI) of Biotin-PEG-b-PLL(Z) were evaluated by gel permeation chromatographic (GPC) system consisting of Waters 2690D separations module and Waters 2410 refractive index detector. Tetrahydrofuran (THF) was used as the eluent at a flow rate of 0.3 mL/min. Fluorescence analysis was performed on a RF-530/PC spectrofluorophotometer (Shimadzu). The particle size and zeta potential were measured using Malvern Zetasizer Nano-ZS ZEN3600. The morphologies of polymer micelles were viewed on transmission electron microscopy (TEM, JEM-

2100).

7. Determination of critical micelle concentration (CMC)

The CMC of Biotin-PEG-b-PLL(Mal)-peptide-DOX micelles was estimated by fluorescence spectra, which was recorded on an RF-530/PC spectrofluorophotometer (Shimadzu) and using pyrene as a hydrophobic fluorescent probe. 50 μL of pyrene solutions (6×10^{-7} M in acetone) were added to containers, after the acetone evaporated, 1 mL aqueous solution of Biotin-PEG-b-PLL(Mal)-peptide-DOX polymeric micelles at particular concentration varying from 1×10^{-4} to 1 mg/mL was added to the container. The sample solutions containing pyrene residues were kept at room temperature for 24 h to reach the equilibrium of pyrene partition between water and micelles. For the pyrene excitation spectra, the emission wavelength was carried out at 390 nm, and the excitation spectra of samples were recorded ranging from 300 nm to 360 nm. The fluorescence intensity ratio of the third and first vibronic bands (I_3/I_1) was plotted against the logarithm of Biotin-PEG-b-PLL(Mal)-peptide-DOX concentrations, and the CMC value was estimated from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration.

8. Cell culture

SCC-7 (squamous cell carcinoma) cancer cells and COS7 normal cells (African green monkey kidney fibroblast cells) were incubated in DMEM medium with 10% FBS and 1% antibiotics (penicillin-streptomycin, 10000 U/mL) at 37 °C in a humidified atmosphere containing 5% CO_2.

9. The MMP-2 protease responsive behaviors of the multifunctional micelles

To investigate the MMP-2 protease responsive property of Biotin-PEG-b-PLL(Mal)-peptide-DOX micelles, the morphology and the size changes of the

micelles were detected after the micelles incubated with the MMP-2 protease for 6 h.

10. *In vitro* drug release study

In vitro drug release experiments were carried out in three different media: 1) 3 mL TCNB buffer solution (composed of 100 mM Tris, 5 mM calcium chloride, 200 mM NaCl, 0.1% Brij35) and 100 μL MMP-2 (2 μg/mL); 2) 3 mL TCNB buffer solution, 100 μL MMP-2 (2 μg/mL) and with the MMP-2 protease inhibitor; and 3) only 3 mL TCNB buffer solution, respectively. For each release study, 1.5 mg of the Biotin-PEG-b-PLL(Mal)-peptide-DOX micelles were dispersed in the above solutions. Then the solutions were put into the dialysis tube (MWCO: 3500 Da) and subsequently immersed into 10 mL incubation medium and maintained at 37 ^{o}C. After particular time intervals, fluorescence intensity of the incubation medium was analyzed by RF-5301PC spectrofluorophotometer. The emission and excitation slit widths were set at 5 nm with λex=470 nm.

11. Co-incubation of Biotin-PEG-b-PLL(Mal)-peptide-DOX micelles with cells

SCC-7 cancer cells and COS7 normal cells were seeded respectively in a glass bottom dish at a density of 1×10^5 cells/well for 24 h. As the negative control, SCC-7 cells and COS7 cells were incubated with the excess biotin (1 mM) for 4 h in advance. Thereafter, the Biotin-PEG-b-PLL(Mal)-peptide-DOX micelles (containing 3 μg/mL of Dox) dispersed in DMEM containing 10% FBS and 1% antibiotics were added and the cells were incubated at 37 °C for another 3 h or 6 h. After removing the medium and washing with PBS, the nucleus were stained with Hoechst 33258 at 37 ^{o}C for 15 min. Then the cells were observed by confocal laser scanning microscopy.

12. Quantitative analysis of cellular uptake DOX by flow cytometry

SCC-7 cancer cells and COS7 normal cells were seeded in 24-well plates at a density of 5×10^4 cells/well and cultured with 1 mL of DMEM containing 10% FBS

for 1 day. As the negative control, SCC-7 cells and COS7 cells were incubated with excess biotin (1 mM) for 4 h in advance. Then all cells were cultured with Biotin-PEG-b-PLL(Mal)-peptide-DOX micelles (containing 3 μg/mL of Dox) for another 3 h or 6 h. After that, the medium was removed and the cells were washed 3 times with PBS. Then all the cells were digested by trypsin and collected in centrifuge tubes by centrifugating at 1000 rpm for 3 mins. The supernatant was discarded and the bottom cells were washed with PBS 3 times to remove excess micelles. Then the suspended cells were filtrated and detected for red fluorescence (PE-A) by flow cytometry (BD FACSAria TM III, USA). Cells untreated with micelles were used as the control. The fluorescence scan was performed with 1×10^4 cells.

13. In vitro cytotoxicity studies

In vitro cytotoxicity was performed with SCC-7 cancer cells and COS7 normal cells by MTT assay. Briefly, SCC-7 and COS7 cells were seeded in 96-well plates at a density of 6000 cells/well, and then cells were incubated in 100 μL DMEM containing 10% FBS and 1% antibiotics for 1 day prior to adding Biotin-PEG-b-PLL(Mal)-peptide-DOX micelles or Biotin-PEG-b-PLL. After co-incubation for 2 days, the medium was replaced with 200 μL of fresh medium. Then 20 μL MTT solutions (5 mg/mL) was added to each well and further incubated for 4 h. After that, the medium was removed and 200 μL DMSO was added. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability was calculated as: cell viability = $(OD_{570\ (samples)}/OD_{570\ (control)}) \times 100\%$, where $OD_{570\ (control)}$ was obtained in the absence of the micelles or Biotin-PEG-b-PLL, and $OD_{570\ (samples)}$ was obtained in the presence of the micelles or Biotin-PEG-b-PLL. Each value was averaged from four independent experiments.

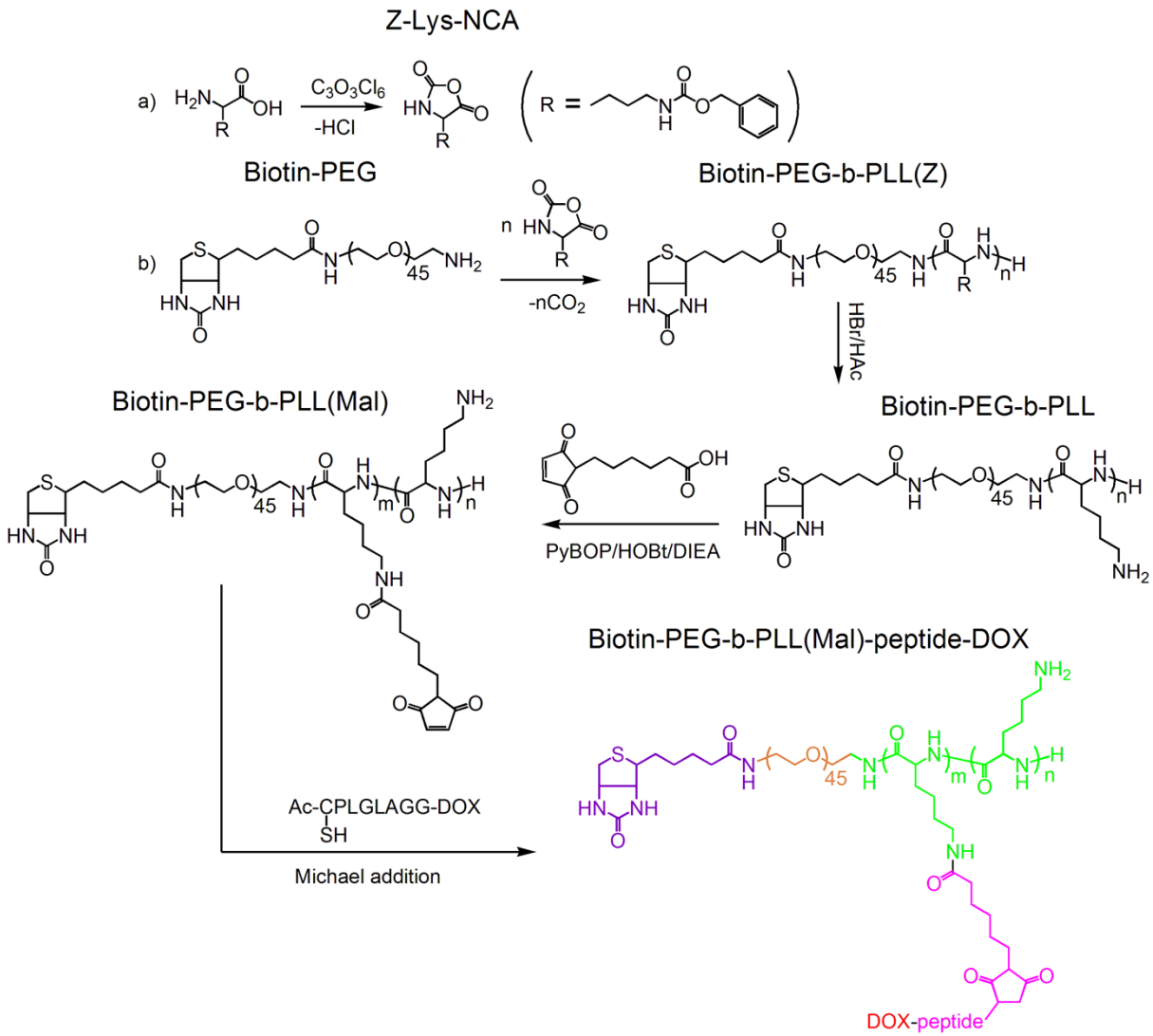
Supplementary References

[S1] W. H. Chen, X. D. Xu, H. Z. Jia, Q. Lei, G. F. Luo, S. X. Cheng, R. X. Zhuo and X. Z. Zhang, *Biomaterials*, 2013, **34**, 8798.

[S2] D. Willner, P. A. Trail, S. J. Hofstead, H. D. King, S. J. Lasch, G. R, Braslawsky, R. S. Greenfield, T. Kaneko and R. A. Firestone, *Bioconjug. Chem.*, 1993, **4**, 521.

[S3] Q. Lin, C. Bao, S. Cheng, Y. Yang, W. Ji and L. Zhu, *J. Am. Chem. Soc.*, 2012, **134**, 5052.

Supplementary Figures and Schemes



Scheme S1. Synthesis of (a) Z-Lys-NCA and (b) Biotin-PEG-PLL(Mal)-peptide-DOX.

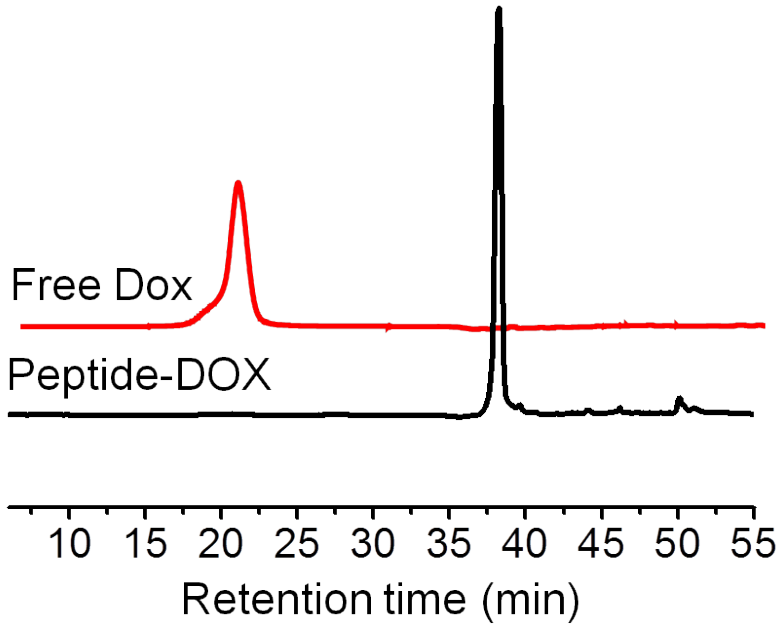


Fig. S1 HPLC profile of peptide-DOX (Ac-Cys-Pro-Leu-Gly-Leu-Ala-Gly-Gly-DOX) and the free DOX as the control. The purity of the peptide-DOX was 95.8%.

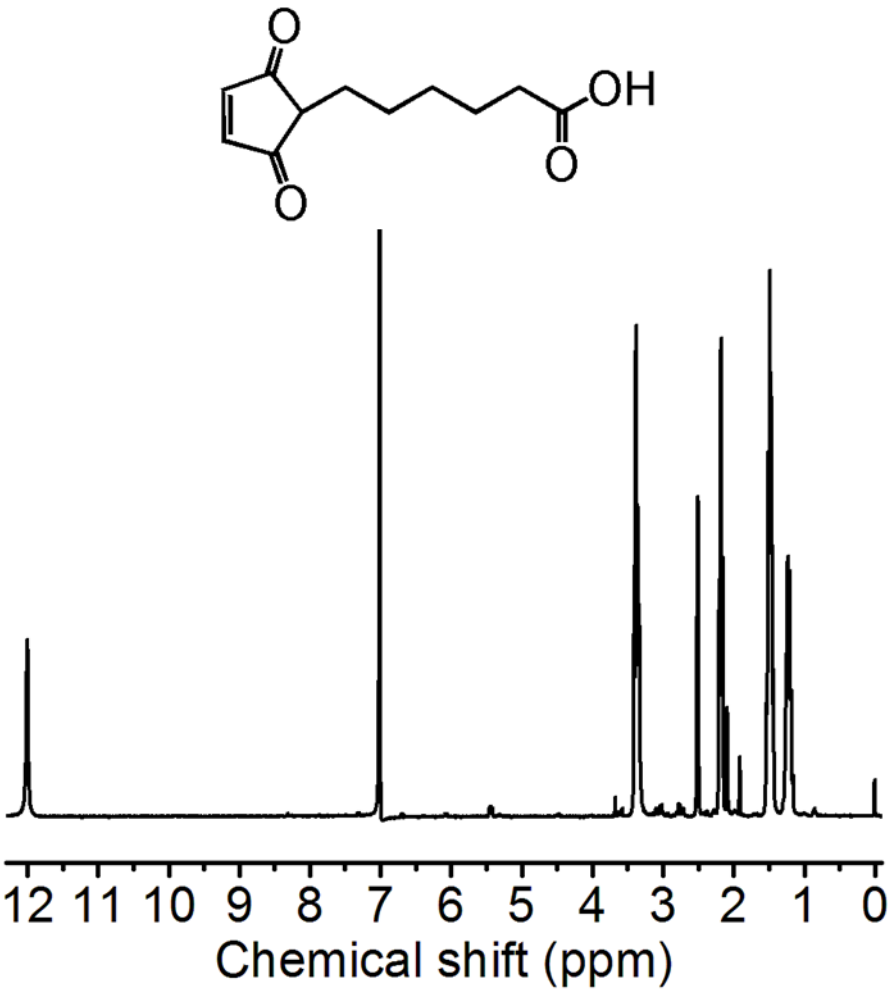


Fig. S2 ^{1}H NMR spectrum of 6-maleimidocaproic acid in DMSO-d_6.

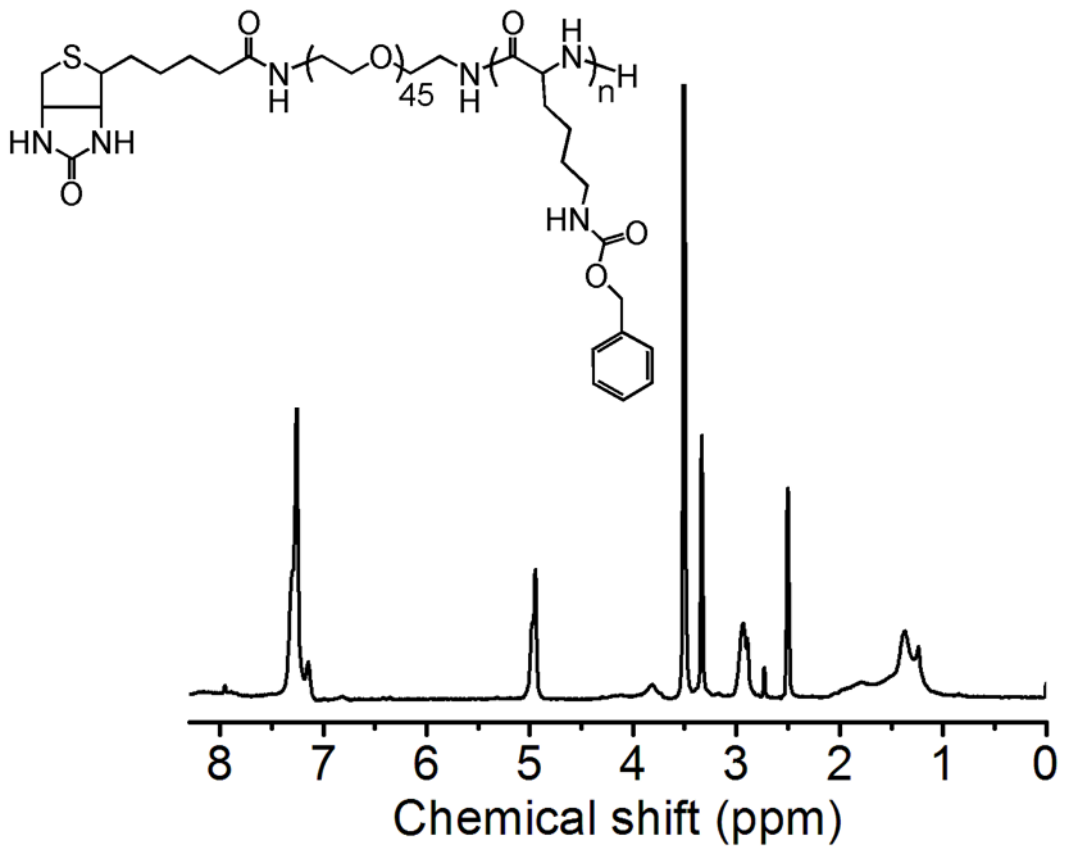


Fig. S3 ^{1}H NMR spectrum of Biotin-PEG-b-PLL(Z) in DMSO-d_6.

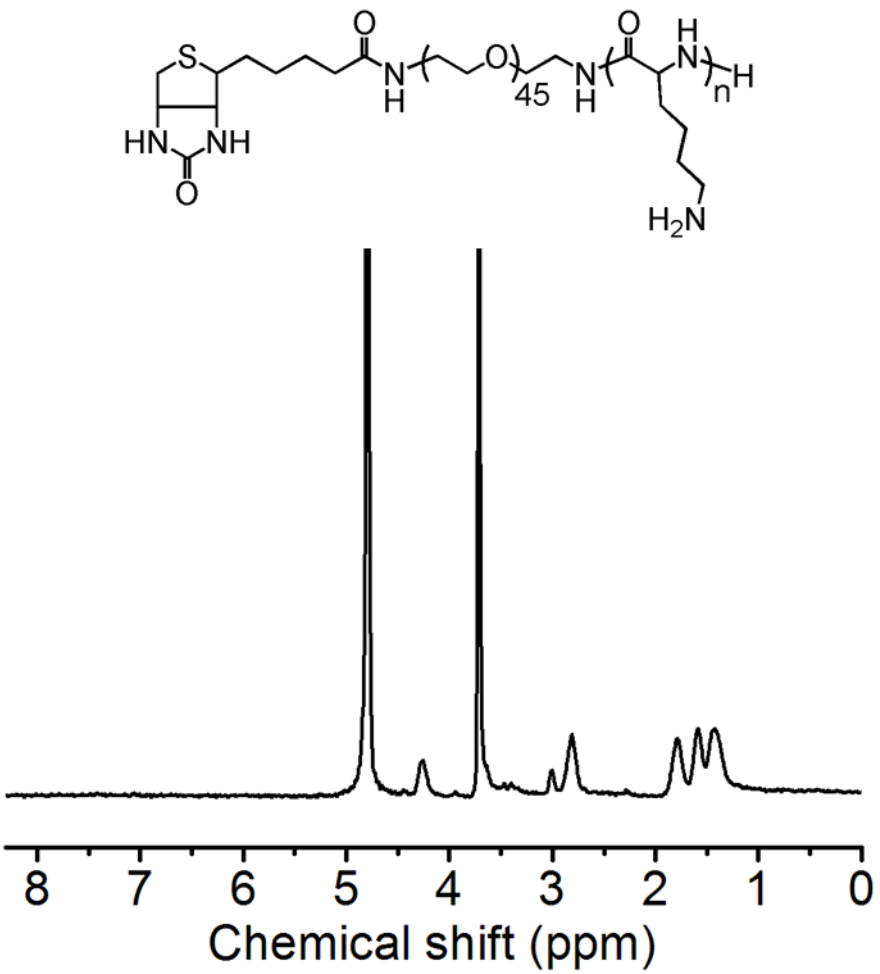


Fig. S4 ^{1}H NMR spectrum of Biotin-PEG-b-PLL in D_2O.

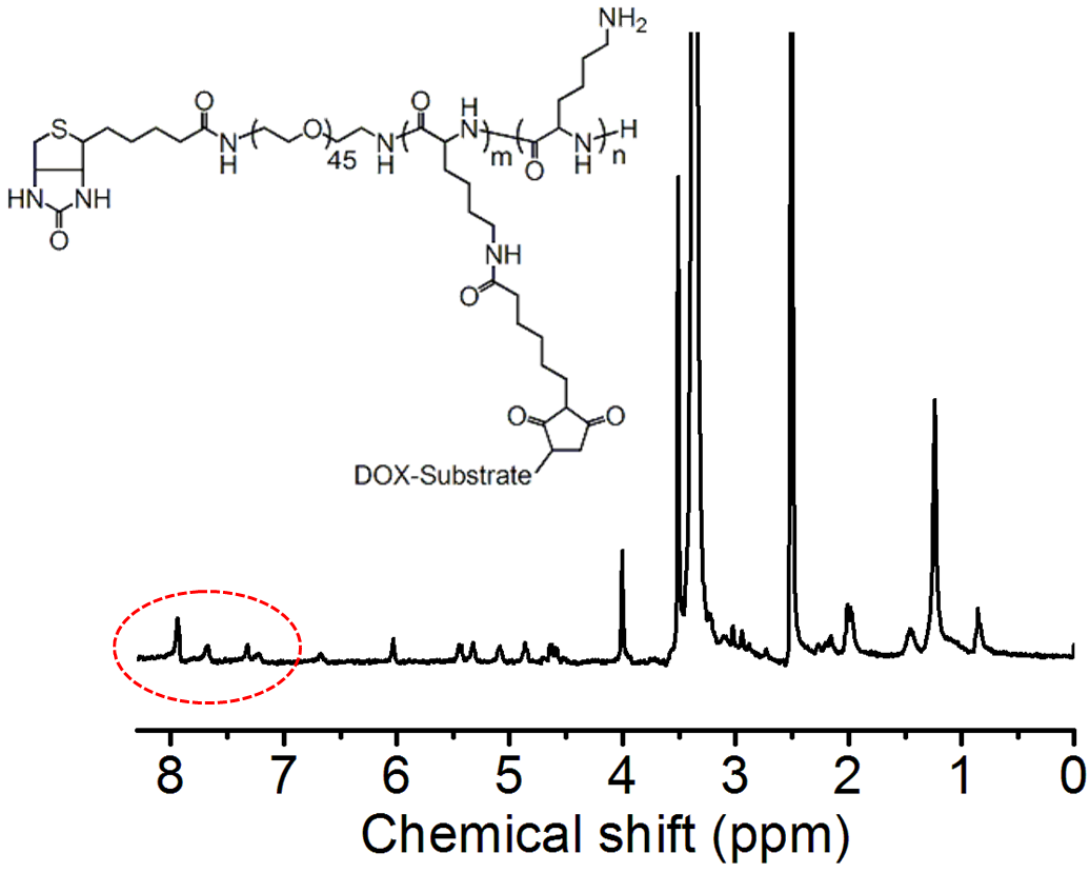


Fig. S5 ^{1}H NMR spectrum of Biotin-PEG-b-PLL(Mal)-peptide-DOX in DMSO-d_6. The red ellipse indicated the successful conjugation of DOX to the Biotin-PEG-b-PLL(Mal) via Michael addition.

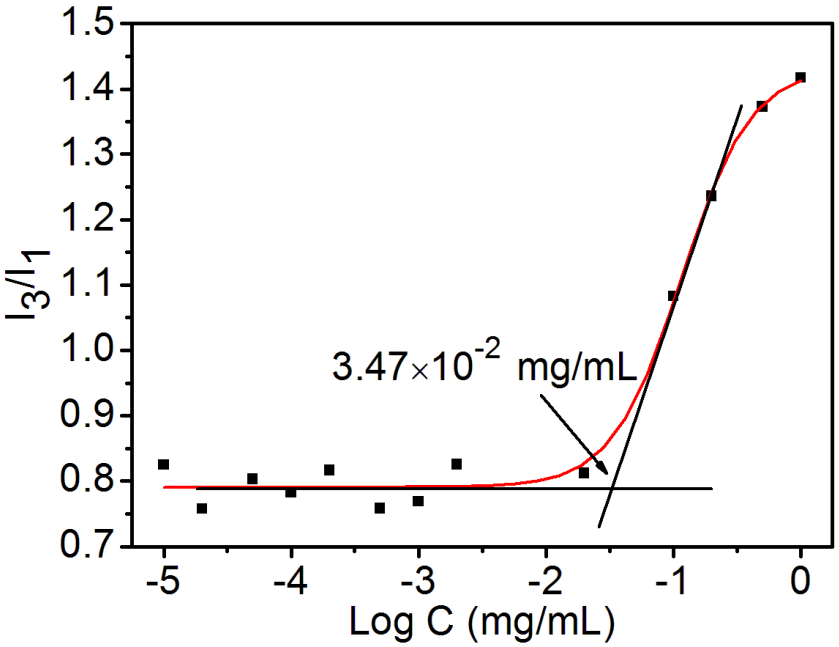


Fig. S6 The CMC of Biotin-PEG-b-PLL(Mal)-peptide-DOX polymeric micelles determined by fluorescence spectra and using pyrene as a hydrophobic fluorescent probe. Plot of the intensity ratio I_3/I_1 vs log C, the tested CMC value was 3.47×10^{-2} mg/mL.

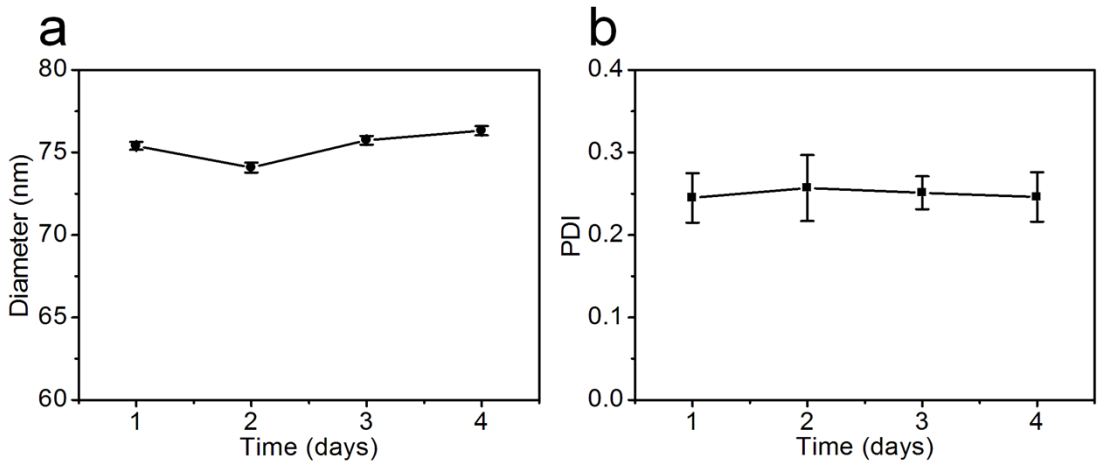


Fig. S7 Monitoring the diameter (a) and PDI (b) changes of Biotin-PEG-b-PLL(Mal)-peptide-DOX polymeric micelles by DLS.

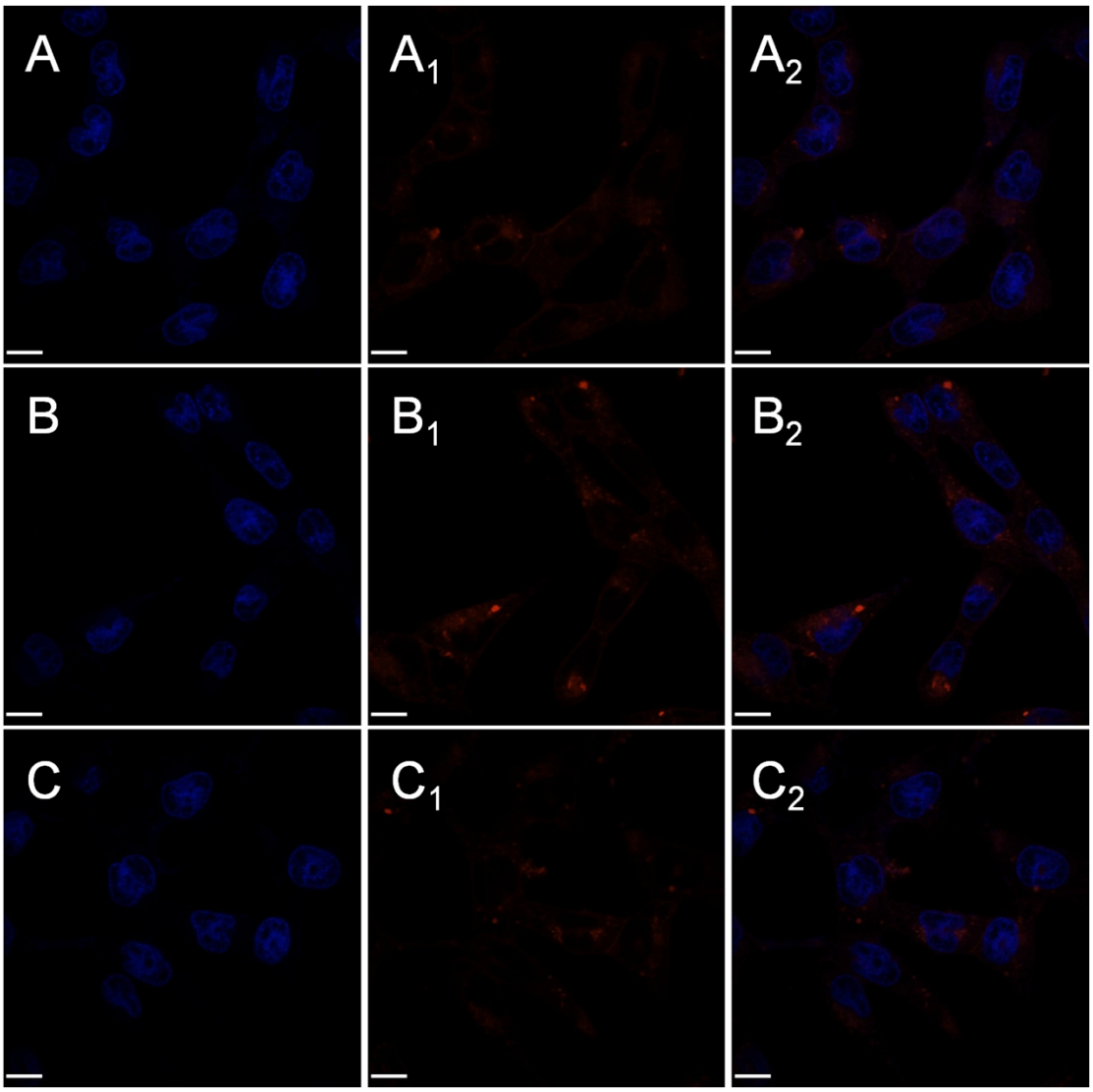


Fig. S8 Confocal laser scanning microscopy (CLSM) images of COS7 normal cells incubated with Biotin-PEG-b-PLL(Mal)-peptide-DOX micelles for 3 h (A-A_2) and 6 h (B-B_2), and 6 h for cells pre-treated with the excess of biotin in advance (C-C_2). (A-C) blue fluorescence images of nuclei; (A_1-C_1) red fluorescence images of DOX; (A_2-C_2) the merge images of blue and red fluorescence. The scale bar is 14 μm.

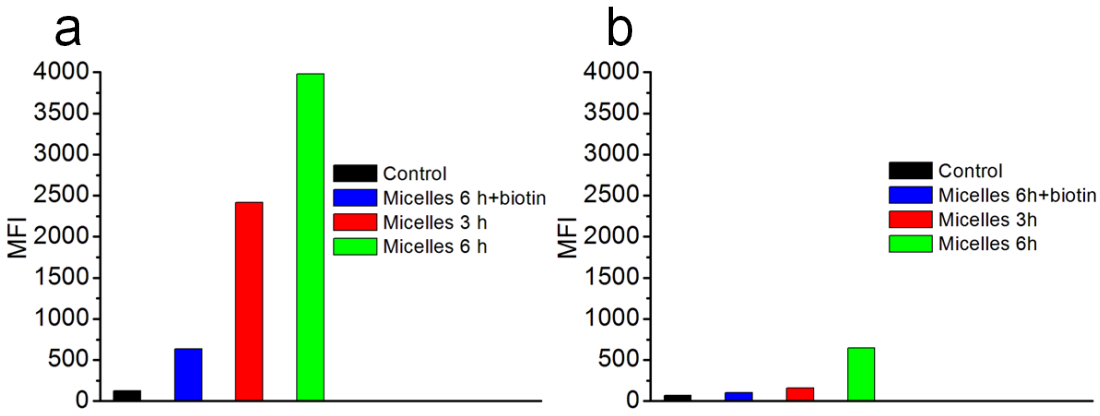


Fig. S9 Quantitative flow cytometry analysis of the cellular DOX red fluorescence mean fluorescence intensity (MFI) values of SCC-7 cancer cells (a) and COS7 normal cells (b), respectively.

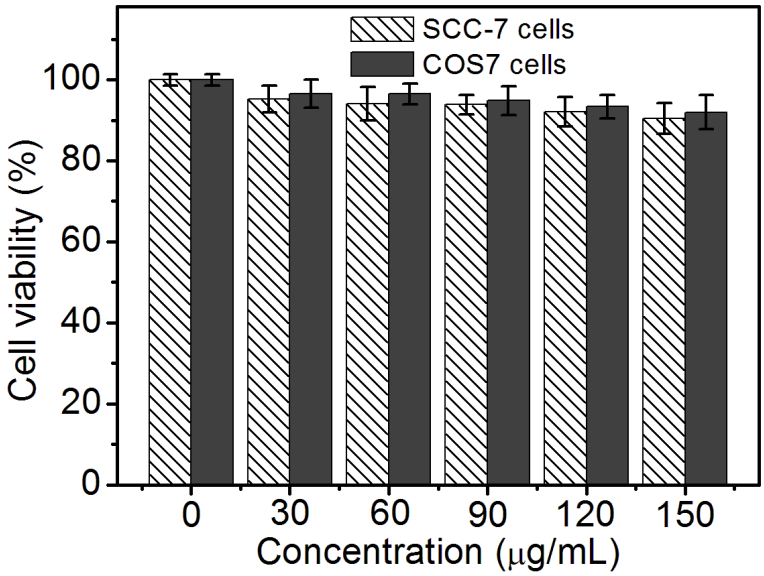


Fig. S10 The cell viability of SCC-7 cancer cells and COS7 normal cells after incubated with Biotin-PEG-b-PLL 48 h by MTT assay.