Electronic Supplementary Material (ESI) for Polymer Chemistry.

Facile construction of stabilized, pH-sensitive micelles based on cyclic statistical copolymers of poly(oligo (ethylene glycol) methyl ether methacrylate-*st*-*N*, *N*-dimethylaminoethyl methacrylate) for *in vitro* anticancer drug delivery

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assay and expressed as % viability compared to control untreated cells. The data were expressed as mean ± SD, n =3.

Figure S12. *In vitro* cytotoxicity of (a) HeLa and (b) HUVEC cells incubated for 72 h with blank C1 and L1 micelles at various concentrations. Cell viability was determined by MTS assay and expressed as % viability compared to control untreated cells. The data were expressed as mean ± SD, n =3.

Figure S13. *In vitro* cytotoxicity of free DOX in HeLa cells for 24 h of incubation. Cell viability was determined by MTS assay and expressed as % viability compared to control untreated cells. The data were expressed as mean ± SD, n =3.

1. Experimental section

Materials

Oligo (ethylene glycol) monomethyl ether methacrylate (OEGMA, Mn = 300 g/mol and 4~5 pendent EO units, Sigma-Aldrich) and 2-(Dimethylamino) ethyl methacrylate (DMAEMA, Aladdin, 99%) were purified by passing through a basic Al_2O_3 alumina column to remove the inhibitor. Triethylamine (TEA) and 2-bromoisobutyryl bromide were purchased from J&K. copper(I) bromide (CuBr, Aladdin, >99.999%) and *N*, *N*, *N*′, *N*″, *N*″-pentamethyldiethylenetriamine (PMDETA, Sigma-Aldrich, 99%). Sodium azide (NaN_3, Sanyou, Shanghai). anisole was purchased from Chengdu Kelong Chemical Reagent Factory (China). Tetrahydrofuran (THF) and n-hexane (Rionlon, Tianjin, china). Doxorubicin hydrochloride (DOX·HCl) with a purity of ≥ 98% was provided by Aladdin, Shanghai, China. All other reagents were of analytical grade and used as received.

Instruments and Measurements

The chemical structures of the synthesized polymers were characterized by ^1H NMR spectra, which recorded on a JNM-ECS spectrometer at 400 MHz using $CDCl_3$ as the solvents. The FT-IR spectroscopic measurements were conducted on a NEXUS 670 FT-IR spectrometer (Nicolet, WI, USA). Samples were pressed into potassium bromide (KBr) pellets prior to the measurements. The molecular weight (MW) and polydispersity index (*Đ*) of all of the synthesized polymer samples were determined by size-exclusion chromatography and multi-angle laser light scattering (SEC-MALLS) and using HPLC-grade DMF containing 0.1 wt % LiBr at 60 °C as the

eluent at a flow rate of 1 mL min^{-1}. The MALLS detector was operated at a laser wavelength of 690.0 nm. The polymer solution was prepared in phosphate buffer solution (PBS, pH 7.4, 150 mM) and saline sodium citrate (SSC, pH 5.0, 150 mM) at a concentration of 1 mg mL^{-1}.

The average hydrodynamic size of the micelles was measured by using dynamic light scattering (DLS) on a Zetasizer (Nano ZS, Malvern, Worcestershire, UK) at a fixed detection angle of 173° at 25 °C. The transmmision electron microscope (TEM) was operated on JEOL JEM-2100 at an accelerating voltage of 80 keV.

Synthesis of Propargyl 2-Bromoisobutyrate

Propargyl 2-bromoisobutyrate was synthesized as described in the literature.[1] Triethylamine (6.8 g, 47.0 mmol) and propargyl alcohol (2.0 g, 39.0 mmol) were dissolved in 50 mL of anhydrous DCM and stirred in a 150 mL flame-dried round-bottom flask under nitrogen at 0 °C. 2-bromoisobutyryl bromide (9.0 g, 47.0 mmol) in 15 mL of anhydrous DCM was added dropwise to the above solution at 0 °C while stirring. After addition, the reaction mixture was stirred at room temperature for 12 h. The solution was filtered, the filtered solution was washed with water for four times, and the solution was dried with Na_2SO_4. After evaporating the solvent by rotary evaporation, and the crude product was purified by column chromatography over silica gel eluting with hexane/ethyl acetate (5:1) to give the product as a colorless oil-like product (yield: 80%).

^{1}H NMR [400 MHz, $CDCl_3$, δ (ppm)]: 4.75 (d, 2H, –$COOCH_2$≡CH), 2.54 (t, 1H, –

$COOCH_2C{\equiv}CH$), and 1.96 (s, 6H, $–C(CH_3)_2Br$).

Preparation and Characterization of Micelles

Generally, 3 mg of copolymers was completely dissolved in 2 mL of DMF, the DMF solution of copolymers was then dialyzed against deionized water to eliminate the DMF using a dialysis bag (molecular weight cut-off (MWCO): 3.5 kDa). Ultimately, micelle solution with a concentration of approximately 0.25 mg mL^{-1} was obtained after 48 h.

Dynamic light scattering (DLS) was used to determine the average hydrodynamic size of micelles on a Zeta sizer (Nano ZS, Malvern, Worcestershire, UK) with the detection angle fixed at 173°. Note that all data were gained from the average of three tests. The sample solution was passed through a Millipore 0.45 μm pore-sized syringe filter prior to measurements. Polymer solutions with concentrations of 0.25 mg mL^{-1} were evaluated.

The morphology of the polymer samples was observed by TEM on JEM-2100 at an accelerating voltage of 200 keV. To prepare specimens for TEM observation, a drop of micelle solution was deposited onto a carbon-coated copper grid. After deposition, excess solution was removed using a strip of filter paper. The phosphotungstic acid (2 % w/w) was used as negative staining and stained the sample for 10 min. Followed, the sample was dried in air.

Fluorescence spectra were recorded on a LS55 luminescence spectrometer (Perkin-Elmer, Waltham, MA, USA). CMC was measured using pyrene as a fluorescence

probe. 0.08 mL of pyrene solution (3×10^{-6} M in acetone) was added to containers, and the acetone was allowed to evaporate. Then 4 mL of polymer aqueous solution at different concentrations were added to the containers containing the pyrene residue and the combined solution of pyrene and copolymers was equilibrated at room temperature in dark for 24 h prior to measurements. The final concentration of pyrene was 6×10^{-8} M in water. Excitation was carried out at 340 nm, and emission spectra were recorded ranging from 340 to 500 nm. Excitation and emission bandwidths were set as 10 nm and 10 nm, respectively. From the pyrene emission spectra, the intensities (peak height) of I_{373}, I_{384} and I_{393} nm were recorded, A CMC value was determined from the intersection of the tangent to the curve at the inflection with the tangent through the points at low concentration.

In Vitro Drug Loading and Drug Release Study

In vitro drug loading and drug release study was performed according to our reported procedures.[2] 1 mg of DOX·HCl was first treated with 100 μL of triethylamine in 2 mL of DMF overnight in dark to get the free DOX base. Next, 10 mg of polymer dissolved in 2 mL of DMF was added to the free DOX base and stirred at room temperature for 1 h. The above mixture was later added dropwise into 4 mL of ultra-purified water under vigorous stirring. After addition, the mixture solution was stirred for another 1 h to realize drug encapsulation followed by transfer to a dialysis tube (MWCO: 3.5 kDa) and dialysis against 5 L of distilled water for 24 h. The water was renewed every 3 h at the initial 12 h to remove DMF, TEA and any unencapsulated free DOX. Finally, the drug-loaded micelles were harvested by lyophilization. To

determine the drug loading content (DLC) and entrapment efficiency (EE), the freeze-dried drug-loaded micelles were re-dispersed in phosphate buffer (PBS, pH=7.4, 150 mM). The concentration of DOX was determined by measuring the absorbance at 485 nm using a Lambda 35 UV-Vis spectrometer (Perkin- Elmer). The DLC and EE were calculated using the following formula,

$$DLC\,(\%) = W_{\text{drug loaded in particles}} / W_{\text{particles}} \times 100\% \qquad (1)$$

$$EE\,(\%) = W_{\text{drug loaded in particles}} / W_{\text{drug fed for encapsulation}} \times 100\% \qquad (2)$$

In vitro drug release study was carried out in phosphate buffer (PBS, pH=7.4, 150 mM) and saline sodium citrate (SSC, pH 5.0, 150 mM) at 37 °C. The freeze-dried drug-loaded nanoparticles was re-dispersed in buffer solution to prepare a drug-loaded micelle solution of concentration at 0.5 mg mL^{-1}. 1 mL of the solution was placed in a dialysis tube (MWCO: 3.5 kDa), and then immersed in a Falcon tube containing 25 mL of release medium of different PHs. The tube was kept in a horizontal laboratory shaker thermostated at a constant temperature of 37 °C and a stirring speed of 120 rpm. Next, 3 mL of release medium in tube was taken out at 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h, and replenished with the equivalent volume of fresh medium was supplemented each time. The drug concentration was calculated by measuring the absorbance at 480 nm according to a standard calibration curve obtained from free DOX·HCl in the corresponding release buffers. The experiment was performed in quadruplicate for each sample.

Cell Viability Study.

The cytotoxicities of various formulations were evaluated *in vitro* using the MTS assay. HeLa cells and Human umbilical vein endothelial cells (HUVEC) were plated in 96-well plates at a density of 2,500 cells per well in 0.1 mL of complete growth medium and incubated in an incubator maintained at 37 °C and 5% CO_2 environment for 24 h. Free DOX, blank copolymers, and drug-loaded micelles of copolymers were prepared in serial dilutions in sterilized water and then diluted in 10-fold in Opti-MEM medium (Invitrogen). The cells were then rinsed once with PBS and incubated with 40 μL of the polymer solutions with different polymer or DOX concentrations at 37 °C for 4 h. cells were rinsed once with PBS and incubated in 1 mL of culture medium. At 24 h, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega) were added to each well. Cells were then incubated at 37 °C, 5% CO_2 for 3 h. The absorbance of each well was measured at 490 nm on a Tecan Safire2 plate reader (Mannerdorf, Switzerland). Cell viability for each treatment condition was determined by normalizing to the cells only signal.

Evaluation of Cellular Uptake by Flow Cytometry (FCM)

The procedures for flow cytometry are same with our previously reported paper.[3] HeLa cells were seeded in 24-well plates at a density of 1×10^6 cells per well in 1.0 mL of complete growth medium and incubated for 24 h at 37 °C in 5% CO_2 environment. Then, fresh MEM containing different samples, was added to replace the original medium, and the cells without drug treatment were set as a control. The DOX concentration for free DOX, L1@DOX and C1@DOX micelles in MEM was

set at 30 μg mL^{-1}. After incubation for 4 h, the polymer solutions were aspirated, and the cells were rinsed twice with PBS. Cells were then harvested by incubation with 200 μL of Trypsin-EDTA, followed by resuspension with 1 mL of complete growth medium. Next, cells were transferred to 1.5 mL microcentrifuge tubes and pelleted at 300 g for 5 min at 4 °C. The supernatant was aspirated, and the cell pellets were resuspended in 200 μL of PBS. Cells were analyzed for uptake of fluorescent samples using a BD Accuri C6 Plus flow cytometer (BD Biosciences) with an excitation wavelength and emission wavelength of 488 nm and 595 nm, respectively. A minimum of 10000 cells was analyzed for each sample with the fluorescence intensity.

Confocal Imaging.

Confocal imaging was studied through our previous reports.[4] Briefly, HeLa cells were seeded in 6-well plates at a plating density of 5×10^5 cells per well in 1 mL of complete growth medium and incubated in a 37 °C, 5% CO_2 environment for 24 h. Solutions of free DOX, L1@DOX and C1@DOX micelles were prepared in complete growth medium at concentrations equal to 25% of their IC_{50} value and then added to the wells and incubated for 4 h at 37 °C. Cells were later rinsed with PBS and fixed with 4% paraformaldehyde (PFA) solution for 20 min at room temperature. Finally, cells were counterstained with 2-(4-amidinophenyl)-6-indolecarbamidine (DAPI). Coverslips were mounted onto glass slides and imaged using Nikon A1R confocal microscope.

References

1. X. He, C. Cao, W. Sun, W. Huang, S. Lin and D. Yan, *Polym. Chem.,* 2013, **51**, 1040–1050.

2. K. Deng, X. Zhao, F. Liu, J. Peng, C. Meng, Y. Huang, L. Ma, B, Wang and H. Wei, *ACS Biomater. Sci. Eng.,* 2019, **5**, 3419–3428.

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4. X. Zhao, K. Deng, F. Liu, X. Zhang, H. Yang, J. Peng, Z. Liu, L. Ma, B. Wang and H. Wei, *ACS Biomater. Sci. Eng.,* 2018, **4**, 566–575.

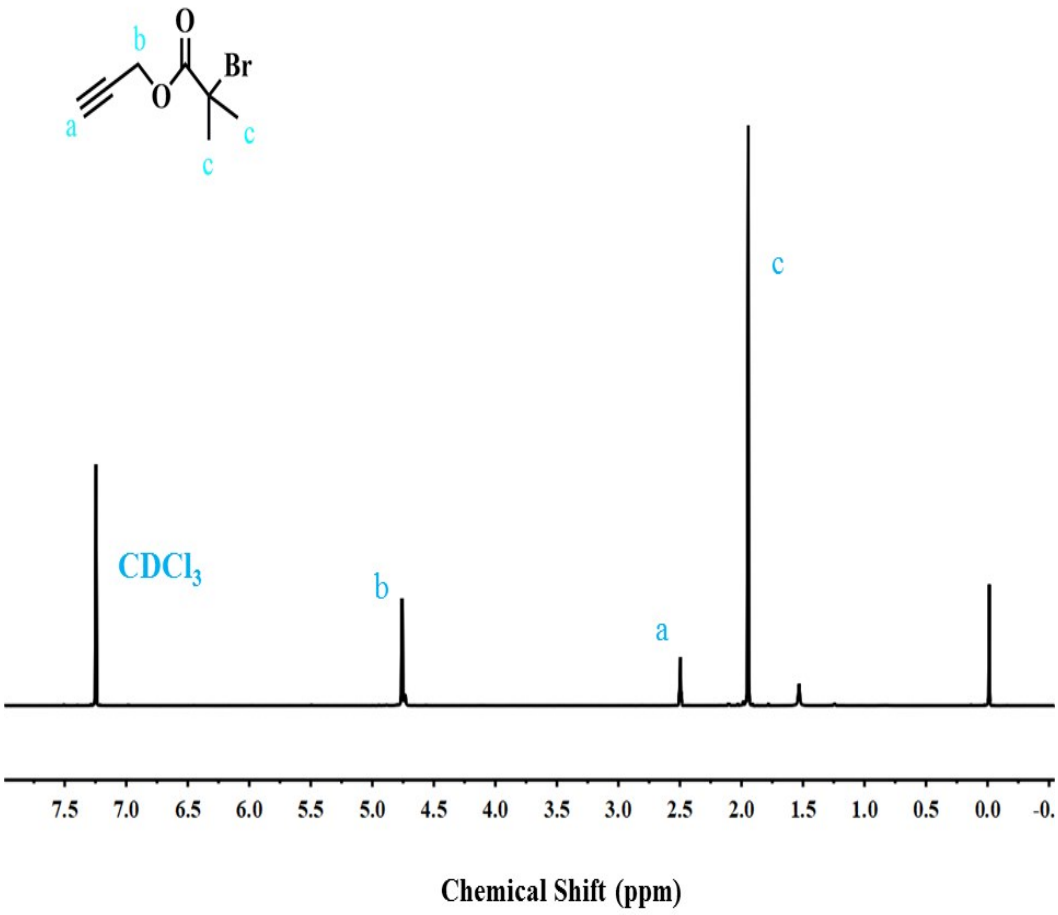

Figure S1. ^{1}H NMR spectrum of alkyne-Br in $CDCl_3$.

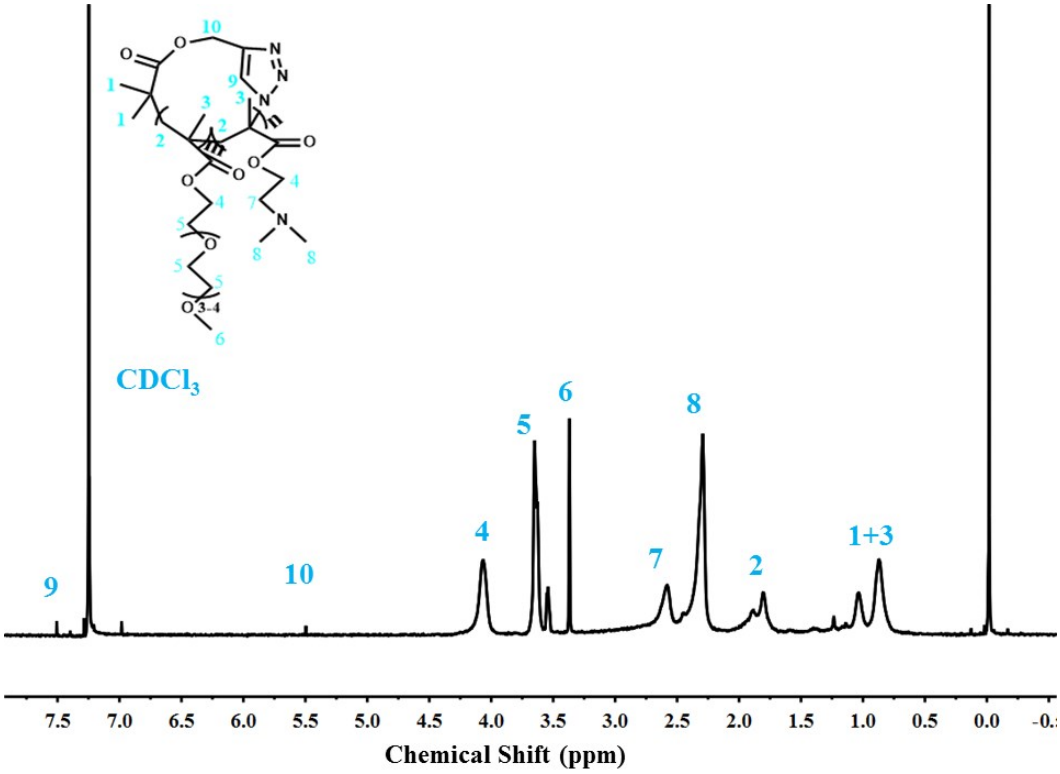

Figure S2. ^{1}H NMR spectrum of *c*-P(OEGMA$_4$-*st*-DMAEMA$_{38}$) in $CDCl_3$.

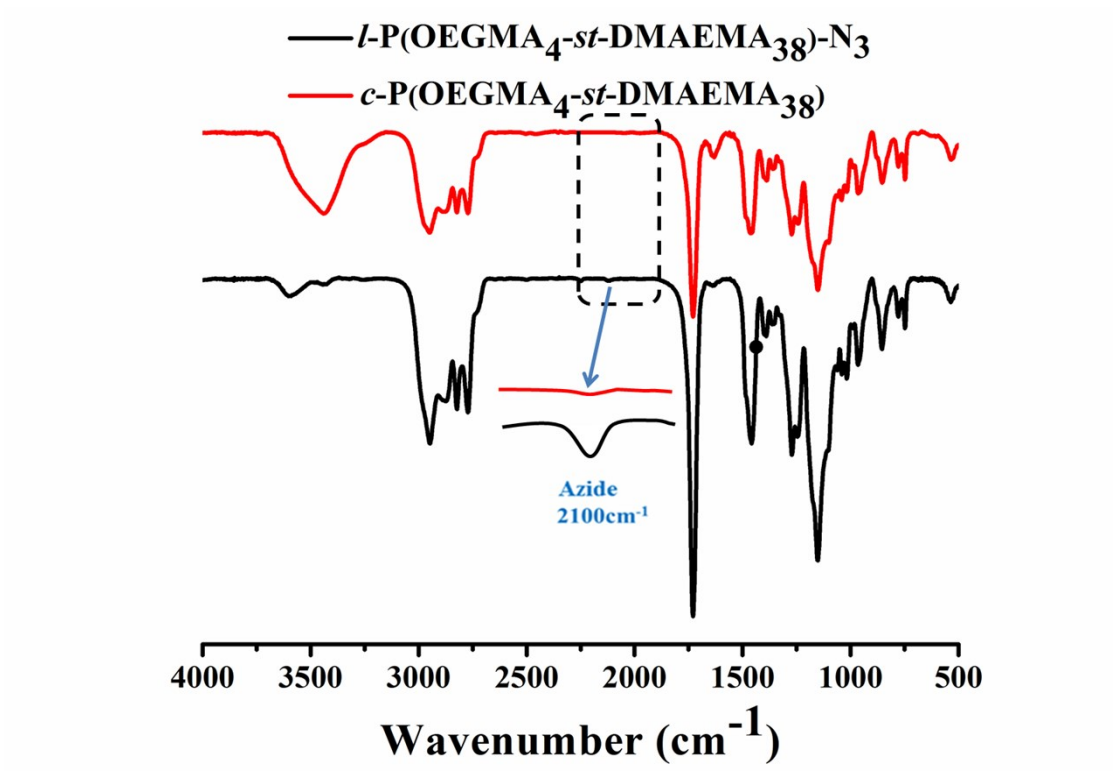

Figure S3. FT-IR spectra of the *l*-P(OEGMA$_4$-*st*-DMAEMA$_{38}$)-N_3 and *c*-P(OEGMA$_4$-*st*-DMAEMA$_{38}$) polymers.

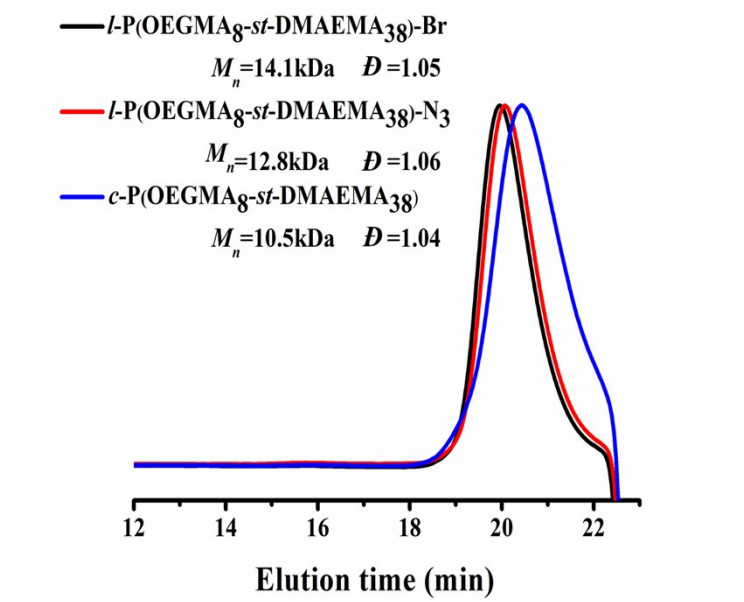

Figure S4. SEC elution traces of *l*-P(OEGMA$_8$-*st*-DMAEMA$_{38}$)-Br, *l*-P(OEGMA$_8$-*st*-DMAEMA$_{38}$)-N$_3$, *c*-P(OEGMA$_8$-*st*-DMAEMA$_{38}$) using DMF as an eluent.

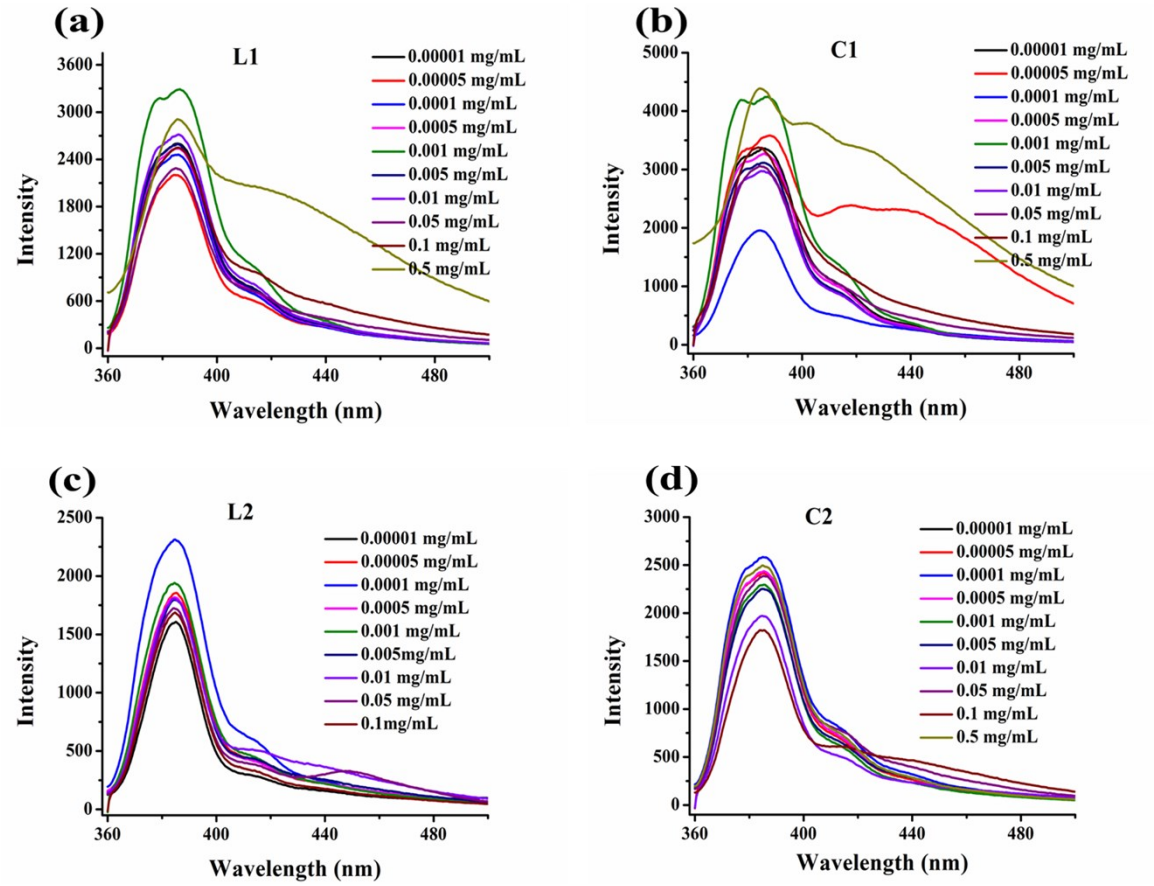

Figure S5. The emission spectra of pyrene of C1, L1, and C2, L2 copolymers.

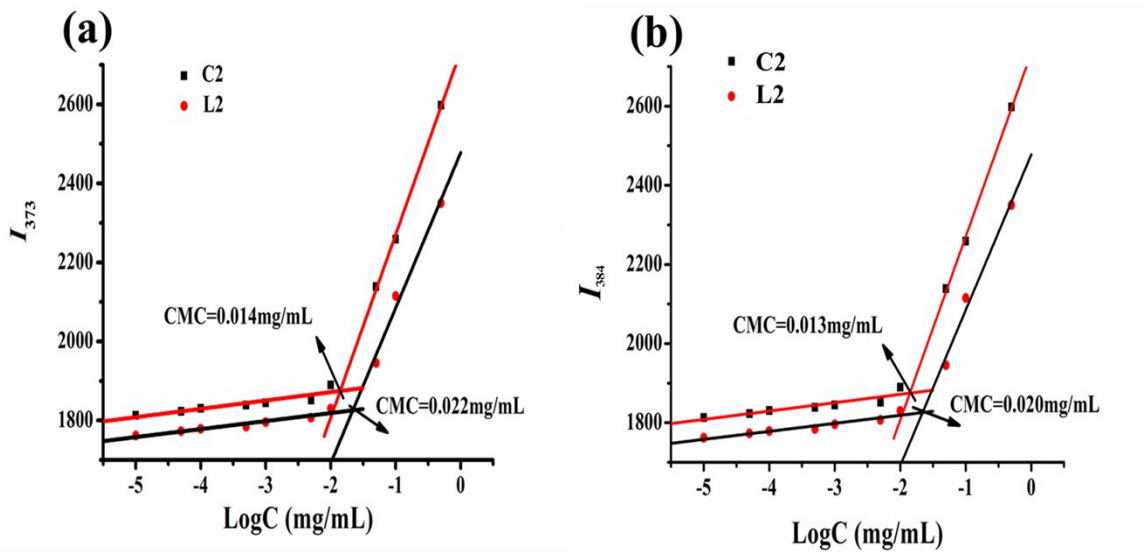

Figure S6. I_{373} (a) and I_{384} (b) in the emission spectra as a function of logarithm of L2 and C2 concentration (λex = 340 nm, [pyrene] = 2×10^{-6} M).

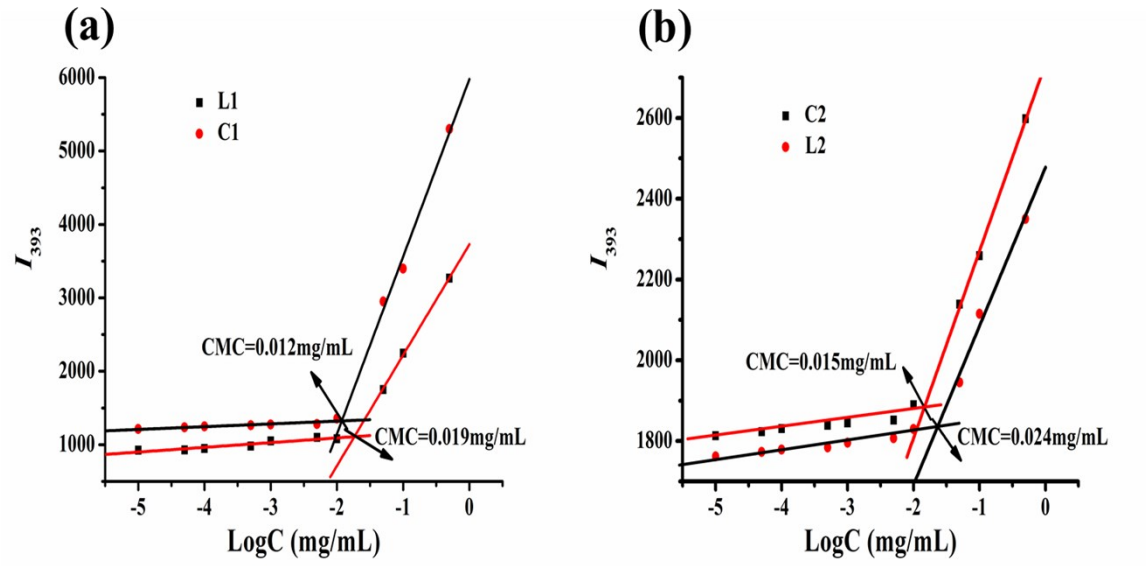

Figure S7. I_{393} in the emission spectra as a function of logarithm of (a) L1, C1 and (b) L2, C2 concentration (λex = 340 nm, [pyrene] = 2×10^{-6} M).

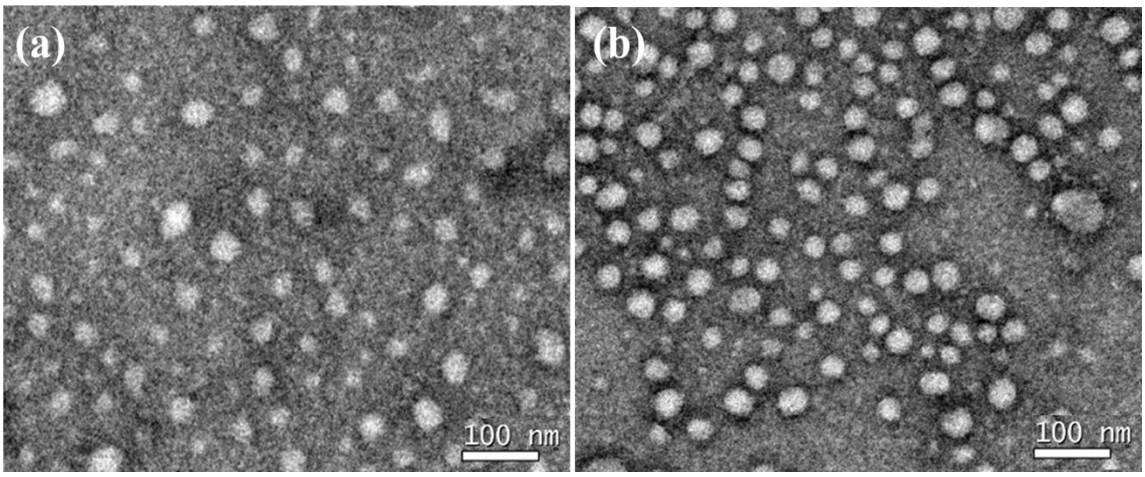

Figure S8. TEM images of (a) L2 micelles, and (b) C2 micelles in an aqueous phase at a polymer concentration of 0.25 mg ml^{-1}.

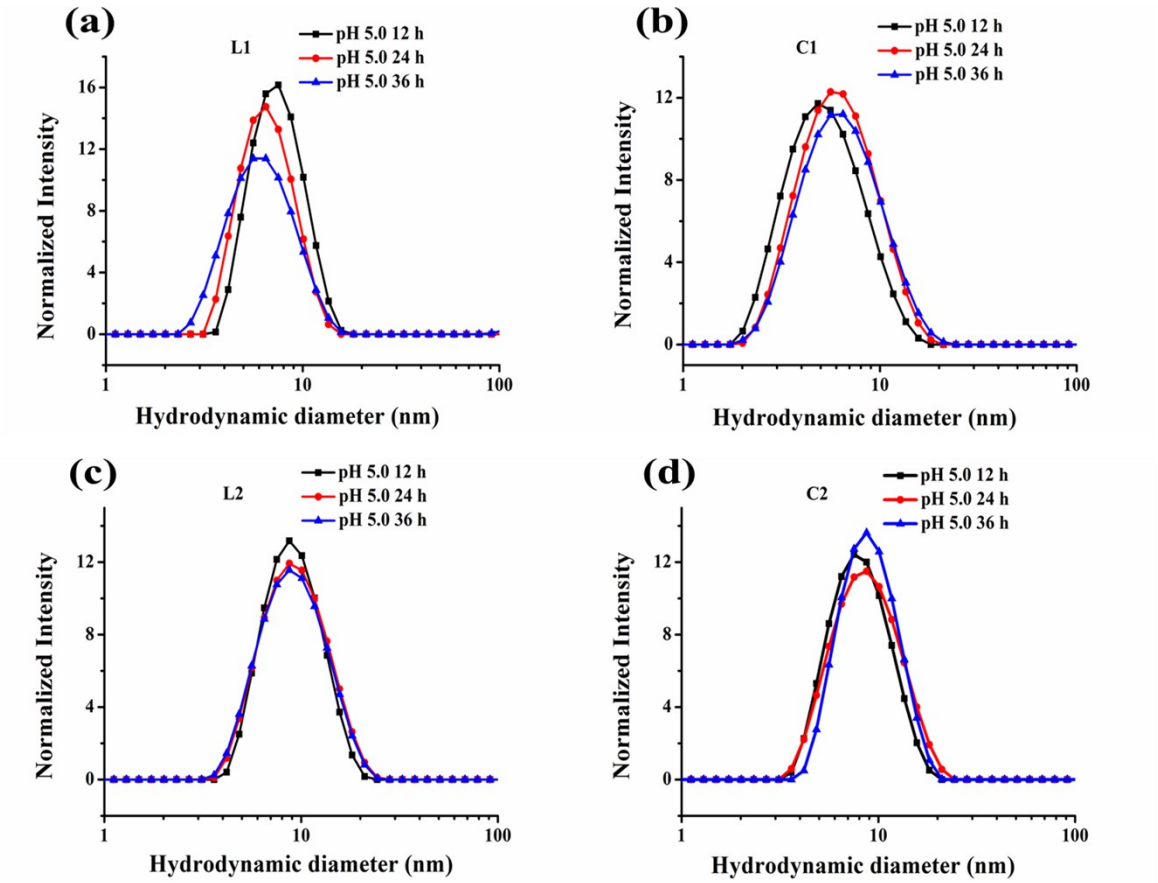

Figure S9. Mean hydrodynamic size of the self-assembled C and L micelles in PBS 5.0 at a polymer concentration of 0.25 mg mL^{-1}.

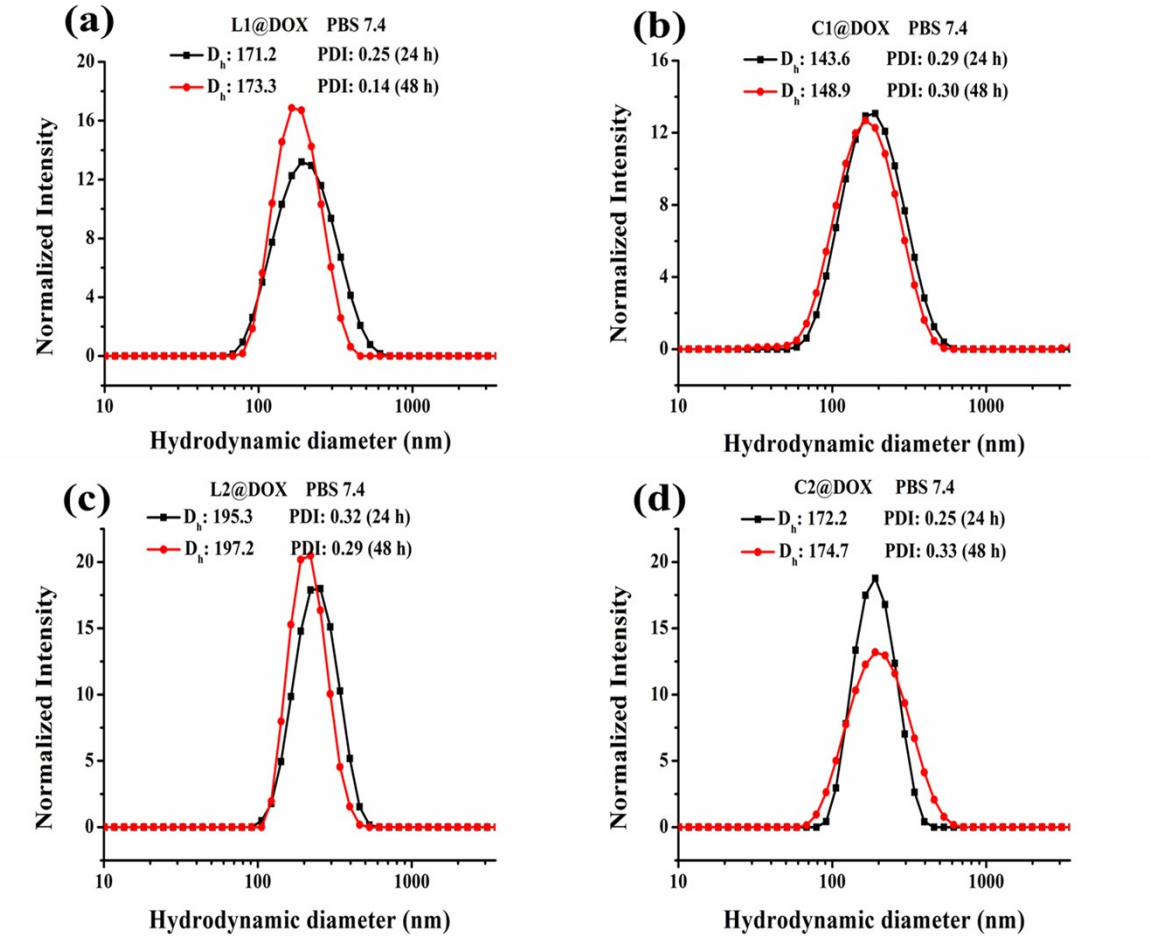

Figure S10. DLS-determined average size and size distribution of L@DOX and C@DOX micelles incubated in PBS (pH 7.4, 150 mM) for 24 and 48 h.

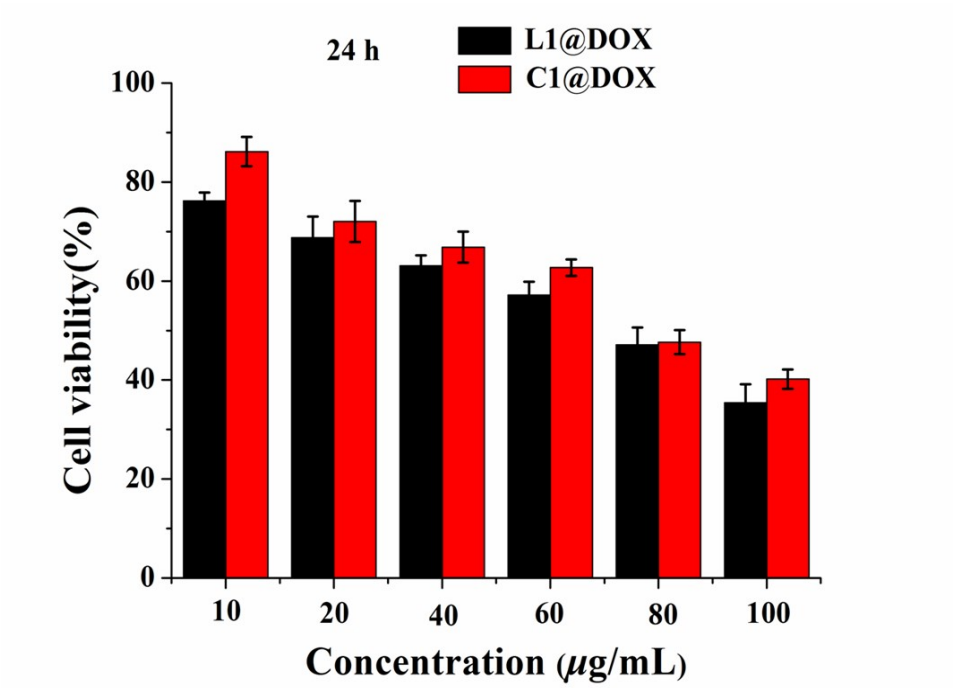

Figure S11. *In vitro* cytotoxicity of HeLa cells incubated for 24 h with L1@DOX and C1@DOX micelles at various concentrations. Cell viability was determined by MTS assay and expressed as % viability compared to control untreated cells. The data were expressed as mean ± SD, n = 3.

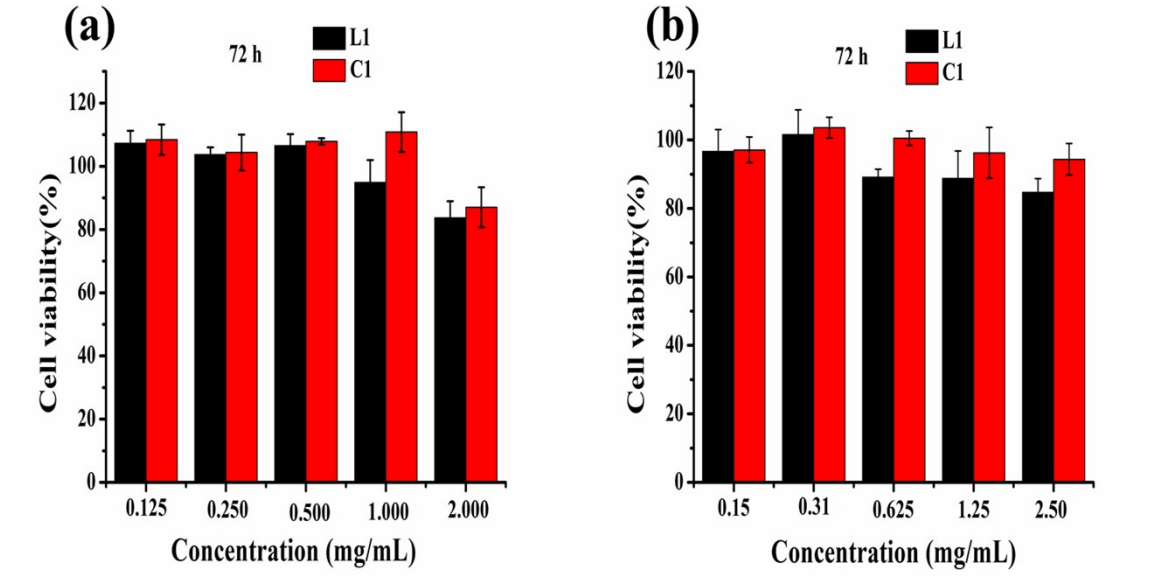

Figure S12. *In vitro* cytotoxicity of (a) HeLa cells (b) HUVEC cells incubated for 72 h with blank C1 and L1 micelles. Cell viability was determined by MTS assay and expressed as % viability compared to control untreated cells. The data were expressed as mean ± SD, n =3.

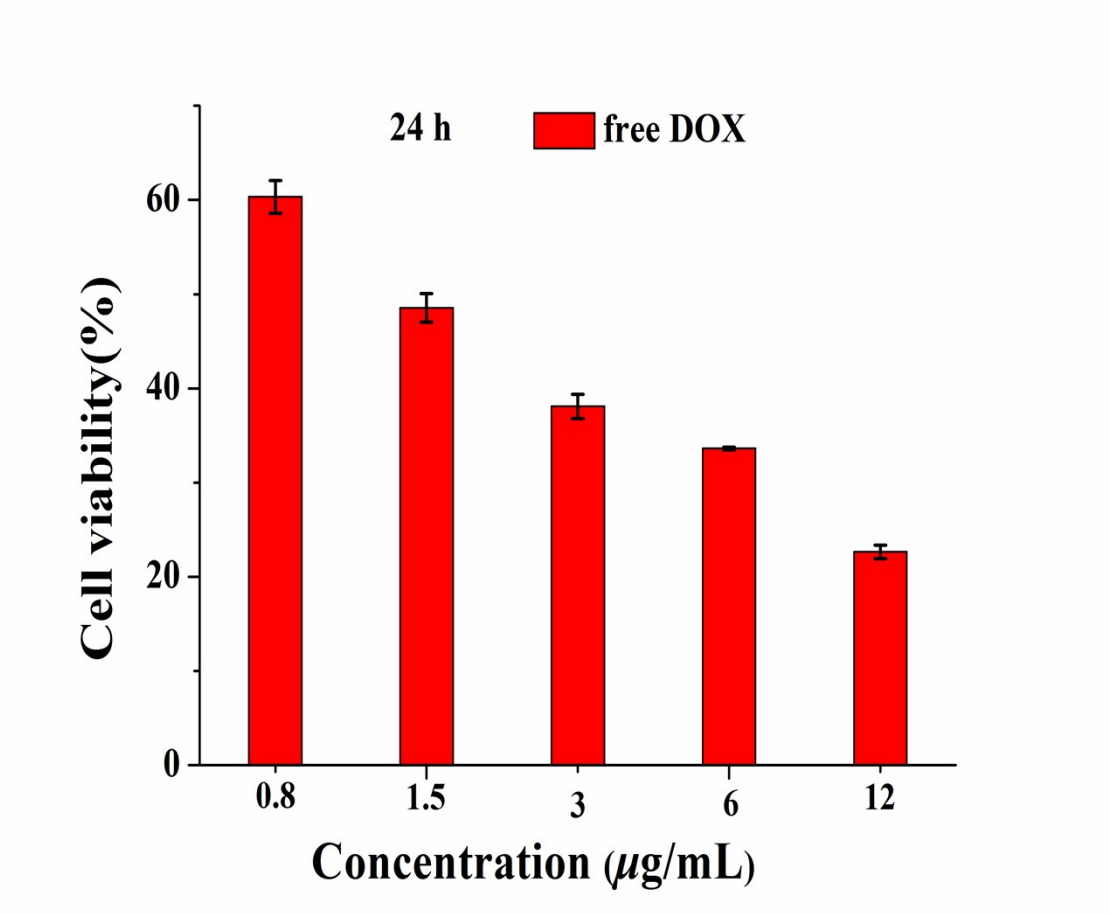

Figure S13. *In vitro* cytotoxicity of HeLa cells incubated for 24 h with free DOX at various concentrations. Cell viability was determined by MTS assay and expressed as % viability compared to control untreated cells. The data were expressed as mean ± SD, n =3.