Supporting information

of

Fabrication of Biocleavable Shell Cross-linked Hybrid Micelles for Controlled Drug Release Using a Reducible Silica Monomer

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1. Experimental Section

Materials. Oligo (ethylene glycol) monomethyl ether methacrylate (OEGMA, average M_n = 300, 4~5 EO units, Sigma-Aldrich) was purified by passing through a column filled with activated basic Al_2O_3 to remove the inhibitor. ε-Caprolactone (ε-CL, J&K, 99%) was dried over CaH_2 and distilled under reduced pressure prior to use. 2,2′-Azobis(isobutyronitrile) (AIBN, J&K, 99%) was recrystallized twice from ethanol. 1,1'-Carbonyldiimidazole (CDI, J&K, 98%), 3-aminopropyltriethoxysilane (APTES, J&K, 98%), *N*,*N*′-dicyclohexylcarbodiimide (DCC, J&K, 98%) and 4-dimethylamino pyridine (DMAP, J&K, 99%) were used without further purification. Stannous (II) octanoate ($Sn(Oct)_2$, 92.5%-100%), *N*,*N*-dimethylacetamide (DMAc) and triethylamine (TEA, 99%) were purchased from Sigma-Aldrich and used as received. Doxorubicin (DOX) was obtained by neutralization of DOX·HCl with $NaHCO_3$/Na_2CO_3 according to the reported procedures.[1] 4-Cyanopentanoic acid dithiobenzoate (CPADB)[2] and 2-((2-hydroxyethyl)disulfanyl)ethyl methacrylate (HSEMA)[3] were synthesized according to the references. MCF-7 cells resistant to DOX (MCF-7/DOX^R) were purchased from Mei Xuan Biotech Co., Ltd. (China), MCF-7 cells and Hela cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), both cells were cultured in RPMI 1640 media containing 10% FBS at 37 °C in a humidified atmosphere with 5% CO2. Dichloromethane (DCM), tetrahydrofuran (THF), toluene, and *n*-hexane were purchased from Tianjin Chemical Reagent Factory (China) and subjected to further purification following the standard protocols.

Instruments. ^{1}H NMR spectra were recorded on a JNM-ECS 400 MHz spectrometer (JEOL, Tokyo, Japan) operated in the Fourier transform mode using deuterated chloroform ($CDCl_3$) as the solvents and tetramethylsilane (TMS) as the internal reference. The size-exclusion chromatography and multi-angle laser light scattering (SEC-MALLS) analyses were used to determine the molecular weight (M_n) and molecular weight distribution ($Đ_M$) of the prepared polymers. SEC using HPLC-grade DMF containing 0.1 wt% LiBr at 60 ^{o}C as the eluent at a flow rate of 1 mL min^{-1}, Tosoh TSK-GEL R-3000 and R-4000 columns (Tosoh Bioscience, Montgomeryville, PA, USA) were connected in series to a Agilent 1260 series (Agilent Technologies, Santa Clara, CA, USA), an interferometric refractometer (Optilab-rEX, Wyatt Technology, anta Barbara, CA, USA) and a MALLS device (DAWN EOS, Wyatt

Technology, Santa Barbara, CA,USA). The MALLS detector was operated at a laser wavelength of 690.0 nm. Dynamic light scattering (DLS) was performed on a Zetasizer (Nano ZS, Malvern, Worcestershire, UK) at a fixed detection angle of 173°. TEM images were recorded on a JNM-2010 instrument operating at an acceleration voltage of 200 keV. The specimens for TEM observation were prepared by placing a drop of micelle solution onto a carbon-coated copper grid. After deposition, excess solution was removed using a strip of filter paper. The sample was further stained using phosphotungstic acid (1% w/w) and dried in air prior to visualization. UV-vis absorbance was measured at room temperature by UV-1780 UV-vis spectrophotometer (Shimadzu Corporation, Japan). Fluorescence spectra were recorded on a LS55 luminescence spectrometer (Perkin-Elmer, Waltham, MA, USA).

Synthesis of 2-hydroxyethyl 4-cyano-4-(phenylcarbonothioylthio)pentanoate (CPADB-OH). CPADB-OH was synthesized according to the reported procedures[4]. Briefly, ethylene glycol (0.49 g, 7.9 mmol), DCC (0.89 g, 4.3 mmol), DMAP (0.05 g, 0.4 mmol) and dry DCM (50 mL) were charged into a 100 mL of round-bottom flask, which was cooled to 0 °C in an ice bath. 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (CPADB, 1.10 g, 3.9 mmol) in 10 mL of dry DCM was later added dropwise over a period of 30 min under vigorous magnetic stirring. After the addition, the mixture was allowed to stir for 0.5 h at 0°C and further stirred for another 4 h at room temperature. After filtration and evaporation of all the solvents, the residues were purified by column chromatography on silica gel (ethyl acetate : hexane = 1:3, v/v) to yield the product as a red oil. (0.85 g, yield: 67.3%). ^{1}H NMR (**Figure S1**, $CDCl_3$, 400 MHz): δ_{ppm}, 7.88 (d, 2H), 7.55 (t, 1H), 7.38 (t, 2H), 4.25 (m, 2H), 3.83 (m, 2H), 2.72 (m, 2H), 2.63 (m, 1H), 2.43 (m, 1H), 1.93 (s, 3H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ_{ppm}, 222.2, 171.8, 144.5, 132.9, 128.6, 126.6, 118.5, 66.6, 61.0, 45.7, 33.4, 29.7, 24.2.

Synthesis of 2-((2-(methacryloyloxy)ethyl)disulfanyl)ethyl 1H-imidazole-1-carboxylate (HSEMA-CDI). 2-((2-hydroxyethyl)disulfanyl)ethyl methacrylate (HSEMA) (2.22 g, 10.0 mmol) was dissolved in 20 mL of dry DCM. CDI (1.95 g, 12.0 mmol) was later added to the above solution and the mixture was stirred at room temperature until the complete reaction of HSEMA. The reaction mixture was washed 3 times with saturated saline solution. The

organic phase was combined and dried over anhydrous Na_2SO_4. After filtration and evaporation of the solvent, HSEMA-CDI was obtained as colorless oil (3.10 g, yield: 98.4%). ^{1}H NMR (**Figure S4**, $CDCl_3$, 400 MHz) : δ_{ppm}, 8.13 (d, 1H), 7.42 (m, 1H), 7.06 (dd, 1H), 6.11 (s, 1H), 5.58 (s, 1H), 4.66 (t, 2H), 4.41 (t, 2H), 3.05 (t, 2H), 2.99 (t, 2H), 1.93 (s, 3H).

Synthesis of 2-((2-(methacryloyloxy) ethyl)disulfanyl)ethyl (3-(triethoxysilyl)propyl)carbamate (TESSPMA).

HSEMA-CDI (14.46 g, 45.7 mmol) and 3-aminopropyltriethoxysilane (APTES, 10.6 g, 48.0 mmol) were dissolved in 100 ml of dry DCM. DMAP (1.4 g, 11.5 mmol) was later added and the mixture was stirred overnight at room temperature. After removal of the solvent, the product was purified by flash column chromatography (ethyl acetate : hexane = 1:3, v/v) to yield the product as a light yellow oil (16.5 g, yield: 76.8%). ^{1}H NMR (**Figure S2**, $CDCl_3$, 400 MHz): δ_{ppm}, 6.12 (s, 1H), 5.58 (s, 1H), 5.07 (s, 1H), 4.38 (t, 2H), 4.27 (t, 2H), 3.78 (q, 2H), 3.17 (m, 2H), 2.94 (t, 2H), 2.89 (t, 2H), 1.93 (s, 3H), 1.61 (m, 2H), 1.19 (t, 3H), 0.60 (t, 2H). ^{13}C NMR (**Figure S3**, $CDCl_3$, 400 MHz): δ_{ppm}, 167.1, 156, 136.2, 126.1, 62.6, 62.2, 58.4, 43.4, 38.0, 36.9, 31.3, 23.2, 18.2, 7.6.

Synthesis of PCL macro-chain transfer agents (PCL-CPADB). PCL-CPADB was synthesized according to the reported procedures.[5, 6] Briefly, CPADB-OH (0.14 g, 0.43 mmol), ε-CL (0.868 g, 7.61 mmol), $Sn(Oct)_2$ (0.01 g, 0.025 mmol) and toluene (2.47 mL) were loaded in a thoroughly dried 10 mL of Schlenk tube with a magnetic stirring bar under nitrogen atmosphere. The reaction tube was degassed by three freeze-pump-thaw cycles and then immersed in an oil bath preheated to 90 °C to start the polymerization. After 24h, the mixture was precipitated in 10-fold ice-cold methanol three times. The purified product was finally dried under vacuum until constant weight (yield: 83%).

Synthesis of PCL-b-PTESSPMA diblock copolymer using PCL-CPADB as a macro-CTA. PCL-*b*-PTESSPMA diblock copolymer was synthesized by RAFT polymerization using PCL-CPADB as a macro-CTA and AIBN as an initiator. The typical procedure was as follows, TESSPMA (0.378 g, 0.81 mmol), PCL-CPADB (0.085 g, 0.027 mmol), AIBN (1.4 mg, 0.009

mmol) and DMAc (1.62 mL) were loaded in a thoroughly dried 10 mL of Schlenk tube with a magnetic stirring bar. After three freeze-pump-thaw cycles, the mixture solution was immersed in an oil bath thermostated at 70 °C for 90 min. The polymerization was stopped by exposing the reaction mixture to air. The reaction mixture was diluted with 1 mL of THF followed by precipitation in10-fold ice-cold hexane three times. The purified product was finally dried under vacuum until constant weight (yield: 69%).

Synthesis of PCL-b-PTESSPMA-b-POEGMA amphiphilic triblock copolymer using PCL-b-PTESSPMA-CPADB as a macro-CTA. PCL-*b*-PTESSPMA-*b*-POEGMA amphiphilic triblock copolymer was synthesized by RAFT polymerization using PCL-*b*-PTESSPMA-CPADB as a macro-CTA and AIBN as an initiator. The typical procedure was as follows, OEGMA (0.363 g, 1.21 mmol), PCL-*b*-PTESSPMA-CPADB (0.025 g, 0.004 mmol), AIBN (0.2 mg, 0.001 mmol) and DMAc (1.21 mL) were loaded in a thoroughly dried 10 mL of Schlenk tube with a magnetic stirring bar. After three freeze-pump-thaw cycles, the mixture solution was immersed in an oil bath thermostated at 70 °C for 53 min. The polymerization was stopped by exposing the reaction mixture to air. The reaction mixture was diluted with 1 mL of THF followed by precipitation in10-fold ice-cold hexane three times. The purified product was further dried under vacuum until constant weight (yield: 75%).

Micelle Preparation. The solution of polymeric micelles was prepared using the classical dialysis method. Taking PCL_{25}-*b*-$PTESSPMA_6$-*b*-$POEGMA_{77}$ as an example, PCL_{25}-*b*-$PTESSPMA_6$-*b*-$POEGMA_{77}$ (10.0 mg) in 1 mL of DMF was added to 8.0 mL of distilled water under vigorous stirring. The mixture solution was next subjected to dialysis (cellulose membrane, molecular-weight cut-off (MWCO), 3.5 kDa) against phosphate buffer solution (PB, pH 7.0, 5.0 mM) for 24 h, during which fresh PB buffer was replenished every 6 h.

Determination of Critical Micelle Concentration (CMC). CMC of PCL-*b*-PTESSPMA-*b*-POEGMA was determined using pyrene as the fluorescence probe. Aliquots of pyrene solutions (3×10^{-6} mol L^{-1} in acetone, 120 μL) were added to containers, and the acetone was allowed to evaporate. Ten milliliter aqueous polymer solutions at various concentrations were

subsequently added to the containers containing the pyrene residue. Note that all the aqueous sample solutions contained excess pyrene residue at an identical concentration of 6×10^{-8} mol L^{-1}. The solutions were kept at room temperature for 24 h to reach the solubilization equilibrium of pyrene in the aqueous phase. Excitation was carried at 339 nm, and emission spectra were recorded ranging from 350 to 600 nm. Both excitation and emission bandwidths were 10 nm. From the pyrene emission spectra, the intensities (peak height) of the first (I_{373}) and the third band (I_{384}) were analyzed as a function of the polymer concentrations. The CMC value was determined from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration.

Preparation of shell cross-linked (SCL) micelle solution. Taking PCL_{25}-*b*-$PTESSPMA_6$-*b*-$POEGMA_{77}$ as an example, PCL_{25}-*b*-$PTESSPMA_6$-*b*-$POEGMA_{77}$ (10.0 mg) in 1 mL of DMF was added to 8.0 mL of distilled water under vigorous stirring. 40 μL of TEA was next added to the above mixture solution. After 4 h, DMF and TEA were removed by dialysis (cellulose membrane, MWCO, 3.5 kDa) against PB buffer solution (pH 7.0, 5.0 mM) for 24 h, during which fresh PB buffer was replenished every 6 h.

In Vitro Drug Loading and Drug Release Study. DOX-loaded cross-linked micelles were prepared following the procedures similar to the preparation of blank micelles. Briefly, DOX (2.0 mg, 0.007 mmol) and PCL_{25}-*b*-$PTESSPMA_6$-*b*-$POEGMA_{77}$ (20 mg) were dissolved in 2.0 ml of DMF. The mixture was next added dropwise into 16.0 ml of distilled water under vigorous stirring. After stirring for 1 h, 80 μL TEA was added to trigger the sol-gel process, which proceeded for 4 h. The mixture solution was later transferred to a dialysis tube with a MWCO of 3.5 kDa and subjected to dialysis against 5.0 L of PB 7.0 buffer solution for 24 h to remove DMF, TEA and unloaded free DOX. The PB buffer was renewed every 3 h during the course of initial 12 h. DOX-loaded noncrosslinked (NCL) micelles were prepared following an identical procedure described above without the addition of TEA.

For in vitro drug release study, the solution of drug-loaded micelles was split in equal volumes into four dialysis tubes with a MWCO of 3.5 kDa, which were then immersed in a Falcon tube containing 25 ml of release medium (PBS, pH 7.4, 150 mM). The tube was kept

in a horizontal laboratory shaker thermostated at a constant temperature of 37 °C and a stirring speed of 120 rpm.[7] At predetermined time intervals, 3 ml of release medium was sampled and replenished with equal volume of fresh medium. The drug concentration was determined by measuring DOX absorbance at 499 nm. The concentration of released DOX from each micelle formulation was calculated based on the standard curves obtained from free DOX·HCl in the corresponding release buffers.

Finally, the encapsulation efficiency (EE) and the drug loading content (DLC) of the drug-loaded micelles were determined using the UV-vis spectrometer at 499 nm. The EE and DLC were calculated using the following equations,

$$EE\ (\%) = W_{DOX\ loaded\ in\ micelles} / W_{DOX\ fed\ for\ encapsulation} \times 100\% \quad (1)$$

$$DLC\ (\%) = W_{DOX\ loaded\ in\ micelles} / W_{DOX\ loaded\ micelles} \times 100\% \quad (2)$$

Evaluation of Cellular Uptake by Flow Cytometry (FCM). HeLa cells were seeded in 24-well plates at a plating density of 40,000 cells per well in 1.0 mL of complete growth medium and incubated for 24 h at 37 °C in 5% CO_2 environment. Next, 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·HCl, DOX-loaded NCL and SCL micelles in MEM was set at 20 μg/mL. After incubation for 4 h, the sample solution was aspirated, and the cells were rinsed twice with PBS. Cells were then harvested by incubation with 200μL of Trypsin-EDTA, followed by resuspension with 1 mL of complete growth medium. Subsequently, cells were transferred to 1.5 mL of microcentrifuge tubes and pelleted at 300g 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 10,000 cells was analyzed for each sample with the fluorescence intensity.

Cell Viability Study. The cytotoxicity of various formulations was evaluated in vitro using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetr zolium (MTS, Promega) assay. HeLa cells were seeded in 96-well plates at a plating density of 2000

cells per well in 100 µl of complete growth medium and incubated in an incubator maintained at 37 °C and 5% CO_2 environment for 24 h. Free DOX·HCl, blank micelles , drug-loaded NCL and SCL micelles were prepared in serial dilutions in PB 7.0 buffer solution and then diluted in 10-fold in Opti-MEM medium (Invitrogen). The cells were then rinsed once with PBS and incubated with 100 µl of the sample solutions with different polymer or DOX·HCl concentrations at 37 °C for various periods of 24, 48 and 72 h. Cells were next rinsed with PBS and the medium was replaced with 100 µl of culture medium. After addition of 20 µl of MTS reagent to each well, cells were then incubated at 37 °C and 5% CO_2 for 3 h. The absorbance of each well was measured at a wavelength of 490 nm on a Tecan Safire2 plate reader (Männerdorf, Switzerland). Cell viability for each treatment condition was determined by normalizing to the cells only signal.

References

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Table S1. Summary of MW, DP and $Đ_M$ of all the synthesized polymers.

Sample	M_n[a] (kDa)	DP[a]	M_n[b] (kDa)	$Đ_M$[b]
PCL_{25}-CPADB (Macro-CTA_1)	3.18	25	3.0	1.08
PCL_{25}-*b*-$PTESSPMA_6$-CPADB (Macro-$CTA_{2\text{-}1}$)	5.99	6	9.3	1.22
PCL_{25}-*b*-$PTESSPMA_6$-*b*-$POEGMA_{77}$ (P_1)	29.1	77	35.1	1.27
PCL_{25}-*b*-$PTESSPMA_{11}$-CPADB (Macro-$CTA_{2\text{-}2}$)	8.34	11	11.8	1.22
PCL_{25}-*b*-$PTESSPMA_{11}$-*b*-$POEGMA_{74}$ (P_2)	30.5	74	44.7	1.27

[a]Determined by ^{1}H NMR;

[b]Determined by SEC-MALLS.

Figure S1-S13

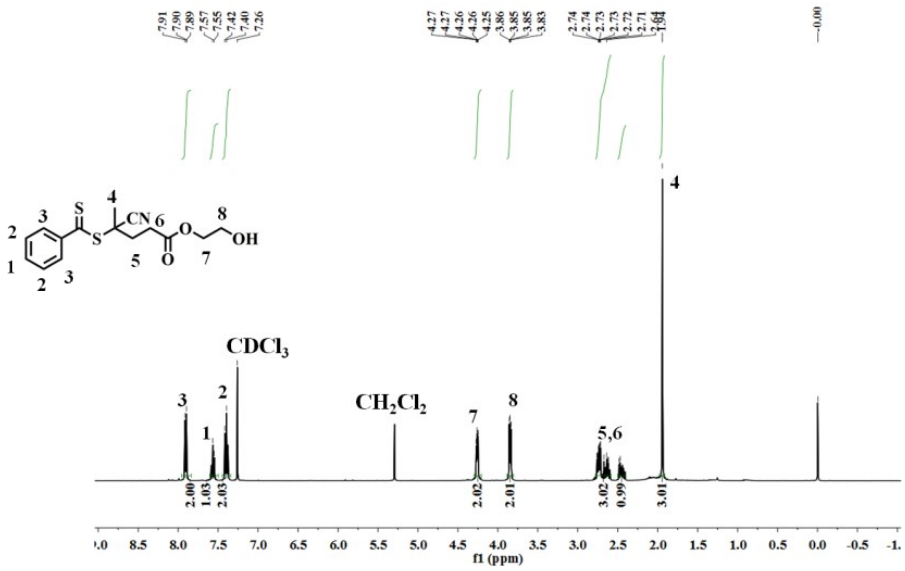

Figure S1. ^{1}H NMR spectrum of CPADB-OH in $CDCl_3$.

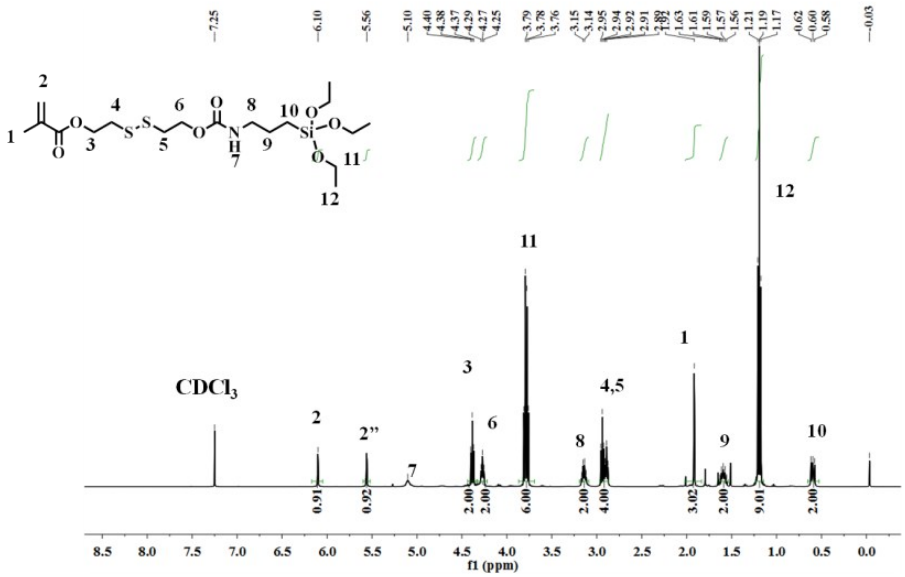

Figure S2. ^{1}H NMR spectrum of TESSPMA in $CDCl_3$.

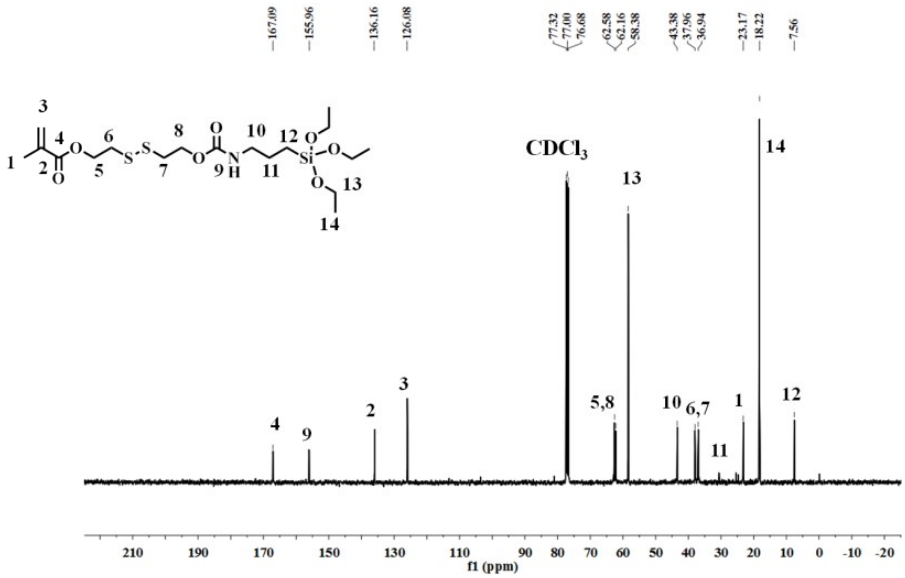

Figure S3. ^{13}C NMR spectrum of TESSPMA in $CDCl_3$.

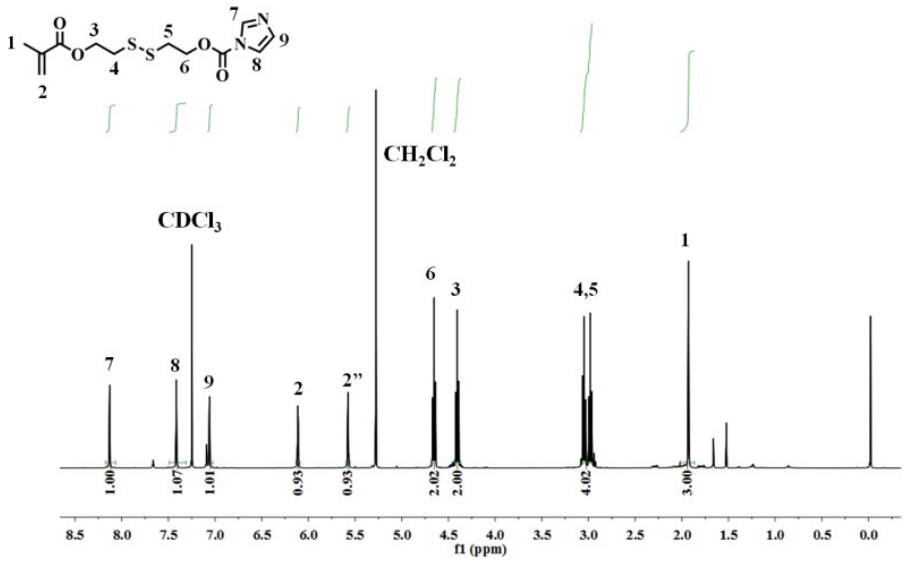

Figure S4. ^{1}H NMR spectrum of HSEMA-CDI in $CDCl_3$

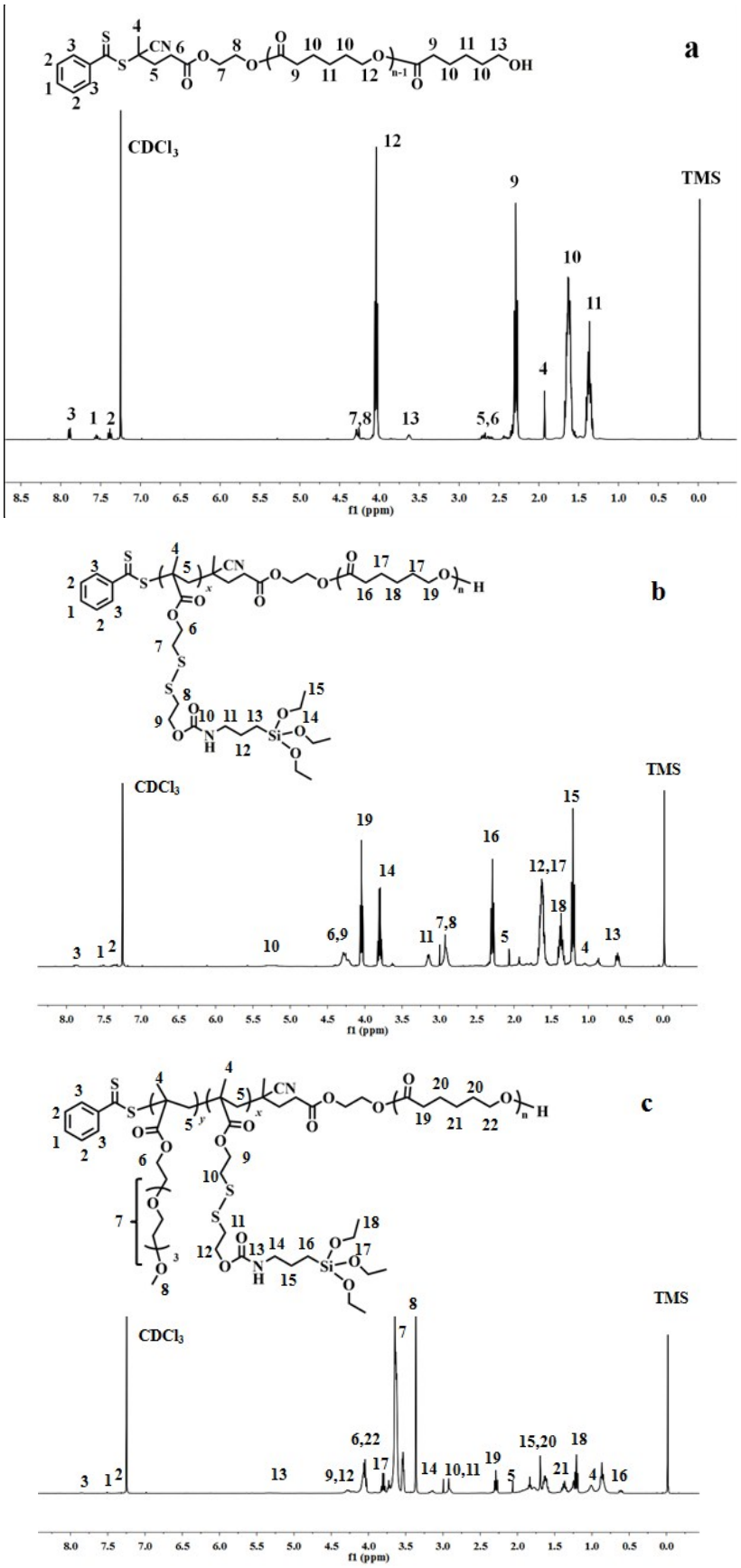

Figure S5. ^{1}H NMR spectra of (a) PCL_{25}-CPADB, (b) PCL_{25}-*b*-$PTESSPMA_6$-CPADB, and (c) PCL_{25}-*b*-$PTESSPMA_6$-*b*-$POEGMA_{77}$ in $CDCl_3$.

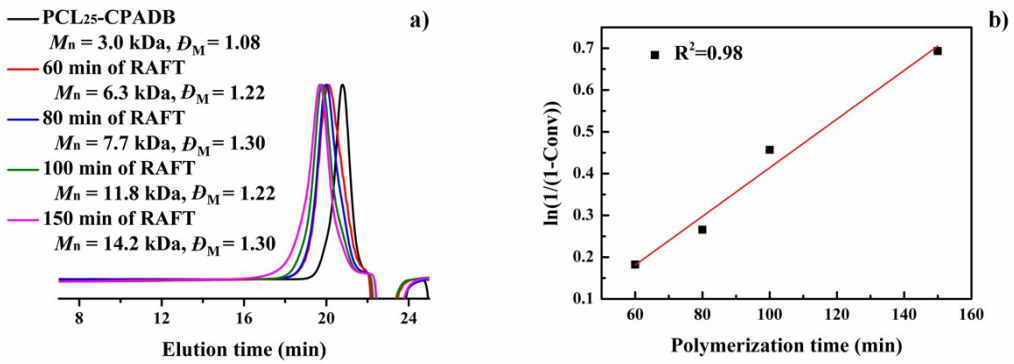

Figure S6. RAFT polymerization presdo-kinetics study of TESSPMA using PCL_{25}-CPADB as a macro-CTA ([M]/ [CTA] /[I] = 30:1:0.33, T = 70 °C, DMAc).

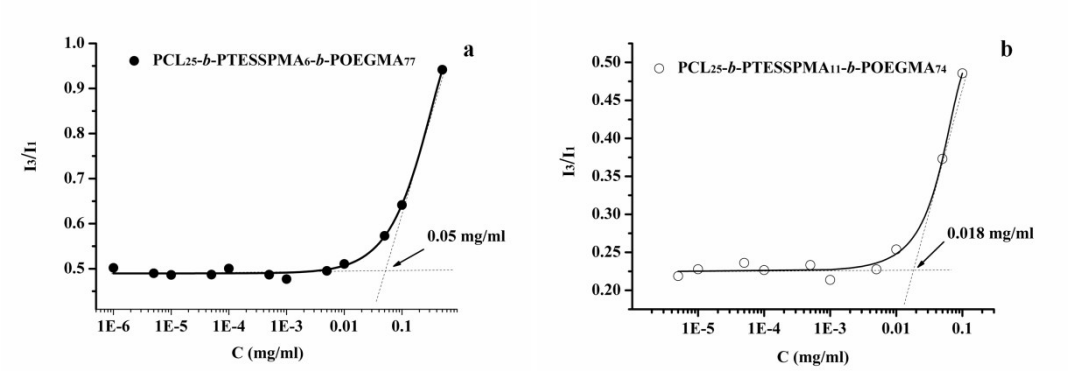

Figure S7. Plots of fluorescence intensity ratios (I_{384}/ I_{373}, I_3/I_1) as function of logarithm of concentrations of (a) PCL_{25}-*b*-$PTESSPMA_6$-*b*-$POEGMA_{77}$, (b) PCL_{25}-*b*-$PTESSPMA_{11}$-*b*-$POEGMA_{74}$.

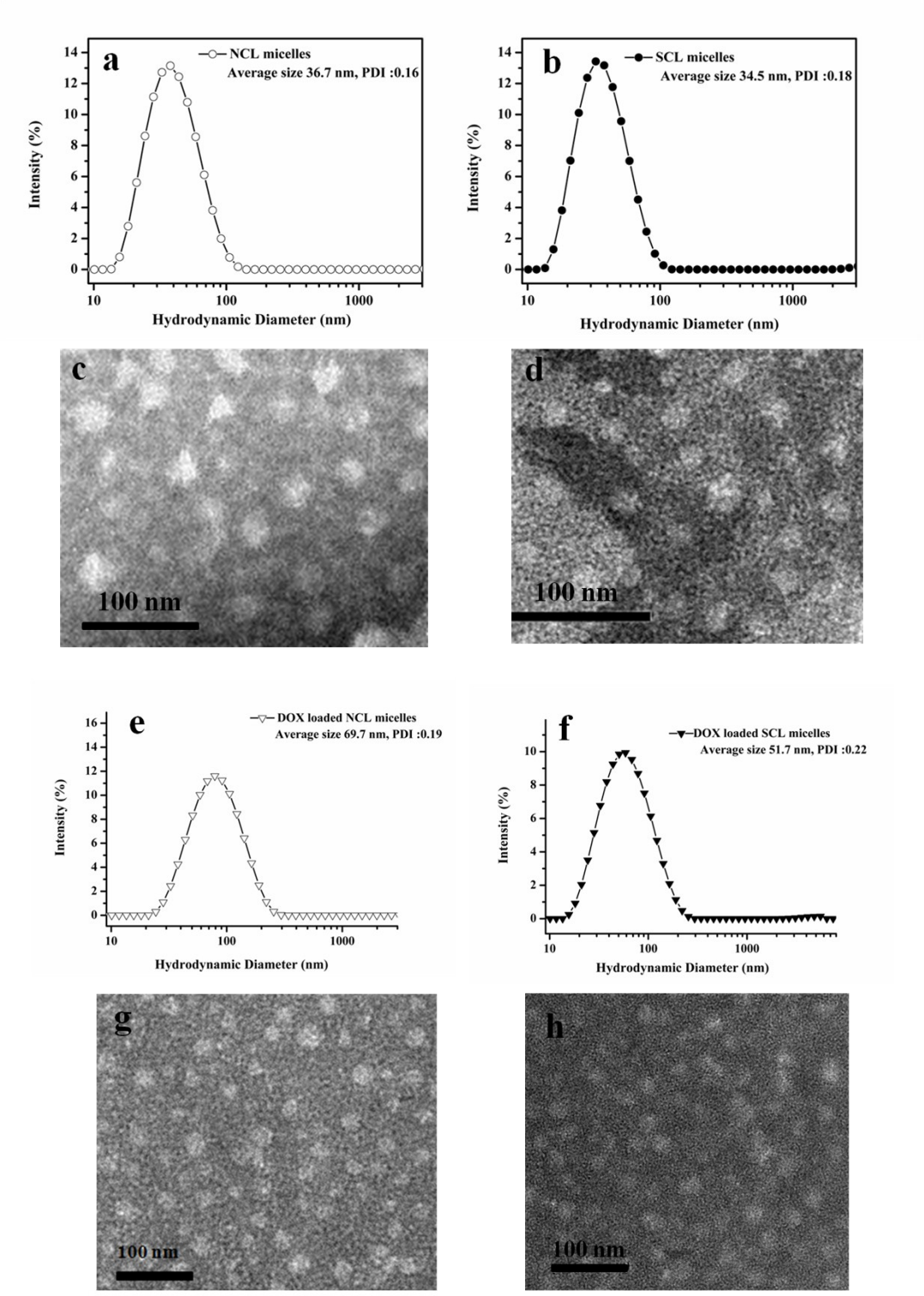

Figure S8. Size distributions and TEM images of PCL_{25}-*b*-$PTESSPMA_6$-*b*-$POEGMA_{77}$ (P_1)-based blank (a & c) NCL and (b & d) SCL micelles, and DOX-loaded (e & g) NCL and (f & h) SCL micelles in an aqueous phase.

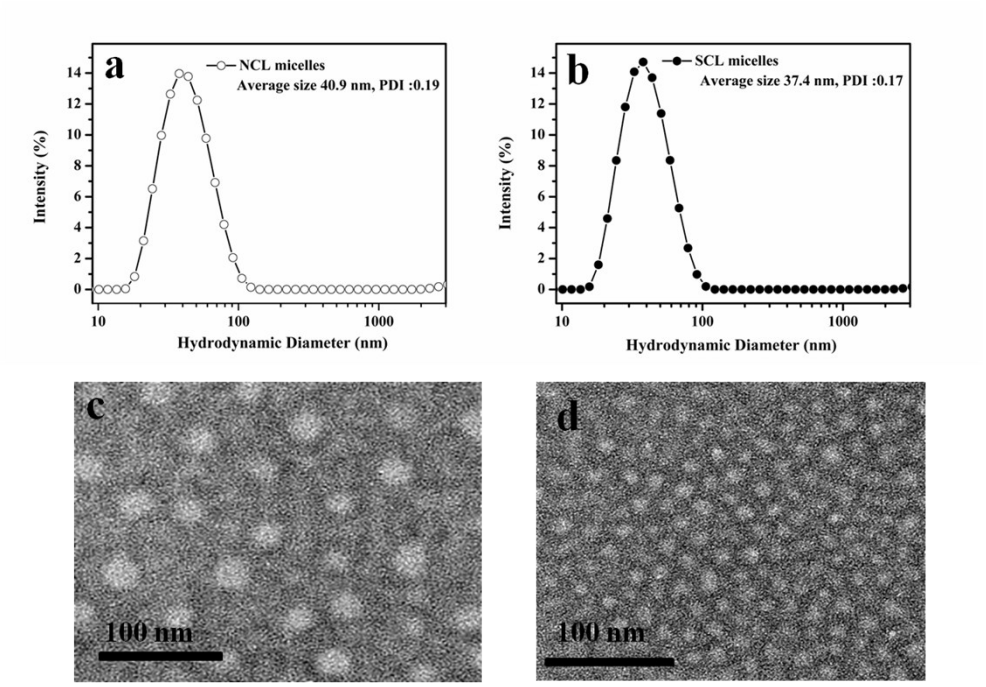

Figure S9. Size distributions and TEM images of (a & c) NCL and (b & d) SCL micelles of PCL_{25}-*b*-$PTESSPMA_{11}$-*b*-$POEGMA_{74}$ in an aqueous phase.

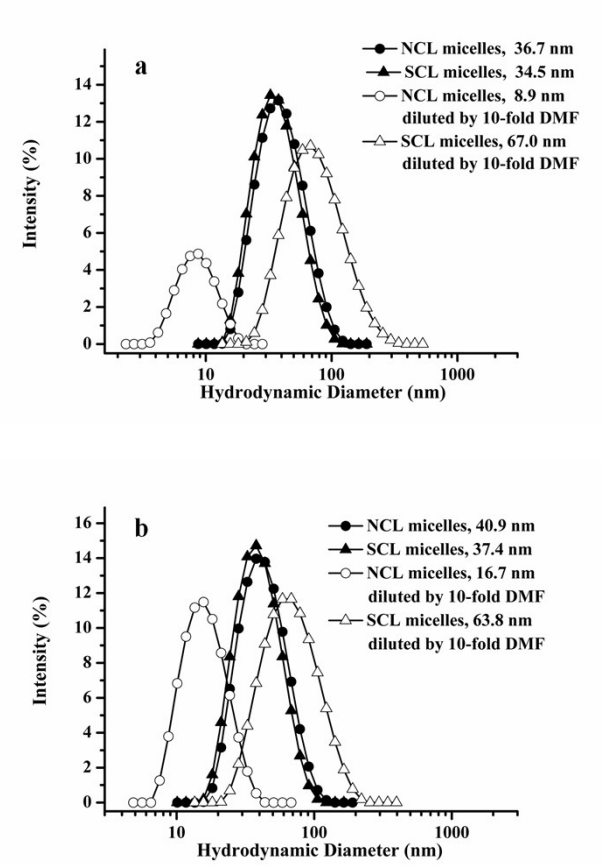

Figure S10. Size distributions of (a) P_1 and (b) P_2-based (●) NCL and (▲) SCL micelles in water, and (○) NCL and (△) SCL micelles diluted by10-fold amount of DMF.

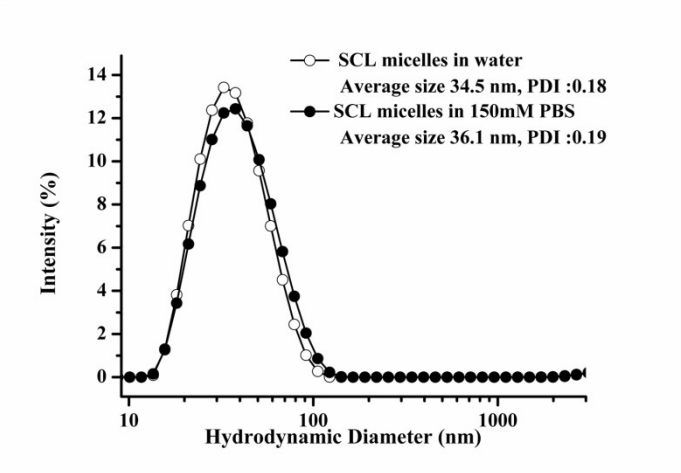

Figure S11. Size distribution of P_1-based SCL micelles in (○) water and (●) PBS (pH 7.4, 150 mM).

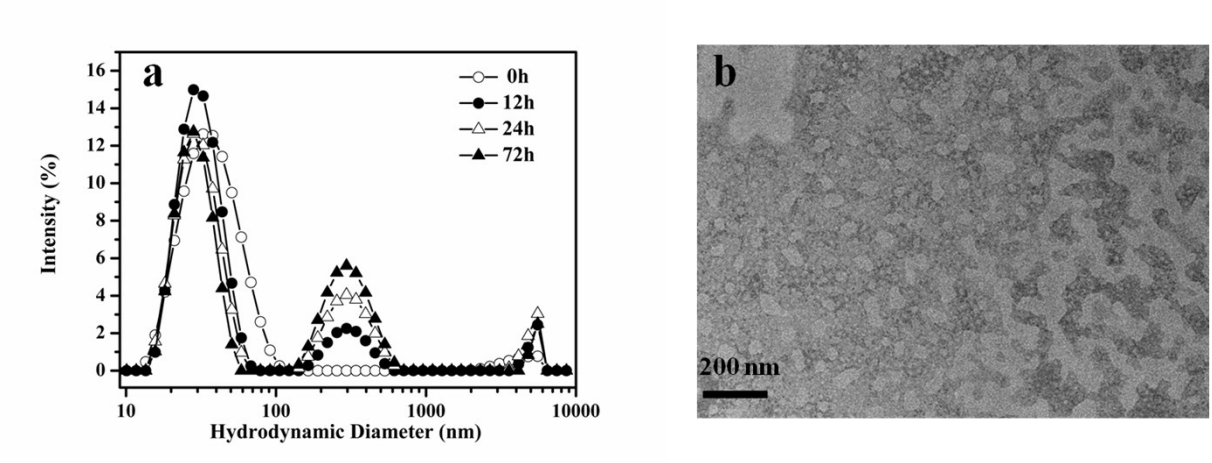

Figure S12. (a) DLS-monitored size variations of P_1-based SCL micelles incubated with 10 mM DTT for different periods of 12, 24, and 72 h, and (b) TEM image of P_1-based SCL micelles after incubation with 10 mM DTT for 72 h.

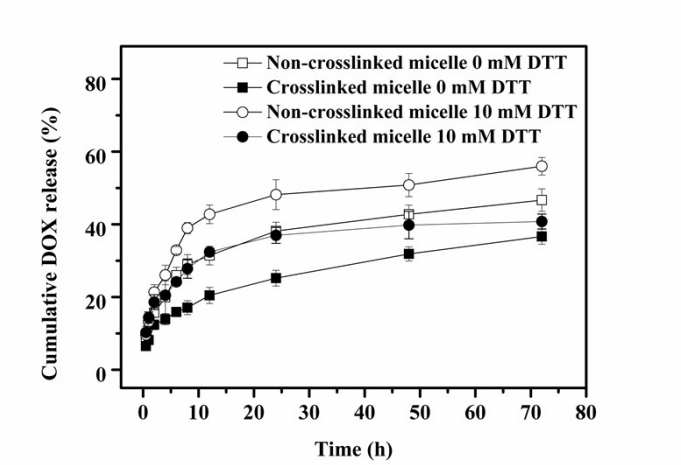

Figure S13. *In vitro* drug release profiles of DOX-loaded in NCL and SCL micelles under various conditions at 37 °C. The data were expressed as mean ± SD, n = 3.

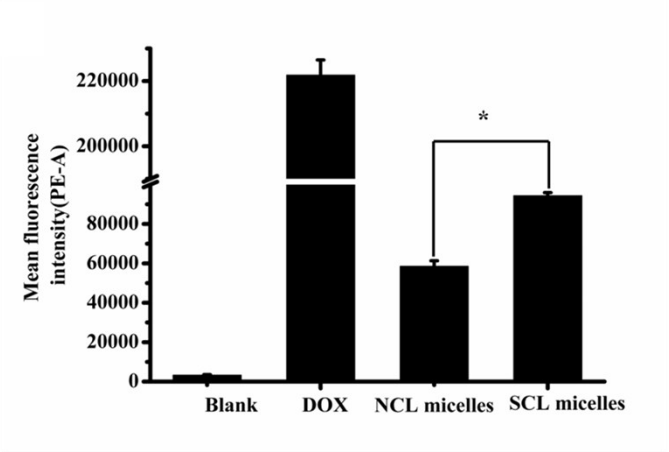

Figure S14. Quantitive measurement of the mean fluorescence intensity after incubation with free DOX·HCl, DOX-loaded NCL and SCL micelles in HeLa cell lines via FCM (4h incubation, DOX concentration ≈ 20 μg/mL, and 10000 cells were counted). The data were expressed as mean ± SD (n = 3; Student's t-test, *p < 0.005).

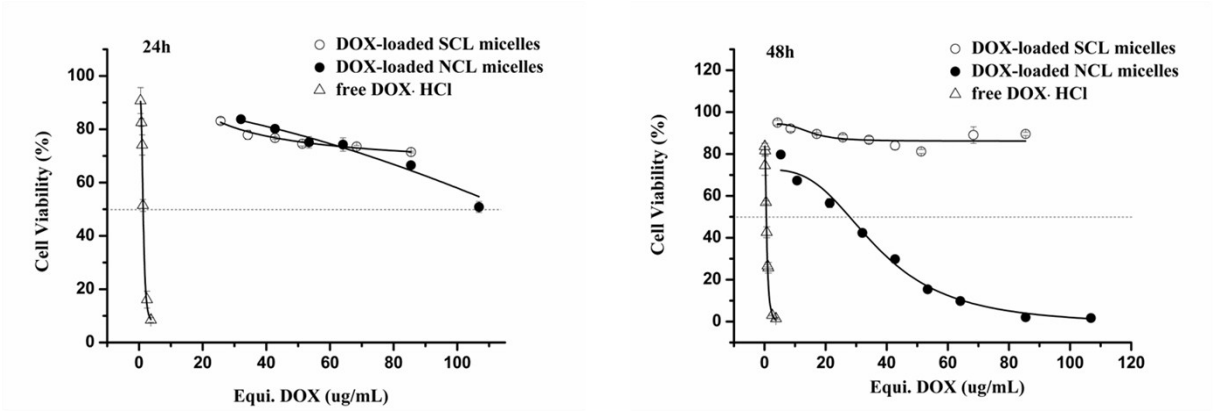

Figure S15. *In vitro* cytotoxicity of free DOX, DOX-loaded SCL and NCL against HeLa cells as determined by MTT assay. Cells were treated with designated regimes for 24 h and 48 h. The data were expressed as mean ± SD, n = 3.

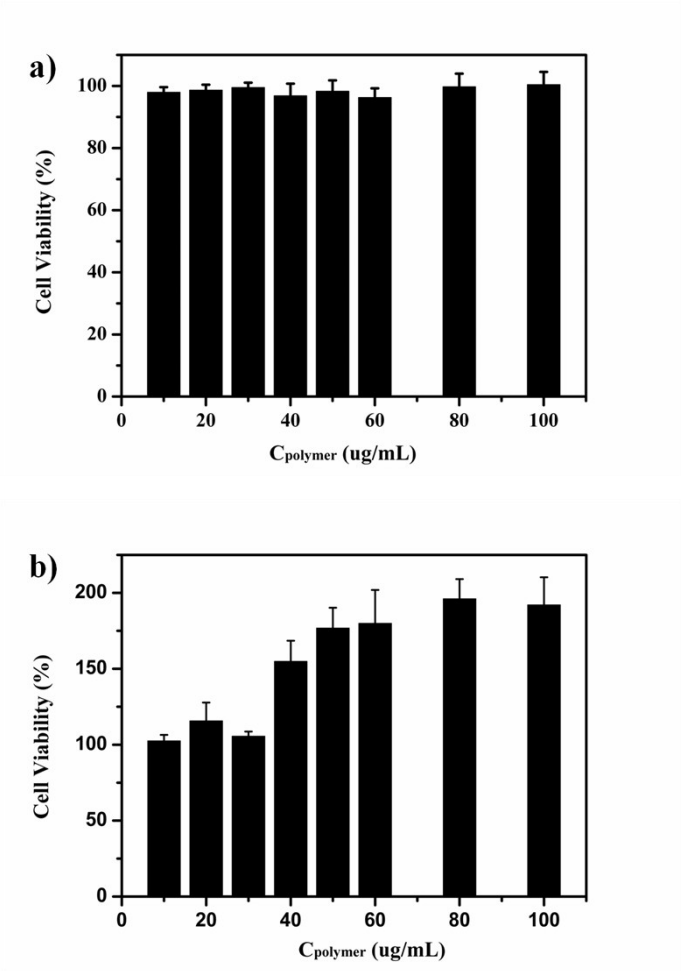

Figure S16. *In vitro* cytotoxicity of P_1-based blank SCL micelles in HeLa (a) and MCF-7 (b) cells. Cell viability was determined by MTS assay and expressed as % viability compared to the untreated cells control. The data were expressed as mean ± SD, n = 3.

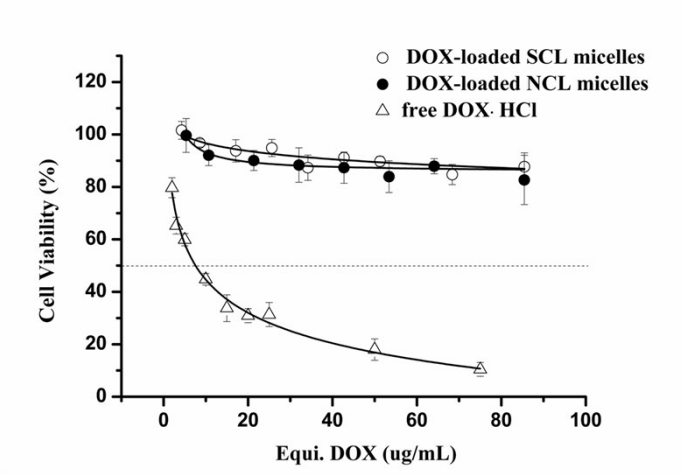

Figure S17. *In vitro* cytotoxicity of free DOX, DOX-loaded SCL and NCL micelles in MCF-7/DOX^R cells. The data were expressed as mean ± SD, n = 3.