Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting information

Promotion of micelle stability via a cyclic hydrophilic moiety

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1. Experimental section

Materials

ε-Caprolactone (CL) purchased from Sigma-Aldrich was dried over CaH_2 and distilled under reduced pressure prior to use. Oligo(ethylene glycol) monomethyl ether methacrylate (OEGMA, M_n= 300 g/mol and 4~5 pendent EO units) from Sigma-Aldrich was purified by passing through a column filled with basic alumina to remove the inhibitor. 2,2'-bis(hydroxymethyl) propionic acid (BMPA, 99%), *p*-toluenesulfonic acid monohydrate (PTSA, 99%), 2,2'-dimethoxypropane (DMP, 98%), 4-dimethylaminopyridine (DMAP, 99%), N, N'-dicyclohexylcarbodiimide (DCC, 99%), Dowex 50W-X4-200 H^+ resin, 2-bromoisobutyric acid (98%) were purchased from J&K , copper(I) bromide (CuBr), bipyridine (bpy), stannous(II) octanoate ($Sn(Oct)_2$) were purchased from Sigma-Aldrich, *N, N, N', N'', N''*-pentamethyldiethylenetriamine (PMDETA) was supplied by Aladdin and used as received. Acetone, ethanol (EtOH), Dichloromethane (CH_2Cl_2, DCM) and toluene were dried by refluxing over sodium and distilled prior to use. Propargyl alcohol were purchased from Tianjin Chemical Reagent Factory (China). Sodium azide (NaN_3, Sanyou, Shanghai), anisole (Kelong, Chengdu, China), ammonia solution (25 %, guangfu, Tianjin, China) and other reagents were used as received without further purification.

Synthesis of Isopropylidene-2,2-bis(methoxy) propionic Acid [1]

BMPA (30.0 g, 223.7 mmol), DMP (41.4 ml, 335.4 mmol) and PTSA (2.1g, 11.1 mmol) were dissolved in acetone (400 ml). The mixture was stirred for 4 h at room temperature before 3.0 ml of mixture of an ammonia solution (25%) and EtOH (50/50, v/v) was added into the reaction mixture to neutralize the catalyst. The solvent was removed by evaporation under reduced pressure at room temperature. The residue was then dissolved in CH_2Cl_2 (600 ml), and extracted with two portions of water (80 ml). The organic phase was dried with anhydrous $MgSO_4$ and evaporated to give white powder (26.03 g, yield: 66.5%). ^{1}H NMR (400 MHz, ppm, $CDCl_3$): δ4.20-4.13 (d, 2H), 3.72-3.64 (d, 2H), 1.47-1.38 (d, 6H), 1.22-1.16 (s, 3H). ^{13}C NMR (400 MHz, ppm, $CDCl_3$): δ180.5, 98.4, 65.9, 41.8, 25.2, 22.1, 18.5.

Synthesis of Propargyl 2,2,5-trimethyl-1,3-dioxane-5-carboxylate

Isopropylidene-2,2-bis(methoxy) propionic acid (4 g, 23.0 mmol) was dissolved in anhydrous DCM (50 ml), and the solution was cooled to 0 °C. DCC (5.27 g, 25.3 mmol) was added to the reaction mixture under N_2 atmosphere followed by adding DMAP (0.88 g, 11.5 mmol) in the mixture after 10 min. Then propargyl alcohol (1.61 ml, 27.6 mmol) was added to the reaction mixture. The reaction mixture was allowed to stir for 30 min in an ice bath and further stirred overnight at room temperature. White dicyclohexyl urea (DCU) precipitate formed during the reaction was removed by vacuum filtration. Then the solvent was removed by rotary evaporation, and the product was purified by flash column chromatography on silica, eluting with hexane (1 L), followed by ethyl acetate/hexane (1/9, v/v) to give propargyl 2,2,5-trimethyl-

1,3-dioxane-5-carboxylate as a pale-yellow oil (4.18 g, 85.8 %). ^{1}H NMR (400 MHz, ppm, $CDCl_3$): δ 4.74 (d, 2H), 4.20 (d, 2H), 3.67 (d, 2H), 2.50 (t, 1H), 1.46-1.37 (d, 6H), 1.22 (s, 3H). ^{13}C NMR (400 MHz, ppm, $CDCl_3$): δ 173.5, 98.2, 75.1, 65.9, 52.4, 42.0, 24.7, 22.7, 18.5.

Synthesis of propargyl 2,2-bis(hydroxymethl) propanoate (PHP)[1]

DOWEX 50W-X4-200 resin (2 g) were added to a solution of Propargyl 2,2,5-trimethyl-1,3-dioxane-5-carboxylate (5 g, 23.6 mmol) in methanol (100 ml) in a 250 ml round bottom flask. The mixture was stirred at 30 ^{o}C for 1h. The resin was filtered off and the filtrate was concentrated and dried under high vacuum to give PHP (3.33 g, yield: 82.0 %). ^{1}H NMR (400 MHz, ppm, $CDCl_3$): δ 4.77 (d, 2H), 4.00-3.88 (d, 2H), 3.79-3.68 (d, 2H), 2.86 (s (br), 1H), 2.51 (t, 1H), 1.1 (s, 3H). ^{13}C NMR (400 MHz, ppm, $CDCl_3$): δ 175.2, 77.4, 75.3, 68.2, 52.6, 49.4, 17.1.

Synthesis of propargyl 3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate (PBH)

PHP (4.00 g, 23.26 mmol), DCC (3.36 g, 16.28 mmol) and DMAP (517.83 mg, 4.24 mmol) were dissolved in anhydrous DCM (30 ml), and the solution was cooled to 0 ^{o}C. 2-bromoisobutyric acid (2.59 g, 15.50 mmol) in 10 ml of DCM was added dropwise to the reaction mixture. The reaction mixture was allowed to stir for 30 min in an ice bath and further stirred overnight at room temperature. After vacuum filtration, the solvent was removed by rotary evaporation, and the product was purified by column chromatography with mixtures of ethyl acetate/hexane (1/4 v/v). The product was isolated by evaporation of the solvents and further dried in a vacuum oven to form a yellow oil (4.60 g, yield: 74.0 %). ^{1}H NMR (400 MHz, ppm, $CDCl_3$): δ 4.75 (d, 2H), 4.45, 4.32 (dd, 2H), 3.77 (d, 2H), 2.5 (t, 1H), 1.93 (s, 6H), 1.29 (s, 3H). ^{13}C NMR (400 MHz, ppm, $CDCl_3$): δ 173.4, 171.7, 77.2, 75.4, 67.0, 65.0, 55.5, 52.7, 48.5, 30.8, 17.4.

Synthesis of (*l*-POEGMA)-OH by ATRP of OEGMA using PBH as an initiator

(*l*-POEGMA)-OH was prepared by ATRP of OEGMA using PBH as an initiator. Typically, PBH (64.18 mg, 0.2 mmol), bpy (62.5 mg, 0.4 mmol) and OEGMA (3.00 g, 10 mmol) were dissolved in anisole (10 ml). After three freeze–pump–thaw cycles, CuBr (28.70 mg, 0.2 mmol) was introduced under the protection of nitrogen flow. After another three freeze–pump–thaw cycles, the reaction mixture was sealed and placed in an oil bath thermostated at 60 ^{o}C to start the polymerization. After 51 min, the reaction was stopped by exposure to air and diluted using THF. The crude product was collected by precipitation in excess ice-cold n-hexane. To remove the copper catalyst and any unreacted monomer, the crude product was dissolved in 2 ml of DMF, placed in a dialysis tube (molecular weight cut-off (MWCO), 3.5 kDa) and then subjected to dialysis against distilled water for 36 h, during which the water was renewed every 12 h. The purified (*l*-POEGMA)-OH was harvested by freeze-drying (yield, 16.4 %).

Synthesis of (*l*-POEGMA)-*b*-PCL via Ring-Opening Polymerization (ROP)

initiated by (*l*-POEGMA)-OH

(*l*-POEGMA)-*b*-PCL was synthesized by $Sn(Oct)_2$-catalyzed ring-opening polymerization (ROP) of *ε*-CL using (*l*-POEGMA)-OH as the macroinitiator and toluene as the solvent. In a typical procedure, (*l*-POEGMA)-OH (85.81 mg, 0.015 mmol), *ε*-CL (171.21 mg, 1.5 mmol) and $Sn(Oct)_2$ (1.22 mg, 0.003 mmol) were dissolved in 500 μl dry toluene and then added into a thoroughly dried tube equipped with a magnetic stirring bar. The tube was connected to a standard Schlenk line and the system was degassed via three freeze–pump–thaw cycles followed by immersing the tube in the oil bath thermostated at 90 ^{o}C. After 250 min, the reaction mixture was cooled down, diluted using THF, and precipitated in 10-fold excess of ice-cold *n*-hexane to yield crude product. The product was purified by re-dissolving/precipitating in THF/*n*-hexane for three times, and further dried under vacuum until constant weight (yield, 35.8 %).

Synthesis of (*c*-POEGMA)-OH by intra-chain click cyclization

First, the linear precursor with azide terminus was obtained as follows, (*l*-POEGMA)-OH (1.20 g, 0.21 mmol) and NaN_3 in a 20-fold molar excess were dissolved in a 1/4 v/v % water/DMF mixed solvent (10 mL) in a round-bottom flask equipped with a magnetic stirrer. The flask was sealed with a rubber septum and was stirred at 45 °C for 48 h. After purification by extensive dialysis to remove residual sodium salts, the linear precursor, alkyne-(*l*-POEGMA)-(OH)-N_3, was obtained by freeze-drying (yield, 85.7 %)

Afterward, in a typical procedure, 750 ml of DMF was placed in a 1 L three-neck flask and degassed by bubbling dry nitrogen gas for 1 h. 20-fold molar equivalents of PMDETA and CuBr were then charged into the flask under the protection of nitrogen flow. A solution of alkyne-(OH)-(*l*-POEGMA)-N_3 linear precursor (500 mg, 84.2 μmol) in degassed DMF (10 mL) was added to the copper catalyst solution via a syringe pump at the rate of 4.63 ml/min. The reaction was carried out at 100 °C in a nitrogen atmosphere for 36 h. At the end of the polymer solution addition, the mixture was allowed to proceed for another 12 h. After the mixture was cooled to room temperature, DMF was removed under reduced pressure, and the concentrated residue was transferred directly to a dialysis tube (molecular weight cut-off (MWCO), 35 kDa) and dialyzed against distilled water to remove the copper catalyst. The resulting cyclic polymer, (*c*-POEGMA)-OH, was harvested by freeze-drying (yield, 86.4 %).

Synthesis of (*c*-POEGMA)-*b*-PCL via Ring-Opening Polymerization (ROP) initiated by (*c*-POEGMA)-OH

The synthesis approach of (*c*-POEGMA)-*b*-PCL was the same as that of (*l*-POEGMA)-*b*-PCL except using (*c*-POEGMA)-OH as the macroinitiator and polymerization time of 305 min.

Characterizations of polymer

^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a JNM-ECS spectrometer at 400 MHz using $CDCl_3$ as the solvent. The size exclusion chromatography and

multi-angle laser light scattering (SEC-MALLS) was carried out to determine the molecular weight and molecular weight distribution of the 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 Tosoh TSK-GEL R-3000 and R-4000 columns (Tosoh Bioscience) were connected in series to a Agilent 1260 series (Agilent Technologies), an interferometric refractometer (Optilab-rEX, Wyatt Technology) and a MALLS device (DAWN EOS, Wyatt Technology). The MALLS detector was operated at a laser wavelength of 690.0 nm.

The FT-IR spectroscopic measurements were conducted on a NEXUS 670 FT-IR spectrometer (Nicolet, WI, USA) and oil samples were pressed into potassium bromide (KBr) pellet prior to the measurements.

Preparation and characterization of micelles

Take (*l*-OEGMA)-*b*-PCL as an example, (*l*-OEGMA)-*b*-PCL (1.5 mg) in 1 ml of DMF was placed in a dialysis tube and dialyzed against distilled water for 24 h. Thereafter, the micelle solution was collected and the micelle concentration was around 0.25 mg/ml. The TEM images were recorded on a JNM-2010 instrument operating at an acceleration voltage of 200 keV. To prepare specimens for TEM observation, a drop of micelle solution was deposited onto a ultra-thin carbon-coated copper grid. After deposition, excess solution was removed using a strip of filter paper. The sample was further stained using phosphotungstic acid (2 % w/w) and dried in air prior to visualization.

The average hydrodynamic size of reduction-responsive micelles was measured by dynamic light scattering (DLS) on a Zeta sizer (Nano ZS, Malvern, Worcestershire, UK) at a fixed detection angle of 173°. The polymer 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 were evaluated. The zeta potentials of both micelles were measured in H_2O using the same instrument.

Fluorescence spectra were recorded on a LS55 luminescence spectrometer (Perkin-Elmer) using pyrene as a fluorescence probe. 1.5 ml of pyrene solution (1.2×10^{-8} M in acetone) was added to containers, and the acetone was allowed to evaporate. Then 3 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^{-9} M in water. Excitation was carried out at 340 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 I_{393nm} were recorded. A CMC value was determined from the intersection of the tangent to the curve at the inflection with the horizontal tangent to the curve through the points at low concentration.

***In vitro* drug loading and drug release study**

DOX•HCl (2 mg) and TEA (728 μl) were dissolved in 4 ml of DMF and stirred overnight in dark at room temperature to obtain DOX. Next, the polymers (20 mg) in

4 ml of DMF was added to the above DOX solution and stirred at room temperature for 1 h. Thereafter, the above mixture was added dropwise into 8 ml of ultra-purified water under vigorous stirring. After stirring for another 1 h, the solution was put into a dialysis tube and dialyzed against 5 L of distilled water for 24 h, during which the water was renewed every 8 h. Finally, the drug-loaded micelles were harvested by freeze-drying. To determine the drug loading content (DLC) and entrapment efficiency (EE), the freeze-dried drug-loaded micelles were re-dispersed in PBS (pH 7.4). The concentration of DOX was determined by measuring the absorbance at 485 nm using a Lambda 35 UV-Vis spectrometer (Perkin-Elmer).

In vitro drug release study was carried out in PBS (pH 7.4, 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 1.0 mg/ml. 1 ml of the solution was loaded in a dialysis tube, and then immersed in a tube containing 25 ml of release medium of different pHs. The tubes were kept in a horizontal laboratory shaker thermos-stated at a constant temperature of 37 °C and a stirring speed of 120 rpm. At predetermined time intervals, 3 ml of release medium was taken out and replenished with equal volume of fresh medium. The drug concentration was determined by measuring the absorbance of DOX at 485 nm using a calibration curve. The amount of released DOX in PBS (pH 7.4) was determined by UV-Vis spectrometer. 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. The cells were seeded in 96-well plates at a density of 2500 cells per well in 100 μl of complete growth medium and incubated in a 37 °C, 5% CO_2 environment for 24 h. Samples were prepared in serial dilutions in water and then diluted 10-fold in Opti-MEM medium (Invitrogen). The cells were then rinsed once with PBS and incubated with 40 μl of the sample solutions with different polymer or Dox concentrations at 37 °C for 4 h. Cells were then rinsed with PBS, and the medium was replaced with 100 μl 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) reagent was 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 (Männerdorf, Switzerland). Cell viability for each treatment condition was determined by normalizing to the cells only signal.

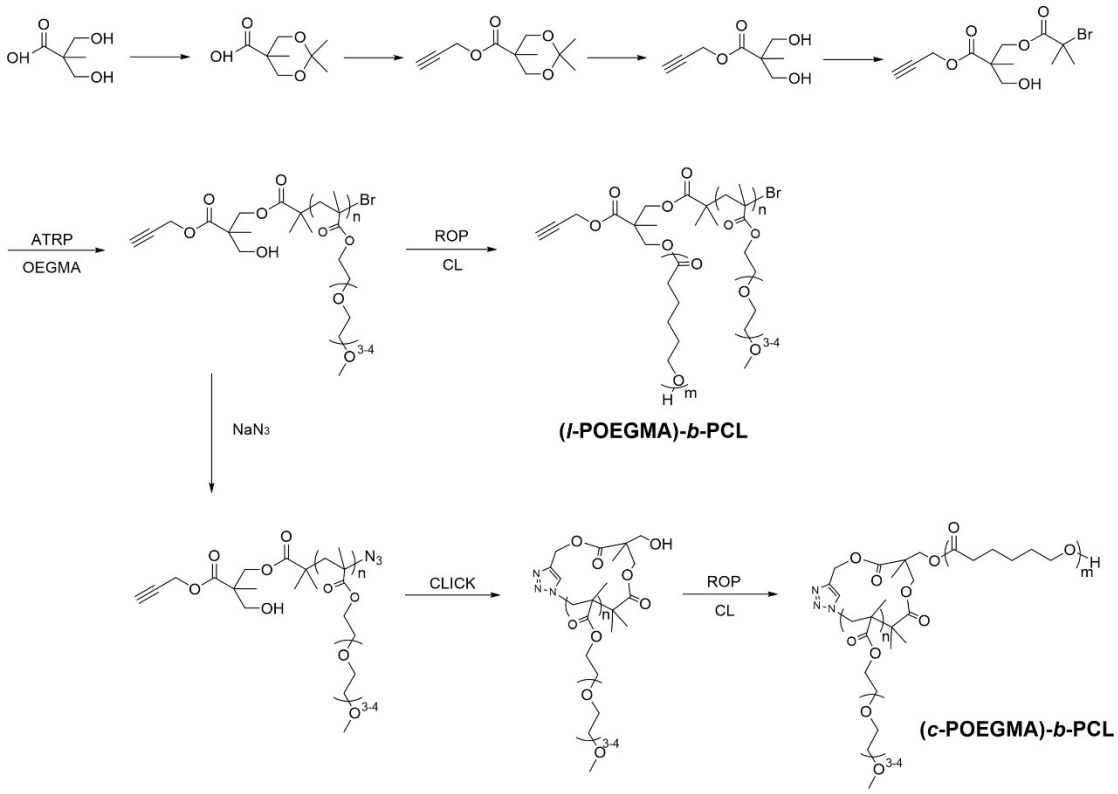
2. Polymer Synthesis

The trible-head initiator was synthesized by four steps including: i) protection of the two hydroxyl groups of BMPA; ii) esterification between isopropylidene-2,2-bis(methoxy) propionic acid and propargyl alcohol; iii) deprotection to release two free hydroxyl groups, and iv) esterification between PBH and 2-bromo-2-methylpropionic acid (Scheme S1). The successful synthesis of the products was confirmed by ^{1}H NMR and ^{13}C NMR analyses (Figure S1 & 2). The (*l*-OEGMA)-OH was synthesized by ATRP using PBH as the initiator, ^{1}H NMR was used to

determined the degree of polymerization (DP). Figure S3 shows the typical ^{1}H NMR spectrum of (*l*-OEGMA)-OH and the DP of OEGMA is determined to be ~18. The molecular weight and molecular weight distribution (PDI) were determined by SEC-MALLS using DMF as an eluent. The SEC elution trace of (*l*-OEGMA)-OH (Figure S5) reveals uni-modal and narrowly distributed molecular weight with M_n = 11.9 kDa and PDI = 1.19, indicating a well-controlled ATRP process.

To obtain (*l*-OEGMA)-*b*-PCL, $Sn(Oct)_2$-catalyzed ROP of ε-CL using (*l*-OEGMA)-OH as the macroinitiator was performed. The composition of the product was determined by comparing the ratio of the integrated intensity of peak 8 assigned to the methylene protons adjacent to carbonyl groups to that of the peak 7 assign to the terminal methyl group of OEGMA monomer (Figure S3b), the DP of PCL is thus calculated to be 26. Moreover, all the elution traces (Figure S5a) reveal uni-model and narrow distribution, confirming the well-controlled ROP processes. The clear shift of the SEC trace toward higher MW for (*l*-OEGMA)-*b*-PCL compared to that of the macroinitiator confirms successful preparation of (*l*-OEGMA)-*b*-PCL by chain extension of PCL block. The PDI is 1.22 and the M_n is 17.5 kDa for (*l*-OEGMA)-*b*-PCL.

The (*c*-OEGMA)-*b*-PCL was prepared by a three-step procedure including: (i) azidation of the alkyne-OEGMA-(OH)-Br to prepare alkyne-OEGMA-(OH)-N_3, (ii) cyclization of the alkyne-(OH)-OEGMA-N_3 by CuAAc and (iii) preparation of the target copolymer (*c*-OEGMA)-*b*-PCL using $Sn(Oct)_2$-catalyzed ROP of ε-CL. The ^{1}H NMR spectra of alkyne-OEGMA-(OH)-N_3 and (c-OEGMA)-OH were similar to that of (*l*-OEGMA)-OH (Figure S4). The DP of PCL in (*c*-OEGMA)-*b*-PCL was determined to be 24 following the calculation method of (*l*-OEGMA)-*b*-PCL mentioned earlier. The successful cyclization is confirmed by a clear shift of the SEC elution trace toward longer retention time for (*c*-OEGMA)-*b*-PCL and the absence of the characteristic band of azide group (~2120 cm^{-1}) in the FT-IR spectrum of (*c*-OEGMA)-*b*-PCL after cyclization (Figure S5b & 6). The PDI is 1.19 and the M_n is 19.4 kDa for (*c*-OEGMA)-*b*-PCL.



Scheme S1. Synthesis route of (*l*-POEGMA)-*b*-PCL and (*c*-POEGMA)-*b*-PCL.

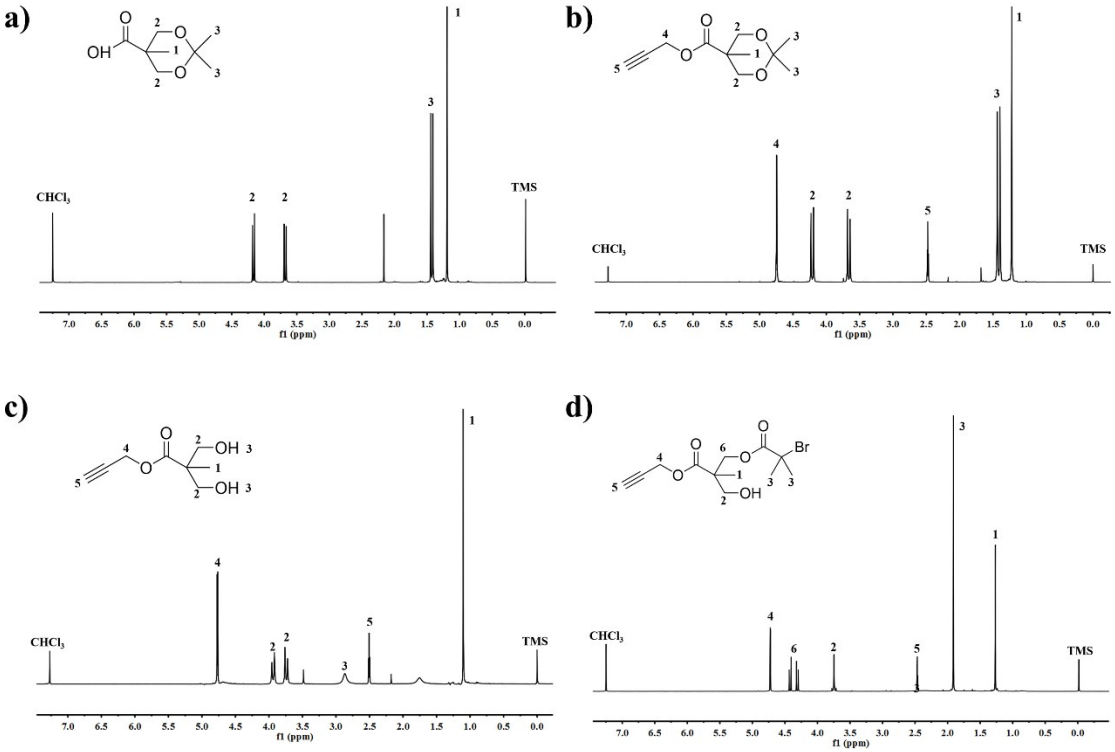


Figure S1. ^{1}H NMR spectra of (a) Isopropylidene-2,2-bis(methoxy) propionic Acid, (b) Propargyl 2,2,5-trimethyl-1,3-dioxane-5-carboxylate, (c) PHP and (d) PBH in $CDCl_3$.

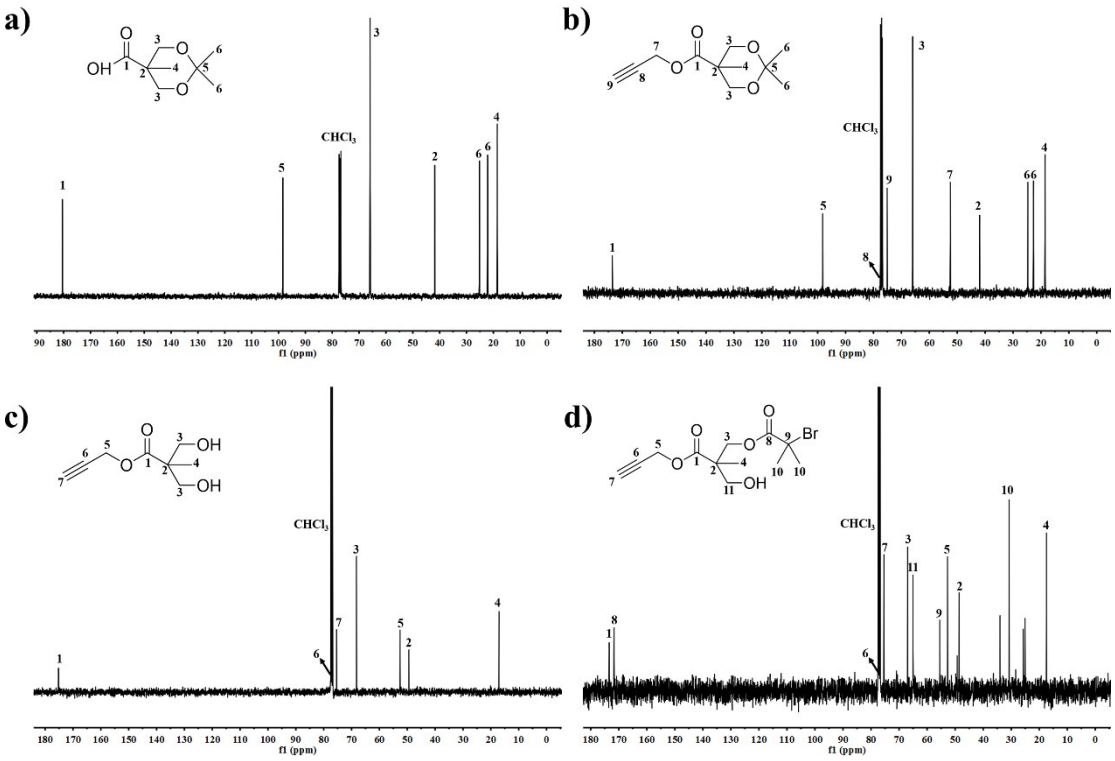


Figure S2. ^{13}C NMR spectra of (a) Isopropylidene-2,2-bis(methoxy) propionic Acid, (b) Propargyl 2,2,5-trimethyl-1,3-dioxane-5-carboxylate, (c) PHP and (d) PBH in $CDCl_3$.

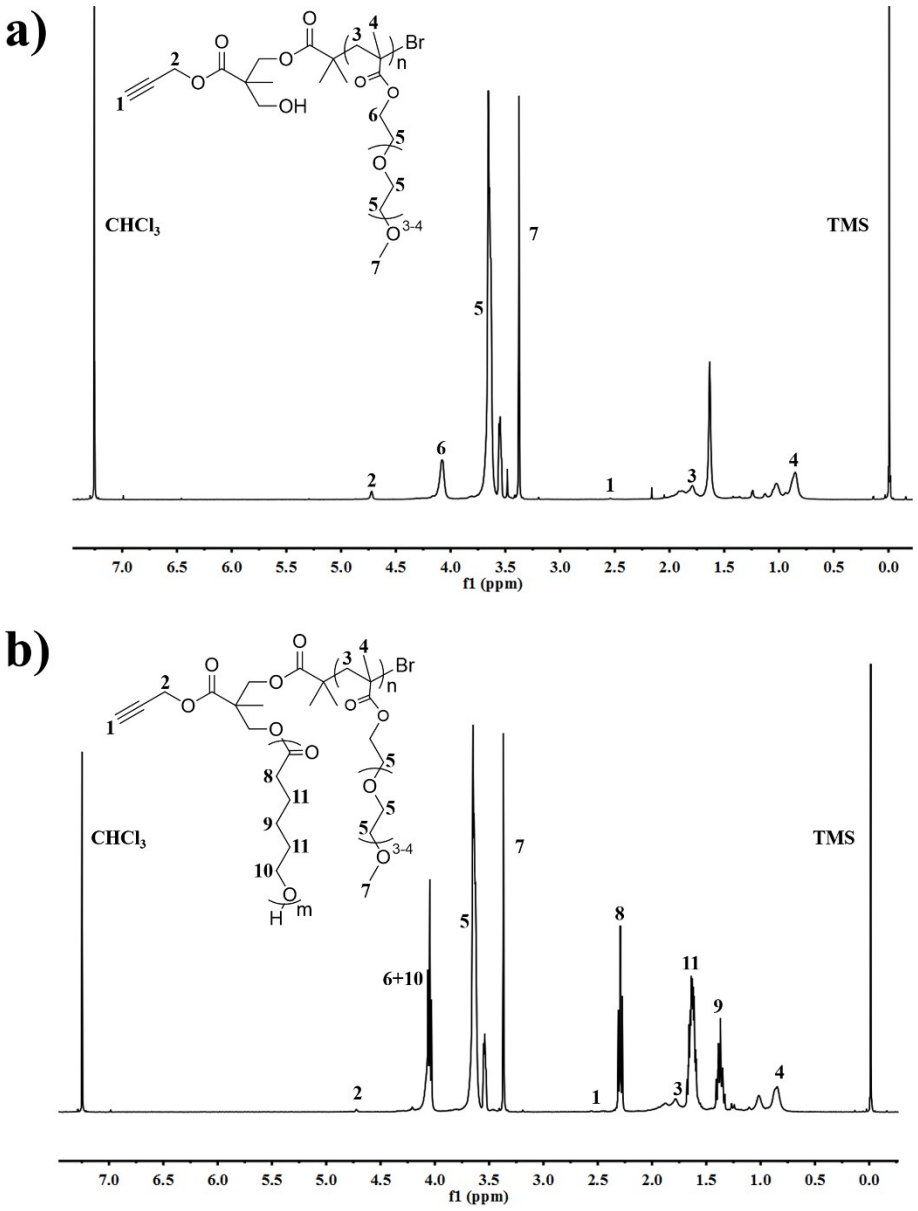


Figure S3. ^{1}H NMR spectra of (a) (*l*-POEGMA$_{18}$)-OH, (b) (*l*-POEGMA$_{18}$)-*b*-PCL$_{26}$ in $CDCl_3$.

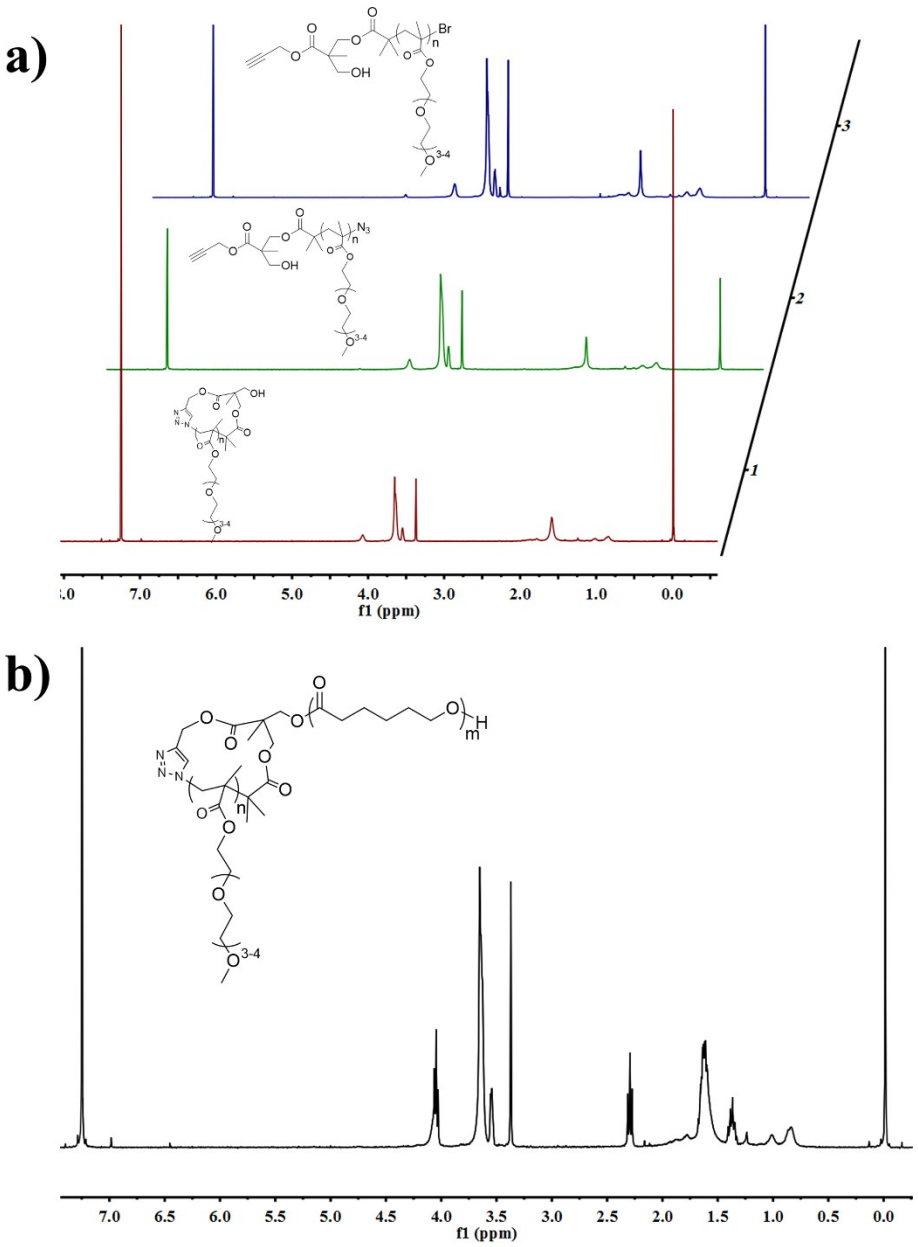


Figure S4. ^{1}H NMR spectra of (a) (*l*-POEGMA$_{18}$)-OH, (*l*-POEGMA$_{18}$)-(OH)-N$_3$, (*c*-OEGMA$_{18}$)-OH, and (b) (*c*-POEGMA$_{18}$)-*b*-PCL$_{24}$ in CDCl$_3$.

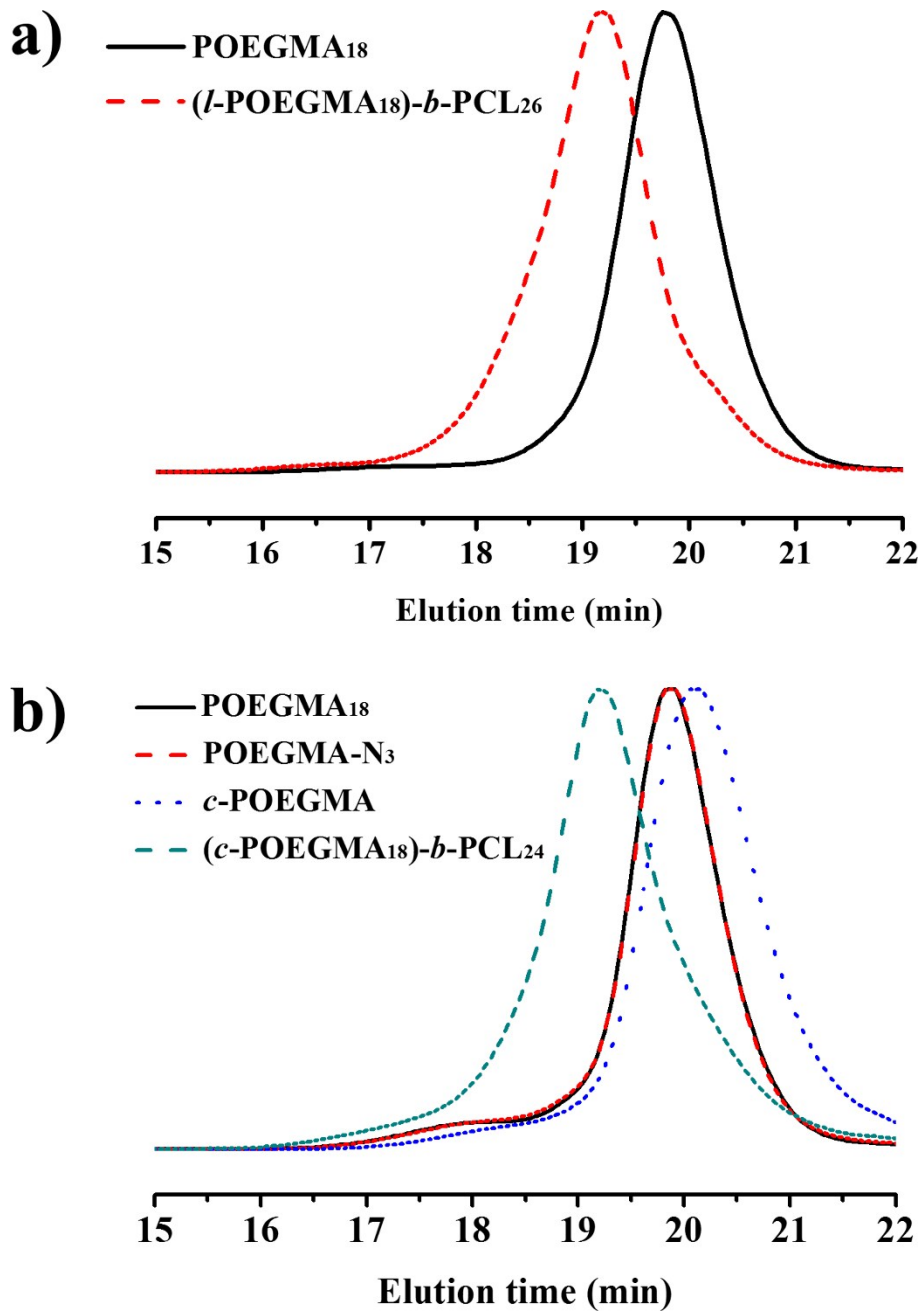


Figure S5. SEC elution traces of (a) (l-POEGMA$_{18}$)-OH, (l-POEGMA$_{18}$)-b-PCL$_{26}$, and (b) alkyne-(l-POEGMA$_{18}$)-(OH)-Br, alkyne-(l-POEGMA$_{18}$)-(OH)-N$_3$, (c-POEGMA$_{18}$)-OH and (c-POEGMA$_{18}$)-b-PCL$_{24}$ using DMF as the eluent.

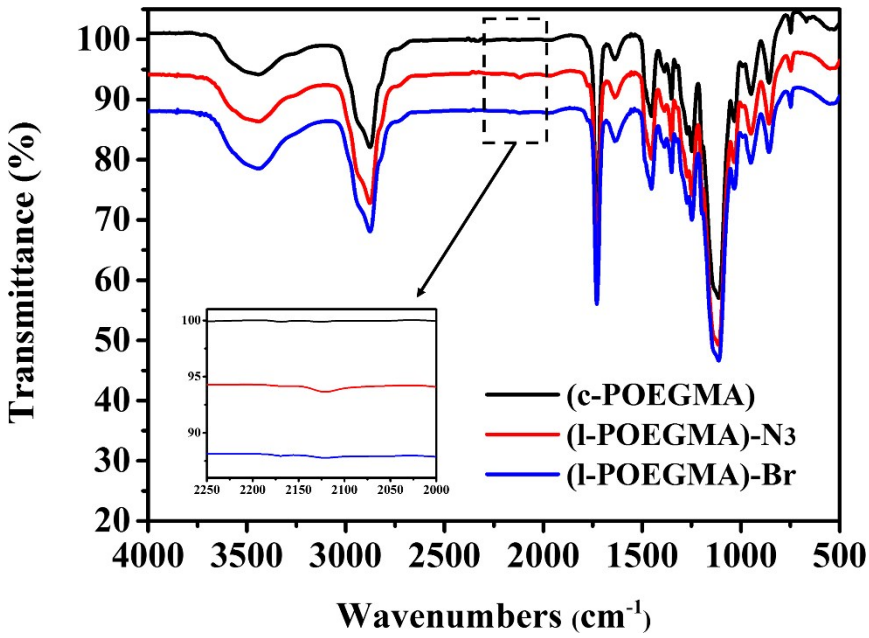


Figure S6. FT-IR spectra of the (*l*-POEGMA)-Br, (*l*-POEGMA)-N_3 and (*c*-POEGMA)-OH verifying the complete loss of the azide (~2120 cm^{-1}) after click coupling.

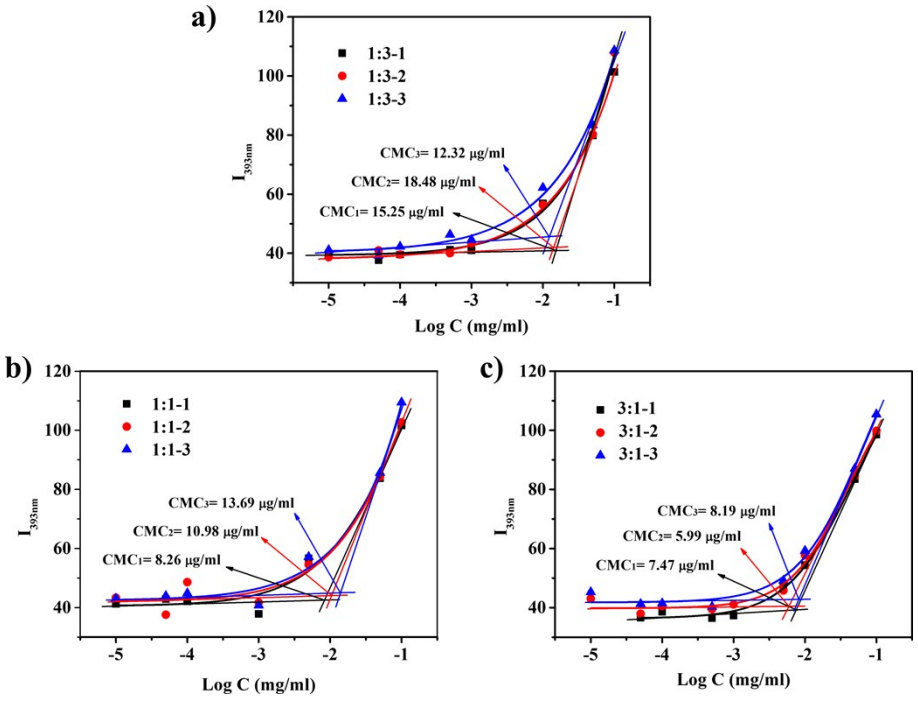


Figure S7. The intensity of I_{393nm} in the emission spectra as a function of logarithm of three mixed micelles. 1:3, 1:1, and 3:1 represent a molar ratio (C: L) of (a) 1:3, (b) 1:1, and (c) 3:1 of the (*c*-POEGMA)-*b*-PCL and (*l*-POEGMA)-*b*-PCL copolymers, respectively.

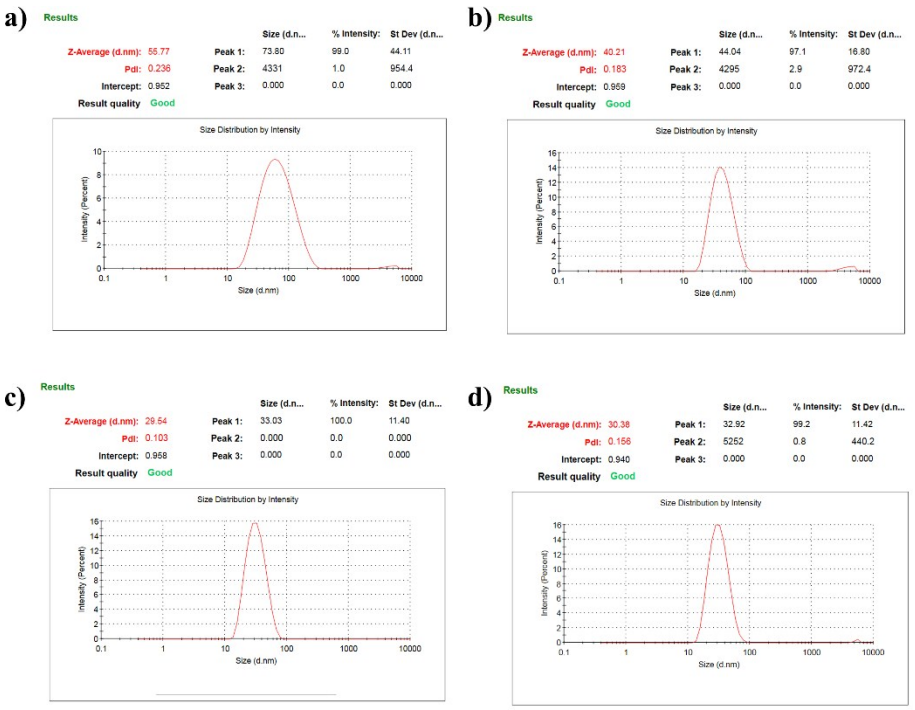


Figure S8. Average size and size distribution of (a & b) L and (c & d) C micelles in H_2O and PBS (pH 7.4) determined by DLS at a polymer concentration of 0.25 mg/ml.

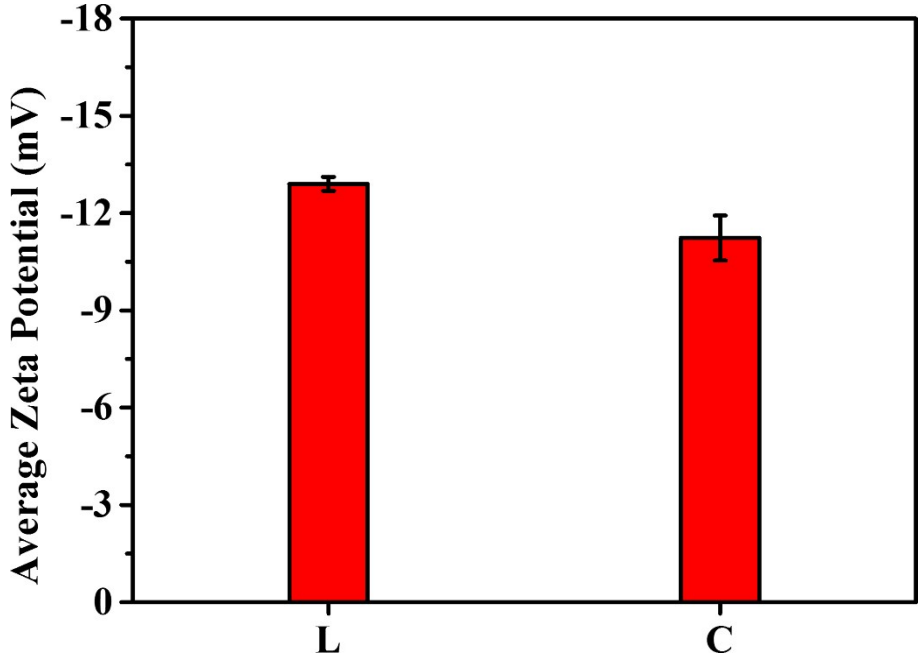


Figure S9. Zeta potentials of L and C micelles in water at a concentration of 1 mg/ml.

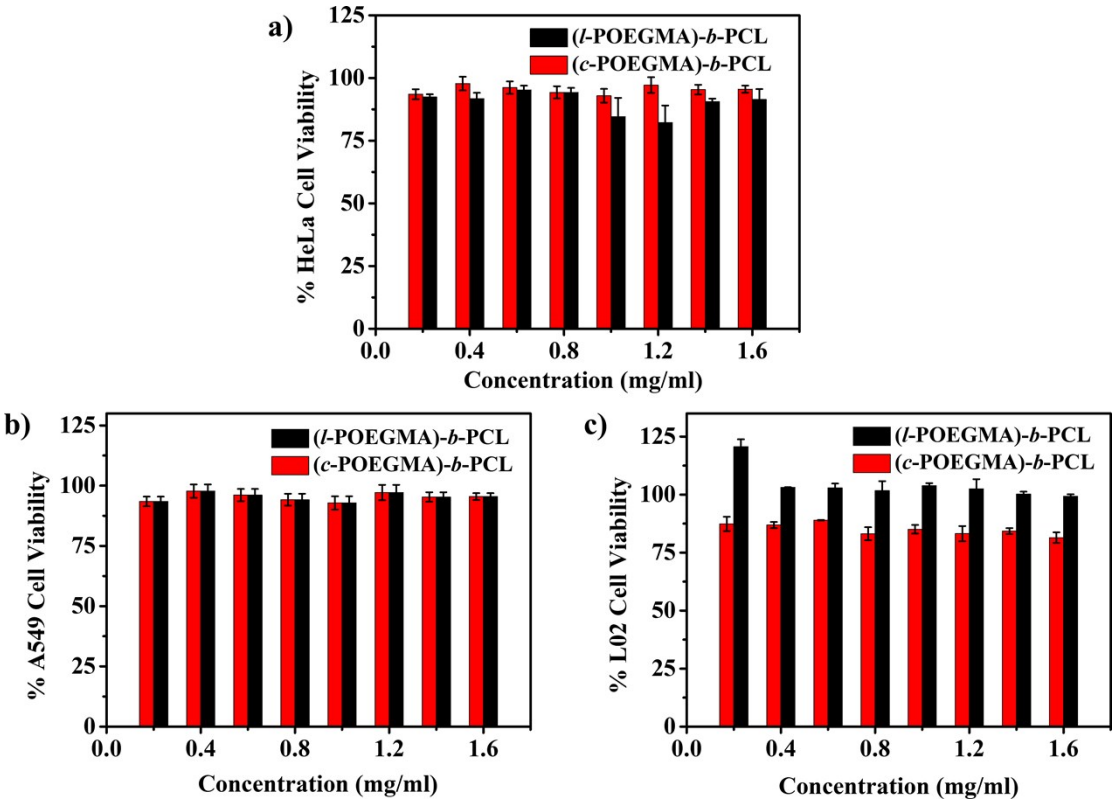


Figure S10. Cell viability of (a) HeLa cells (b) A549 cells and (c) L02 cells incubated with blank L and C micelles. Cell viability was determined by MTS assay and expressed as % viability compared to control untreated cells.

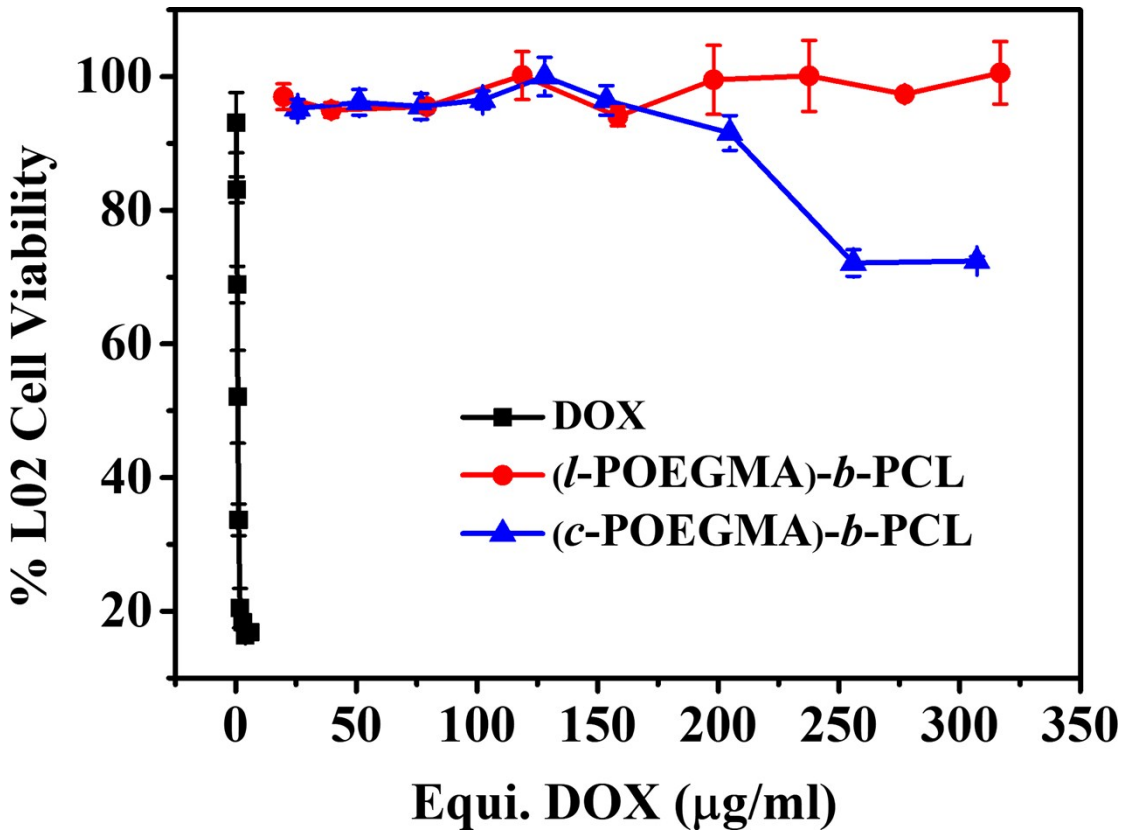


Figure S11. Cell viability of L02 cells exposed to Dox, Dox-loaded L and C micelles at various concentrations for 24 hours. Cell viability was determined by MTS assay and expressed as % viability compared to control untreated cells.

References

1. H. Ihre, A. Hult, J. M. J. Fréchet, and I. Gitsov. *Macromolecules*, 1998, **31**, 4061.