Supporting Information

Fabrication of Cyclic Brush Copolymers with Heterogeneous Amphiphilic Polymer Brushes for Controlled Drug Release

Zhe Liu,[a] Yupeng Huang,[a] Xiaolong Zhang,[a] Xiaoyan Tu,[a] Mingqi Wang,[a] Liwei Ma,[a] Baoyan Wang,[a] Jinlin He,[b] Peihong Ni,[b] and Hua Wei[a,]*

[a]State Key Laboratory of Applied Organic Chemistry, Key Laboratory of Nonferrous Metal Chemistry and Resources Utilization of Gansu Province, and College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou, Gansu 730000, China

[b]College of Chemistry, Chemical Engineering and Materials Science, State and Local Joint Engineering Laboratory for Novel Functional Polymeric Materials, Suzhou Key Laboratory of Macromolecular Design and Precision Synthesis, Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, Soochow University, Suzhou 215123, China

*Corresponding author

E-mail address: weih@lzu.edu.cn (H. Wei)

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Experimental section

Materials

Oligo (ethylene glycol) monomethyl ether methacrylate (OEGMA, M_n = 300 g/mol, 4~5 pendent EO units, Sigma-Aldrich) and 2-hydroxyethyl methacrylate (HEMA, 99%, Sigma-Aldrich) were purified by passing through a basic Al_2O_3 column to remove the inhibitor.ε-Caprolactone (CL) (Sigma-Aldrich) was dried over CaH_2 and distilled under reduced pressure prior to use. 2-Bromoisobutyryl bromide (α-iBuBr, >98%, Sigma-Aldrich, Steinheim, Germany), anisole (≥ 99.0, Kelong, Chengdu, China), n-hexane (99.5%, analytical grade, Rionlon, Tianjin, China), dichloromethane (DCM, 99.5%, analytical grade, Rionlon, Tianjin, China), *N,N′*-dimethylformamide (DMF, 99.5%, analytical grade, Rionlon, Tianjin, China), anhydrous ethyl ether (99.0%, analytical grade, Rionlon, Tianjin, China), tetrahydrofuran (THF, 99.0%, analytical grade, Rionlon, Tianjin, China), 2,2′-bipyridyl (bpy, 98.0%, Sigma-Aldrich, Steinheim, Germany), *N,N,N′,N″,N″*-pentamethyldiethylenetriamine (PMDETA, 99%, Aladdin, Shanghai, China), copper(I) bromide (CuBr, >99.999%, Aldrich, Steinheim, Germany). Sodium azide (NaN_3, 98.0%, Sigma-Aldrich, Steinheim, Germany) was used as received. Propargyl alcohol was purchased from Tianjin Chemical Reagent Factory (China). Doxorubicin hydrochloride (DOX·HCl) with a purity of ≥ 98% was provided by Aladdin, Shanghai, China and used as received. Other reagents were used as received without further purification.

Characterization of Polymers

^{1}H NMR spectra were recorded on a JNM-ECS 400 MHz spectrometer (JEOL, Tokyo, Japan) operated in the Fourier transform mode. Dimethyl sulfoxide (DMSO-d_6), and deuterated chloroform ($CDCl_3$) were used as the solvents, respectively. The FT-IR spectroscopic measurements were conducted on a NEXUS 670 FT-IR spectrometer (Nicolet, WI, USA) and solid samples were pressed into potassium bromide (KBr) pellet prior to the measurements. The size-exclusion chromatography and multi-angle laser light scattering (SEC-MALLS) analyses were used to determine the molecular weight (MW) and polydispersity (PDI) of the prepared polymers. SEC using HPLC-grade DMF containing 0.1 wt % LiBr at 60 ℃ as the eluent at a flow rate of 1 mL/min. 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. Absorbance spectra were evaluated on a PerkinElmer Lambda 35 UV-vis spectrometer (Perkin-Elmer, Waltham, MA, USA). The polymer solution was prepared in phosphate buffer solution (PBS, pH 7.4, 150 mM) at a concentration of 1 mg/mL.

MALDI-TOF MS were acquired on an UltrafleXtreme MALDI TOF mass spectrometer equipped with a 1 kHz smart beam-II laser. The

trans-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]-malononitrile (DCTB, Sigma-Aldrich, >98%) served as the matrix and was prepared in CH2Cl2 at a concentration of 10 mg mL^{-1}. The cationizing agent sodium trifluoroacetate (NaTFA, Sigma-Aldrich, 98%) or silver trifluoroacetate (AgTFA, Sigma-Aldrich, 98%) was prepared in ethanol at a concentration of 10 mg mL^{-1}. The matrix and cationizing salt solutions were mixed in a ratio of 10/1 (v/v). All samples were dissolved in THF at a concentration of 10 mg mL^{-1}. The sample preparation involved depositing 0.5 μL of matrix and salt mixture as well as 0.5 μL of sample on the wells of a 384-well ground-steel plate. After evaporation of the solvent, the plate was inserted into the MALDI mass spectrometer. The attenuation of the laser was adjusted to minimize undesired polymer fragmentation and to maximize the sensitivity. The mass scale was calibrated externally using the peaks obtained from a poly(methyl methacrylate) (PMMA) standard at the molecular weight range under consideration. Data analyses were conducted using the Bruker's flex analysis software.

ATRP Synthesis of Linear alkyne-P(OEGMA)-Br

Linear P(OEGMA)-Br was prepared by ATRP of OEGMA monomer in anisole, using propargyl 2-bromoisobutyrate[1] as the initiator and bpy/Cu(I)Br as the catalyst. Typically, propargyl 2-bromoisobutyrate (41 mg, 0.2 mmol), bpy (63 mg, 0.4 mmol) and OEGMA (3 g, 10 mmol) were dissolved in anisole (20 mL), which were transferred to a thoroughly dried polymerization tube equipped with a magnetic stirring bar. The

tube was connected to a standard Schlenk line. After three freeze-pump-thaw cycles, CuBr (28.7mg, 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 100 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 5 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 24 h, during which the water was renewed every 8 h. The purified P(OEGMA)-Br was harvested by freeze-drying (yield, 75%). ^{1}H NMR ($CDCL_{3,}$ 400 MHz): δ 0.80-1.10 (s, -OCO(C**H$_3$**)$_2$CH$_2$-, -CH$_2$C(**CH$_3$**)COO-), 1.75-1.95 (s, -C**H$_2$**C(CH$_3$)COO-), 2.52 (s, C**H**C-), 3.37-3.39 (s, -O**CH$_3$**), 3.60-3.75 (s, -OCH$_2$C**H$_2$**O-) 4.05-4.15 (s, -OC**H$_2$**CH$_2$O-), 4.62-4.66 (m, CHCC**H$_2$**O-)

ATRP Synthesis of Linear alkyne-P(OEGMA)-*b*-P(HEMA)-Br Using alkyne-P(OEGMA)-Br as a Macro-initiator

Linear P(OEGMA)-*b*-P(HEMA)-Br (*l*-P(OEGMA)-*b*-P(HEMA)-Br) was synthesized by ATRP of HEMA in DMF, using P(OEGMA)-Br as the macro-initiator and bpy/Cu(I)Br as the catalyst. In a typical procedure, a 10 mL Schlenk flask was charged with P(OEGMA)-Br (0.75 g, 0.1 mmol), HEMA (0.13 g, 1 mmol), bpy (0.032 g, 0.2 mmol) to obtain a molar feed ratio of HEMA and initiator

([HEMA]/[Initiator]) of 10.The above mixture was dissolved in DMF to obtain a 0.5 mol/l HEMA solution. After three freeze-pump-thaw cycles, CuBr (28.7 mg, 0.1 mmol) was added under the protection of nitrogen flow. After another three freeze-pump-thaw cycles, the flask was sealed and placed in an oil bath thermostated at 65 ^{o}C to start the polymerization. After a short polymerization time of 10 min, the reaction mixture was quenched by exposing to the air. The reaction mixture was diluted with DMF, followed by precipitation in cold anhydrous ethyl ether to collect the crude products by centrifugation. The crude products were later subjected to dialysis against distilled water to remove the copper catalyst. The purified *l*-P(OEGMA)-*b*-P(HEMA)-Br was harvested by freeze-drying (yield, 60%).^{1}H NMR (DMSO-d_6, 400 MHz) δ 0.70-1.00 (s, -CO(C**H**$_3$)$_2$CH$_2$-, -CH$_2$C(**CH**$_3$)COO-)**,** 1.65-1.95 (s, -C**H**$_2$C(CH$_3$)COO-), 3.24-3.27 (s, -O**CH**$_3$), 3.40-3.65 (s, -OCH$_2$C**H**$_2$O-, -OCH$_2$C**H**$_2$OH), 3.86-3.93 (s, -OC**H**$_2$CH$_2$OH), 3.96-4.05 (s, -OC**H**$_2$CH$_2$O-), 4.73-4.81 (s, -OCH$_2$CH$_2$O**H**)

Synthesis of Linear Precursor with Azide Terminus, alkyne-P(OEGMA)$_{25}$-*b*-P(HEMA)$_5$-N$_3$ and Cyclic P(OEGMA)$_{25}$-*b*-P(HEMA)$_5$ (*c*-P(OEGMA)$_{25}$-*b*-P(HEMA)$_5$) by Intra-Chain Click Cyclization of Linear alkyne-P(OEGMA)$_{25}$-*b*-P(HEMA)$_5$-N$_3$ Precursor

l-P(OEGMA)$_{25}$-*b*-P(HEMA)$_5$-N$_3$ and *c*-P(OEGMA)$_{25}$-*b*-P(HEMA)$_5$ were prepared according to the procedures reported in our previous studies.[2] In a typical procedure for the synthesis of *c*-P(OEGMA)$_{25}$-*b*-P(HEMA)$_5$ by intra-chain click cyclization, 750

mL of DMF was placed in a 1000 mL 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 *l*-P(OEGMA)$_{25}$-*b*-P(HEMA)$_5$-N$_3$ linear precursor (0.5 g) in degassed DMF (10 mL) was added to the copper catalyst solution via a syringe pump at the rate of 0.007 mL/min. The reaction was carried out at 100°C in a nitrogen atmosphere for 24 h. At the end of the polymer solution addition, the mixture was allowed to proceed for another 24 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 (MWCO: 3.5 kDa) and dialyzed against distilled water to remove the copper catalyst. The resulting cyclic polymer, *c*-P(OEGMA)$_{25}$-*b*-P(HEMA)$_5$, was harvested by freeze-drying. Yield: 80% (0.4 g).

ROP Synthesis of *cb* Copolymers and Their *bb* Analogues

The *cb* copolymers and their *bb* analogues were both synthesized by Sn(Oct)$_2$ -catalyzed ROP of ε-CL using *c*-P(OEGMA)-*b*-P(HEMA) and *l*-P(OEGMA)-*b*-P(HEMA)-Br as the initiator respectively. Taking the synthesis of *bb* analogue as an example, *l*-P(OEGMA)-*b*-P(HEMA)-Br (0.082 g, 0.01 mmol) , ε-CL (0.17 g, 1.5 mmol) and 330 μl of anhydrous DMF were added into a thoroughly dried Schlenk flask equipped with a magnetic stirring bar. The system was degassed *via* three freeze-pump-thaw cycles, and then the flask was placed in an oil bath preheated at 100

^{o}C for a couple of minutes to obtain a homogeneous mixture. $Sn(Oct)_2$ (10 mg, 0.025 mmol) in 165 μl of anhydrous DMF was later added to the above mixture under the protection of nitrogen flow and three freeze–pump–thaw cycles were applied again, followed by immersing the flask in the oil bath thermostated at 100 ^{o}C. After 1 h, the reaction mixtures were cooled down, diluted using THF, and precipitated in 10-fold excess of ice-cold ethyl ether to yield the crude product, which was purified twice by re-dissolving/precipitating in THF/ethyl ether, and further dried under vacuum until constant weight (yield, 50%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.80-1.10 (s, -CO(C**H**$_3$)$_2$CH$_2$-, -CH$_2$C(**CH**$_3$)COO-) 1.34-1.44 (m, -OCOCH$_2$CH$_2$C**H**$_2$CH$_2$CH$_2$-) 1.60-1.70 (m, -OCOCH$_2$C**H**$_2$CH$_2$C**H**$_2$CH$_2$-) 1.75-1.95 (s, -C**H**$_2$C(CH$_3$)COO-) 2.25-2.40 (m, -OCOC**H**$_2$CH$_2$CH$_2$CH$_2$CH$_2$-) 3.36-3.38 (s, -OC**H**$_3$) 3.60-3.68 (s, -OCH$_2$C**H**$_2$O-) 4.02-4.15 (s, -OC**H**$_2$CH$_2$O-, -CH$_2$O**H**)

cb copolymer was prepared following the identical procedures mentioned above. The detailed reaction conditions were summarized in Table S1.

Preparation and Characterization of Self-Assembled Micelles

Take *bb* copolymeras an example, *bb*-P(OEGMA$_{25}$)-*b*-P(HEMA-*b*-OCL$_6$)$_5$ (1 mg) was dissolved in 1 mL of DMF. The polymer solution was transferred to a dialysis tube and and dialyzed against distilled water for 24 h to obtain a micelle solution with a concentration of approximate 0.5 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, the freeze-dried micelles were first re-dispersed in water, and a drop of the micelle solution was deposited 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 (2% w/w) and dried in air prior to visualization.

The average hydrodynamic size of micelles was measured by dynamic light scattering (DLS) on a Zetasizer (Nano ZS, Malvern, Worcestershire, UK) at a fixed detection angle of 90°. The polymer solution was passed through a Millipore 0.45 μm pore-sized syringe filter prior to measurements. The polymer solution with a concentration of 0.25 mg/mL was used.

Critical Micelle Concentration (CMC) Determination

Fluorescence spectra were recorded on a LS55 luminescence spectrometer (Perkin-Elmer, Waltham, MA, USA). Pyrene was used as a hydrophobic fluorescent probe. The same amount of pyrene solutions (3×10^{-6} in acetone, 80 μl) were added to each container, and acetone was allowed to evaporate. Then 4 mL of polymer aqueous solution at different concentrations were added to the containers containing the pyrene residue. The combined solution of pyrene and polymers was equilibrated at room temperature in dark for 24 h to reach the solubilization equilibrium of pyrene in an aqueous phase. Excitation was carried at 340 nm, and emission spectra were recorded ranging from 350 to 500 nm. Both excitation and emission bandwidths were 10 nm.

From the emission spectra, the intensity (peak height) of the first (I_{373}) and the third band (I_{384}) 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

DOX-loaded nanoparticles were prepared following a classical dialysis method. Briefly, 1.0 mg of DOX·HCl was dissolved in 2.0 mL of DMF, and then 0.26 g of TEA was added and stirred overnight in dark at room temperature to remove the hydrochloride. *cb* or *bb* copolymers (10 mg) in 2.0 mL of DMF was added to the above DOX solution and stirred for 1 h. Thereafter, the mixture was added dropwise into 4.0 mL of deionized (DI) water under vigorous stirring and kept stirring for another 1 h to realize drug encapsulation. The resulting solution was transferred to a dialysis tube with a MWCO of 3.5 kDa and subjected to dialysis against 5.0 L of DI water for 24 h, which was renewed every 3 h during the course of initial 12 h to remove any unencapsulated free DOX. Finally, the drug-loaded nanoparticles were harvested by lyophilization. To determine the encapsulation efficiency (EE) and the drug loading content (DLC), a known amount of lyophilized DOX-loaded nanoparticles was dissolved in a certain volume of PBS and the absorbance of the solution was measured using the UV-vis spectrometer at 485 nm. The EE and DLC were calculated using the following Equations,

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

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

In vitro drug release study was carried out in 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 with a concentration of 1mg/mL. 1 mL of the solution was placed in a dialysis tube, 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. 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 standard calibration curve. The amount of DOX released in PBS (pH 7.4) or SSC (pH 5.0) was determined by UV-Vis spectrometer. The experiment was performed in triplicate for each sample.

Determination of Quantum Yield of DOX in free and encapsulated states

DOX·HCl and DOX-loaded *cb*$_2$ micelles were dissolved in distilled water to prepare free and encapsulated DOX solutions, respectively with an equivalent DOX concentration of 1 mg/mL. Both solutions were diluted to the detection limit of the instrument. The quantum yield of DOX in free and encapsulated states was

determined by measuring the fluorescence of the aqueous solutions of DOX·HCl and DOX-loaded *cb*$_2$ micelles at an excitation wavelength of 485 nm on the FLS 920 transient/steady-state fluorescence spectrophotometer (Edinburgh Instruments, EI, UK).

Evaluation of Cellular Uptake by Flow Cytometry (FCM)

HeLa cells were seeded in 24-well plates at a 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. Then, fresh MEM containing free DOX or NPs (DOX) was added to replace the original medium, and the cells without drug treatment were set as a control. The DOX concentration for free DOX and NPs (DOX) in MEM was set at 24 μg/mL. 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. Cells were transferred to 1.5 mL 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 polymer using a BD Accuri C6 Plus flow cytometer (BD Biosciences) with an excitation wavelength and emission wavelength of 488 nm and 595 nm. A minimum of 10 000 cells was analyzed 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 density of 2500 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, blank copolymers, and drug-loaded micelles of *cb* and *bb* 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 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. After further incubation for 20 h, 20 μl of MTS reagent was added to each well. Cells were then incubated with 5% CO_2 for 3 h at 37 °C. 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

(1) Wang, C. E.; Wei, H.; Tan, N.; Boydston, A. J.; Pun, S. H. Sunflower Polymers for Folate-Mediated Drug Delivery. *Biomacromolecules* **2016**, *17*, 69-75.

(2) Wei, H.; Wang, C. E.; Tan, N.; Boydston, A. J.; Pun, S. H. ATRP Synthesis of Sunflower Polymers using Cyclic Multimacroinitiators. *ACS Macro Lett*. **2015**, *4*, 938-941.

Table S1. Summary of ROP synthesis of *bb* and *cb* copolymers.

Sample	Molar feed ratio of [initiating site]:[CL]:$Sn(Oct)_2$	DP of OCL[a]	Polymerization time (h)	Reaction temperature (^oC)
bb_1	1:20:0.2	3	3	100
cb_1	1:100:0.2	3	12	120
bb_2	1:20:0.2	6	6	100
cb_2	1:100:0.2	6	24	120

[a]Determined by ^1H NMR

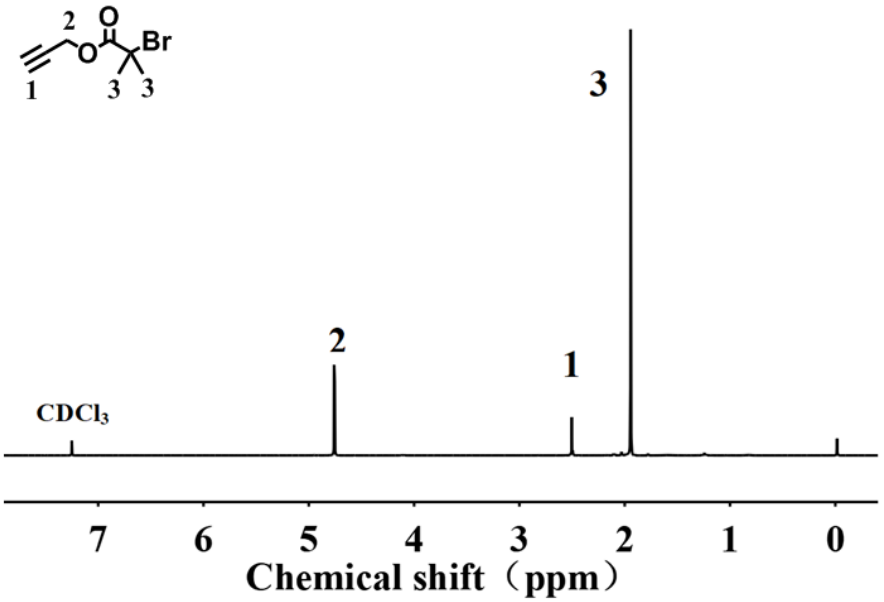

Figure S1. ^{1}H NMR spectrum of alkyne-Br in $CDCl_3$.

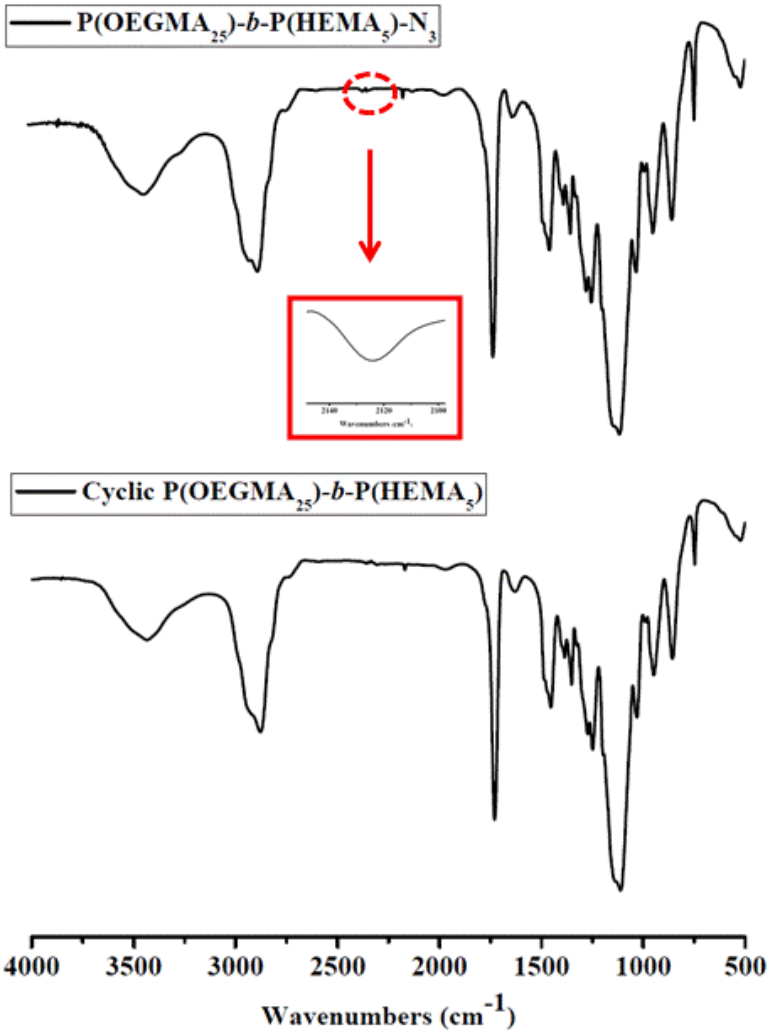

Figure S2. FT-IR spectra of the P(OEGMA$_{25}$)-*b*-P(HEMA$_5$) and Cyclic P(OEGMA$_{25}$)-*b*-P(HEMA).

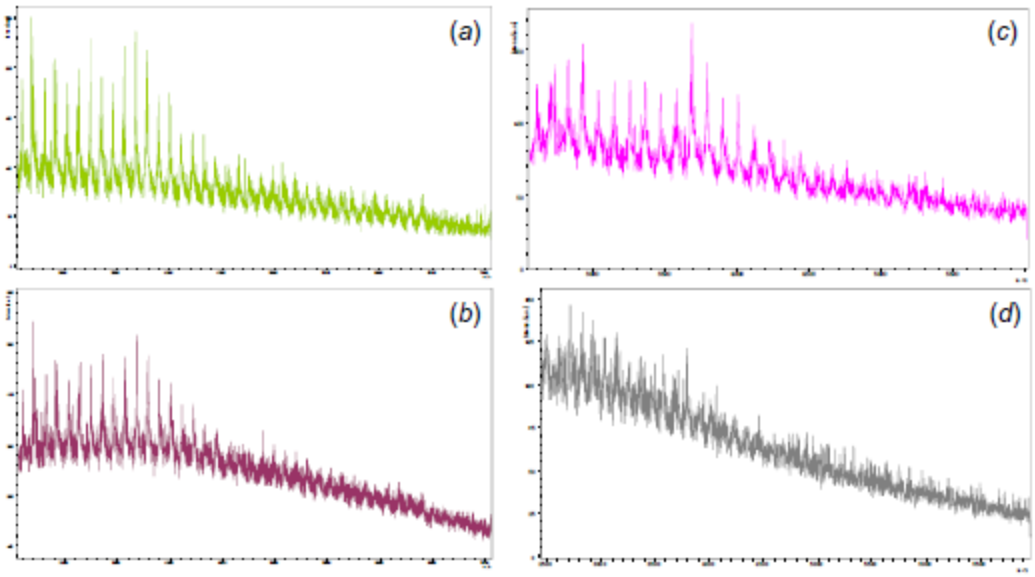

Figure S3. MALDI-TOF mass spectra of (a) *l*-P(OEGMA)$_{25}$-*b*-P(HEMA)$_5$-N$_3$, (b) *c*-P(OEGMA)$_{25}$-*b*-P(HEMA)$_5$, (c) *bb*$_2$, and (d) *cb*$_2$.

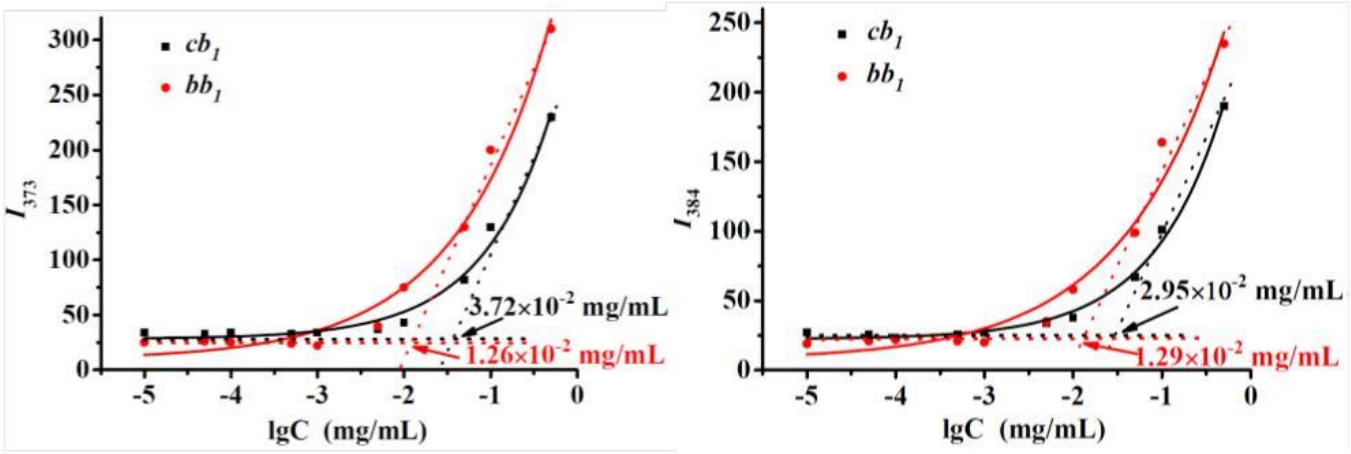

Figure S4. I_{373} and I_{384} in the emission spectra as a function of logarithm of cb_1 and bb_1 concentration. (λ_{ex} = 340 nm, [Pyrene] =2 × 10^{-6} M).

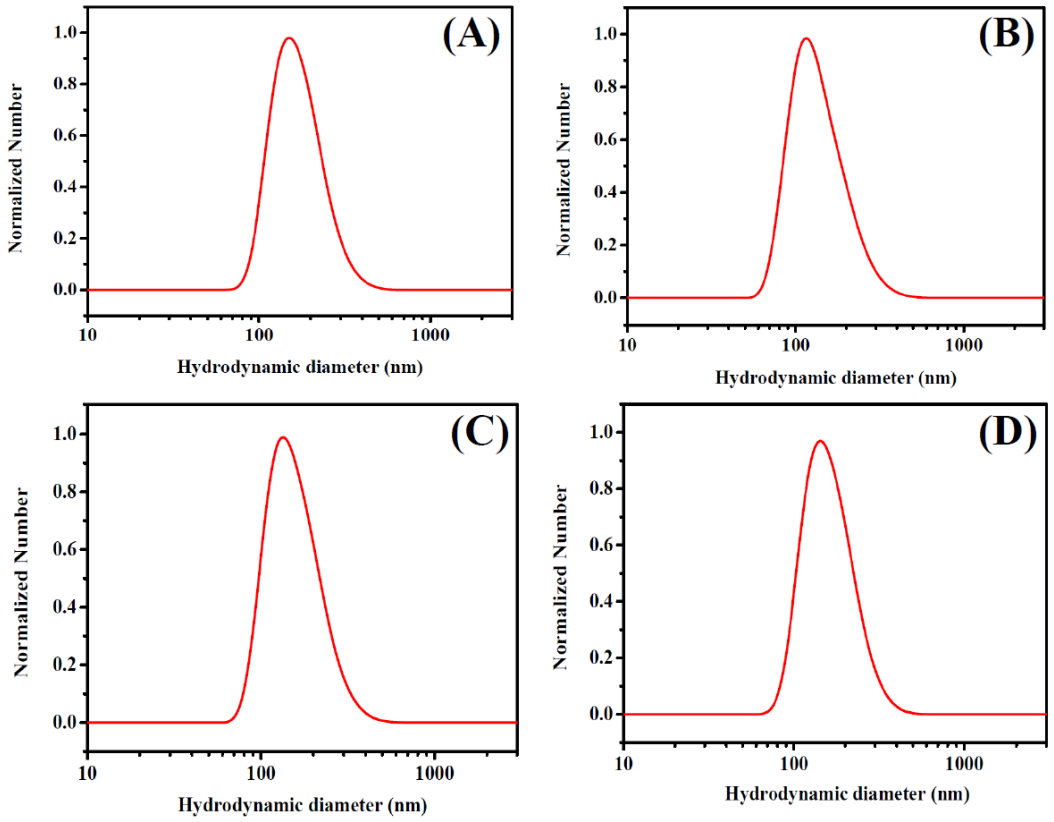

Figure S5. Number-size distributions of (A) *bb*$_2$ (B) *cb*$_2$ (C) *bb*$_1$ (D) *cb*$_1$ micelles in aqueous solution at a polymer concentration of 0.25 mg/mL.

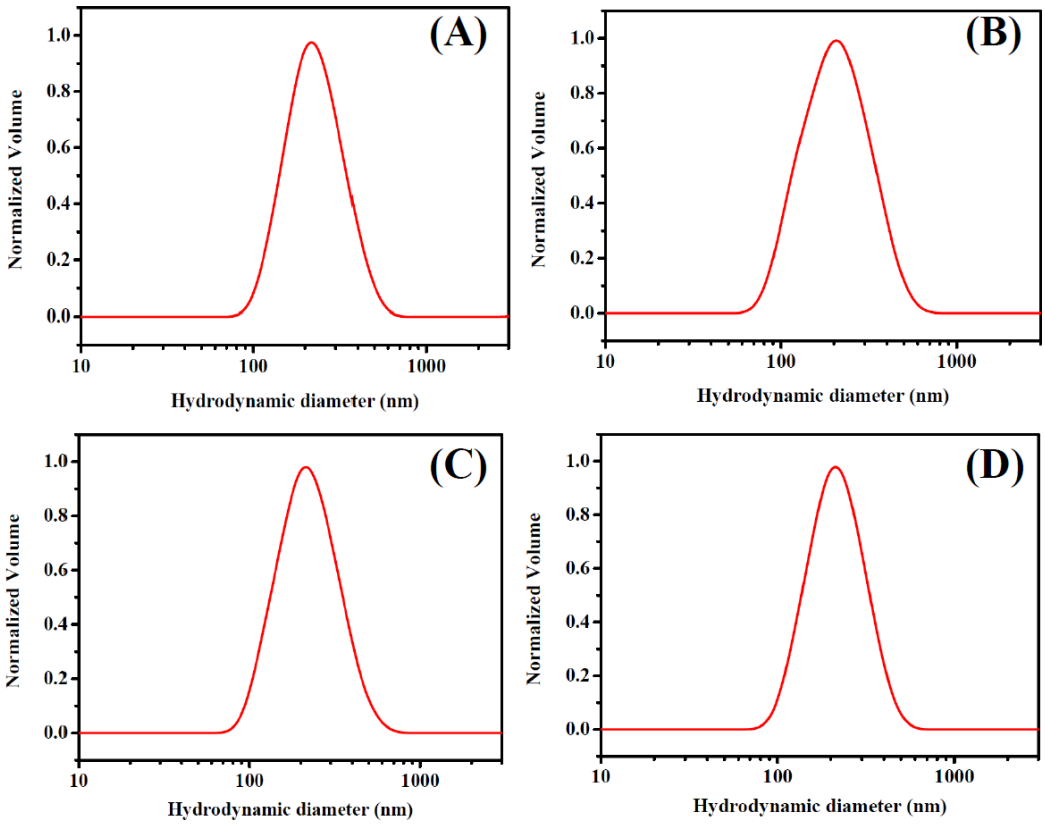

Figure S6. Volume-size distributions of (A) bb_2 (B) cb_2 (C) bb_1 (D) cb_1 micelles in aqueous solution at a polymer concentration of 0.25 mg/mL.

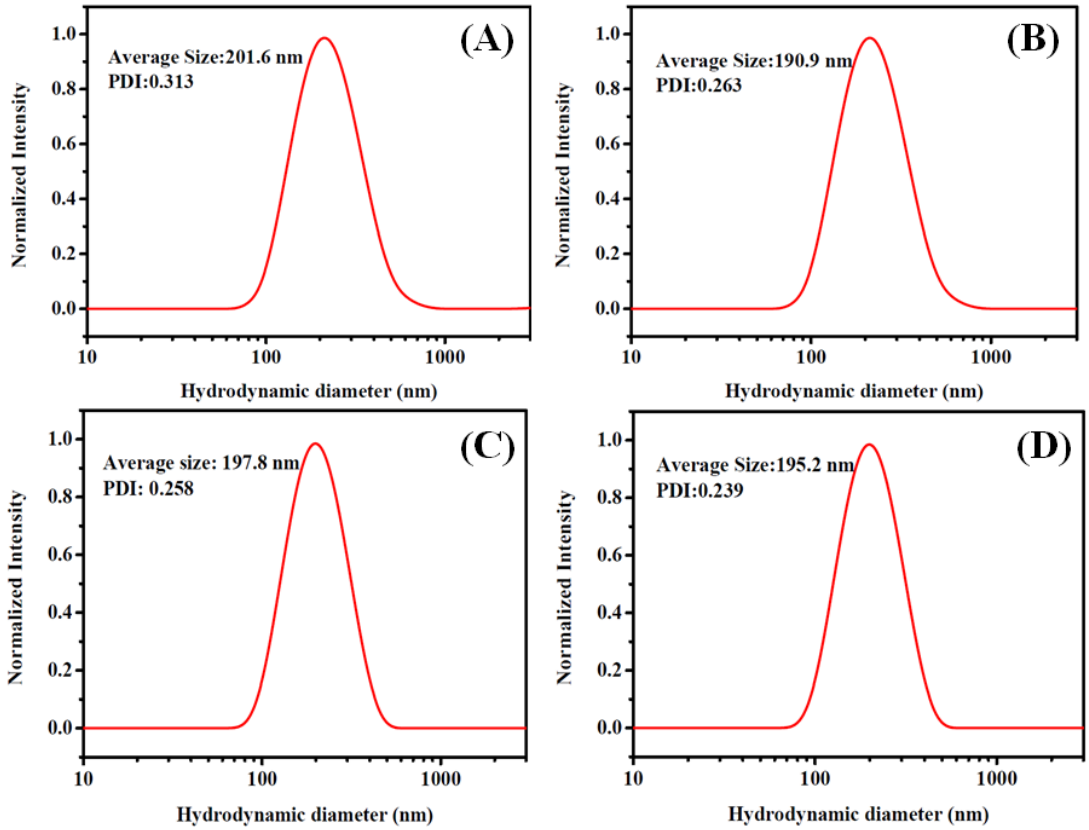

Figure S7. Size distributions of (A) *bb*$_2$ (B) *cb*$_2$ (C) *bb*$_1$ (D) *cb*$_1$ micelles in PBS (pH 7.4, 150 mM) at a polymer concentration of 0.25 mg/mL.

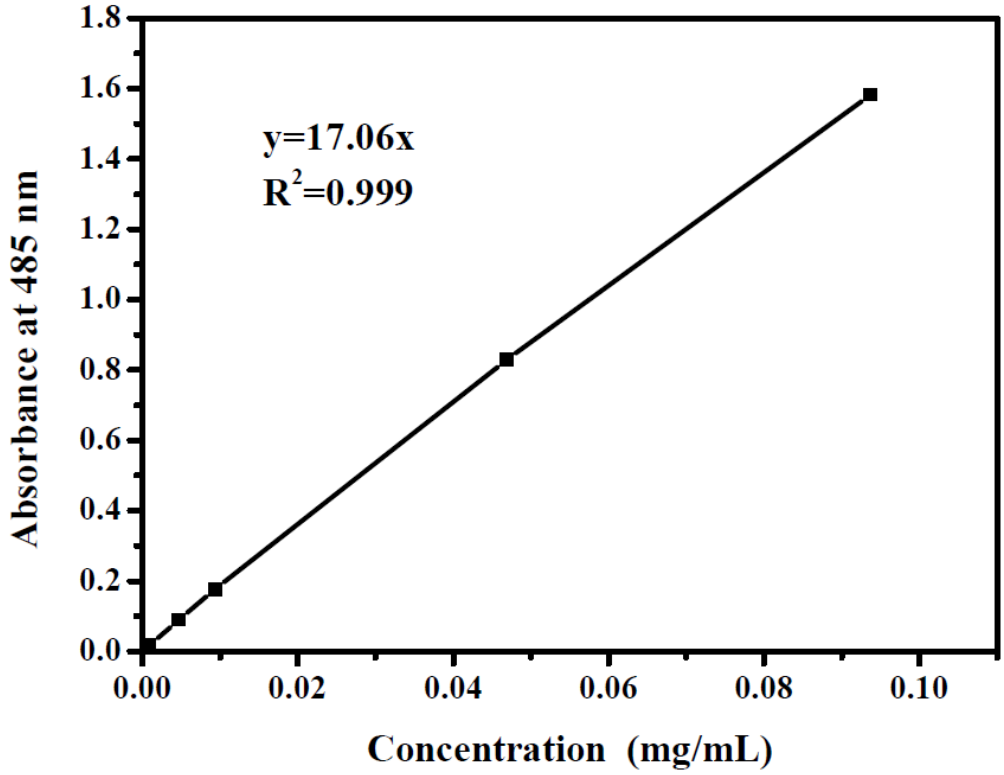

Figure S8. Standard calibration curve plotted by the absorbance at 485 nm against various DOX/DMF concentrations.

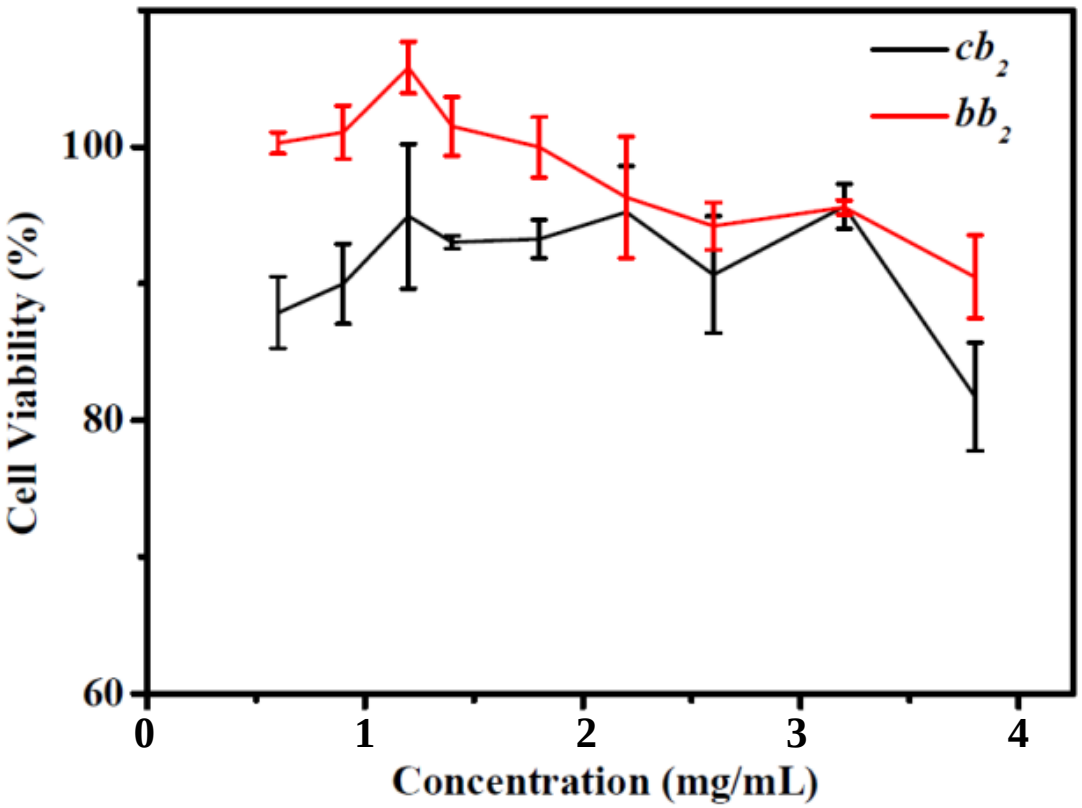

Figure S9. *In vitro* cytotoxicity of blank bb_2 and cb_2 copolymers in HeLa cells. Cell viability was determined by MTS assay and expressed as % viability compared to control untreated cells.

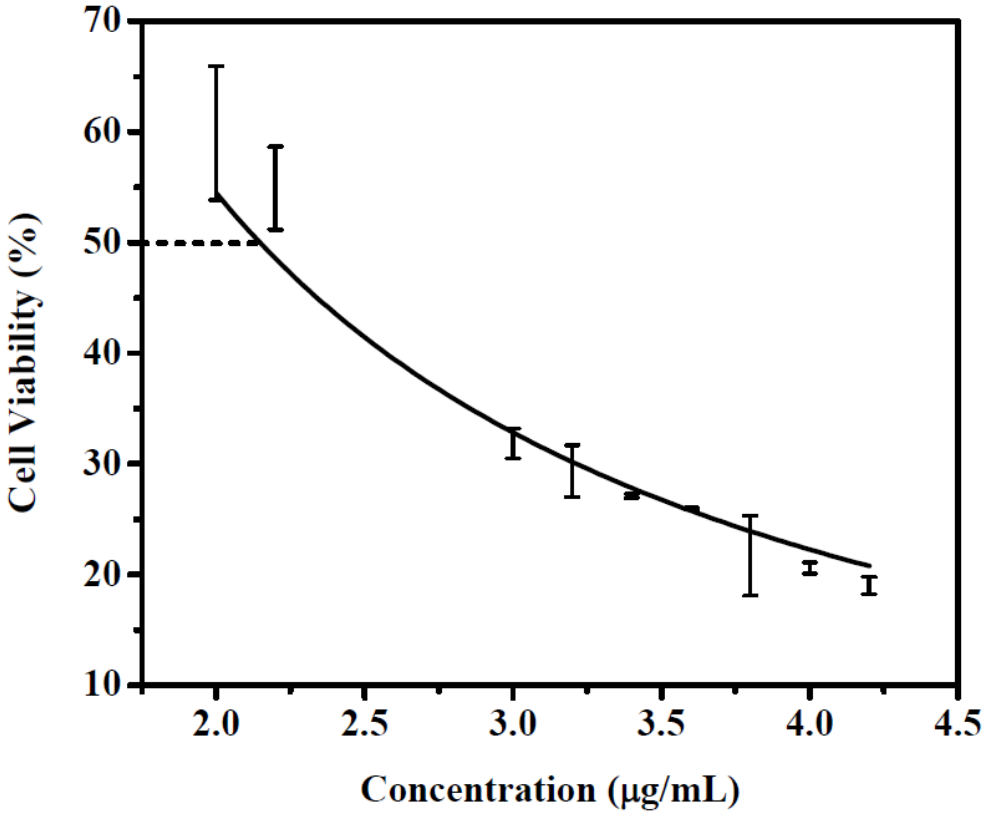

Figure S10. *In vitro* cytotoxicity of free DOX in HeLa cells. Cell viability was determined by MTS assay and expressed as % viability compared to control untreated cells.