Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting Information

Fabrication of Theranostic Amphiphilic Conjugated Bottlebrush Copolymers with Alternating Heterografts for Cell Imaging and Anticancer Drug Delivery

Fangjun Liu†,[a], Xuezhi Zhao†,[a], Xiaolong Zhang[a], Xianshuo Zhang[a], Jinlei Peng[a], Huiru Yang[a], Kaicheng Deng[a], Liwei Ma[a], Cong Chang[b], 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] Department of Pharmaceutics, School of Pharmacy, Hubei University of Chinese Medicine, Wuhan, Hubei 430065, China

† These authors contributed equally to this paper.

*Corresponding authors

E-mail address: weih@lzu.edu.cn (H. Wei) or 1126@hbtcm.edu.cn (C. Chang)

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Experimental section

Materials

Stannous (II) octanoate ($Sn(Oct)_2$), copper(I) bromide (CuBr), triethylamine (TEA), 2-bromoisobutyryl bromide, oligo (ethylene glycol) monomethyl ether methacrylate (OEGMA, M_n = 300 g/mol) and ε-caprolactone (ε-CL) were purchased from Sigma-Aldrich. ε-CL was dried over CaH_2 overnight, and then distilled under reduced pressure prior to use. OEGMA was passed through a basic alumina column to remove the inhibitor. Tetrabutylammoniumbromide (TBAB), 1,6-dibromohexane, 2-(2-(2-chloroethoxy)ethoxy)ethanol, 2,7-dibromofluorene, and tetrakis-(triphenylphosphine)-palladium(0) were purchased from J&K and used as received without further purification. *N,N,N′,N″,N″*-Pentamethyldiethylenetriamine (PMDETA) (Aladdin), bis(pinacolato)diborane, [Pd(dppf)Cl_2] (dppf = 1,1′-bis (diphenylphosphanyl) ferrocene)) (Aladdin), tetrahydrofuran (THF), *N,N*-dimethylformamide (DMF) and propargyl alcohol (Tianjin Chemical Reagent Factory (China)), sodium azide (NaN_3, Sanyou, Shanghai), anisole (Kelong, Chengdu, China), and other reagents were used as received without further purification. 2,7-Diiodo-9H-fluorene,[1] 2,7-dibromo-9,9-bis(6-bromohexyl)-fluorene,[2] 2,7-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9,9-bis(6′-bromohexyl)fluorine,[3] 2,7-bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9,9-bis(6′-azidohexyl)fluorine,[4] and 2,7-diiodo-9,9-bis(2-(2-(2-hydroxyethoxy)ethoxy) ethyl)–fluorene[5] were synthesized according to the reported procedures.

Synthesis of 2,7-Dibromo-9,9-bis(6-bromohexyl)-fluorene

2,7-dibromofluorene (6.61 g, 20 mmol) and tetrabutylammoniumbromide (TBAB) (0.65 g, 1 mmol) were dissolved in 1, 6-dibromohexane (32 mL, 200 mmol), and 50% KOH solution (20 mL) was injected. After stirring for 1.5 h at 75^oC, dichloromethane (DCM) (100 mL×3) was used to extract the mixture, and the organic phase was combined, washed with saturated $NaHCO_3$ solution (100 mL×3), brine solution (100 mL), dried over anhydrous Mg_2SO_4, filtered,

and rotary evaporated under reduced pressure to remove DCM and excessive 1,6-dibromohexane. Finally, the crude product was purified by column chromatography on silica gel (eluent: hexane:dichloromethane = 95:5, v/v), affording a white solid (10.6 g, yield: 81.5 %). ^{1}H NMR (400 MHz, $CDCl_3$), δH [ppm]: 7.53 (d, 2H, J = 8.0), 7.46 (dd, J = 1.6.0 and 8.0, 2 H), 7.43 (d, 2 H, J = 1.6), 3.30 (t, 4 H, J = 6.8), 1.92 (m, 4 H), 1.67 (m, 4 H), 1.19 (m, 4 H), 1.10 (m, 4 H), 0.58 (m, 4 H).

Synthesis of 2,7-Bis (4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)- 9,9-bis (6′-bromohexyl) fluorine

2,7-Dibromo-9,9-bis(6-bromohexyl)-fluorene (3.25 g, 5 mmol), bis(pinacolato)diborane (3.08 g, 12 mmol), KOAc (3.61 g, 35 mmol), and [Pd(dppf)Cl_2] (0.37 g, 0.25mmol, dppf =1,1′-bis(diphenylphosphanyl)ferrocene) were dissolved in dioxane (50 mL). After three freeze–pump–thaw cycles to replace air with nitrogen, the mixture was kept for 24 h at 85 °C. Dioxane was distilled out, and the residual was dissolved in DCM, washed with water, dried over anhydrous Na_2SO_4, filtered, and rotary evaporated under reduced pressure to remove the solvent. Finally, the crude product was purified by column chromatography on silica gel (eluent: hexanes / dichloromethane = 2/1, v/v), affording a white solid (2.78 g, 74.6 %). ^{1}H NMR (400 MHz, $CDCl_3$), δH [ppm]: 7.83-7.81 (m, 2 H), 7.73-7.71 (m, 4 H), 3.03 (t, 4 H, J=7.2 Hz), 2.00 (m, 4 H), 1.58 (m, 4 H), 1.39 (s, 24 H), 1.11 (m, 4 H), 1.05 (m, 4 H), 0.55 (m, 4 H).

Synthesis of 2,7-Bis (4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9,9- bis(6′-azidohexyl) fluorine (1)

2,7-Bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9,9-bis(6′-bromohexyl) fluorine (3.72 g, 5 mmol) and sodium azide (0.99 g, 15 mmol) were dissolved in DMSO (35 mL). After being stirred at 75 °C for 4 h, water (150 mL) was added, and DCM was used to extract the mixture. The organic phase was combined, washed with water 5 times, dried over anhydrous Mg_2SO_4, filtered,

and rotary evaporated under reduced pressure to remove the solvent by. Finally, the crude product was purified by column chromatography on silica gel (eluent: hexanes / dichloromethane = 1/1, v/v), affording a white solid (2.60 g, yield: 77.7 %). ^{1}H NMR (400 MHz, $CDCl_3$), δH [ppm]: 7.82-7.80 (m, 2 H), 7.73-7.71 (m, 4 H), 3.10 (t, 4 H, J=6.8 Hz), 2.00 (m, 4 H), 1.39 (s, 24 H), 1.34 (m, 4 H),1.07 (m, 8 H), 0.55 (m, 4 H).

Synthesis of 2,7-Diiodo-9H-fluorene

To a mixture solution of acetic acid (350 mL) and 20 % H_2SO_4 (32 mL), fluorine (5.54 g, 32.64 mmol), KIO_3 (2.82 g, 13.06 mmol), and I_2 (8.97 g, 35.26 mmol) was added in turn. After being stirred for 20 h at 80 ^{o}C, the reaction mixture was cooled to room temperature, and saturated $Na_2S_2O_4$ (300 mL) was added. DCM (150 mL×3) was later added to extract the mixture, and the organic phase was combined, washed with saturated $NaHCO_3$ solution (100 mL×3), brine solution (100 mL), dried over anhydrous Mg_2SO_4, filtered, and rotary evaporated under reduced pressure to remove the solvent. Finally, the crude product was recrystallized with DCM and dried under vacuum to give white solids (11.11 g, 81 %). ^{1}H NMR (400 MHz, $CDCl_3$), δH [ppm]: 7.87 (s, 2 H), 7.70 (d, J = 8.00 Hz, 2 H), 7.49 (d, J = 8.00 Hz, 2 H), 3.83 (s, 2 H).

Synthesis of 2,7-Diiodo-9,9-bis(2-(2-(2-hydroxyethoxy)ethoxy)ethyl) –fluorene (2)

2,7-Diiodo-9H-fluorene (5.18 g, 12.4 mmol), 2-(2-(2-chloroethoxy)ethoxy)ethanol (4.06 mL , 27.04 mmol), KOH (1.64 g, 24.92 mmol) and KI (0.80 g, 4.8 mmol) were dissolved in *N,N*-dimethylformamide (DMF) (60 mL). After being stirred for 8 h at 80 ^{o}C, the solvent was removed by rotary evaporation under reduced pressure. DCM was used to dissolve the residue, followed by being washed with diluted hydrochloric acid solution, brine, and dried over anhydrous Mg_2SO_4, filtered, and rotary evaporated under reduced pressure to remove the solvent. Finally, the crude product was purified by column chromatography on silica gel (eluent: dichloromethane/methanol =

98/2, v/v), affording a pale yellow oil (2.28 g, yield: 54 %). ^{1}H NMR (400 MHz, $CDCl_3$), δH [ppm]: 7.76 (d, J = 1.60 Hz, 2 H), 7.68 (dd, J = 8.00 Hz and 1.60 Hz, 2 H), 7.40 (d, J= 8.00 Hz, 2 H), 3.69 (t, 4 H), 3.52-3.43 (m, 8 H), 3.24 (t,4 H), 2.84 (t, 4 H), 2.32 (t, 4 H).

Synthesis of hydroxyl and azido-functionalized Polyfluorene (PF-((*g*-N_3)-*alt*-(*g*-OH))) by Suzuki coupling reaction

To a mixture solution of toluene (5 mL) and water (3 mL), 2,7-Bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9,9-bis(6′-azidohexyl) fluorine (0.34 g, 0.5 mmol), 7-Diiodo-9,9-bis(2-(2-(2-hydroxyethoxy)ethoxy) ethyl)-fluorene (0.33 g, 0.5 mmol), $Pd(PPh_3)_4$ (10 mg), K_2CO_3 (0.84 g, 6 mmol), and Alquat 336 (0.016 mg, 0.04 mmol) was added. After three freeze–pump–thaw cycles to replace air with nitrogen, the mixture was kept for 24 h at 85 °C. After reaction, the mixture was cooled to room temperature and extracted with THF. The organic phase was combined, dried over anhydrous $MgSO_4$, filtered, and rotary evaporated under reduced pressure to remove the solvent. The residue was dissolved in 2 mL of THF, and the mixture was added dropwise into cold methanol (20 mL) to precipitate the product, which was later harvested by centrifugation. The process was repeated again to purify the product. Finally, the precipitates were desiccated in vacuum to afford a yellow solid (95 mg, yield: 45.1 %).

Synthesis of Alkyne-PCL_{30}-OH by ring-opening polymerization

Alkyne-PCL_{30}-OH was synthesized according to the procedures previously reported for the synthesis of 4-arm star-shaped PCL-*b*-POEGMA block copolymers[6] except for the use of propynol as the starting initiator (Yield, 81.7 %). The degree of polymerization (DP) of PCL was calculated to be 30 by ^{1}H NMR analyses.

Synthesis of Alkyne-PCL_{30}-$OOCCH_3$

Alkyne-PCL_{30}-$OOCCH_3$ was synthesized according to the procedures previously reported for the synthesis of reduction-responsive star-shaped amphiphilic block copolymers.[7] In this study, anhydrous acetic acid was first reacted with oxalyl chloride, followed by the addition of alkyne-PCL_{30}-OH (Yield, 92.5 %).

Synthesis of conjugated *bb* copolymers PF_{13}-((*g*-PCL_{30}-$OOCCH_3$)-*alt*- (*g*-OH)) by click reaction through "grafting to" approach

PF_{13}-((*g*-N_3)-*alt*-(*g*-OH)) (60 mg, 0.14 mmol $-N_3$), alkyne-PCL_{30}-$OOCCH_3$ (421 mg, 0.12 mmol), PMDETA (13.48 mg, 0.077 mmol), and $CuBr_2$ (1.58 mg, 0.007 mmol) were dissolved in DMF (5 mL). After three freeze−pump−thaw cycle, CuBr (10.04 mg, 0.07 mmol) was loaded under the protection of nitrogen flow. After another three freeze−pump−thaw cycle, the mixture was sealed and kept at 45 °C for 48 h. The reaction was quenched by exposure to air, followed by dialysis against distilled water. Because of the hydrophobicity of the product, formation of precipitates was observed in the dialysis tube. The precipitates were harvested by centrifugation and freeze-drying to afford a pale yellow solid (yield, 85.2 %).

Synthesis of conjugated polymer bottlebrushes ATRP macroinitiator PF_{13}-((*g*-PCL_{30}-$OOCCH_3$)-*alt*-(*g*-Br))

PF_{13}-((*g*-PCL_{30}-$OOCCH_3$)-*alt*-(*g*-Br)) was synthesized according to the procedures previously reported for the synthesis of four-arm star-shaped PCL-*b*-POEGMA block copolymer[6] (Yield, 93.7 %).

Synthesis of conjugated amphiphilic *bb* copolymers PF_{13}-((*g*-PCL-$OOCCH_3$)-*alt*-(*g*-POEGMA)) by ATRP through "grafting from" approach

PF_{13}-((*g*-PCL_{30}-$OOCCH_3$)-*alt*-(*g*-Br)) (30 mg, 0.008 mmol -Br), PMDETA (1.46 mg, 0.008 mmol), and OEGMA (250 mg, 0.8 mmol) were dissolved in anisole (4.16 mL). After three freeze−pump−thaw cycle, CuBr (1.21 mg, 0.008

mmol) was loaded under the protection of nitrogen flow. After another three freeze−pump−thaw cycle, the mixture was sealed and kept at 60 °C. The reaction mixture was quenched by being exposed to air at predetermined time intervals and poured into ice-cold diethyl ether to precipitate the prodcuct. The crude product was collected by centrifugation, followed by dialysis against distilled water to remove any unreacted monomer and copper catalyst. The purified product was harvested by freeze-drying.

Characterization of Polymers

The samples were dissolved in $CDCl_3$ for ^1H NMR measurements on a JEOL-ECS 400 MHz NMR spectrometer with TMS as an internal reference. The molecular weight (MW) and molecular weight distribution ($Đ_M$) of the prepared polymers were determined by the size exclusion chromatography and multiangle laser light scattering (SEC-MALLS). SEC was performed using HPLC-grade DMF containing 0.1 wt % LiBr at 60 °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.

Preparation and Characterization of Self-Assembled Micelles

Taking P_4 as an example, P_4 (0.5 mg) was dissolved in 1 mL of deionized water and the micelle solution with a concentration of 0.5 mg/mL was obtained. The TEM images were recorded on a JNM-2010 instrument operating at an acceleration voltage of 200 keV. To get the specimens for TEM observation, a 20 μL droplet of micelle solution was dripped onto a carbon-coated copper grid. After deposition for 15 min, excess solution was removed by a strip of filter paper. The sample was further stained using phosphotungstic acid (1 % w/w) and dried in air prior to visualization.

Dynamic light scattering (DLS) was used to determine the average hydrodynamic size of micelles on a Zetasizer (Nano ZS, Malvern, Worcestershire, UK) with the detection angle fixed at 173°. The sample solution was passed through a Millipore 0.45 μm pore-sized syringe filter prior to measurements.

Spectral Feature Evaluation

The absorption spectra of various samples were recorded on a PerkinElmer Lambda 35 UV−vis spectrometer (PerkinElmer, Waltham, MA, United States). The concentrations of the samples were fixed at 0.1 mg/mL in both water and DMF. Fluorescence spectra were recorded on a LS55 luminescence spectrometer (PerkinElmer). The concentration was 0.001 mg/mL in both water and DMF. Excitation wavelength was fixed at 365 nm. Emission spectrum was recorded from 350 to 600 nm. The bandwidths of excitation and emission were both 5 nm.

The fluorescence quantum yields of the polymers in water and DMF were calculated using quinine sulfate in 0.5 mol/L H_2SO_4 ($\Phi_F = 0.55$) as the standard. The emission spectrum was recorded with an excitation wavelength of 365 nm. The relative fluorescence quantum yield was calculated based on the following expression,

$$\Phi_S = F_S/F_R \times (1 - 10^{-A_R})/(1 - 10^{-A_R}) \times \eta_S^2/\eta_S^2 \times \Phi_R \quad (1)$$

The subscripts R and S respectively refer to the reference and sample. Φ is fluorescence quantum yield, and Φ_R is equal to 0.55. F is the integrated fluorescence intensity. A is the absorbance at the excitation wavelength, and η is the solvent refractive index.

***In Vitro* Drug Loading and Drug Release**

To obtain the free DOX base, DOX·HCl (1 mg) and TEA (0.26 g) were dissolved in 2 mL of DMSO. After stirring overnight in dark at room

temperature, the polymer (10 mg) dissolved in 2 mL of DMSO was added to the above solution. After stirring for another 1 h at room temperature, the mixture was added dropwise to 4 mL of ultrapurified water under vigorous stirring. After stirring for another 1 h, the mixture was dialyzed against distilled water for 24 h, and 5 L water was refreshed every 8 h. Finally, the drug-loaded micelle was collected by freeze-drying. To determine the drug loading content (DLC) and entrapment efficiency (EE), the drug-loaded micelles were re-dissolved in PBS (pH 7.4). The concentration of DOX was detected on a Lambda 35 UV−vis spectrometer (PerkinElmer) at 485 nm. DLC and EE were calculated as follows,

$$DLC\ (\%) = W_{\text{drug loaded in particles}}/W_{\text{particles}} \times 100\ \% \qquad (2)$$

$$EE\ (\%) = W_{\text{drug loaded in particles}}/W_{\text{drug fed for encapsulation}} \times 100\ \% \qquad (3)$$

The in vitro drug release study was investigated respectively in PBS (pH 7.4, 150 mM) and saline sodium citrate (SSC, pH 5.0, 150 mM) at 37 °C. The drug-loaded micelles were re-dispersed in PBS buffer, and the drug-loaded micelle solution with a concentration of 1 mg/mL was obtained. 1 mL of the solution was loaded in the dialysis bag (MWCO 3,500 kDa), which was immersed in a tube loaded with 25 mL of release medium. The tube was placed in a horizontal laboratory shaker thermostated with a constant temperature of 37 °C and a stirring speed of 120 rpm. At the predetermined time intervals, 3 mL of the release medium in tube was taken out and equal volume of fresh medium was added. The drug concentration was calculated by measuring the absorbance at 485 nm according to a standard calibration curve. The experiment was performed in triplicate for each sample.

Cell Imaging

HeLa cells were seeded in 6-well plates with a plating density of 5×10^5 cells per well in 1 mL of complete growth medium and incubated in the 37 °C, 5 % CO_2 environment for 24 h. The solutions of DOX and DOX-loaded micelles

were prepared in complete growth medium at the concentration equal to 25 % of their respective IC_{50} values and were later added to the wells and incubated for 24 h at 37 °C. Then, cells were rinsed with PBS and fixed with 4 % paraformaldehyde (PFA) solution for 20 min at room temperature. Finally, cells were counterstained with acridine orange (AO). Coverslips were mounted onto glass slides and imaged using a Nikon A1R confocal microscope.

Cell Viability Study

The cytotoxicities of various formulations were investigated *in vitro* using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay. The cells were seeded in the 96-well plates with the density of 2500 cells per well in 100 μL of complete growth medium and incubated in 37 °C, 5 % CO_2 environment for 24 h. Samples were prepared in serial dilutions in water and then diluted 10-fold in OptiMEM medium (Invitrogen). The cells were then rinsed once with PBS and incubated with 40 μL of the sample solutions with different polymers 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-carbox-ymethoxyphenyl)-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.

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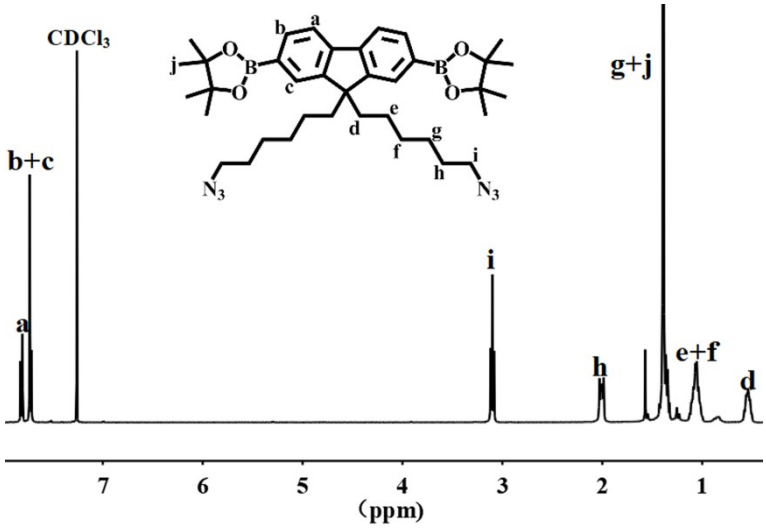

Figure S1. [1]H NMR spectrum of 2,7-Bis (4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-9,9-bis(6′-azidohexyl) fluorine in $CDCl_3$.

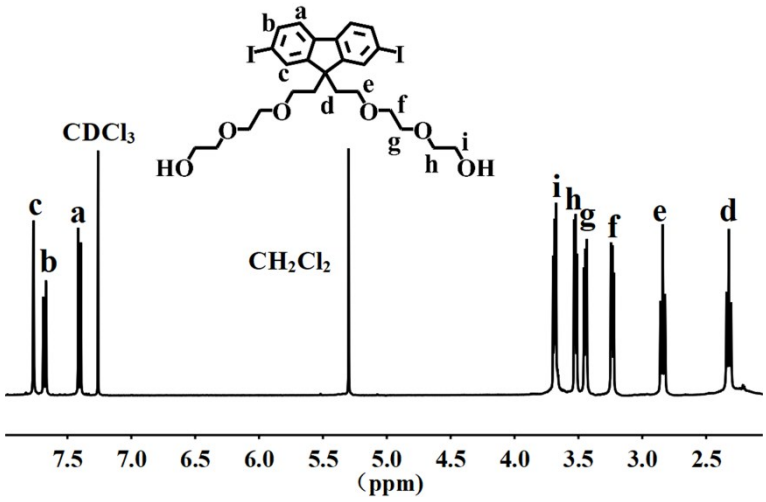

Figure S2. [1]H NMR spectrum of 2,7-Diiodo-9,9-bis(2-(2-(2-hydroxyethoxy)ethoxy)ethyl) –fluorene in $CDCl_3$.

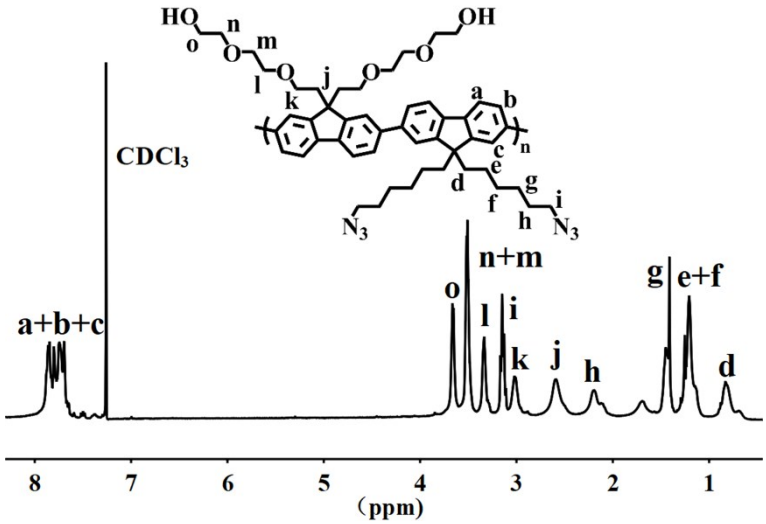

Figure S3. ^{1}H NMR spectrum of PF_{13}-((*g*-N_3)-*alt*-(*g*-OH)) in $CDCl_3$.

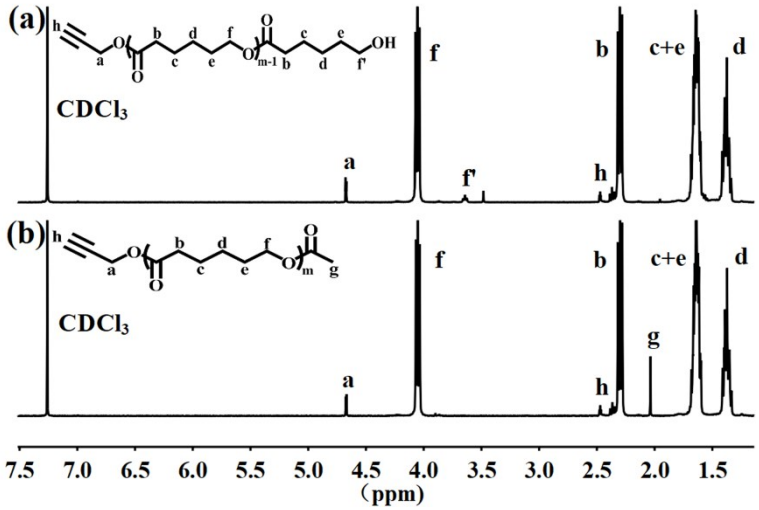

Figure S4. ^{1}H NMR spectra of (a) alkyne-PCL_{30}-OH and (b) alkyne-PCL_{30}-$OOCCH_3$ in $CDCl_3$.

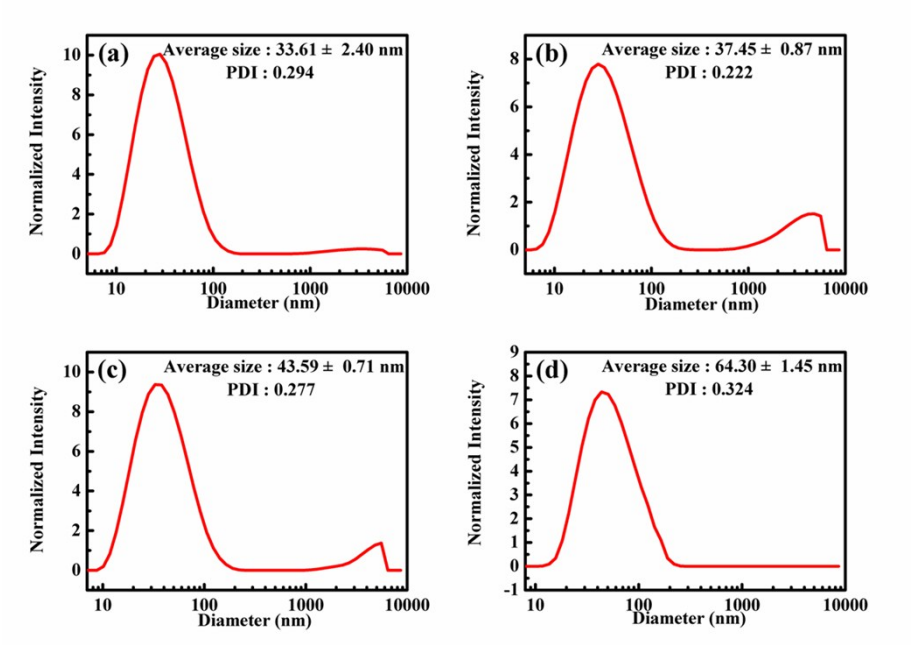

Figure S5. Size distributions of (a) P_1, (b) P_2, (c) P_3 and (d) P_4 micelles in DMF at a polymer concentration of 0.1 mg/mL.

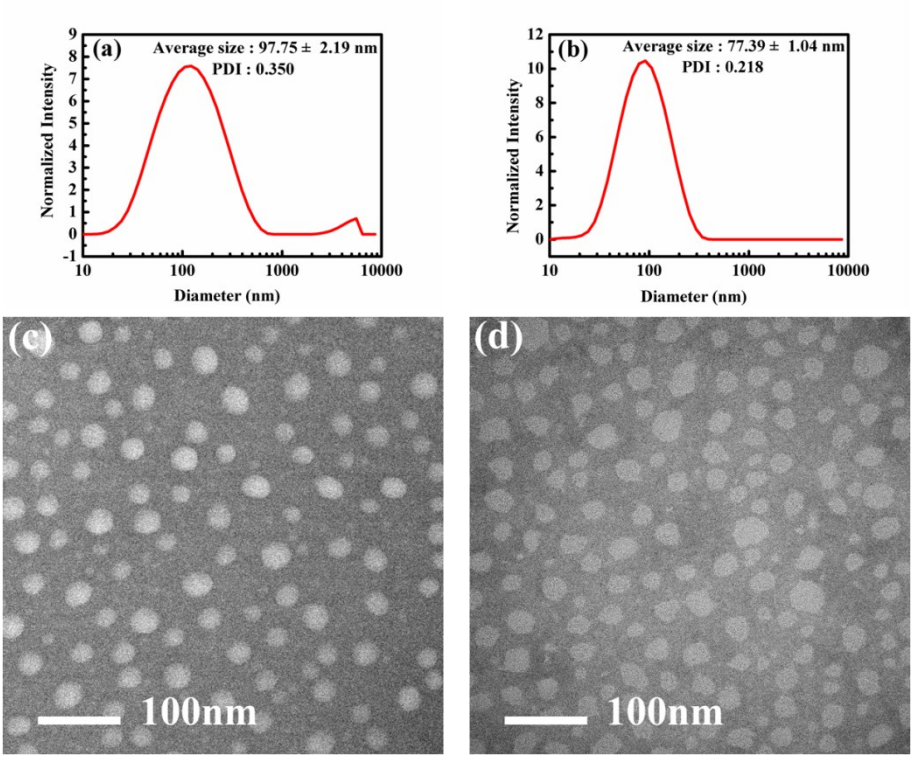

Figure S6. Size distributions and TEM images of (a & c) P_1 and (b & d) P_2 micelles in water at a polymer concentration of 0.1 mg/mL.

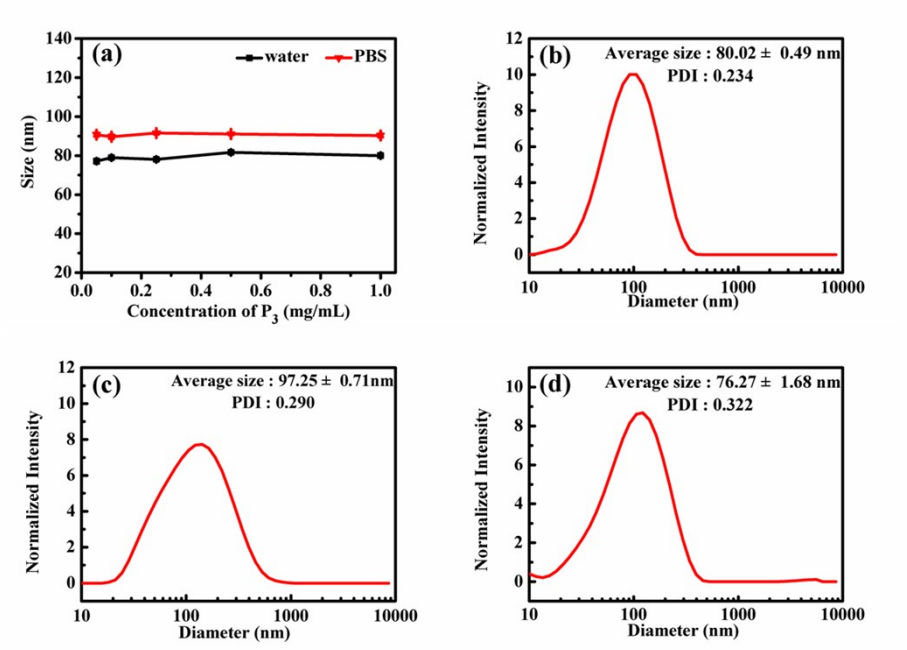

Figure S7. (a) Average size of P_3 at various concentrations in water and PBS (pH7.4, 150mM) determined by DLS, size distributions of (b) P_3 in PBS, (c) DOX@P_3 in PBS and (d) DOX@P_3 in the presence of 10% FBS at a polymer concentration of 1 mg/mL.

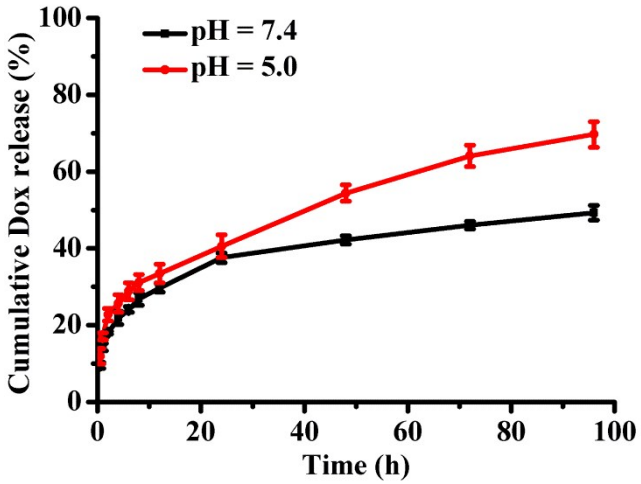

Figure S8. In vitro drug release profiles of DOX@P_3 micelles at different pHs of 7.4 and 5.0 at 37 °C.

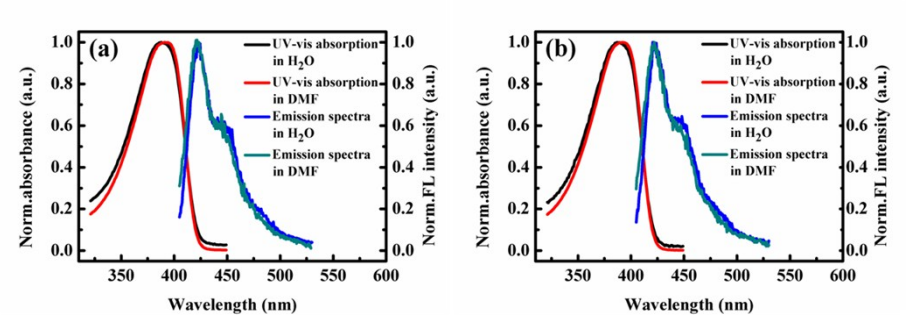

Figure S9. UV-vis absorption and emission spectra of (a) P_1 and (b) P_2 in water and DMF.

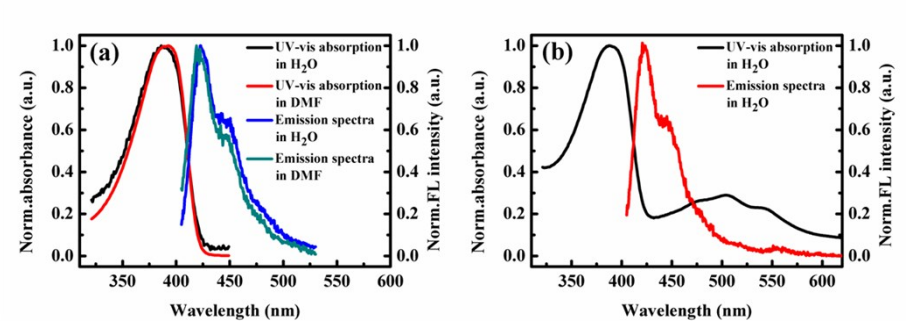

Figure S10. UV-vis absorption and emission spectra of (a) P_3 in water and DMF, and (b) DOX@P_3 in water.

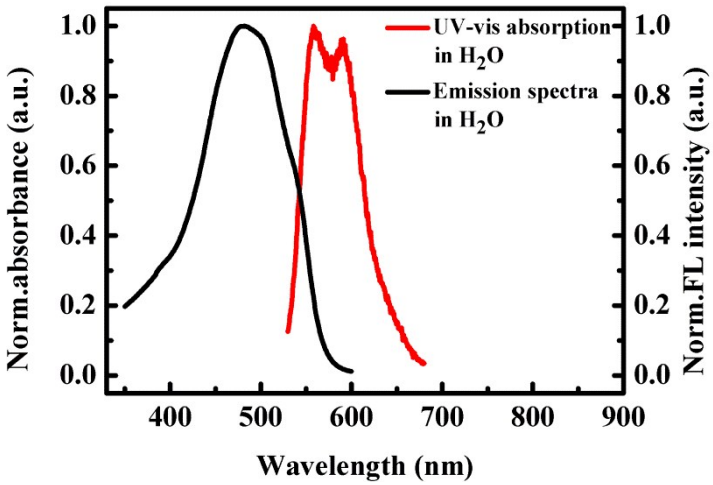

Figure S11. UV-vis absorption and emission spectra of free DOX in water.

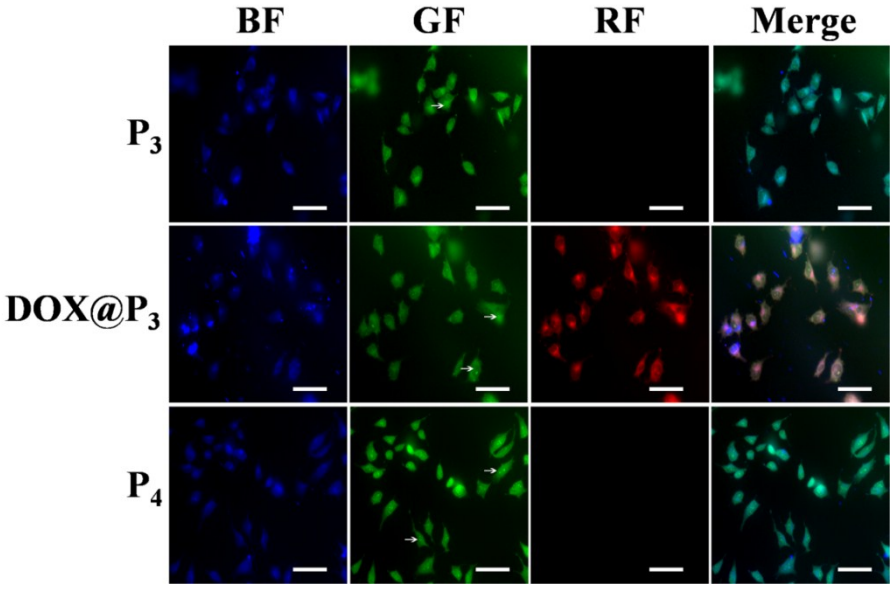

Figure S12. Fluorescence microscopy images of P_3, DOX@P_4 and P_4 (blue for PF moiety) micelles uptake in HeLa cells (cytoplasm stained green with AO). The scale bar represents 50 μm.

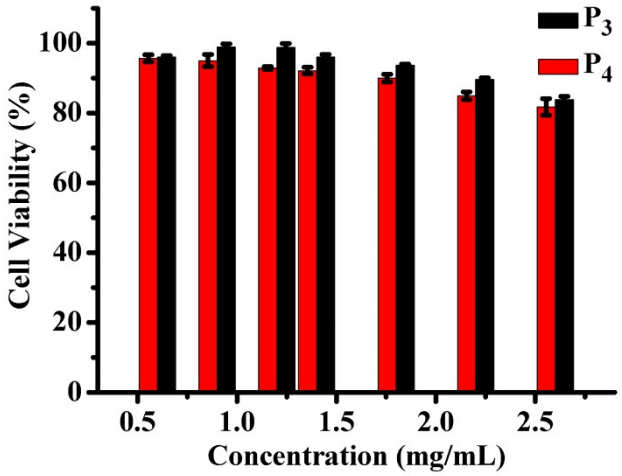

Figure S13. *In vitro* cytotoxicity of blank P_3 and P_4 micelles in HeLa cells.

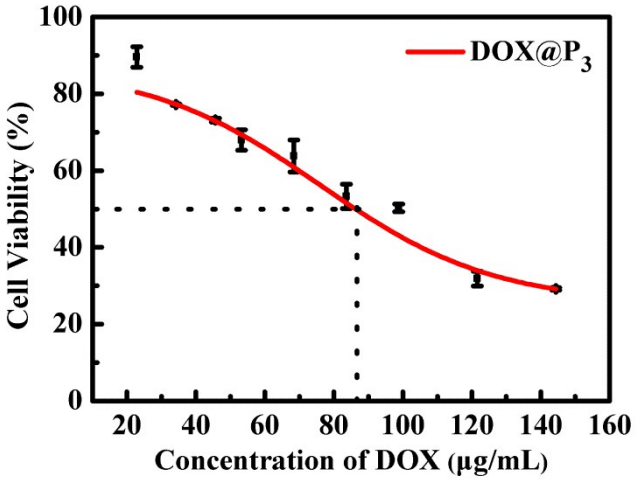

Figure S14. *In vitro* cytotoxicity of DOX@P_3 micelles in HeLa cells.

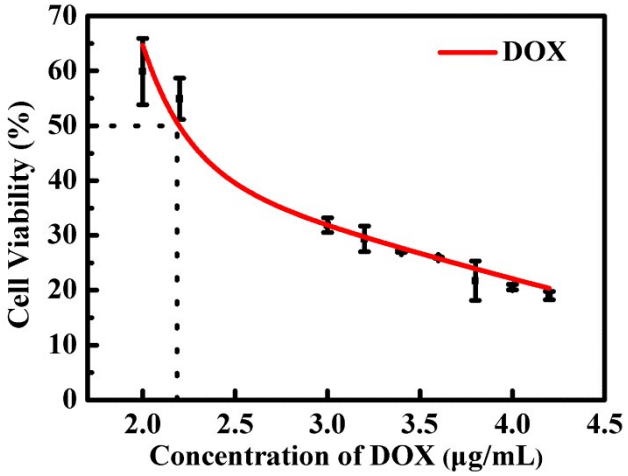

Figure S15. *In vitro* cytotoxicity of free DOX in HeLa cells.