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Electronic Supplementary Information

Unimolecular Micelles of Amphiphilic Cyclodextrin-Core Star-like Block Copolymers for Anticancer Drug Delivery

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Reagents and Materials. All chemical reagents including β-cyclodextrin (β-CD, 99.7 %), α-bromoisobutyryl bromide (BIBB, 98 %), 3,6-dimethyl-1,4-dioxane-2,5-dione (DL-lactide, LA), Dimethyl aminopyridine (DMAP, 99.0 %), triethylamine (TEA, 99 %), Copper(I) bromide (Aldrich, 98.0 %), N, N, N′, N″, N″-pentamethyldiethylenetriamine (PMDETA, 99.0 %) and Poly(ethylene glycol) methyl ether methacrylate (OEGMA, M_n=500) were purchased from Sigma-Aldrich (USA) and used as received unless otherwise noted. Copper(I) bromide was purified by washing with acetic acid and methanol three times, respectively, then stored in glove box. OEGMA was passed through a column of activated basic alumina to remove inhibitors before using. Doxorubicin hydrochloride (DOX·HCl) was obtained from Beijing HuaFeng United Technology CO. Ltd (Beijing, China). All anhydrous solvents including anisole and tetrahydrofuran (THF) were also provided by sigma-aldrich (USA) and used directly. All the other solvents were analytical grade and provided by the Ctech Global Pte Ltd (Singapore). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin /streptomycin mixture, phosphate buffered saline (PBS), Alexa Fluor® 633 phalloidin, TrypLE™ Express Enzyme (1×), DAPI (4',6-diamidino-2-phenylindole) and PrestoBlue cell viability reagent were purchased from Life Technologies (Singapore). Deionized (D.I.) water was prepared from Millipore (Bedford, MA, USA).

Characterization. ^{1}H NMR spectra were recorded on a Bruker AV 300 NMR spectrometer (Rheinstetten, Germany) using tetramethylsilane as an internal standard at 25 ℃. The size distribution and Zeta-potentials of resulting micelles were determined by dynamic light scattering (DLS) using a BI-200SM (Brookhaven, USA) with angle detection at 90^o. The morphology of the samples was recorded by a JEM-1230EX transmission electron microscopy (TEM) (Japan). Samples for TEM measurements were prepared by the following method: for the micelle samples prepared in water, one droplet of the aliquot was cast on carbon-coated copper grids held by tweezers and dried in air at room temperature; for the micelle samples prepared in DMF, one droplet of the aliquot was cast on carbon-coated copper grids held by tweezers and the excess solution was wicked off with filter paper, and the process was repeated three times to ensure enough sample coverage on the grids. The Fourier transform infrared (FT-IR) spectra were acquired on a Perkin Elmer FT-IR spectrophotometer (USA) using KBr pellets. The number-

average molecular weight (M_w) and molecular weight distribution (M_w/M_n) were measured by gel permeation chromatography (GPC) system (Agilent 1260, USA) equipped with waters 1260 pump and a Agilent 1260 refractive index detector and a styragel® HT column. Tetrahydrofuran (THF) was used as the eluent (1 mL/min), and polystyrene was used as the standard for calibration. Fluorescence spectra were recorded on a Perkim Elmer LS-55 fluorescence spectrometer (Perkim Elmer,USA). Absorbance spectra were carried out using a Shimadzu UV-2450 visible spectrophotometer (Shimadzu, Japan). The fluorescence images of cells were taken using a confocal laser scanning microscopy (LSM 780, Carl Zeiss, Germany).

Synthesis of β-CD-PLA. The hydrophobic core β-CD-PLA was synthesized as follows: β-CD (60 mg, 0.05 mmol, 1.05 mmol OH) was completely dried in a vacuum at 80 °C over calcium oxide overnight and immediately transferred into a flask in glove box. Then, lactide (LA, 7.57 g, 52.5 mmol) and DMAP (250 mg, 2.1 mmol) were weighted into with magnetic stirring. The reaction temperature was maintained at 120 °C for 30 min. The resulting syrup-like polymer was diluted with THF and then poured into ether, leading to the precipitation of an off-white polymer and denoted as β-CD-PLA, this process repeated three times. The solvents were evaporated under vacuum, and resulting white product was dried under vacuum for 48 h (Yield: 60%, $M_{n,\ GPC}$=72.3 KDa, M_w/M_n=1.12, Figure S5). The actual DPs of LA segment was determined to be 24 by ^{1}H NMR results (Figure S3) and shown in Table S1.

Synthesis of β-CD-PLA-Br. Typically, β-CD-PLA (3.0 g, 0.04 mmol) was dissolved in anhydrous THF solution (20 mL), and then TEA (6.3 mmol, 880 uL) was added under an argon atmosphere at 0 °C, after 0.5 h, the BIBB (4.2 mmol , 520 uL) in 5 mL THF was added dropwise to the mixture with magnetic stirring. The reaction was carried out at 0 °C for 2 h and then stirred for 72 h at room temperature. Then THF was removed using a rotary evaporator. Then, CH_2Cl_2 was added and washed with saturated $NaHCO_3$ aqueous solution and deionized water. The organic phase was collected and the activated carbon and $MgSO_4$ were added into the CH_2Cl_2 solution. After being stirred for a night, the CH_2Cl_2 solution was collected by filtration, and concentrated by using a rotary evaporator. Then, ether was added to precipitate the product and dried in vacuum at

room temperature for 48 h. The resulting polymer was denoted as β-CD-PLA-Br (Yield: 80 %, $M_{n,\ GPC}$=80.0 KDa, M_w/M_n=1.14, Figure S5).

Synthesis of β-CD-PLA-POEGMA by ATRP. Typically, the polymer of β-CD-PLA-POEGMA was synthesized as follows: β-CD-PLA-Br (0.16 g, 0.042 mmol Br), CuBr (6.1 mg, 0.042 mmol), and OEGMA (M_n = 500, 1.0 g, 2.1 mmol) were dissolved in 3 mL anisol in a 25 mL two-neck flask, and the solution was degassed with three freeze−pump−thaw cycles. Then the PMDETA (8.0 uL, 0.042 mmol) was injected into the above solution, and the mixture was degassed with two freeze−pump−thaw cycles. The polymerization was carried out at 60 °C for different desired times. The reaction mixture was diluted with THF (10 ml) and then passed through a short neutral Al_2O_3 column to remove the copper catalyst. The resulting solution was concentrated and poured into hexane to precipitate the product. (The GPC results for β-CD-poly (LA_{24}-$OEGMA_5$) (CPP-1) is $M_{n,\ GPC}$=128 KDa, M_w/M_n=1.34; β-CD-poly (LA_{24}-$OEGMA_{12}$) (CPP-2) is $M_{n,\ GPC}$=168 KDa, M_w/M_n=1.26 and β-CD-poly (LA_{24}-$OEGMA_{24}$) (CPP-3) is $M_{n,\ GPC}$=203 KDa, M_w/M_n=1.24, as shown in Figure 1b and Table S1).

Preparation of β-CD-PLA-POEGMA unimolecular micelles in different solvents.

Typically, β-CD-PLA-POEGMA unimolecular micelles in DMF were obtained by direct dissolving corresponding copolymer in DMF and stirred for 12 h with a final concentration of 1.0 mg/mL. The resulting solution was further analyzed by DLS and TEM to corroborate a successful unimolecular micelles formation.

Furthermore, β-CD-PLA-POEGMA micelles in water were prepared by a dialysis method. Briefly, 1 mL solution of 5.0 mg/mL β-CD-PLA-POEGMA in DMF was added into 10 mL of deionized water by pump under sonication with a rate of 1.0 mL/min, followed by dialysis (MWCO 3500) against deionized water (2L × 3) for 72 h to remove organic solvent, then the β-CD-PLA-POEGMA micelles solution with a concentration of 0.5 mg/mL was further analyzed by DLS and TEM.

Preparation of DOX loaded β-CD-PLA-POEGMA micelles. DOX as a model drug was loaded into β-CD-PLA-POEGMA micelles by a dialysis method. Typically, DOX·HCl (4.0 mg), 1 equimolar amounts of triethylamine (about 45.0 uL) and β-CD-PLA-POEGMA block copolymer

(30 mg) were dissolved in 3.0 mL of DMF, and the mixture was strried at room temperature for 2 h. Then 15 mL of deionized water were added under sonication. The organic solvent and free DOX was removed by dialysis against deionized water for 72 h at room temperature (MWCO 3500) again DI water (2L×3). The concentration of resulting β-CD-PLA-POEGMA micelles were adjust to 0.5 mg/mL for further experiments. The amount of drugs in β-CD-PLA-POEGMA/DOX micelles was determined by a Perkim Elmer LS-55 fluorescence spectrometer. The loading content (LC %) and entrapment efficiency (EE %) of DOX was calculated by the following equations:

$$\text{LC \%} = \frac{\text{amount of drug in micelles}}{\text{amount of micelles}} \times 100\%$$

$$\text{EE \%} = \frac{\text{amount of drug in micelles}}{\text{total amount of drug in feed}} \times 100\%$$

In vitro release profiles of DOX were evaluated by the dialysis method. First, A dialysis bag (MWCO 3500) was filled with 2 mL of above CPP-1/DOX, CPP-2/DOX or CPP-3/DOX micelles soaked in a tube containing 50 mL of PBS 7.4 or 5.0 in a water bath with gentle shaking at 37 ± 1 °C. At predetermined time intervals, 3 mL of the external buffer was withdrawn and it was replaced with 3 mL of fresh PBS 7.4 or 5.0. Then release concentration of free DOX was calculated based on a calibration curve by fluorescence spectroscopy with an excitation spectrum at 488 nm and emission spectrum at 586 nm. The released total amount of DOX was calculated on the basis of the formula. Above release experiments were tested in triplicate.

$$m_{\text{t-act}} = (C_{\text{t}} + \frac{\text{V}}{\text{V}} \sum_{0}^{\text{t-1}} C_{\text{t}})\text{V}$$

Where $m_{t\text{-act}}$ is the actual quantity of DOX·HCl released at time t, C_t is the drug concentration in release fluid at time t measured on fluorescence spectrometer, ν is the sampled volume taken at a predetermined time interval, and V is the total volume of release fluid.

The preparation of CPP-2 and CPP-2/DOX in deuterium oxide (D_2O).

The preparation of CPP-2 solution in D_2O: Briefly, the polymer of CPP-2 (10 mg) was dissolved in 0.5 mL $CHCl_3$, after evaporation the solvent, 0.5 mL D_2O was added into and stirried

at room temperature for 3 h, and the resulting CPP-2 solution in D_2O was determinted by ^1H NMR. The CPP-2 solution in $CDCl_3$ was the control sample. (The result were shown in Figure S7).

The preparation of CPP-2/DOX solution in D_2O: Briefly, we first prepared the CPP-2/DOX micelles using the above methods of DOX-loaded β-CD-PLA-POEGMA micelles. Then the obtained CPP-2/DOX solution was freeze drying and the obatined red solid was redispersed into 1.0 mL D_2O for ^1H NMR characterization. The free DOX in D_2O and the CPP-2 solution in D_2O were as the control samples. (The result were shown in Figure S13).

Cell culture. HeLa cells and HEK 293T cells were regularly cultured and passaged using EMEM medium and DMEM medium supplemented with 10 % FBS and 1 % penicillin-streptomycin at 37 ^oC with 5 % CO_2 in a humidified incubator.

In vitro cytotoxicity. PrestoBlue assay was performed to test the cell viability, in which a nonfluorescent blue compound called resazurin ($\lambda_{max.abs}$ = 600 nm) in PrestoBlue® reagent can be reduced by live cells to resorufin ($\lambda_{max.abs}$ = 570 nm) which is red in color and highly fluorescent. By measuring the absorbance at 570 and 600 nm, cell viability can be calculated relative to the control cells without drug treatment. The cytotoxicity of CPP-2/DOX in HeLa cells was studied as below, in which free DOX was also studied for comparision . Firstly, HeLa cells were seeded into a 96-well plate (5000 cells per well) and cultured at 37 °C with 5 % CO_2. After 12 h for attachment, the culture medium was removed and replaced with fresh medium containingfree DOX and CPP-2/DOX micelles with varied concentrations for desired time, respectively. Then the culture medium was removed and PrestoBlue reagent diluted by EMEM medium were added to each wells and incubated at 37 °C with 5 % CO_2. At the same time, PrestoBlue reagents diluted by EMEM medium were also added to blank wells without cells as control. After 1 h incubation, the absorbance at 570 nm (reference wavelength is 600 nm) was detected by Plate Reader (Tecan Infinite M200 series Pro, Tecan Asia, Singapore). All samples were tested in triplicates. Cells without treatment were used as control and corresponding cell viability was set as 100%. Data were analyzed according to the protocol. Meanwhile, the cytotoxicity of blank micelles was investigated using HeLa cells and HEK 293T cells through the similar mehtod and protocol.

In vitro cellular uptake by confocal laser scanning microscopy. HeLa cells were seeded into a 6-well plate and cultured in 37 °C with 5 % CO_2. After 12 h for attachment, free DOX and CPP-2/DOX (final DOX concentration: 50 μg/mL) were added to the medium and incubated with HeLa cells. After 2 h or 6 h incubation, the cell culture medium was removed and each well was washed with 1×PBS for five times. After that, cells were fixed by formalin solution for 20 min and then washed by 1×PBS extensively for three times. Then cells were permeabilized with 0.1 % (vol/vol) Triton X-100 in 1×PBS for 5 minutes at room temperature. After washing twice by 1×PBS, cells were blocked for 30 min in 1×PBS containing 1 % (wt/vol) BSA. Then Alexa Fluor® 633 phalloidin in 1×PBS was added to stain filamentous actin (F-actin) cytoskeleton for 1 h at room temperature. After washing three times, cell nucleus was stained by DAPI for 1 min at room temperature and then the samples were washed three times and then added in fresh 1×PBS. Lasers of 405, 488, and 633 nm were used to excite DAPI, DOX, and Alexa Fluor® 633 phalloidin, respectively. The corresponding fluorescence emissions were recorded by a confocal laser scanning microscopy (LSM 710, Carl Zeiss, Germany) using a band-pass filter combination including 410-507 nm, 493-634 nm, and 638-747 nm for imaging in three individual channels (Objective: EC Plan-Neofluar 10x/0.30 M27; dimension is 1024 ×1024.) .

In Vitro Cellular Uptake by Flow Cytometry. HeLa cells were seeded on a 6-well plate and cultured in 37 °C with 5% CO_2. After 12 h for attachment, free DOX and CPP-2/DOX (DOX final concentration: 10 μg/mL) were added to the medium and incubated with HeLa cells at 37 °C with 5 % CO_2 for 2 h and 6 h. After desired time, excess drugs were washed by 1×PBS extensively for three times. Then the cells were detached by TrypLE Express and then the culture medium was added to stop trypsinization. After centrifugation, the supernatant were thrown away and 1×PBS (500 μL) were added to resuspend the cells for flow cytometry (LSRII, BD Biosciences). The fluorescence of free DOX and CPP-2/DOX were detected using FITC (excitation: 488 nm, emission: 500-560 nm) channel with around 10000 gated cells. The cells without treatment were used as control. The flow cytometry data were analyzed using FlowJo software.

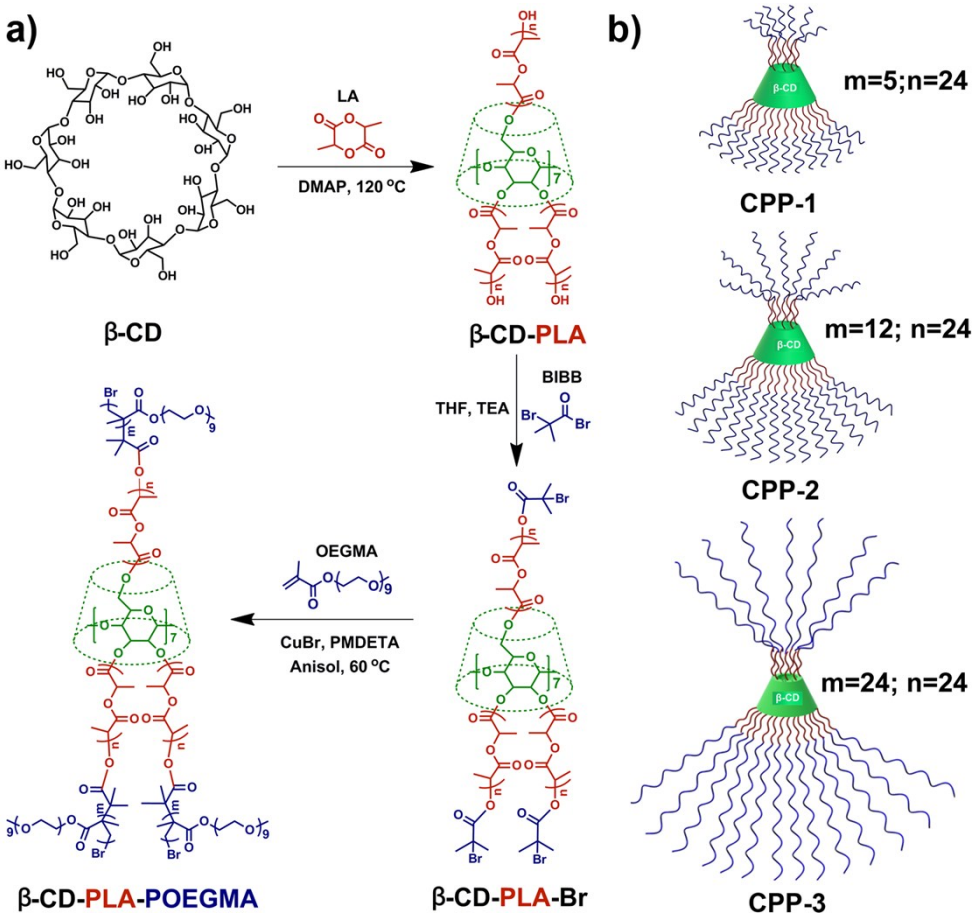

Figure S1. (a) Synthetic route to amphiphilic β-CD-PLA-POEGMA polymers by ring opening polymerization (ROP) and the following atom transfer radical polymerization (ATRP); (b) Schematic illustration of the structure of β-CD-poly(LA_{24}-$OEGMA_5$) (CPP-1), (β-CD-poly(LA_{24}-$OEGMA_{12}$) (CPP-2) and (β-CD-poly(LA_{24}-$OEGMA_{24}$) (CPP-3). The m and n represent the degree of polymerization (DP) of OEGMA and LA, respectively.

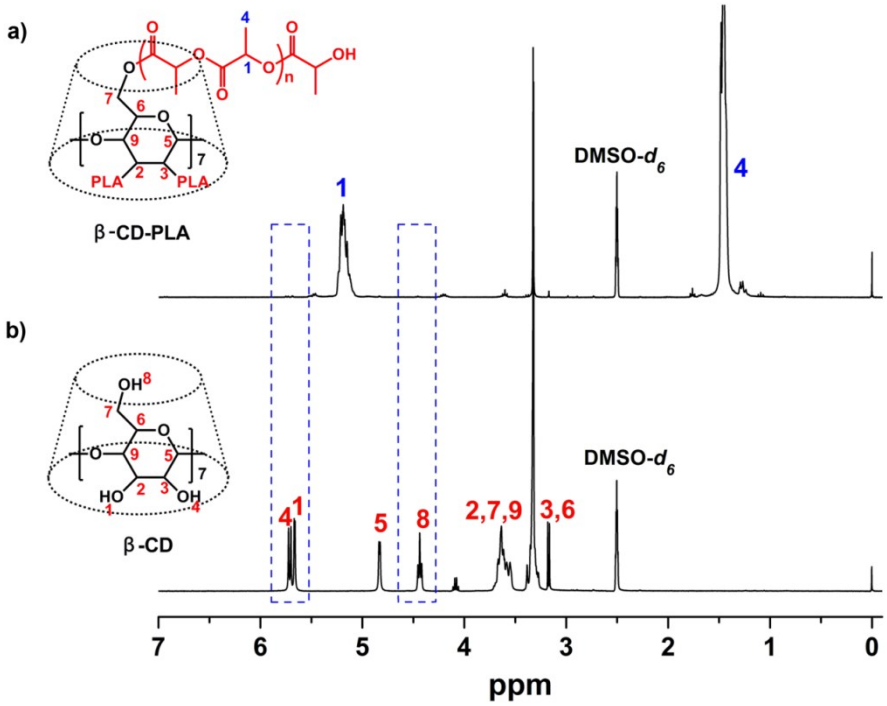

Figure S2. ^{1}H NMR spectra (300 MHz) of β-CD (a) and β-CD-PLA (b) in DMSO-d_6.

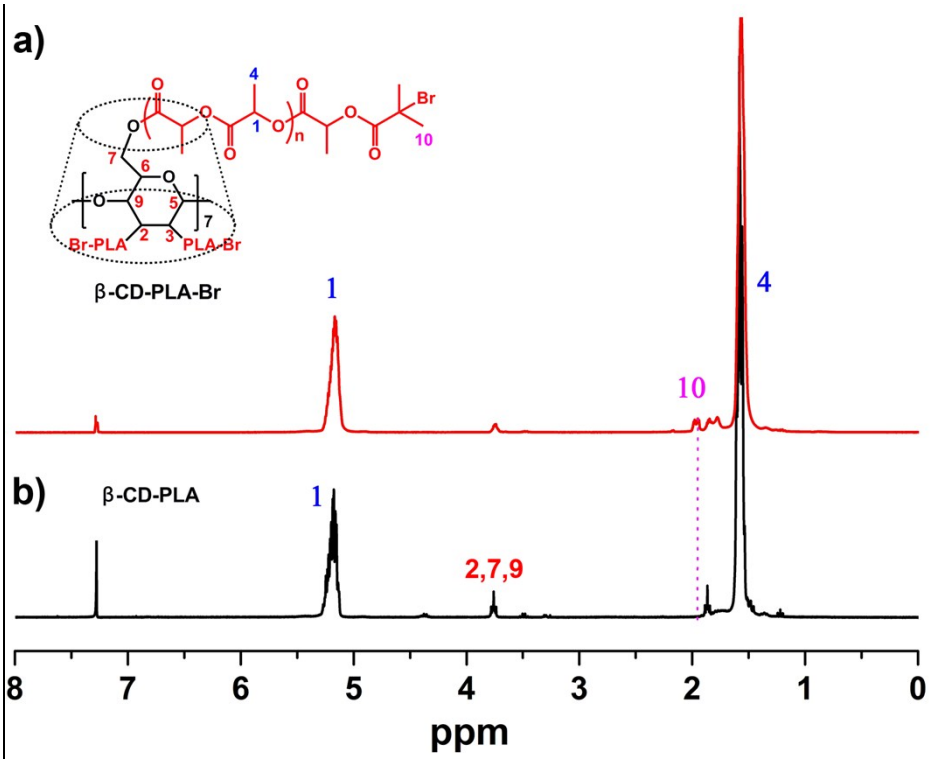

Figure S3. ^{1}H NMR spectra (300 MHz) of β-CD-PLA (a) and β-CD-PLA-Br (b) in $CDCl_3$. The number-average degree of polymerization (DP) of LA was determined using the integral ratio of LA (peak 1) and β-CD (peak 2, 7, 9).

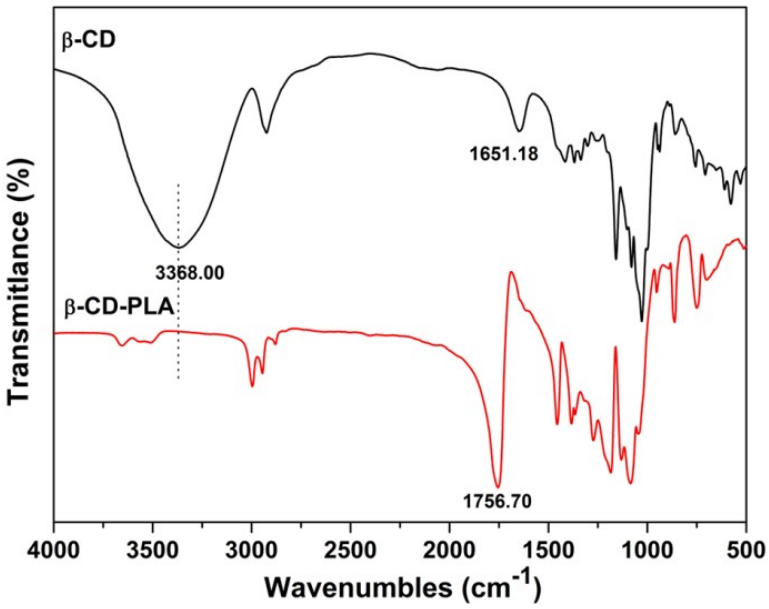

Figure S4. The FT-IR spectra of β-CD and β-CD-PLA.

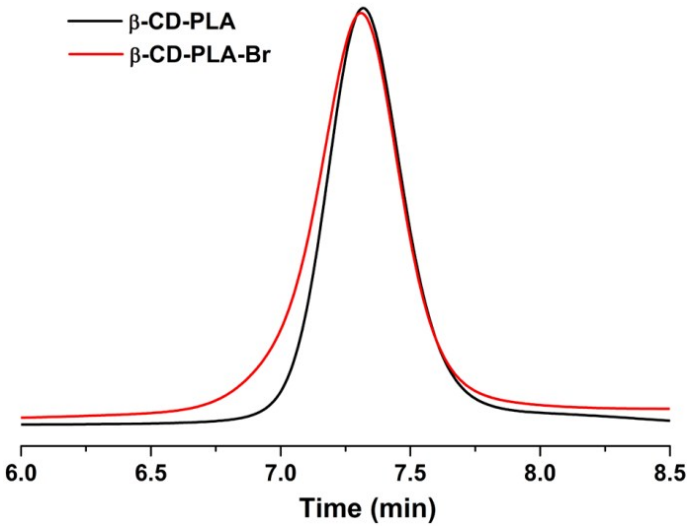

Figure S5. GPC traces of β-CD-PLA polymer ($M_{n,}$= 72.3 KDa, M_w/M_n = 1.12), and β-CD-PLA-Br polymer ($M_{n,}$= 80.0 KDa, M_w/M_n = 1.14).

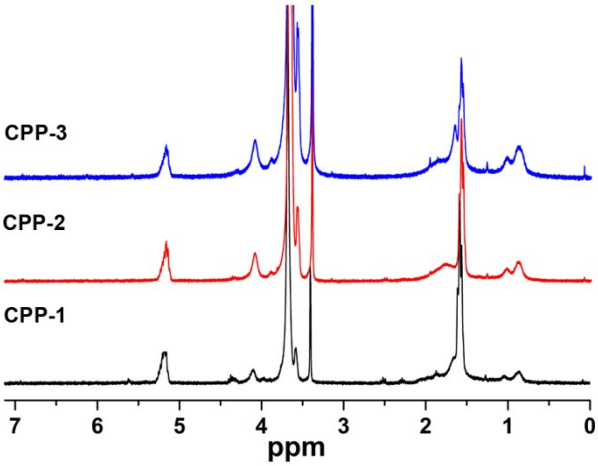

Figure S6. ^{1}H-NMR spectra of β-CD-poly (LA_{24}-$OEGMA_{5}$) (CPP-1), β-CD-poly (LA_{24}-$OEGMA_{12}$) (CPP-2), β-CD-poly (LA_{24}-$OEGMA_{24}$) (CPP-3) in $CDCl_3$.

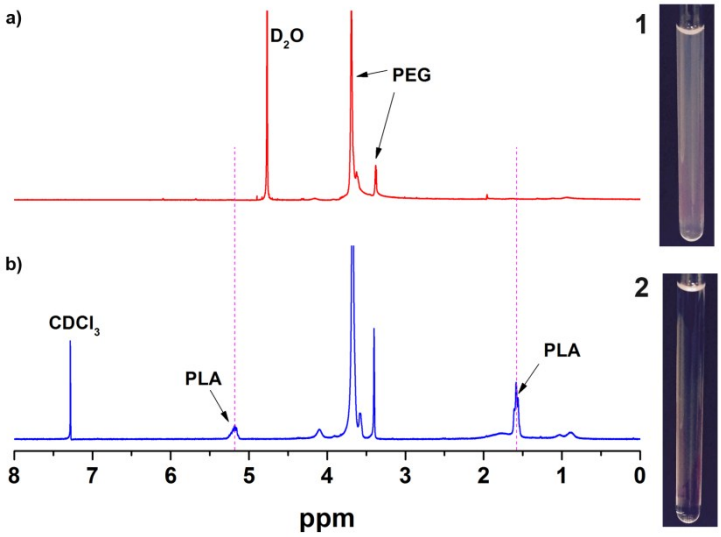

Figure S7. ^{1}H NMR spectra (300 MHz) of CPP-2 in (a) D_2O and (b) $CDCl_3$. The insets show digital photographs of the corresponding CPP-2 in D_2O (1) and in $CDCl_3$ (2).

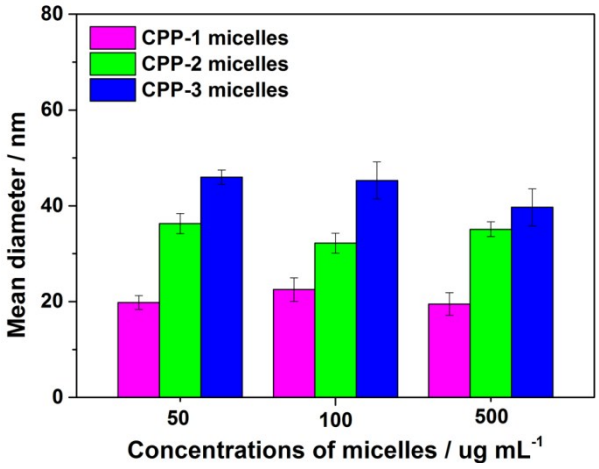

Figure S8. DLS results of CPP-1, CPP-2 and CPP-3 micelles in water with different concentrations.

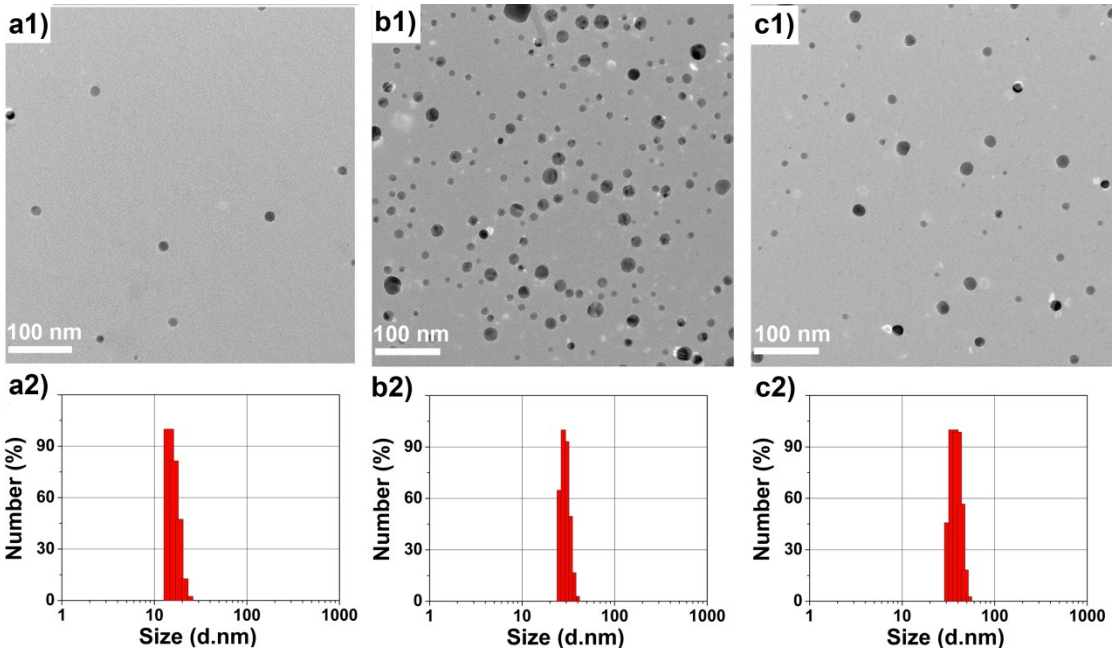

Figure S9. TEM images (a1, b1, c1) and DLS histograms (a2, b2, c2) of CPP-1 (a1-a2), CPP-2 (b1-b2) and CPP-3 (c1-c2) micelles in DMF. Concentration of β-CD-PLA-POEGMA polymer: 1 mg/mL.

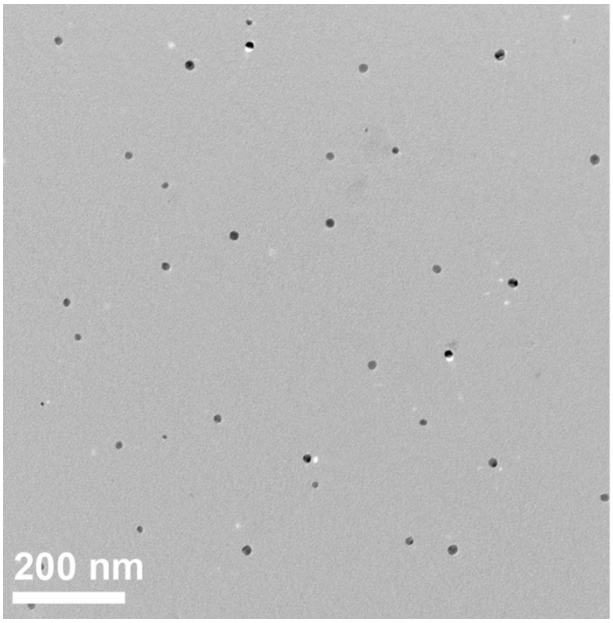

Figure S10. Low magnification TEM image of CPP-1 micelles in DMF.

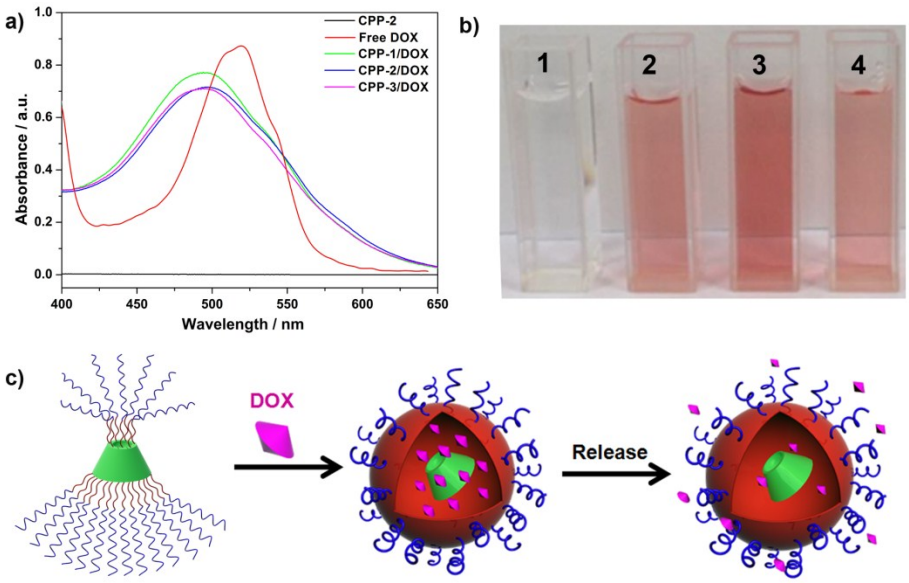

Figure S11. a) UV−vis spectra of CPP-2, free DOX, CPP-1/DOX, CPP-2/DOX and CPP-3/DOX in water with same concentrations; b) photograph for the corresponding (1) β-CD-PLA-POEGMA, (2) CPP-1/DOX, (3) CPP-2/DOX and (4) CPP-3/DOX water solution under ambient light; c) Schematic representation for the drug loading and release of β-CD-PLA-POEGMA micelles.

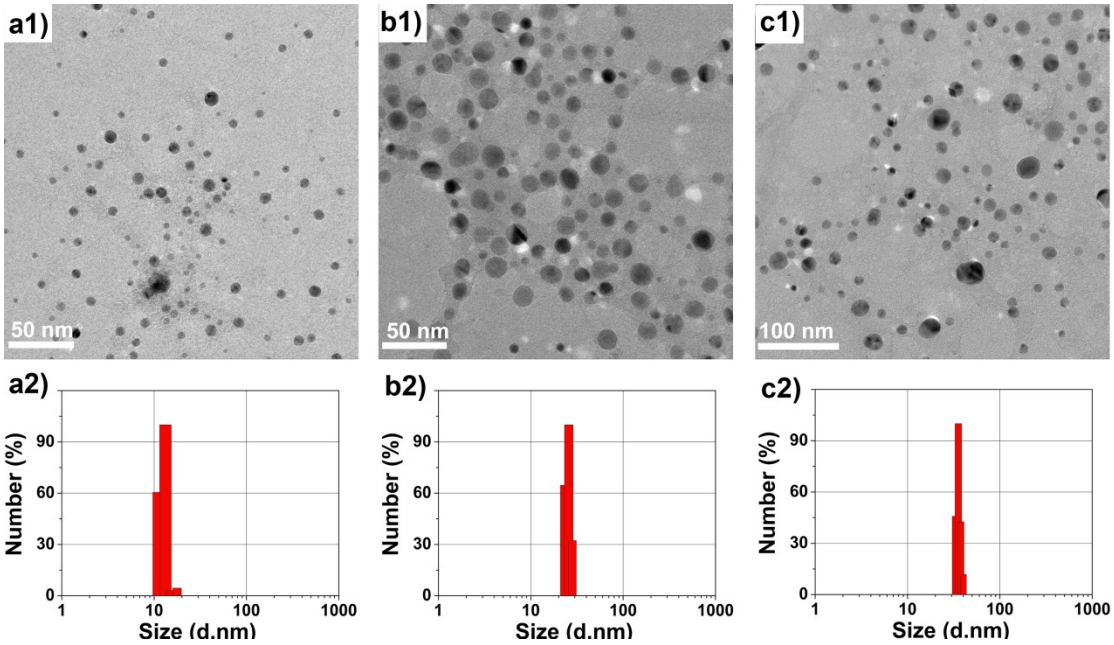

Figure S12. TEM images (a1, b1, c1) and DLS histograms (a2, b2, c2) of CPP-1/DOX (a1-a2), CPP-2/DOX (b1-b2) and CPP-3/DOX (c1-c2) micelles in water.

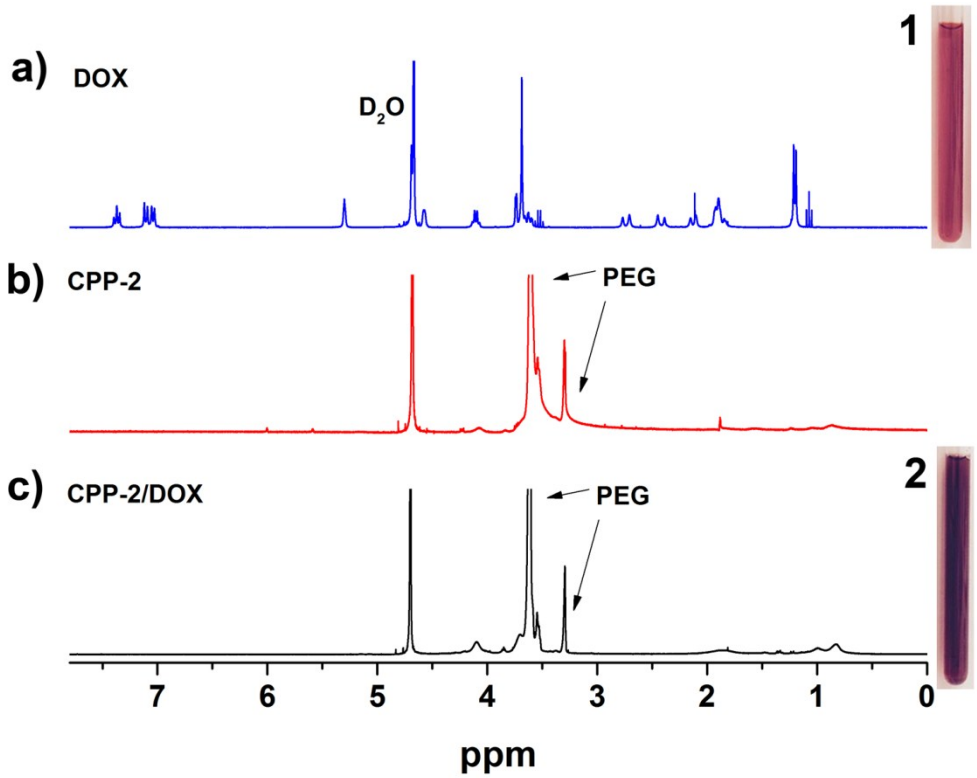

Figure S13. ^{1}H NMR spectra (300 MHz) of DOX in (a) D_2O, (b) CPP-2 in D_2O and (c) CPP-2/DOX in D_2O. The insets show digital photographs of the corresponding (1) DOX in D_2O and (2) CPP-2/DOX in D_2O.

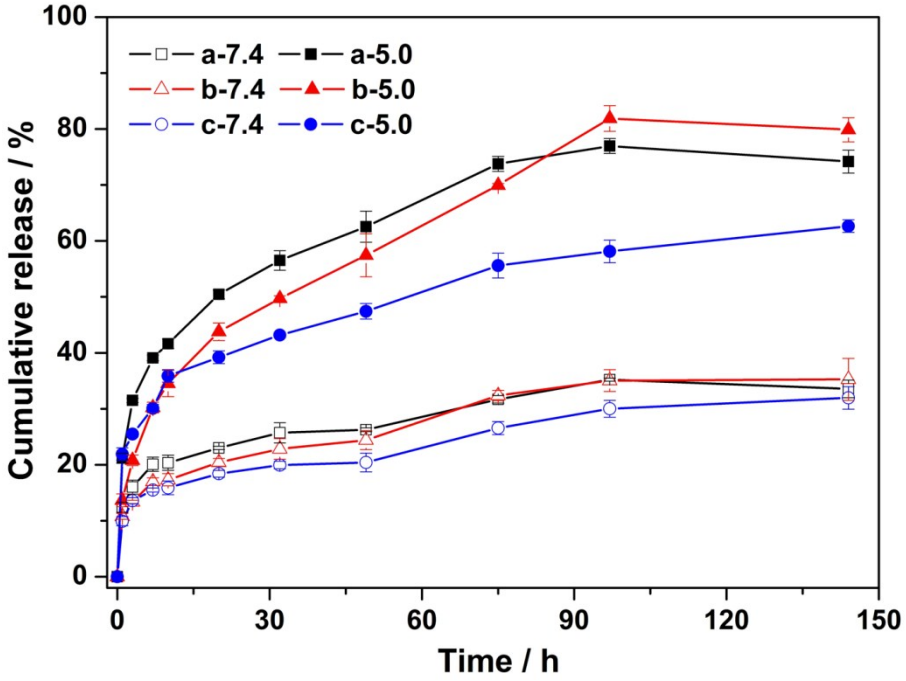

Figure S14. *In vitro* release of DOX from the CPP-1/DOX (a), CPP-2/DOX (b) and CPP-3/DOX (c) micelles in PBS (pH 7.4 or 5.0) at 37 °C. Data are presented as the average ± standard deviation (n = 3).

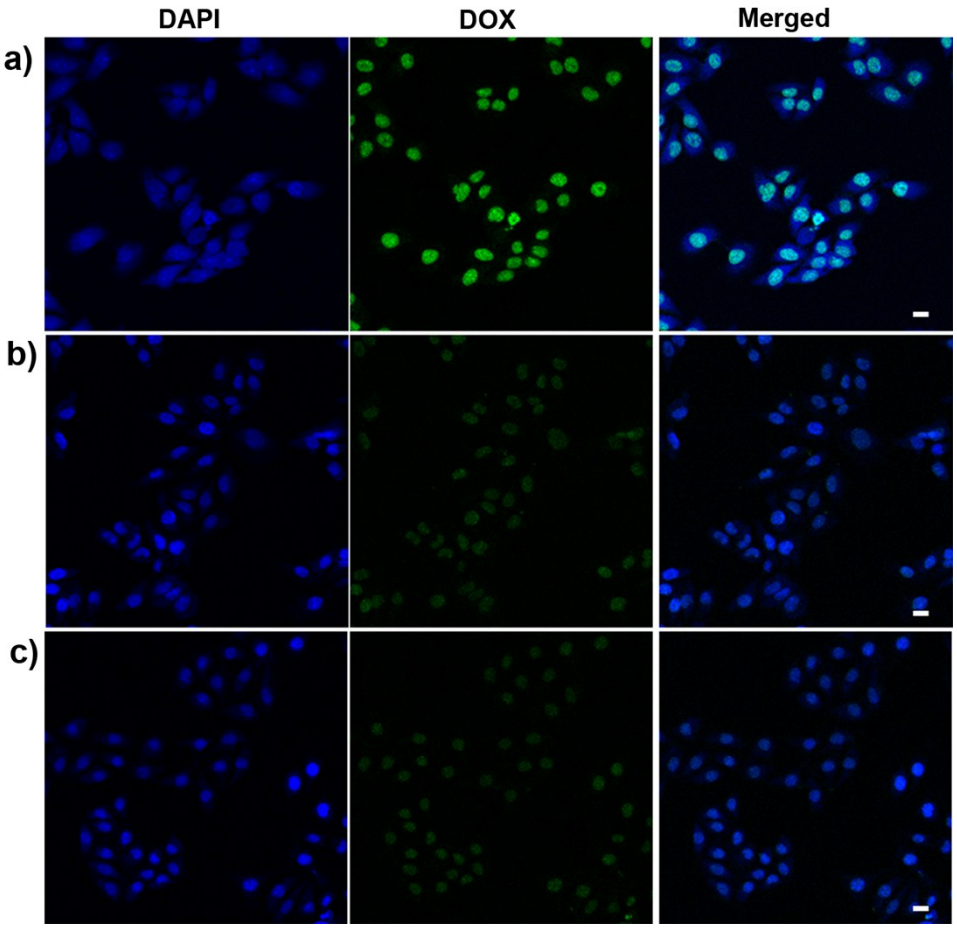

Figure S15. CLSM images of HeLa cells after treatment with free DOX for 2 h (a), CPP-2/DOX for 2 h (b) and CPP-2/DOX for 6 h (c). The fluorescences of DAPI and DOX were pseudo-labeled with blue and green. Scale bars: 20 μm. Merged images of DAPI and DOX indicated both free DOX and CPP-2/DOX can enter nucleus.

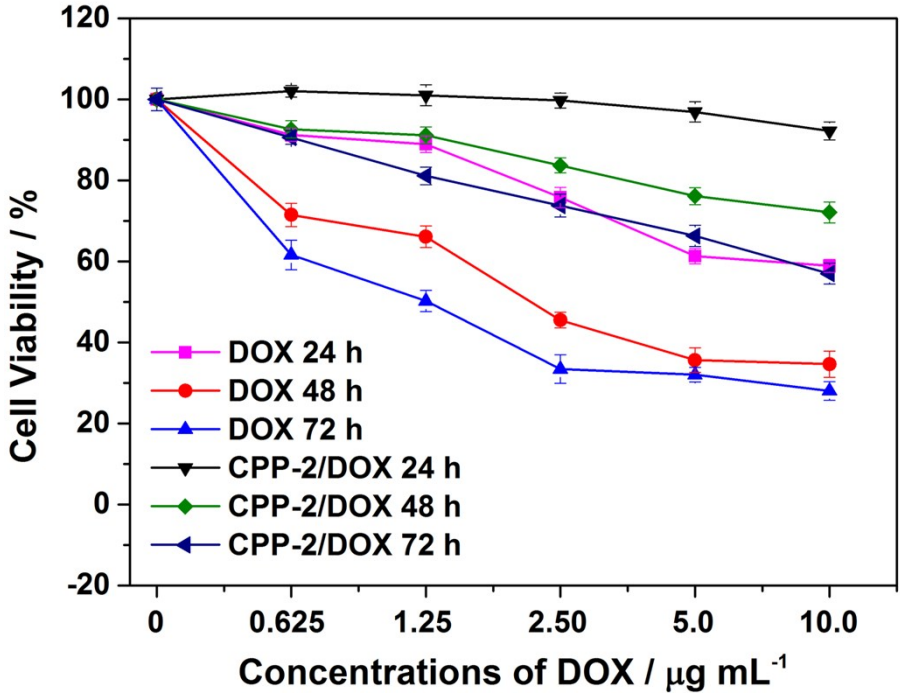

Figure S16. Cell viability of HeLa cells tested by PrestoBlue assay after treatment with different concentrations of free DOX and CPP-2/DOX over 24, 48, and 72 h, respectively. Cells without treatment were used as control. Data were shown as means ± SD (n=3).

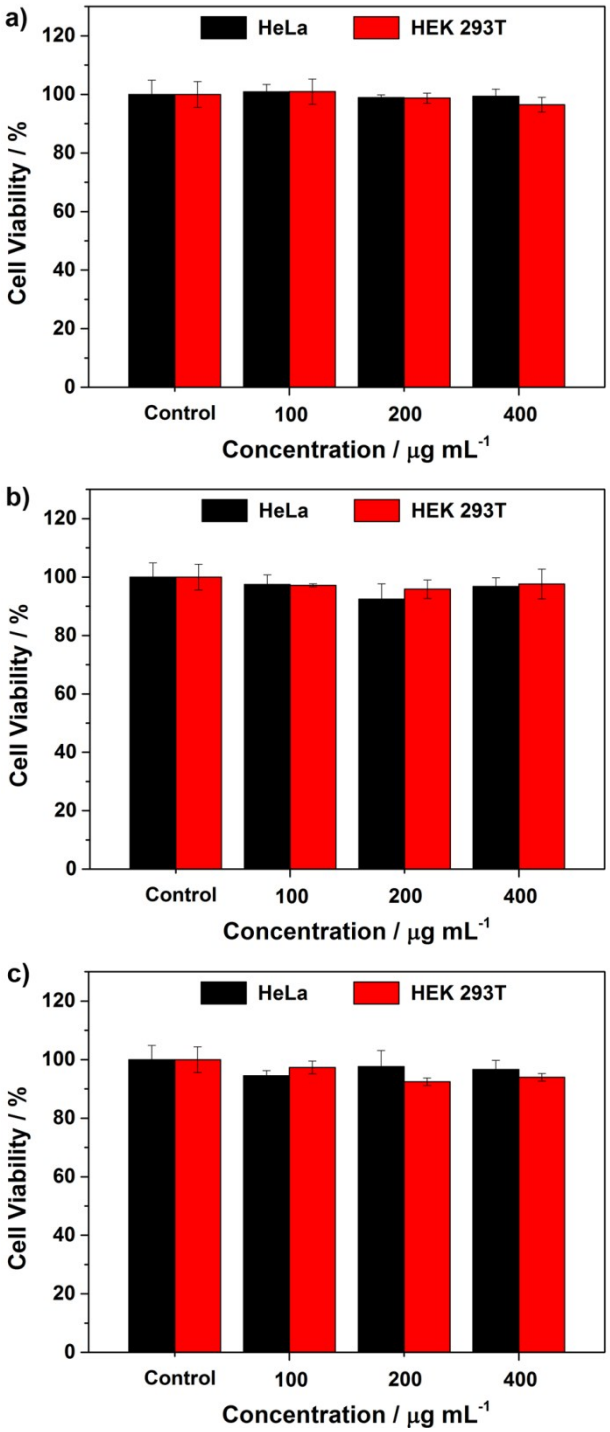

Figure S17. Cell viability of HeLa cells and HEK 293T cells after treatment with CPP-1 (a), CPP-2 (b) and CPP-3 (c) at various concentrations for 72 h tested by PrestoBlue assay. Cells without treatment were used as control. Data were shown as mean ± SD (n = 3).

Table S1. Structural characterization of the star-like polymers.

Polymer	Time (min) [a]	Conv (%) by NMR	$M_{n,NMR}$ [b]	$M_{n,GPC}$ [c]	M_w/M_n [d]	$F_{hydrophilic}$ [e]
β-CD-PLA	/	/	44 200	72 300	1.12	0
β-CD-PLA-Br	/	/	47 300	80 000	1.14	0
CPP-1	30	10	101 000	128 000	1.34	56.3 %
CPP-2	60	24	174 000	168 000	1.26	74.6 %
CPP-3	90	48	309 000	203 000	1.24	85.7 %

[a] β-CD-PLA-POEGMA polymers with different hydrophilic chain lengths were synthesized at different reaction periods using β-CD-PLA-Br as the ATRP initiator; [b] Number-average molecular weight, $M_{n,NMR}$ calculated from ^{1}H NMR data.[c] Number-average molecular weight, $M_{n,GPC}$ determined by GPC. [d] Polydispersity index, PDI determined by GPC. [e] $f_{hydrophilic}$ refers to hydrophilic fraction based on $M_{n,NMR}$ values.

Table S2. Molecular weights of amphiphilic star polymers of β-CD-PLA-POEGMA

Polymer	$M_{n,PLA}$ [a] (DP_{PLA})	$M_{n,PEG}$ [b] (DP_{PEG})	*LC (%)*[c]	*EE (%)*[d]	ζ-potential [e]
CPP-1	1 700 (24)	2 500 (5)	7.6	58.4	-8.41±1.06
CPP-2	1 700 (24)	6 000 (12)	10.0	76.6	-9.74±1.03
CPP-3	1 700 (24)	12 000 (24)	6.3	48.7	-5.05±0.45

[a,b] Number-average molecular weight, M_n of each PLA or PEG chain calculated from ^{1}H NMR data. DP_{PLA} and DP_{PEG} are the degree of polymerization of LA or OEGMA, and calculated from ^{1}H NMR data; [c,d] The drug loading (LD) and encapsulation efficiency (EE) of CPP-1/DOX ,CPP-2/DOX and CPP-3/DOX. [e]The zeta potential of CPP-1, CPP-2 and CPP-3 micelles in water.

Table S3. Size and polydispersity index (PDI) of β-CD-PLA-POEGMA micelles measured by DLS and TEM

Sample	Size[a] (nm)	PDI[a]	D[b] (nm)	SD[b] (nm)
CPP-1 (water)	18.5	0.25	10.8	4.1
CPP-2 (water)	30.9	0.23	17.7	5.5
CPP-3 (water)	38.1	0.21	21.5	4.3
CPP-1 (DMF)	19.3	0.27	12.7	3.7
CPP-2 (DMF)	31.8	0.36	14.7	5.3
CPP-3 (DMF)	38.9	0.35	16.2	5.5
CPP-1/DOX (water)	16.5	0.23	8.4	2.3
CPP-2/DOX (water)	25.6	0.19	15.4	4.3
CPP-3 /DOX (water)	36.1	0.16	18.6	5.2

[a] Size and PDI of β-CD-PLA-POEGMA micelles were measured by DLS; [b]Average diameter (D) and standard deviation (SD) were calculated by measuring 100 micelles in a representative TEM image.

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Table S4: IC_{50} value of free DOX and CPP-2/DOX of different time.[a]

Sample	IC_{50} (24 h) μg mL^{-1}	IC_{50} (48 h) μg mL^{-1}	IC_{50} (72 h) μg mL^{-1}
Free DOX	> 10	2.25	1.25
CPP-2/DOX	> 10	> 10	> 10

[a)] IC_{50} value of free DOX and CPP-2/DOX determined in HeLa cells by PrestoBlue assay, and cells were incubated with samples for different periods.