Electronic Supplementary Information (ESI)

A NIR-triggered gate-keeper of a supramolecular conjugated unimicelle with two-photon absorption for controlled drug release

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1. Materials

The hyperbranched conjugated polymer (HCP) and HCP with hydroxyl terminal groups (HCP-CH_2OH) were prepared according to our previous work.[1,2] Sodium borohydride ($NaBH_4$, C.P. grade, Shanghai Chemical Reagent Co.), succinic anhydride (SA, 99.8%, Adamas-beta®), β-cyclodextrin (CD, C.P. grade, Sigma-Aldrich), poly(ethylene glycol) monomethyl ether (PEG, Mw = 2000, Sigma-Aldrich), 3-hydroxy-4'-dimethylamino-azobenzene (99.8%, TCI), *p*-toluene sulfonyl chloride (99.8%, Adamas-beta®), sodium hydroxide (NaOH, C.P. grade, Shanghai Chemical Reagent Co.), potassium hydroxide (KOH, C.P. grade, Shanghai Chemical Reagent Co.), potassium carbonate (K_2CO_3, C.P. grade, Shanghai Chemical Reagent Co.), ethylenediamine (99.8%, Adamas-beta®), 1-(4-dimethylaminopropyl)-4-ethylcarbodiimide hydrochloride (EDC, 99.8%, Sigma-Aldrich), *N*-hydroxysuccinimide (NHS, 99.8%, Sigma-Aldrich), anhydrous pyridine (Sigma-Aldrich) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) were directly utilized. Chloroform, *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), ethanol, and dichloromethane were further dried before use. All other reagents were purchased from commercial suppliers like Alfa, Sigma-Aldrich, TCI, J&K and Adamas-beta® and used as received.

2. Measurements

Varian Mercury plus 400 NMR spectrometer (400 MHz) was used to record ^{1}H NMR spectra with deuterium oxide (D_2O), $CDCl_3$ or dimethyl sulfoxide-d_6 (DMSO-d_6) or *N,N*-dimethylformamide-d_7 (DMF-d_7) as solvents at 25 ^oC. Fourier transform infrared (FTIR) spectra were recorded on a Paragon 1,000 instrument by KBr sample holder method. Liquid chromatography-mass spectrometry (LC-MS) was performed on a Water ACQUITY UPLC system equipped with a binary solvent delivery manager and a sample manager, coupled with

a Waters Q-TOF Premier mass spectrometer equipped with an electrospray interface (Waters Corporation, Milford, MA). The number-average molecular weight (M*n*) and the polydispersity (M*w*/M*n*) were determined by gel permeation chromatography/multi-angle laser light scattering (GPC-MALLS). The gel permeation chromatography system consisted of a Waters degasser, a Waters 515 HPLC pump, a 717 automatic sample injector, a Wyatt Optilab DSP differential refractometer detector, and a Wyatt miniDAWN multi-angle laser light scattering detector. Three chromatographic columns (styragel HR3, HR4, and HR5) were used in series. THF was used as the mobile phase at a flow rate of 1 mL/min at 30 °C. The refractive index increment *dn/dc* was determined with Wyatt Optilab DSP differential refractometer at 690 nm. Data analysis was performed with Astra software (Wyatt Technology). Dynamic light scattering (DLS) measurements were performed under a Malvern Zetasizer 3,000 HS (Malvern Instruments, Ltd.) equipped with 125 mW laser light and operated at $\lambda = 633$ nm. All samples were measured at a scattering angle of 90^{o}. Transmission electron microscopy (TEM) studies were performed with a JEOL 2010 instrument operated at 200 kV. Samples were prepared by drop-casting micelle solutions onto carbon-coated copper grids, and then air-drying at room temperature before measurement. Thermo Electron-EV300 UV-vis spectrophotometer was used to measure UV-vis absorption of the materials at 25 ^{o}C. PTI-QM/TM/IM steady-state & time-resolved fluorescence spectrofluorometer was used to record the fluorescence emission spectra. Two-photon irradiation and two-photon fluorescence measurement were excited by the femtosecond (fs) pulses (iHR550, HORIBA) with different intensities at wavelength of 800 nm. BD LSRFortessa flow cytometer and CELLQuest software were used for flow cytometry analysis. Two-photon laser scanning fluorescence microscopy (TLSM) imaging was measured by LEICA TCS SP8 MP System. Perkin-Elmer 1420 Multi-label counter was used to measure the absorbance with an excitation wavelength of 490 nm for the MTT test.

3. Methods

3.1. Synthesis of carboxyl-terminated hyperbranched conjugated polymer (HCP-COOH)

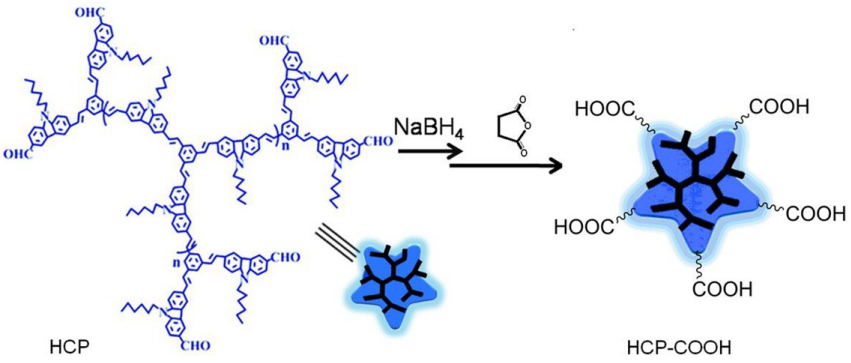

Figure S1. The synthetic route of HCP-COOH.

HCP and HCP-CH_2OH with hydroxyl terminal group was firstly synthesized according to the method described in our previous papers.[1-3] The carboxyl-terminated HCP-COOH was synthesized through an esterification of HCP-CH_2OH and succinic anhydride (SA). Briefly, both HCP-CH_2OH (0.5 g, 0.12 mmol) and SA (0.3 mmol) were placed into a flask with pyridine and stirred at 60 °C for 24 h. Then the product was subjected to reduced pressure distillation to remove pyridine, dissolved in THF and purified by dialysing in deionized water for 96 h (MWCO = 3.5 kDa). Subsequently, the polymer HCP-COOH was dried by freeze drying. Yield: 76.2 %.

^{1}H NMR ($CDCl_3$, 400 MHz, 25 °C) δ: 0.54-0.93 (br, -C*H*$_3$), 0.96-1.43 (br, -C*H*$_2$C*H*$_2$CH$_3$), 1.51-1.91 (br, -NCH$_2$C*H*$_2$C*H*$_2$-), 2.11-2.72 (-C*H*$_2$CH$_2$COOH), 3.88-4.33 (br, -NC*H*$_2$CH$_2$-), 4.91-5.43 (br, -CH$_2$C*H*$_2$COOH), 6.68-8.37 (br, Ar-*H*) ppm.

^{13}C NMR ($CDCl_3$, 400 MHz, 25 °C) δ: 13.56, 18.26, 22.43, 26.62, 29.14, 29.21, 29.55, 29.69, 29.83, 29.97, 34.27, 34.41, 34.55, 34.68, 34.82, 34.96, 35.10, 42.83, 56.71, 66.70, 109.50,

109.83, 118.90, 120.87, 122.72, 123.00, 125.03, 126.05, 126.83, 129.02, 130.15, 138.99, 140.79, 162.04, 162.24, 162.63, 170.56, 173.69 ppm.

IR (KBr): 3391, 3015, 2953, 2926, 2851, 1731, 1622, 1601, 1581, 1485, 1382, 1347, 1244, 1196, 1151, 1150, 1091, 1044, 955, 877, 807, 692 cm^{-1}.

3.2. Synthesis of amino-modified *β*-cyclodextrin (CD-$NHCH_2CH_2NH_2$)

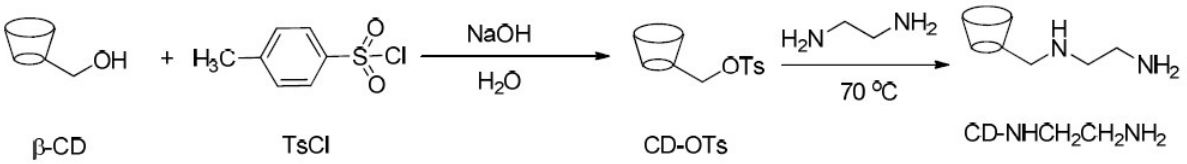

Figure S2. The synthetic route of CD-$NHCH_2CH_2NH_2$.

According to the literature,[4] β-CD (6.0 g) was dissolved in 50 mL of 0.4 M aqueous sodium hydroxide (NaOH) and cooled down to 0 °C. Subsequently, *p*-toluene sulfonyl chloride (TsCl, 874.0 mg) was added dropwise to the solution under vigorous stirring. The resulting suspension was stirred for 30 min below 5 °C, and then filtered quickly. The filtrate was neutralized to pH 8.5 with hydrochloric acid and stirred for another 1 h. The resultant precipitate (CD-OTs) was filtered off, washed three times with water and dried at 60 °C for 96 h. Yield: (40.1%).

^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 7.75 (d, 2H), 7.43 (d, 2H), 5.86-5.59 (m, 14H), 4.83 (br, 4H), 4.77 (s, 3H), 4.53-4.15 (m, 6H), 3.74-3.45 (m, 28H), 3.41-3.20 (m), 2.43 (s, 3H).

ESI-MS (m/z): calcd 1288.3809, found 1289.3815 ($M+H^+$).

CD-OTs (1.0 g) and ethylenediamine (4.0 mL) were placed into a flask and stirred at 70 °C for 4 h. After that, the product was precipitated for three times with 50 mL cold absolute ethyl alcohol and filtered off. The resultant product (CD-$NHCH_2CH_2NH_2$) was dried at 60 °C for 48 h. Yield: (88.7%).

^{1}H NMR (400 MHz, DMSO-d_6), δ (ppm): 4.96 (br, 4H), 4.81 (br, 3H), 4.53-4.15 (m, 6H), 3.75-3.17 (m, 28H), 2.88-2.58 (m. 2H), 1.96-1.67 (m, 2H), 1.11-0.92 (m, 2H).

ESI-MS (m/z): calcd 1177.0789, found 1177.4404 ($M+H^+$)

3.3. Synthesis of *β*-cyclodextrin-grafted HCP (HCP-CD)

Figure S3. The synthetic route of HCP-CD.

HCP-COOH (1.0 g), 1-(4-dimethylaminopropyl)-4-ethylcarbodiimide hydrochloride (EDC, 0.4 g), *N*-hydroxysuccinimide (NHS, 0.8 g) and 35 mL of dried DMF were placed into 100 mL flask and stirred in nitrogen atmosphere. CD-$NHCH_2CH_2NH_2$ (1.0 mmol) was dissolved into 5.0 mL of dried DMF and added dropwise to the reaction solution. Then the reaction mixture was stirred at 30 °C in nitrogen atmosphere for 36 h. After that, the product (HCP-CD) was purified by dialysing in deionized water for 96 h (MWCO = 3.5 kDa). Yield: (80.6%).

^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.61-0.95 (br, -CH_3), 0.93-1.42 (br, -$CH_2CH_2CH_3$), 1.45-1.79 (br, -$NCH_2CH_2CH_2$-), 2.12-2.93 (br, -$COCH_2CH_2$-), 3.22-4.07 (br, CD-*H*), 4.28-5.36 (br, -CH_2OCO-), 7.15-8.21 (br, Ar-*H*) ppm.

^{13}C NMR ($CDCl_3$, 400 MHz, 25 °C) δ: 14.53, 22.51, 26.70, 29.18, 29.32, 29.46, 29.88, 30.01, 31.55, 34.31, 34.45, 34.57, 34.72, 34.86, 34.99, 35.13, 42.91, 60.90, 66.75, 72.83, 73.43, 73.82, 82.51, 102.91, 109.59, 109.89, 118.96, 120.94, 122.79, 123.08, 125.11, 126.13, 126.91,127.31, 129.10, 130.26, 138.58, 139.07, 140.86, 162.04, 162.23, 162.43, 171.68, 173.63, 173.80 ppm.

IR (KBr): 3394, 3026, 2968, 2939, 2853, 1728, 1637, 1621, 1592, 1477, 1374, 1352, 1247, 1201, 1165, 1147, 1086, 1028, 963, 878, 790, 693 cm^{-1}.

3.4. Synthesis of 4-(dimethylamino)azobenzene-terminated poly(ethylene glycol) (Azo-PEG)

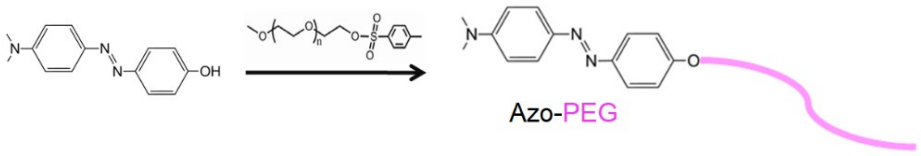

Figure S3. The synthetic route of Azo-PEG.

According to our previous work,[5] we first synthesized the poly(ethylene glycol) monomethyl ether p-toluene sulfonate (PEG-OTs). Briefly, PEG monomethyl ether (M_n = 2000, 10.0 g, 5 mmol) was dissolved in 200 mL of CH_2Cl_2 and cooled to 0 °C. Then, *p*-toluene sulfonyl chloride (TsCl, 1.65 g, 6 mmol) and KOH powder (0.9 g, 16 mmol) were added into the solution, respectively. The mixture was heated to room temperature overnight. After the reaction was completed, the mixture was filtered, washed with water and CH_2Cl_2. The organic phase was dried with anhydrous magnesium sulfate, concentrated under reduced pressure and precipitated into cold diethyl ether for three times, and filtered and dried to obtain a white solid of PEG-OTs (91% yield). PEG-OTs, 3-hydroxy-4'-dimethylamino-azobenzene, and K_2CO_3 were placed into 200 mL flask with the molar ratio of 1.2:1.0:1.2, then 100 mL of anhydrous acetonitrile was added into the mixture and stirred at room temperature for 36 h. The reaction mixture was concentrated under reduced pressure and precipitated into cold diethyl ether for three times. The resultant product Azo-PEG was filtered and dried at 40 °C for 48 h. Yield: 87.5 %.

^{1}H NMR ($CDCl_3$, ppm): 7.01-8.11 (m, Ar-*H*), 3.47-4.41 (m, -OCH_2CH_2O-), 3.19 (s, -OCH_3), 2.81 (s, -CH_3).

IR (KBr): 3430, 2917, 2875, 1938, 1736, 1643, 1455, 1354, 1297, 1253, 1102, 943, 842, 553 cm^{-1}.

3.5. HCP-CD/Azo-PEG (HCP-PEG) preparation, self-assembly and its DOX encapsulation

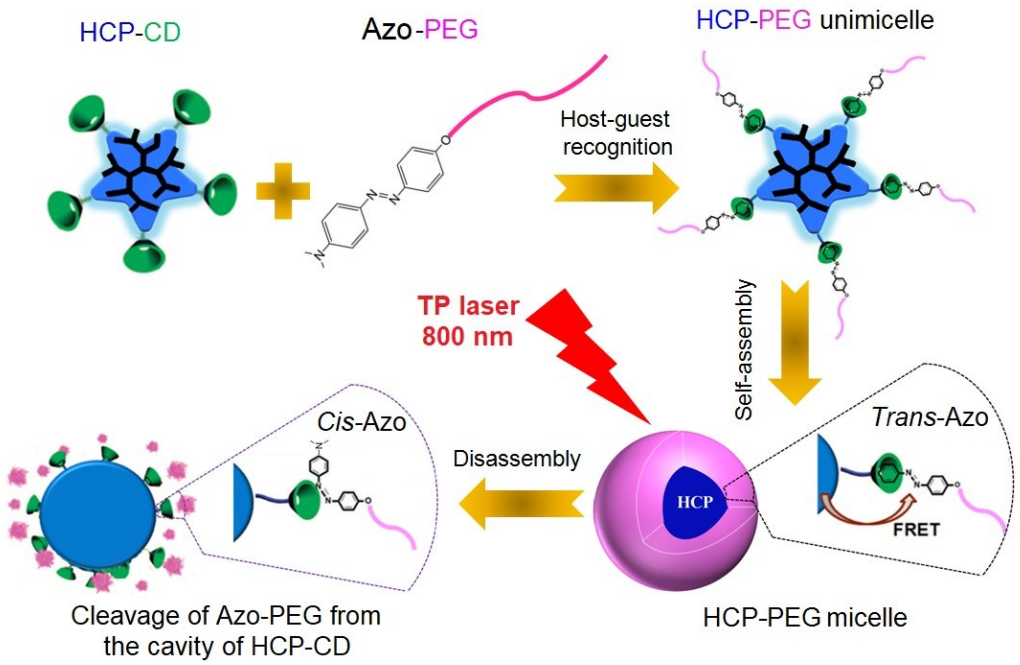

Figure S4. The synthetic route of HCP-PEG micelle and its NIR-triggered photoisomerization.

Both HCP-CD and Azo-PEG were dissolved in DMSO (4 mL) and stirred for 6 h. Later the mixture was added dropwise into 4 mL of ultrapure water with stirring, followed by dialysis (MWCO = 3500) against deionized water to remove DMSO. After 24 h, the HCP-PEG solution was diluted with deionized water to the desired concentration for further experiments. All of the above reactions were done in the dark.

DOX·HCl and an equal molar amount of triethylamine (TEA) were dissolved in 2 mL of DMSO and added to the mixture solution of HCP-CD/Azo-PEG in DMSO (4 mL). Then the mixture was added dropwise to 5 mL of PBS (50 mM, pH 7.4). After being stirred for an additional 6 h, the solution was dialyzed against deionized water for 24 h (MWCO = 3500).

For the determination of drug loading content, the DOX-loaded HCP-PEG particle solution was lyophilized and then dissolved in DMSO. The UV absorbance at 486 nm was measured to determine the DOX concentration. Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following formula:

$$DLC\ (wt\%) = \frac{Weight\ of\ loaded\ DOX}{Weight\ of\ polymers} \times 100\%$$

$$DLE\ (wt\%) = \frac{Weight\ of\ loaded\ DOX}{Weight\ of\ DOX\ in\ feed} \times 100\%$$

3.6. *In vitro* light-triggered drug release

Two equal samples of DOX-loaded HCP-PEG particle solutions were transferred to the dialysis bags (MWCO = 3500), respectively. One was placed in dark and the other was treated by two-photon laser irradiation (λ = 800 nm, iHR550, HORIBA, the mean energy per pulse 3.0 mJ).[6] Both of them were immersed in the phosphate buffer (pH 7.4) in a shaking water bath at 37 °C to acquire sink conditions. At predetermined time intervals, 2 mL of the external buffer was withdrawn and replenished with an equal volume of fresh medium. The amount of released DOX was measured with fluorescence spectrophotometer with the excitation at 480 nm. The release experiments were conducted in triplicate, and the results were the average data.

3.7. Cell Cultures

HeLa cancer cells and L929 normal cells were cultured in Dulbecco's modified Eagle's medium (DMEM). The culture media contain 10 % fetal bovine serum and antibiotics (50 units/mL penicillin and 50 units/mL streptomycin) at 37 ^{o}C under a humidified atmosphere containing 5 % CO_2. And the confluent cells were sub-cultured every 2 days following standard procedure.

3.8. *In vitro* cytotoxicity of HCP-CD, Azo-PEG and HCP-PEG micelle

The MTT method was performed in normal L929 cells and cancer HeLa cells to evaluate the cytotoxicity of HCP-CD, Azo-PEG and HCP-PEG micelle. Cells were seeded into 96-well plates with a density of 5×10^3 cells/well in 200 μL medium and cultured until 70~80 % confluence. After incubation for another day, the culture medium was carefully removed and 200 μL of medium with 50 μL serial concentrations of HCP-CD, Azo-PEG and HCP-PEG micelle were added, respectively. After 48 h incubation, 20 μL of MTT assays stock solution with a concentration of 5 mg/mL in PBS was added to each well. After 4 h incubation, the supernatant was removed, followed by the addition of 200 μL of DMSO to dissolve the formazan crystals. Perkin-Elmer 1420 Multi-label counter was used to measure the absorbance with an excitation wavelength of 490 nm.

3.9. Cellular uptake study of DOX-loaded HCP-PEG micelles

The cellular uptake of DOX-loaded HCP-PEG micelles was studied by using flow cytometry in cancer HeLa cells. HeLa cells were seeded in 6-well plates at 5.0×10^5 cells per well in 2 mL of complete DMEM and cultured for 24 h. Then the solution of DOX-loaded HCP-PEG micelles was diluted with DMEM culture medium at a final DOX concentration of 20 μg/mL. The diluted solution was added to different wells and the cells were incubated at 37 °C for 30, 120 and 240 min. Cells without treatment of micelles were used as control. Thereafter, culture medium was removed and cells were washed with PBS for three times and treated with trypsin. Data for 1.0×10^4 gated events were collected and analysis was performed by means of a BD LSRFortessa flow cytometer.

3.10. Cell imaging studies of DOX-loaded HCP-PEG micelles

The confocal laser scanning microscopy (CLSM) measurement was performed to further evaluate the cellular uptake of DOX-loaded HCP-PEG micelles in HeLa cells. Cells were seeded in 6-well plates with a density of 3×10^5 cells/well in 2 mL of complete DMEM. After 24 h incubation, culture medium was removed and 2 mL DMEM medium containing DOX-

loaded HCP-PEG micelles with DOX concentration of 20 μg/mL was added. The cells were cultured at 37 ^{o}C for desired time, followed by being rinsed with cold PBS buffer, fixed with 4 % paraformaldehyde for 30 min at room temperature, and the slides were rinsed with PBS three times. Finally, the resulting slides were mounted and observed with a LEICA TCS SP8 MP System. As for the two-photon laser scanning fluorescence microscopy (TLSM) imaging in the study of NIR-induced intracellular drug release of DOX-loaded HCP-PEG micelles, cells were seeded in the cell imaging plate with a density of 3×10^5 cells/well in 2 mL of complete DMEM. After 24 h incubation, culture medium was removed and 2 mL DMEM medium containing DOX-loaded HCP-PEG micelles with DOX concentration of 20 μg/mL was added. The cells were cultured at 37 ^{o}C for 4 h, followed by being rinsed with cold PBS buffer for three times. The plate was mounted on the LEICA TCS SP8 MP imaging system, and then the cells were irradiated with two-photon laser for desired time. Finally, the images were captured by the LEICA TCS SP8 MP imaging system. In the measurement, the two-photon laser at 800 nm was used for HCP excitation, and one photon laser at 488 nm was used for DOX excitation. Signals from the HCP and DOX were recorded in channel blue and channel red, respectively.

3.11. *In vitro* phototoxicity of DOX-loaded HCP-PEG micelle under laser irradiation

The phototoxicity of DOX-loaded HCP-PEG micelle under laser irradiation was performed by MTT method in cancer HeLa.[7] Briefly, cells were seeded into the glass bottom multiwell plate with a density of 2.5×10^3 cells/well in 50 μL medium and cultured until 70~80 % confluence. Then the culture medium was carefully removed and fresh medium with (or without) DOX-loaded HCP-PEG micelle (DOX concentration of 20 μg/mL) were added. After 24 h incubation, cells were washed twice with PBS, maintained in fresh culture medium, and then submitted (or not) to laser irradiation; with the LEICA TCS SP8 MP Microscope (the mean energy per pulse 2.5 mJ). The well was illuminated at 800 nm by 3 scans of 1 s duration

in 5 different areas of the well. Cells were further incubated for 24 h, and then the cell viability was evaluated by MTT assay as mentioned in Method 3.8.

4. Figures

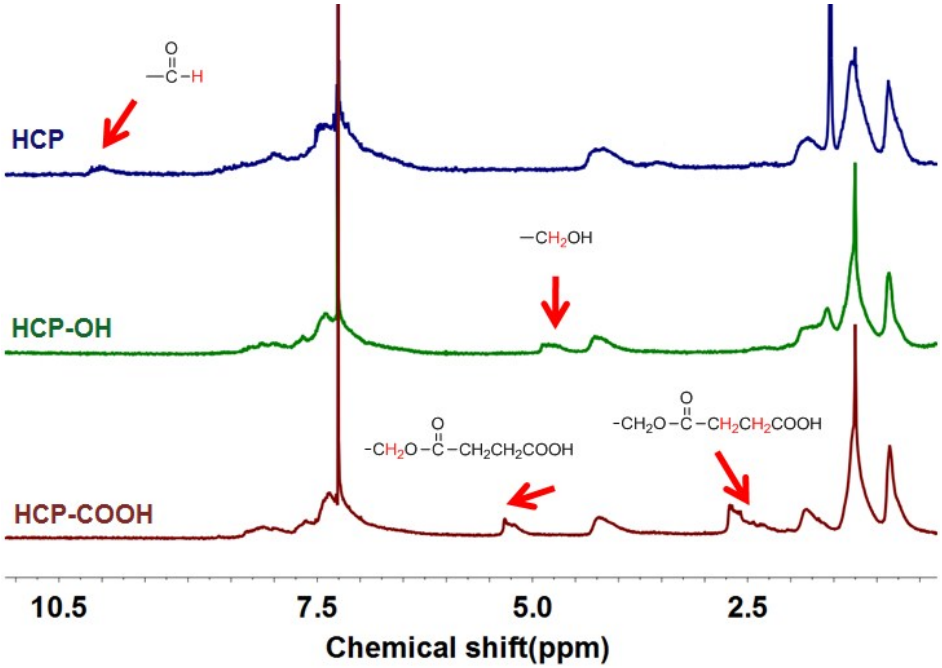

Figure S5. ^{1}H NMR spectra of HCP, HCP-OH and HCP-COOH in $CDCl_3$.

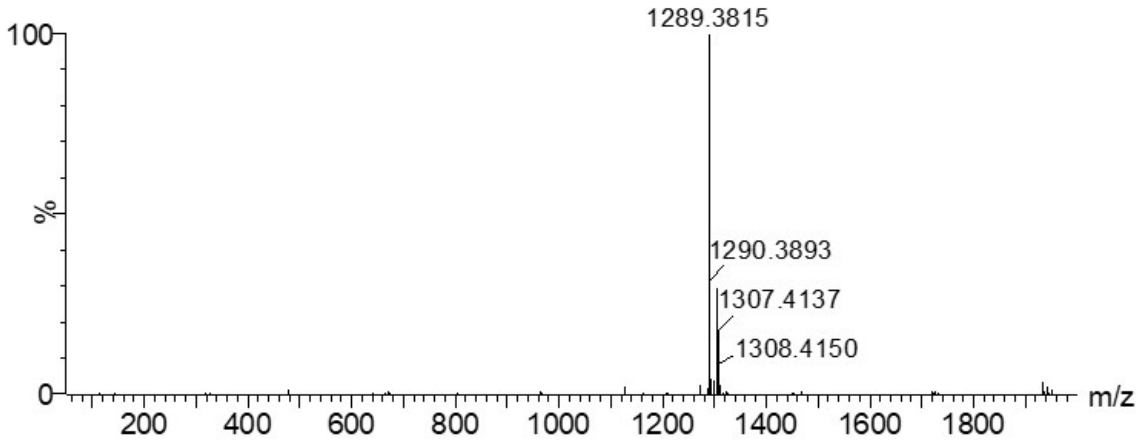

Figure S6. Mass spectrum of CD-OTs.

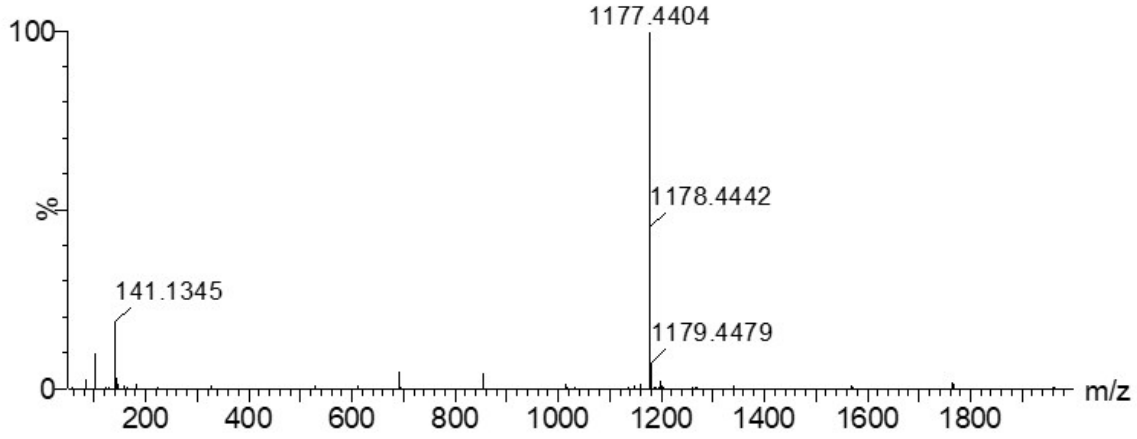

Figure S7. Mass spectrum of CD-$NHCH_2CH_2NH_2$.

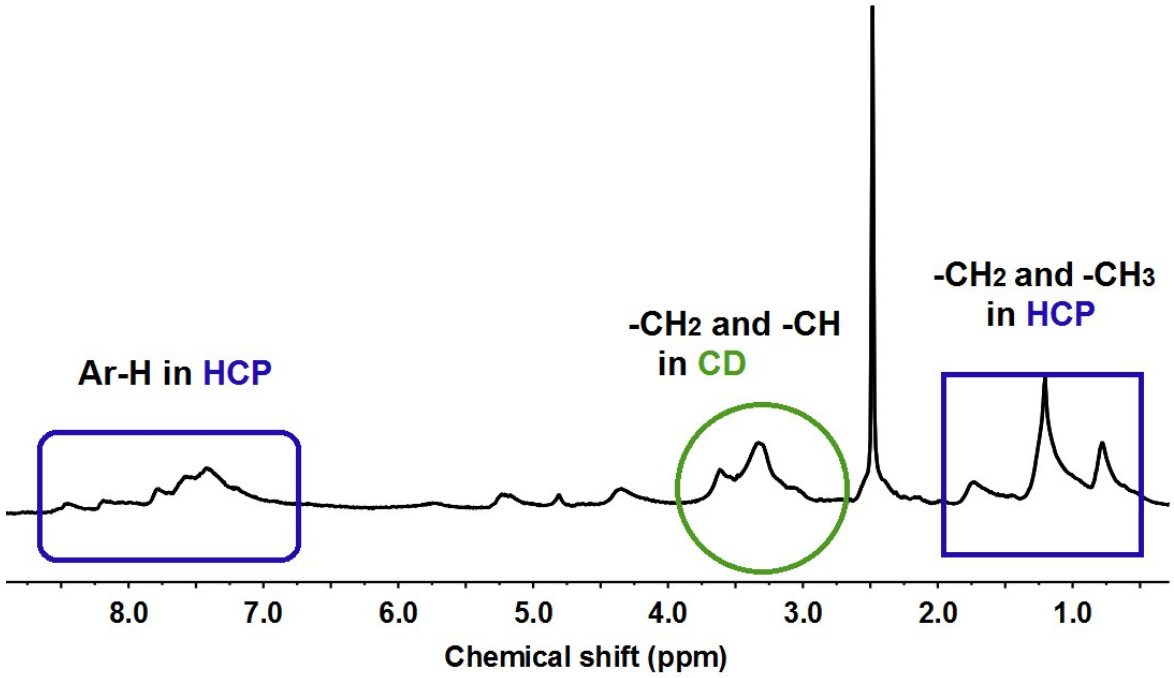

Figure S8. ^{1}H NMR spectrum of HCP-CD in DMSO-d_6.

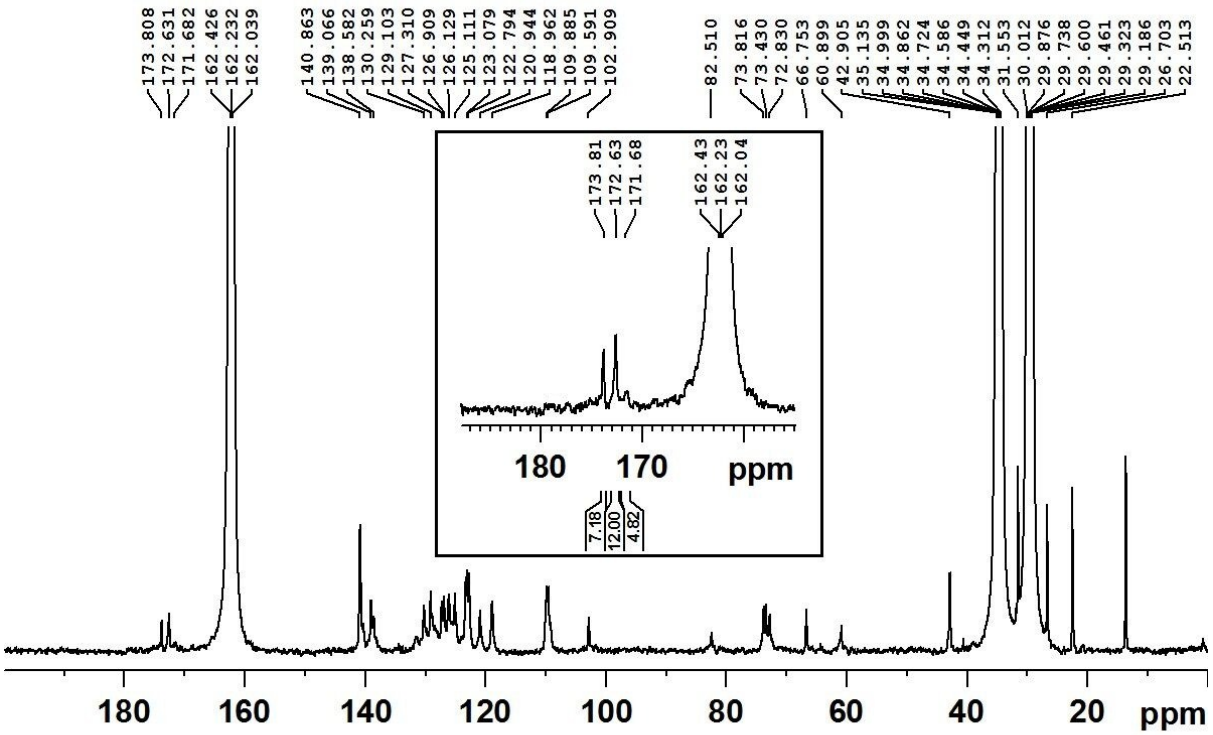

Figure S9. Quantitative ^{13}C NMR spectrum of HCP-CD included a relaxation reagent, chromium acetylacetonate ($Cr(acac)_3$) in DMF-d_7.

The proton signals of 171.68, 172.63, and 173.81 ppm were belong to the carbon of amido bond (-$CH_2OCOCH_2CH_2$***C***ONH-, generated by the reaction of HCP-COOH with CD-$NHCH_2CH_2NH_2$), the carbon of ester bond (-CH_2O***C***OCH_2CH_2CONH-, came from the total HCP-COOH), and the carbon of carboxy group (-$CH_2OCOCH_2CH_2$***C***OOH, came from the un-consumed HCP-COOH), respectively. Thus, the grafting degree of CD on the surface of HCP was calculated by the integral area of the corresponding peak: 4.82/12.00 × 100 % = 40.2 %. According to the molecular weight M_n = 4300 of HCP in Table S1, it was estimated that the total terminal groups number of –CHO was about 12, therefore the grafted CD group was 12 × 40.2 % ≈5.

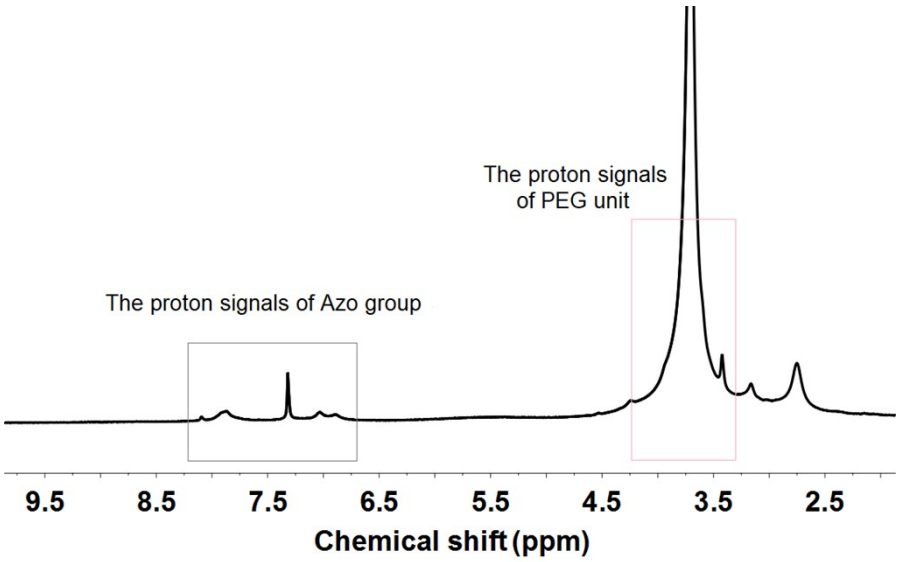

Figure S10. ^{1}H NMR spectrum of Azo-PEG in $CDCl_3$.

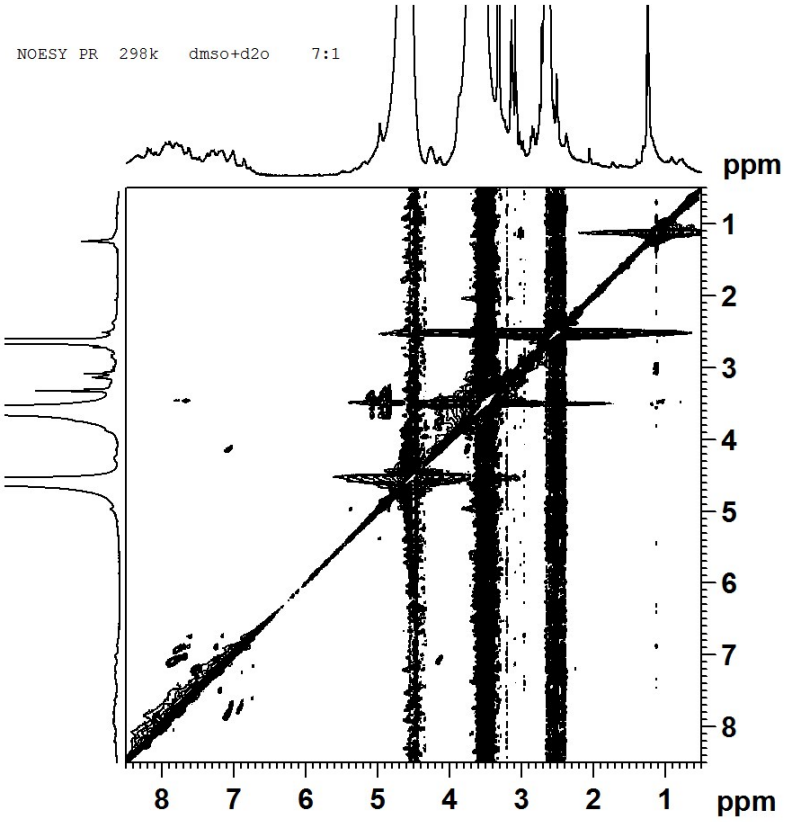

Figure S11. 2D NOESY ^{1}H NMR spectrum of HCP-CD and Azo-PEG at molar ratios of 1:7 in DMSO-d_6/D_2O (1/1, v/v) at 25 ^{o}C.

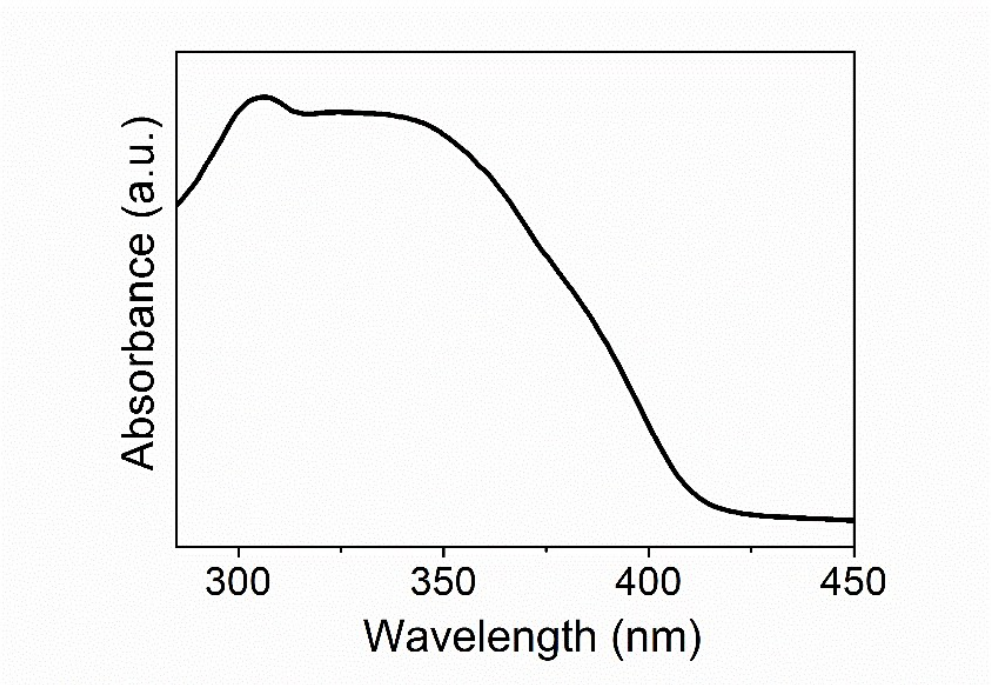

Figure S12. UV-Vis spectrum of HCP-CD in THF.

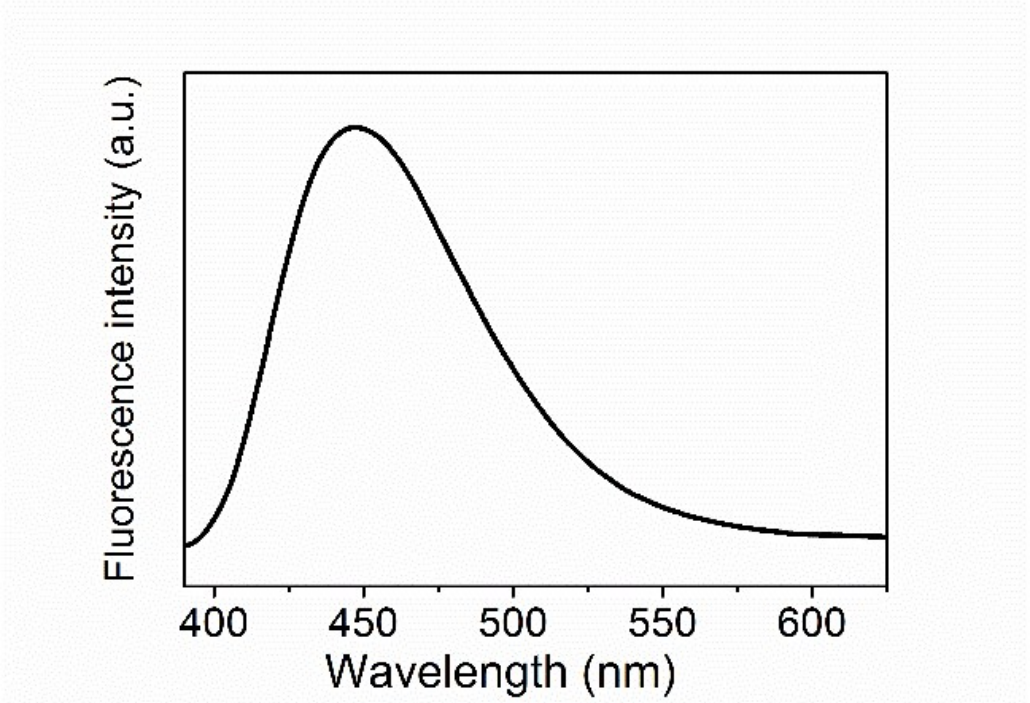

Figure S13. The fluorescence emission of HCP-CD in THF under two-photon laser excitation at 800 nm.

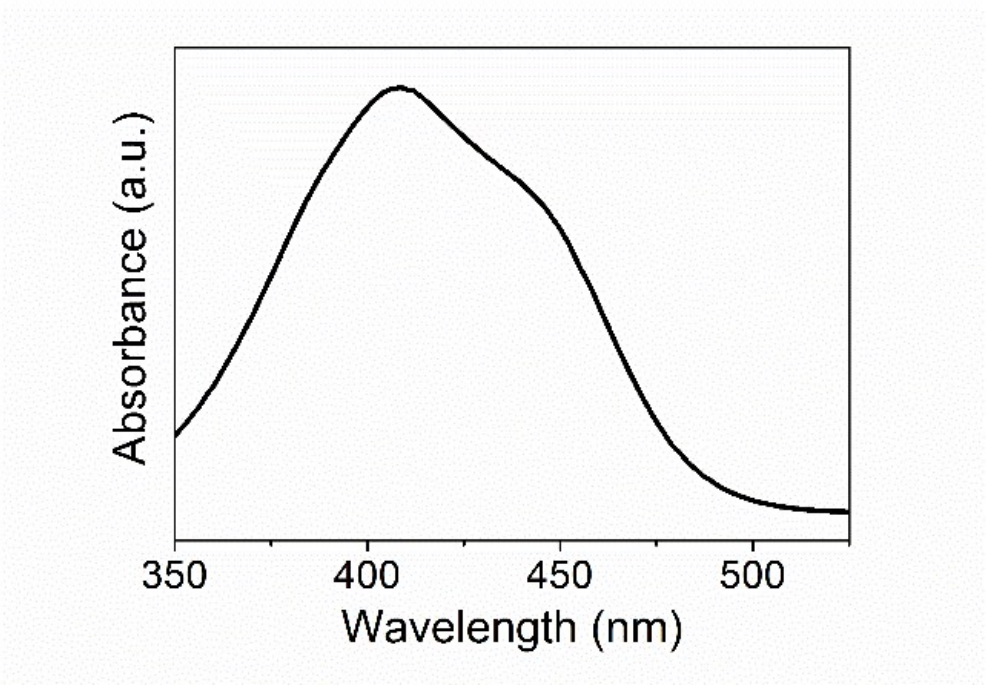

Figure S14. UV-Vis spectrum of Azo-PEG in H_2O.

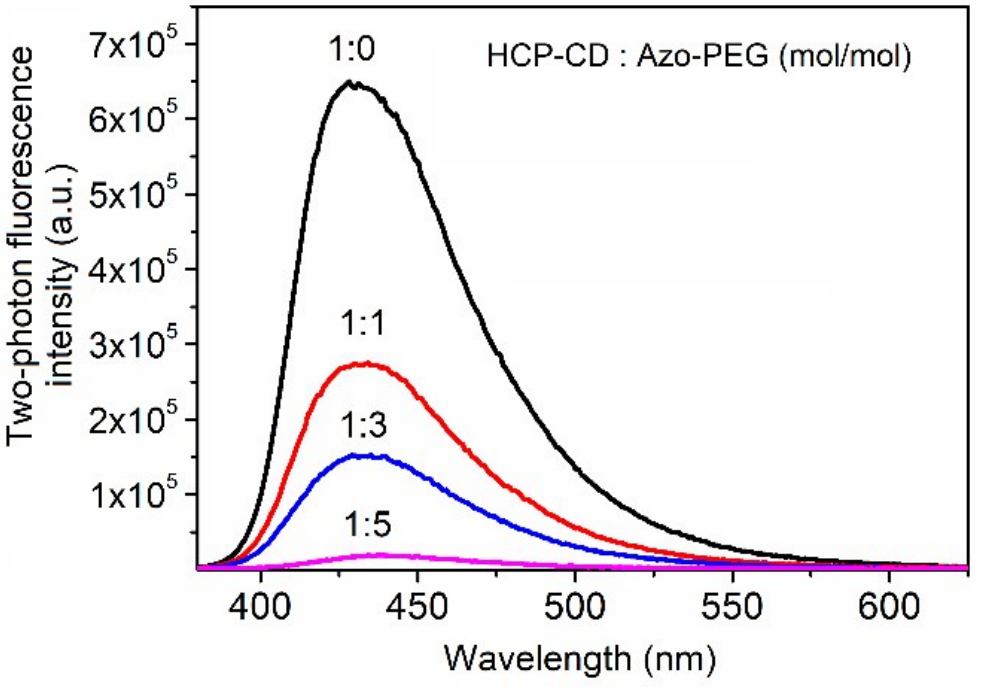

Figure S15. The fluorescence intensity of HCP-CD with adding different content of Azo-PEG under two-photon laser excitation at 800 nm.

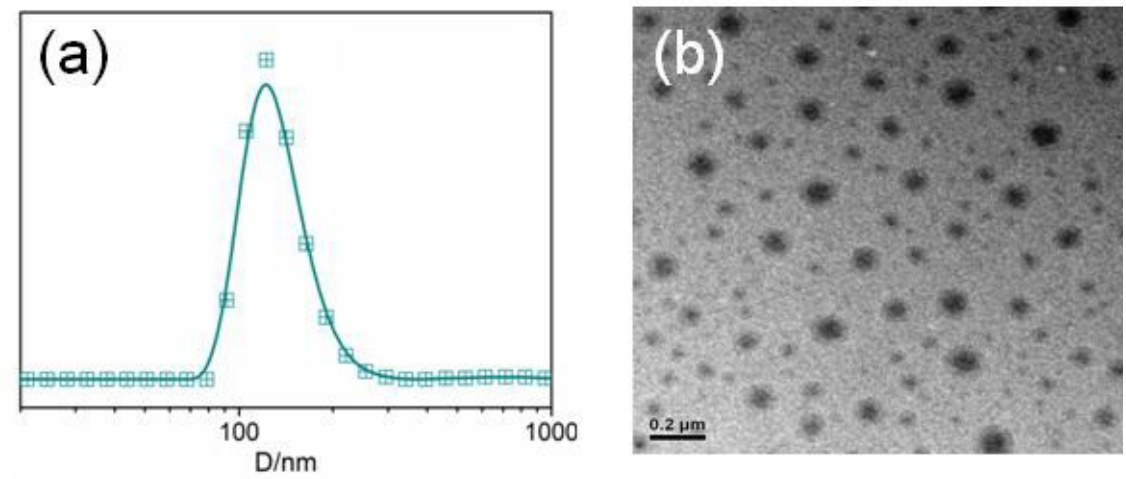

Figure S16. DLS plot (a) and TEM image (b) of DOX-loaded HCP-PEG micelles. Scale bar represents 200 nm.

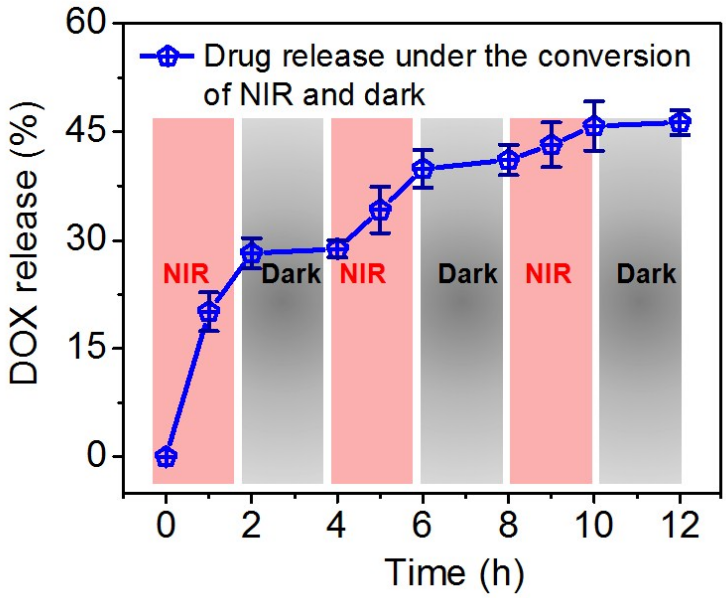

Figure S17. Drug release profile in PBS buffer by alternating periods of NIR light exposure and dark conditions. Every duration of NIR irradiation and dark condition is 2 h.

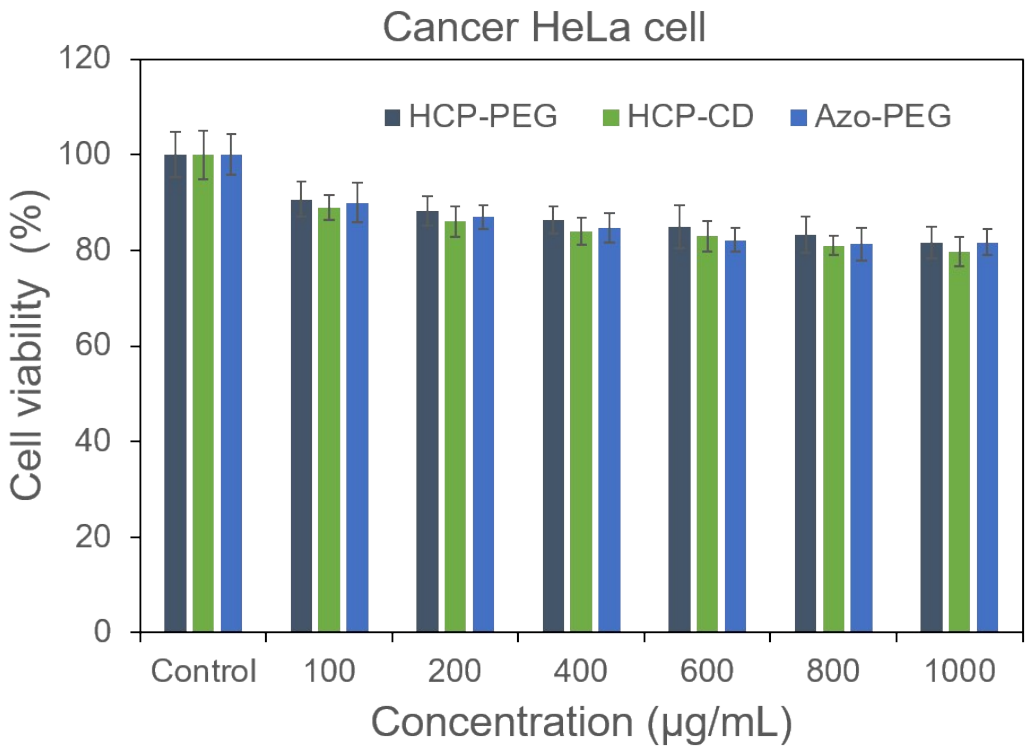

Figure S18. Cell viability of HCP-CD, Azo-PEG and HCP-PEG micelles in cancer HeLa cells.

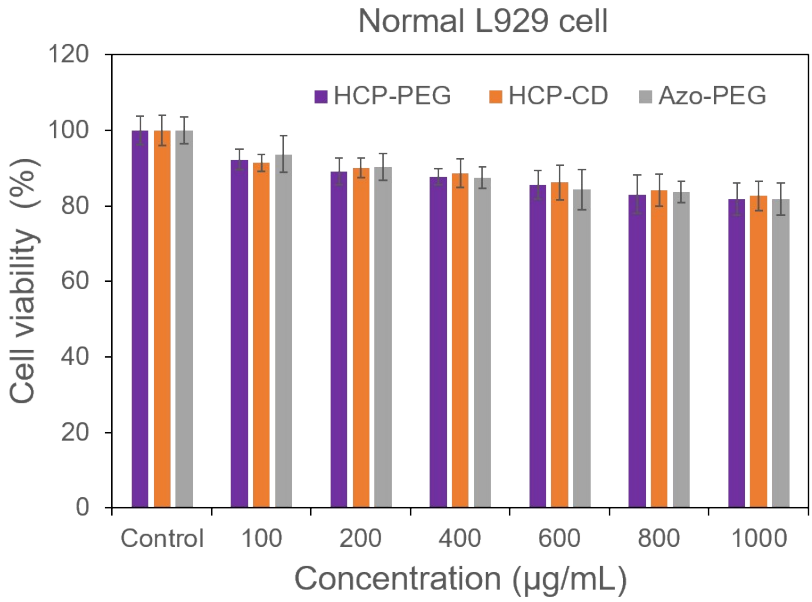

Figure S19. Cell viability of HCP-CD, Azo-PEG and HCP-PEG micelles in normal L929 cells.

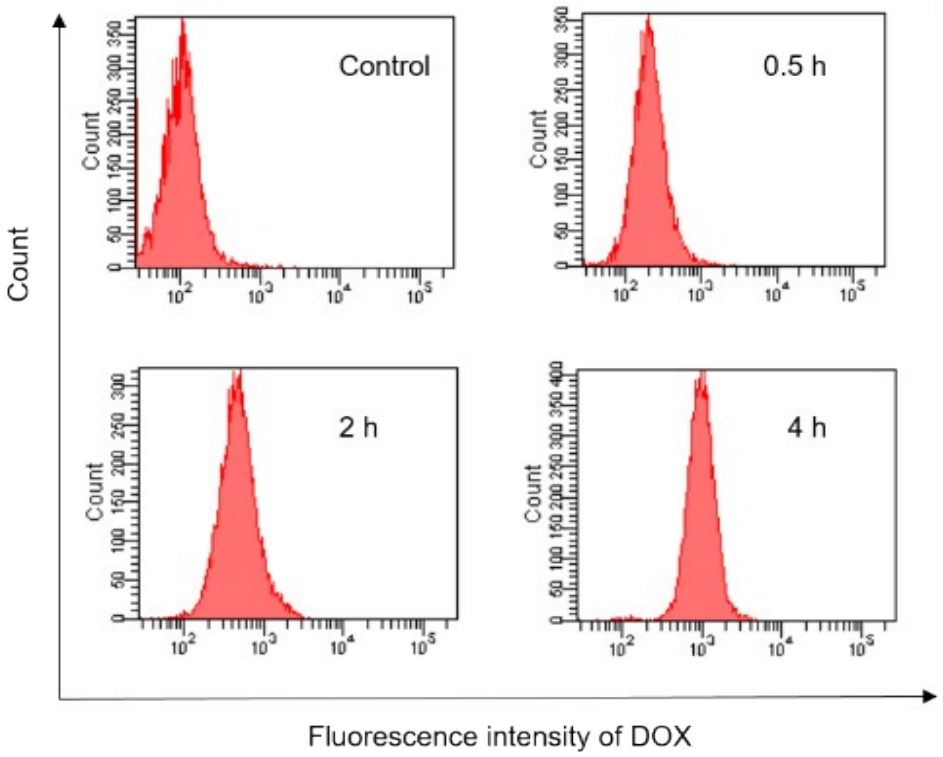

Figure S20. Flow cytometry histogram profiles of HeLa cells incubated with DOX-loaded HCP-PEG micelles.

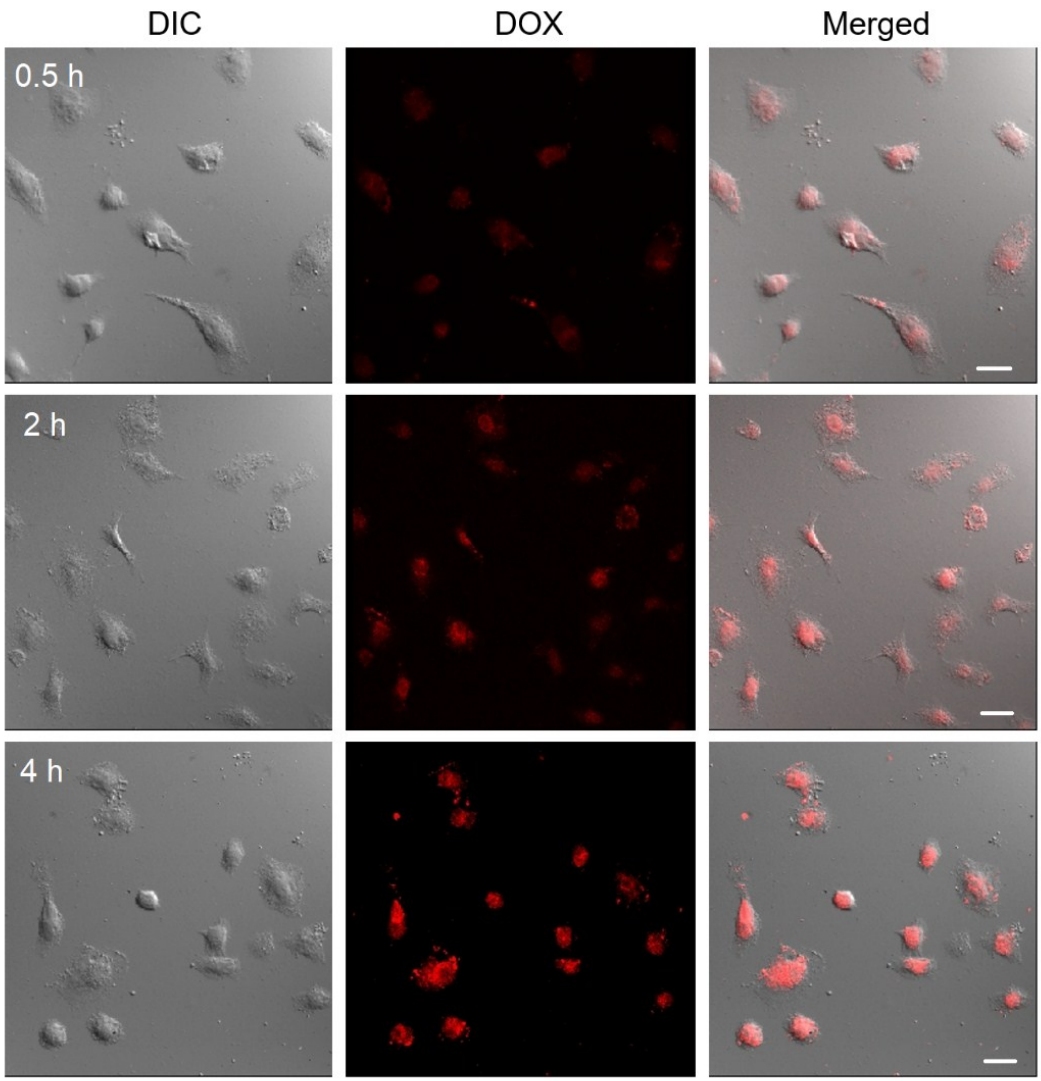

Figure S21. The confocal laser scanning microscopy (CLSM) images of HeLa cells incubated with DOX-loaded HCP-PEG micelles for 0.5 h, 2 h and 4 h at 37 ^{o}C. Red fluorescence comes from DOX. Scale bars represent 20 μm.

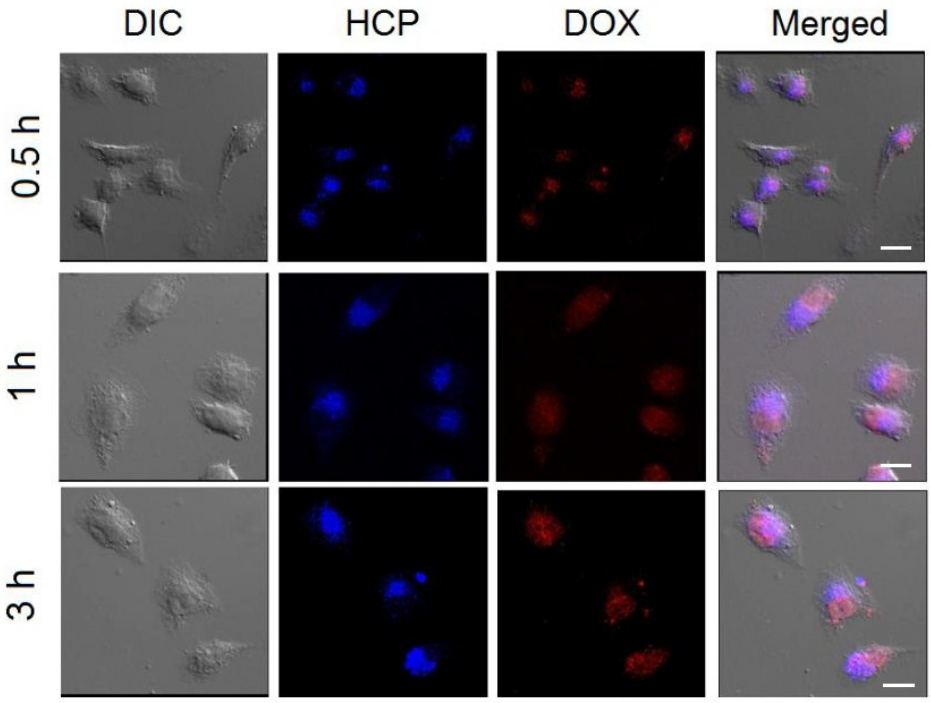

Figure S22. The confocal laser scanning microscopy (CLSM) images of HeLa cell that pretreated with DOX-loaded HCP-PEG micelles for 4 h under the TP laser irradiation for 0.5 h, 1 h and 3 h, respectively.

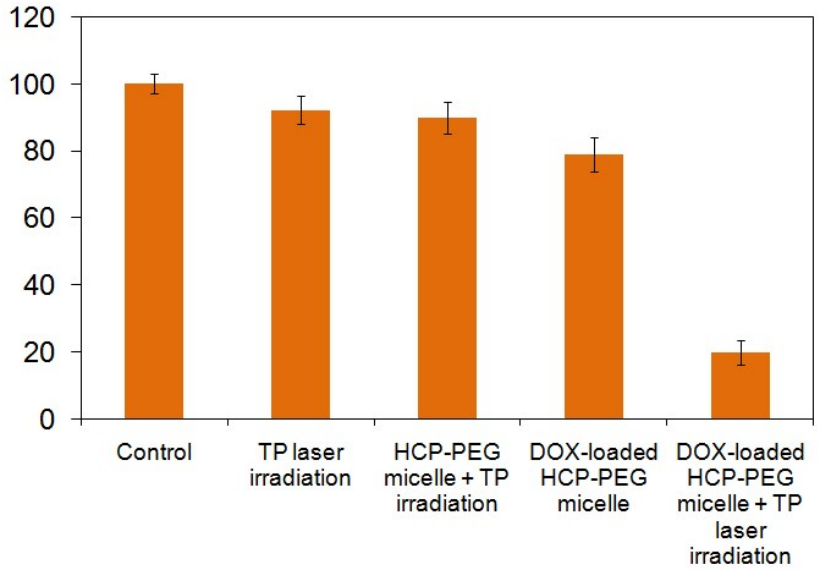

Figure S23. *In vitro* cytotoxicities of TP laser, HCP-PEG micelle with TP laser irradiation, DOX-loaded HCP-PEG micelle without and with TP laser irradiation.

5. Tables.

Table S1. Molecular weights and polydispersities of HCP and HCP-CD

Polymers	M_n (× 10^4)	M_w (× 10^4)	PDI
HCP	0.43±0.02	0.99±0.01	2.30±0.12
HCP-CD	1.07±0.03	2.81±0.02	2.61±0.14

Table S2. Representative fluorescence properties of HCP and HCP-CD in THF

Polymers	Quantum yield (%)	Emission lifetimes (ns)	Two-photon cross-sections (GM)
HCP	27.31 ± 0.18	5.62 ± 0.16	1335 ± 177
HCP-CD	25.79 ± 0.25	5.34 ± 0.19	1309 ± 193

6. References

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