pH responsive supramolecular prodrug micelles based on cucurbit[8]uril for intracellular drug delivery

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Experimental section

Materials

Monomethoxypoly(ethylene glycol) (PEG, *M*n = 2.0 kDa) were purchased from Sigma-Aldrich and dried by azeotropic distillation in the presence of dry toluene. 2-Naphthylacetic acid was supplied by Aladdin Industrial Corporation. 6-maleimidocaproyl-doxorubicin (Mal-DOX) [1] and *N*-(10-Mercaptodecyl)-*N'*-methyl-4, 4'-bipyridinium chloride (MV-SH) [2] were synthesized according to the literatures. All other reagents and solvents were of analytical grade and used as received without further purification.

Synthesis of naphthaline-terminated poly(ethylene glycol) (PEO-Np)

A dried three neck flask was charged with PEG (3 g, 0.6 mmol), 2-naphthylacetic acid (0.13 g, 0.7 mmol), DCC (0.144 g, 0.7 mmol), DMAP (24 mg, 0.2 mmol) and 20 mL of anhydrous DCM. After completely dissolving, the reaction was allowed to take place for 48 h. Then the solution was filtered, followed by evaporating to dryness. The solid was dissolved in small amount of methanol

and recrystallized to afford the product. After the process was repeated for three times, the solid was dried in vacuum.

Synthesis of methyl viologen functioned doxorubicine (MV-DOX)

MV-DOX was prepared using the thiol - maleimide reaction between Mal-DOX and MV-SH. Mal-DOX (0.45g, 0.6 mmol) and MV-SH (0.31g, 0.75 mmol) were added to a flask containing 10 mL of methanol. After complete dissolving, the reaction was catalyzed by a drop of triethylamine and allowed to take place for 24 h. The red solid product was obtained by precipitation of the solution into acetonitrile and dried in vacuum for 24 h at 30 ^{o}C.

Preparation of supramolecular prodrug micelles

PEO-Np, MV-DOX, and CB[8] in an equimolar ratio were added into 10 mL of deionized water. After being sonicated for a period, the resultant solution was vigorously stirred for 12 h. Then the micellar solution was dialyzed against water for 1 days (MWCO = 3.5 kDa) and passed through a 1 μm Millipore filter to remove the dust. In order to determine the DOX content, 1 mL micellar solution was lyophilized and treated with 1 N HCl for 24 h. The solution was diluted to 10 mL with distilled water. Then DOX content was measured using a fluorescence spectroscopy (λ_{ex} =480 nm, λ_{em}=560 nm, slit width=10 nm).

Controlled release of DOX from the supramolecular prodrug system

The controlled release experiment was conducted in PBS buffer at different pH (pH 7.4, 5.0) at 37 °C. Typically, 1 mL prodrug solution was transferred to a dialysis bag (MWCO = 1 kDa) and immersed in a jar containing 10 mL PBS under stirring (120 rpm). At predetermined time intervals,

2 mL of liquid was sampled from the outer solution and then replaced with the same volume of release medium. The drug concentration was detected using a fluorescence spectroscopy (λ_{ex} =480 nm, λ_{em}=560 nm, slit width=10 nm) against calibration curves. Experiments were performed in triple.

Cell culture

Human hepatocellular carcinoma cells (HepG2 cells) were cultured with high-glucose Dulbecco's modified Eagle medium (DMEM). The cell growth media was supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin, and cultured at 37 °C in a 5% CO_2 humidified environment.

Cytotoxicity assays

Cytotoxicity was performed by the standard MTT assay. To determine cell viability, the HepG2 cells were plated at a density of 6×10^3 cells per well in a 96-well plate and cultured for 24 h. The medium was replaced with fresh medium containing varying concentrations of the prodrug micelles. After treatment for 48 h, the wells were washed with PBS and the medium was replaced with fresh medium, 20 μL MTT (5 mg mL^{-1}) was added to each well and the cells were further cultured at 37 °C for 4 h. The dark blue formazan crystals generated by the mitochondria dehydrogenase in the live cells were dissolved with 150 μL DMSO to measure the absorbance at 490 nm by a microplate reader (MODEL 550, Bio Rad). Experiments were performed in quintic.

The intracellular drug release was determined using fluorescence microscopy and flow cytometry.

Fluorescence microscopy

HepG2 cells were inoculated into 24 well plates at 2×10^4 cells per well in DMEM medium for 24 h. The prodrug and the free DOX with a final concentration of 10 μg mL^{-1} were added. Then, the cells were cultured for 1, 3 and 5 h and washed with PBS three times. Finally, the cells were fixed with 4 % paraformaldehyde and stained with 4′, 6-diamidino-2-phenylindole (DAPI). Images were obtained using fluorescence microscopy.

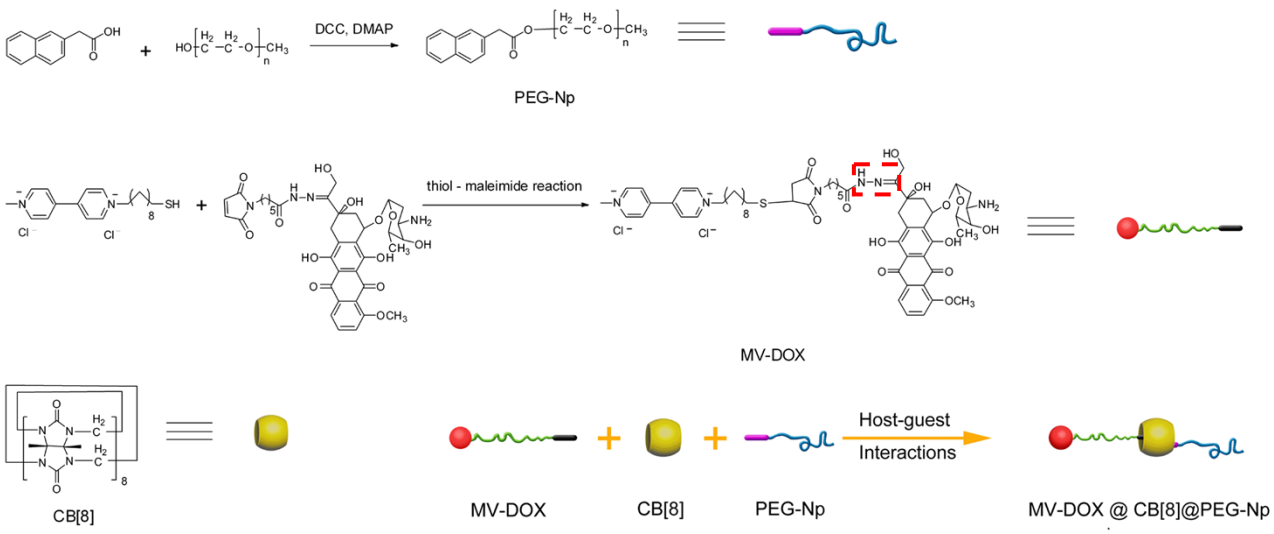
Flow cytometry

HepG2 cells were incubated into 24 well plates at a density of 2×10^5 cells per well in DMEM medium for 24 h. The prodrug with a final concentration of 10 μg mL^{-1} was added. Then, the cells were cultured for 1, 3 and 5 h and washed three times with PBS. Cells were treated with trypsin and centrifuged for 5 min at 1000 rpm. Then the cells were suspended in 0.5 mL of PBS and analyzed using a FACScan flow cytometer.

Characterizations

The ^1H NMR spectra were recorded on a Bruker DMX500 spectrometer operating at 500 MHz using DMSO-d_6 or CD_3OD-d_4 or $CDCl_3$ as the solvent. The size of the micelles was measured using dynamic light scattering (DLS). Measurements were performed using Zetasizer Nano-ZS from Malvern Instruments equipped with a He-Ne laser at wavelength of 633 nm with a angle of 173^o (25 °C). The samples were cleaned using a 0.45 μm Millipore filter before measurements. The sizes and morphologies of the resultant samples were also characterized by HT7700 transmission electron microscopy (TEM) at an accelerating voltage of 100 kV, whereby a carbon-coated copper EM grid (230 mesh) was immersed into the micellar solution for a while and dried at room temperature at atmospheric pressure. UV-visible spectra were

obtained using a UV–vis Shimadzu UV-2505 spectrophotometer.



Scheme S1. Detailed synthetic route of PEO-Np and MV-DOX with the molecule structure of CB[8]. The acid-labile hydrazone bond was labeled by the red rectangle.

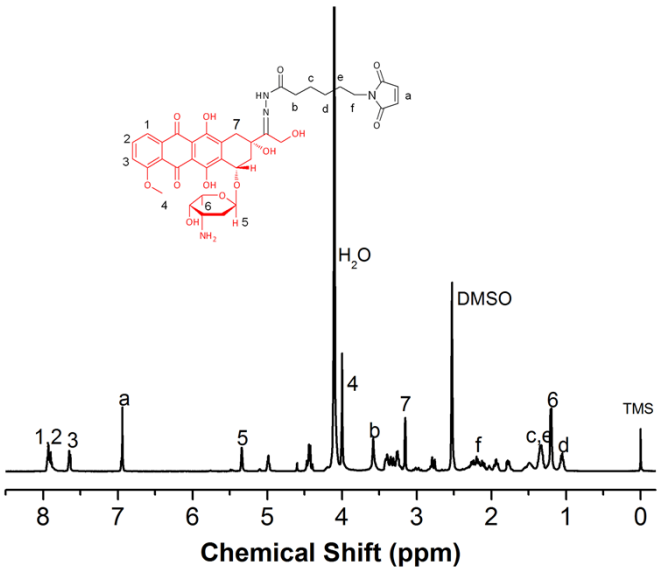


Figure S1. ^{1}H NMR spectrum of Mal-DOX in DMSO-d_6.

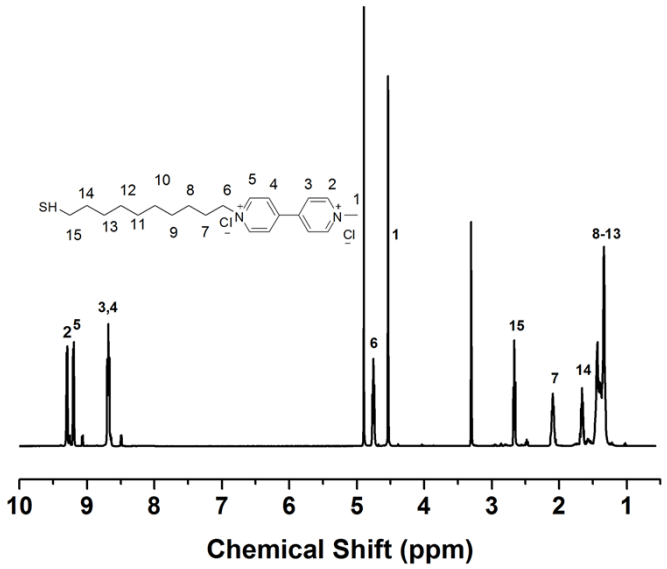


Figure S2. ^{1}H NMR spectrum of MV-SH in CD_3OD-d_4.

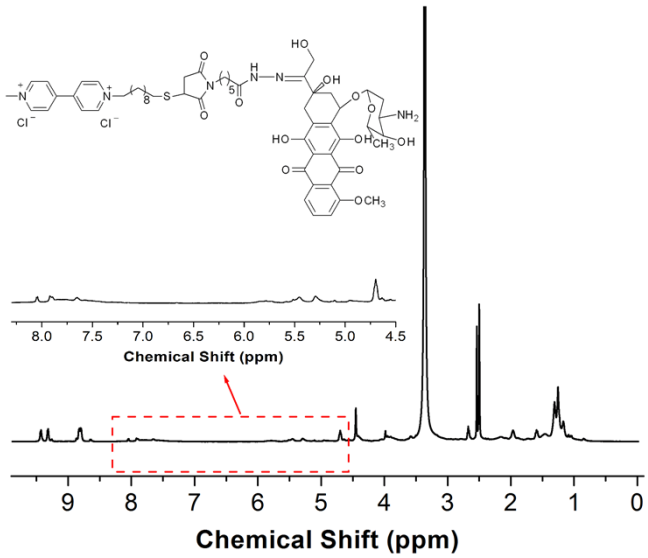


Figure S3. ^{1}H NMR spectrum of MV-DOX in DMSO-d_6. The peak assigned to double bonds at around 7 ppm disappeared (Labeled by a in Figure S1). Integration ratio of the peaks corresponding to MV groups (9.3-9.4 ppm) and to DOX (7.5-8.0 ppm) were approximately 4:3, suggesting that MV-DOX had been synthesized successfully.

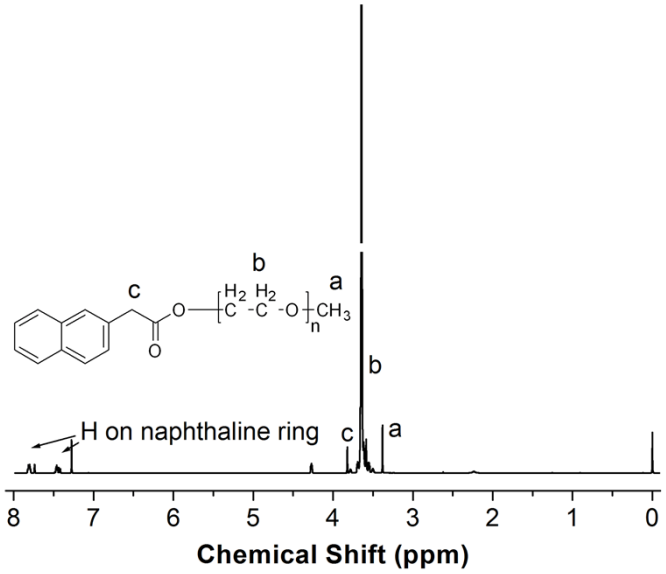


Figure S4. ^{1}H NMR spectrum of PEG-Np in $CDCl_3$.

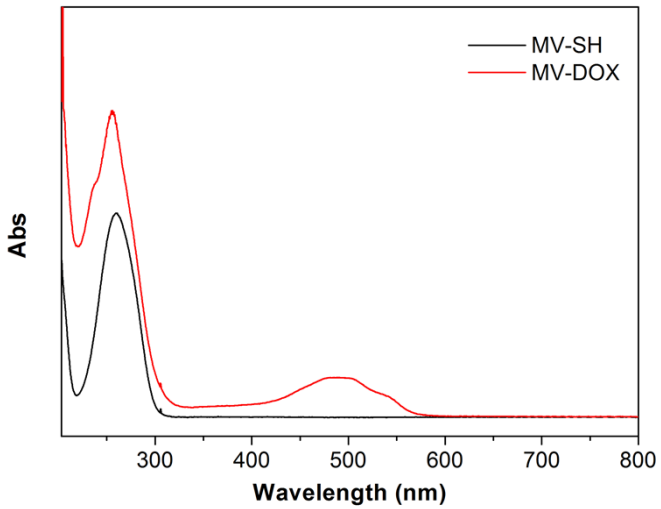


Figure S5. UV-vis spectra of MV-SH and MV-DOX.

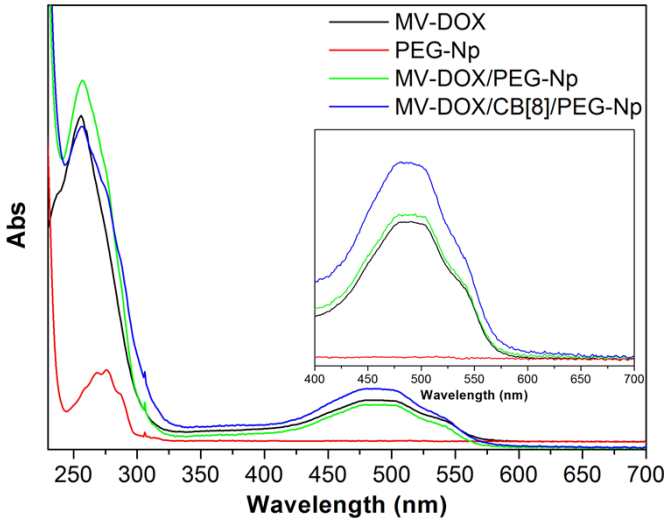


Figure S6. UV-vis spectra of MV-DOX, PEG-Np, MV-DOX/PEG-Np and the ternary complex.

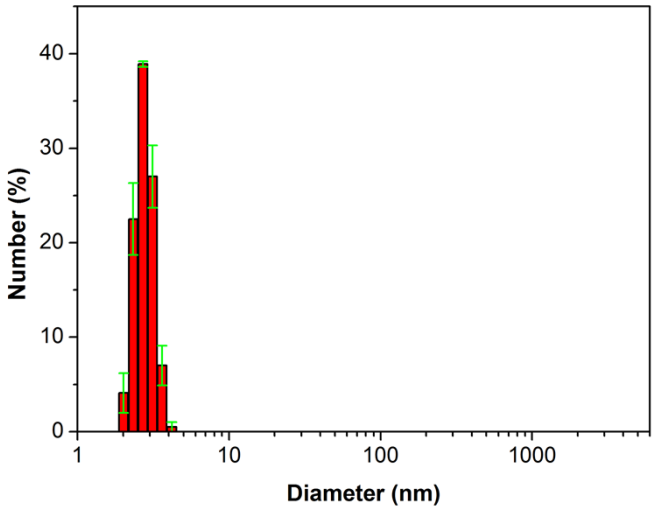


Figure S7. DLS plot of PEG-Np.

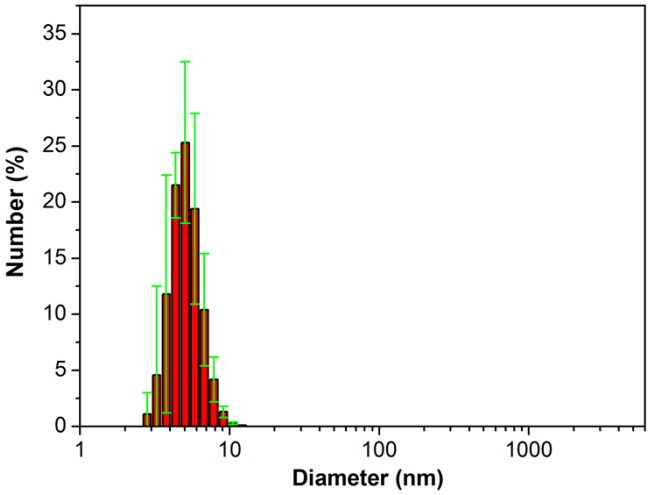


Figure S8. DLS plot of MV-SH.

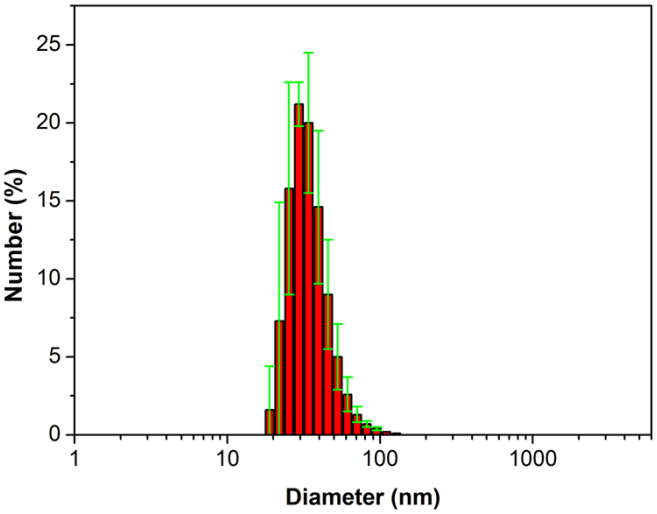


Figure S9. DLS plot of PEG-Np@CB[8]@MV-SH. The slight increase of diameter may be caused

by the formation of disulfide bond between one PEG-Np@CB[8]@MV-SH chain and another.

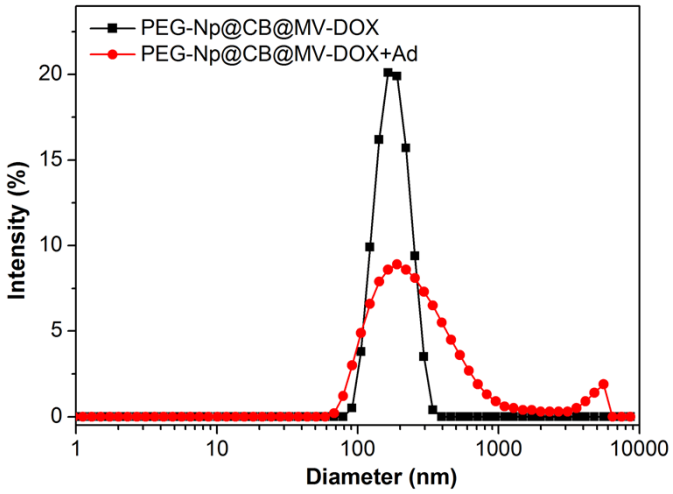


Figure S10. DLS plots of supramolecular micelles before and after treated with admantadine(Ad) for 5 min.

Flow cytometry was a potent way to reveal the cellular uptake efficiency of nanocarriers. So it was carried out to trace the efficiency of the prodrug micelles. From the data displayed in Figure S11, it can be concluded that the relative geometrical mean fluorescence intensities of cells treated with prodrug micelles increased as the incubation time was prolonged from 1 h to 5 h. This increase of fluorescence signals was attributed to the internalization of prodrug micelles by HepG2 cells.

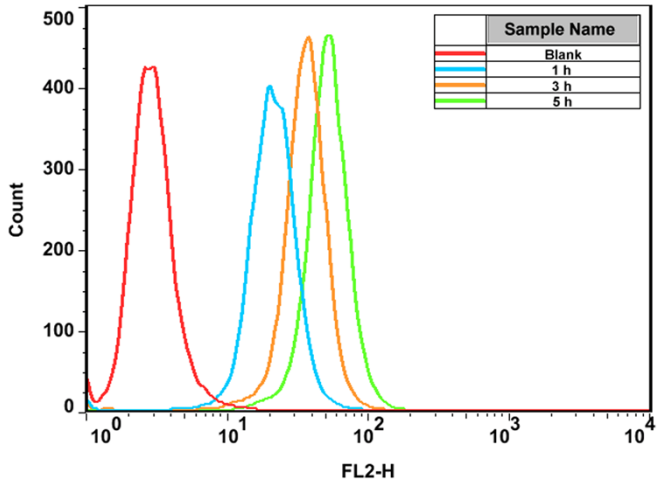


Figure S11. Flow cytometric profiles of HepG2 cells incubated with prodrug micelles (10 μg mL^{-1}) for 1 h, 3 h or 5 h;

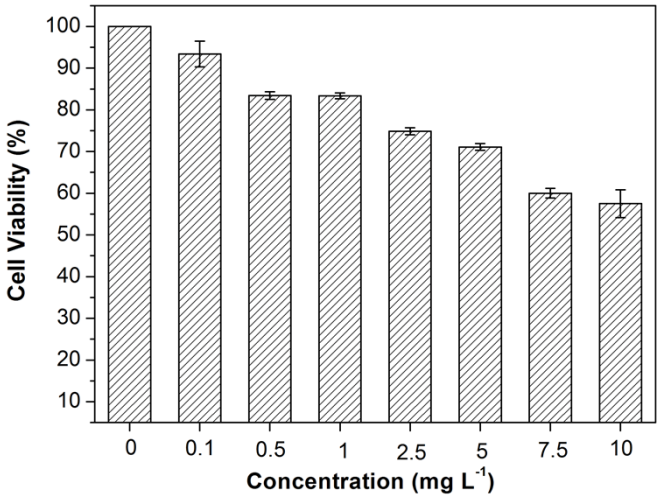


Figure S12. Cell viability of HepG2 cells incubated with various concentrations of MV-SH for 48 h. The concentration adopted in this figure is derived from Figure 3. The concentration of MV-SH used was the same for these two experiments.

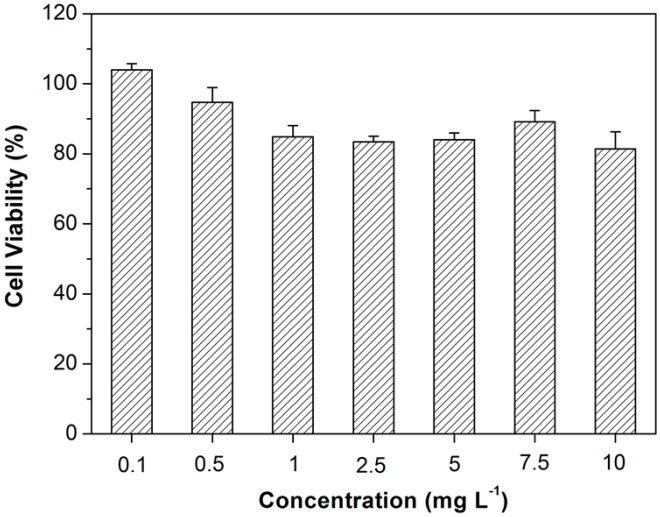


Figure S13. Cell viability of HepG2 cells incubated with various concentrations of PEG-Np@CB[8]@MV-SH for 48 h. The concentration adopted in this figure is derived from Figure 3. The concentration of PEG-Np@CB[8]@MV-SH used was the same for these two experiments.

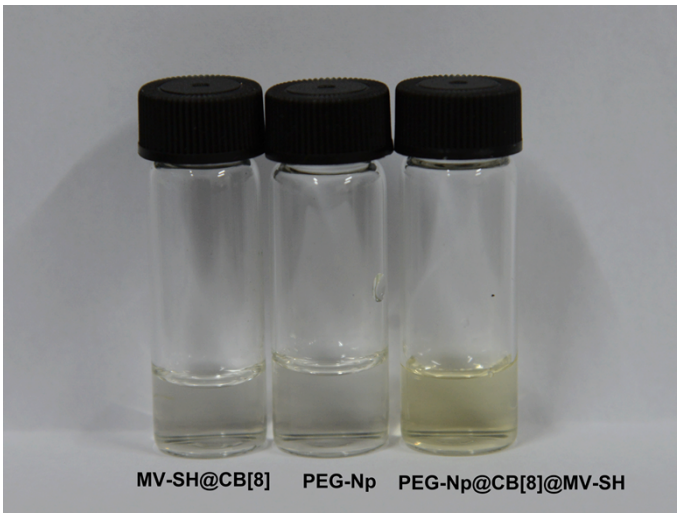


Figure S14. Digital photos of MV-SH@CB[8], PEG-Np and PEG-Np@CB[8]@MV-SH aqueous solution.

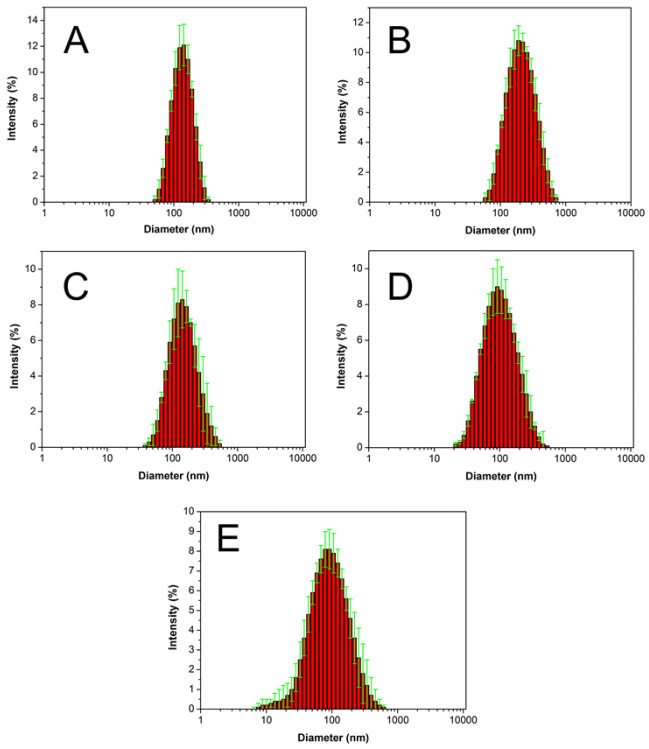


Figure S15. DLS plots of supramolecular prodrug micelles in water (A), 1M PBS (B), 20X dilution (C), DMEM (D) and DMEM with 10 % FBS (E).

References

1. D. Willner, P. A. Trail, S. J. Hofstead, H. D. King, S. J. Lasch, G. R. Braslawsky, R. S. Greenfield, T. Kaneko and R. A. Firestone, *Bioconjugate Chem.*, 1993, **4**, 521.
2. F. Tian, N. Cheng, N. Nouvel, J. Geng and O. A. Scherman, *Langmuir*, 2010, **26**, 5323.