

## Supporting information

# Pillar[5]arene based supramolecular prodrug micelles as a feasible candidate for pH induced aggregate nanocarrier for intracellular drug delivery

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### Experimental section

#### Materials

Water-soluble pillar[5]arene was kindly supplied by Professor Feihe Huang (Department of Chemistry, Zhejiang University) and the molecular structure was shown in Figure S1.<sup>1</sup> Methyl viologen functioned doxorubicin (MV-DOX) was synthesized according to the literature and the molecular structure was shown in Figure S1.<sup>2</sup> All other reagents and solvents were of analytical grade and used as received without further purification.

#### Synthesis of methyl viologen functioned doxorubicine (MV-DOX)

6-maleimidocaproyl-doxorubicin (Mal-DOX)<sup>3</sup> and *N*-(10-Mercaptodecyl)-*N'*-methyl-4, 4'-bipyridinium chloride (MV-SH)<sup>4</sup> were synthesized according to the literatures. 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 °C.

#### Preparation of supramolecular prodrug micelles

WP5 and MV-DOX in an equimolar ratio were added into 10 mL of deionized water. After 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  $\mu$ m 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 ( $\lambda_{\text{ex}}$  =480 nm,  $\lambda_{\text{em}}$  =560 nm, slit width=10 nm). The preparation of PEG-Np@CB[8]@MV-DOX prodrug micelles was according to the published literature.

#### 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<sup>-1</sup> penicillin, and 100 mg mL<sup>-1</sup> streptomycin, and cultured at 37 °C in a 5% CO<sub>2</sub> 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  $6 \times 10^3$  cells per well in a 96-well plate and cultured for 24 h. Then the medium was replaced with fresh medium (pH 6.5) containing varying concentrations of the prodrug micelles or MV-SH or WP5@MV-SH. After incubated for 6 h, the wells were washed with PBS and replaced with fresh media (pH 7.4). After treatment for another 48 h, the wells were further washed with PBS and replaced with fresh media (pH 7.4), 20  $\mu$ L MTT ( $5 \text{ mg mL}^{-1}$ ) 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  $\mu$ L DMSO to measure the absorbance at 490 nm by a microplate reader (MODEL 550, Bio Rad). Experiments were performed in quintic. For comparison with CB[8] based prodrug micelles, the MTT assay was carried out in the same way expect the pH of the culture media is 7.4.

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

#### Fluorescence microscopy

HepG2 cells were inoculated into 24 well plates at  $2 \times 10^4$  cells per well in DMEM medium for 24 h. The prodrug with a final concentration of  $10 \mu\text{g mL}^{-1}$  was added after the wells were washed with PBS and replaced with fresh media (pH 6.5). Then, the cells were cultured for 0.5, 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  $2 \times 10^5$  cells per well in DMEM

medium for 24 h. The prodrug with a final concentration of  $10 \mu\text{g mL}^{-1}$  was added after the wells were washed with PBS and replaced with fresh media (pH 6.5). Then, the cells were cultured for 0.5, 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.

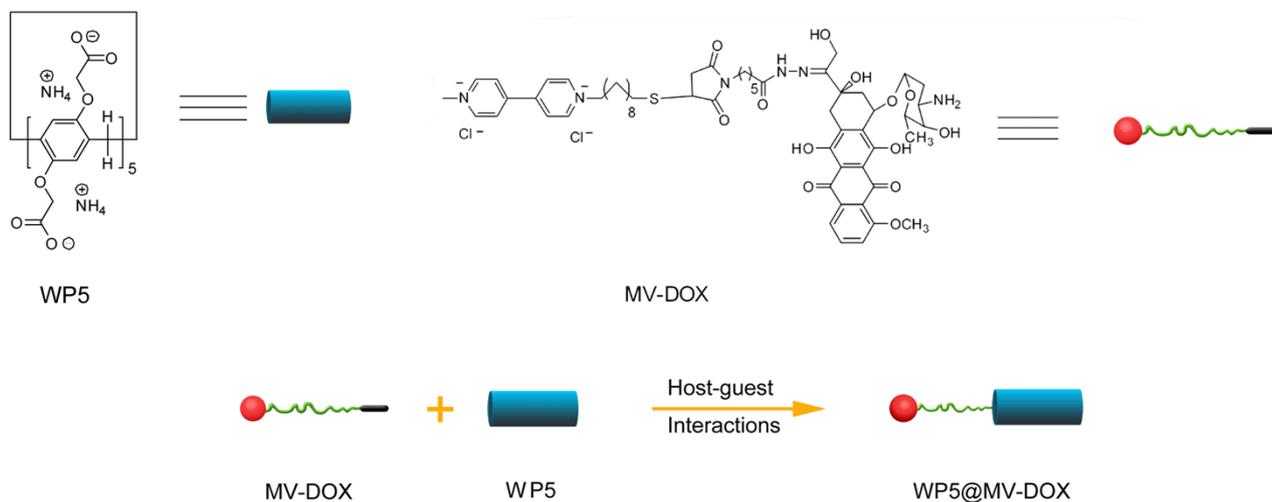
#### Retention test of supramolecular prodrug micelles in cancer cells

HepG2 cells were incubated into 24 well plates at a density of  $2 \times 10^5$  cells per well in DMEM medium for 24 h. Then, two kinds of prodrug micelles (WP5@MV-DOX and PEG-Np@CB[8]@MV-DOX) with a final concentration of  $10 \mu\text{g mL}^{-1}$  was added after the wells were washed with PBS and replaced with fresh media (pH 7.4). After incubation for 2 h, the cells were further washed by PBS and replaced with fresh media (pH 7.4) to culture for 2 or 4 h. Cells were treated with trypsin and centrifuged for 5 min at 1000 rpm after being washed three times with PBS. Then the cells were suspended in 0.5 mL of PBS and analyzed using a FACScan flow cytometer.

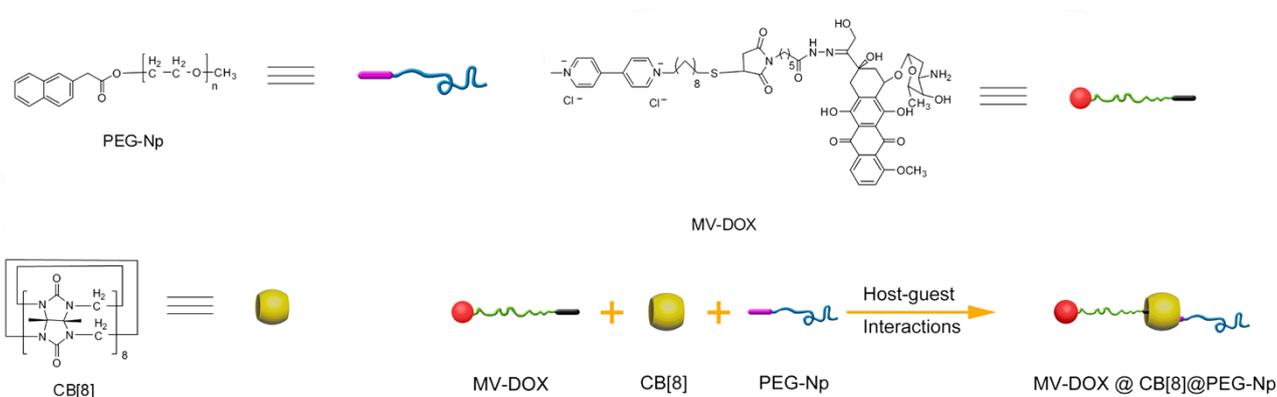
#### Characterizations

The  $^1\text{H}$  NMR spectra were recorded on a Bruker DMX500 spectrometer operating at 500 MHz using DMSO- $d_6$  or  $\text{D}_2\text{O}$  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  $173^\circ$  ( $25^\circ\text{C}$ ). The samples were cleaned using a  $0.45 \mu\text{m}$  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

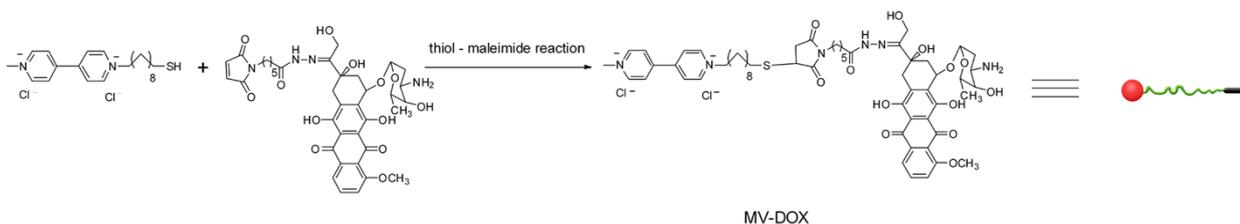
grid (230 mesh) was immersed into the micellar solution for a while and dried at room temperature at atmospheric pressure.



Scheme S1. Schematic illustration for preparation of WP5 @MV-DOX and molecular structures of WP5 and MV-DOX.



Scheme S2. Schematic illustration for preparation of PEG-Np@CB[8]@MV-DOX and molecular structures of PEG-Np, CB[8] and MV-DOX.<sup>2</sup>



Scheme S3. Synthetic route of MV-DOX.

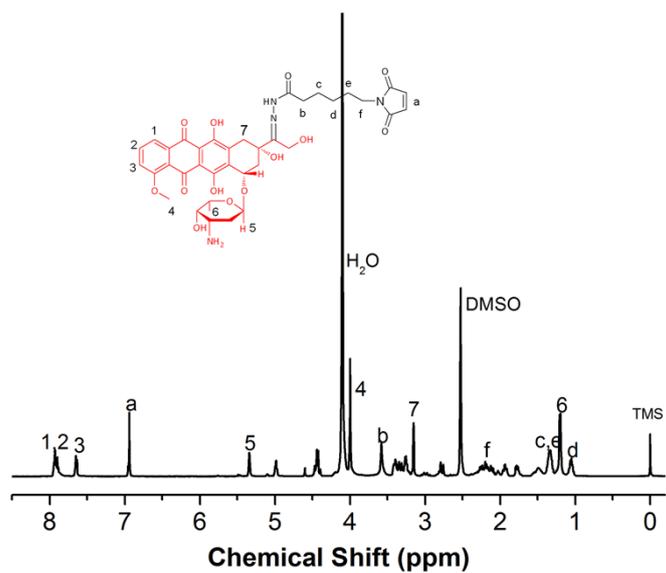


Fig. S1 <sup>1</sup>H NMR spectrum of Mal-DOX in DMSO-*d*<sub>6</sub>.

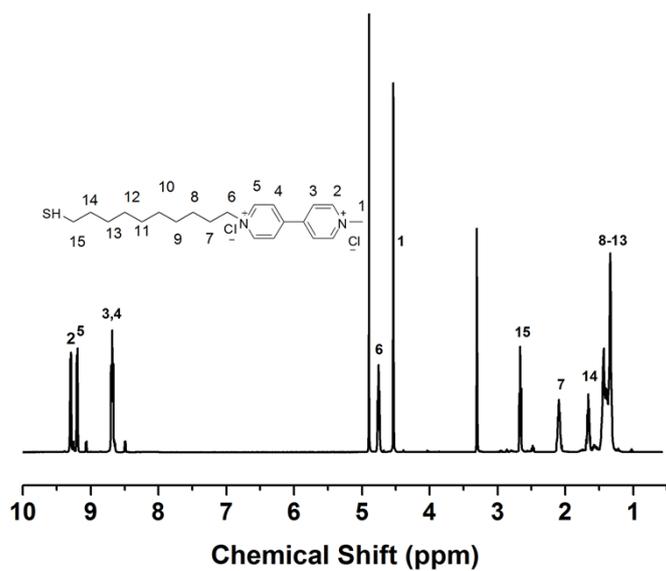


Fig. S2 <sup>1</sup>H NMR spectrum of MV-SH in CD<sub>3</sub>OD-*d*<sub>4</sub>.

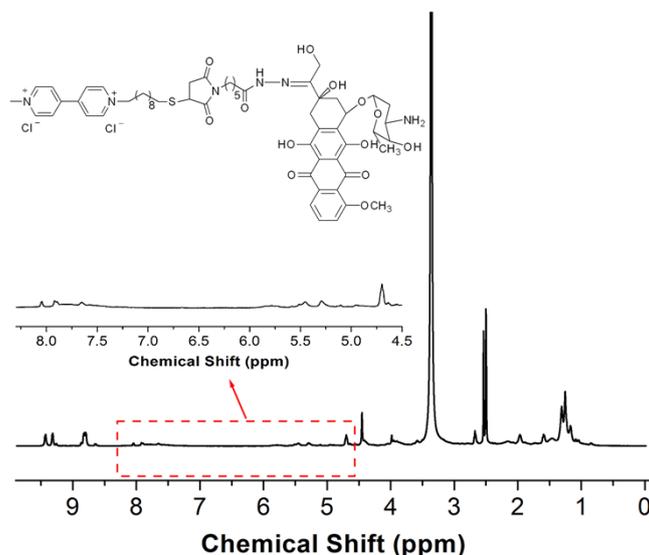


Fig. S3  $^1\text{H}$  NMR spectrum of MV-DOX in  $\text{DMSO-}d_6$ . The peak assigned to double bonds at around 7 ppm disappeared (Labeled by a in Figure S1). Integration ratio of the peaks corresponding to MV groups (9.3-9.4 ppm) and to DOX (7.5-8.0 ppm) were approximately 4:3, suggesting that MV-DOX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

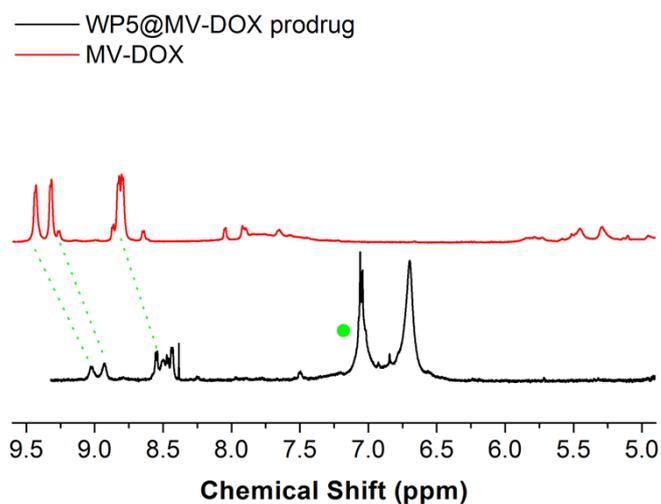


Fig. S4  $^1\text{H}$  NMR spectra of MV-DOX and WP5@MV-DOX prodrug in  $\text{D}_2\text{O}$ . The peak labeled with green dot is attributed to WP5.

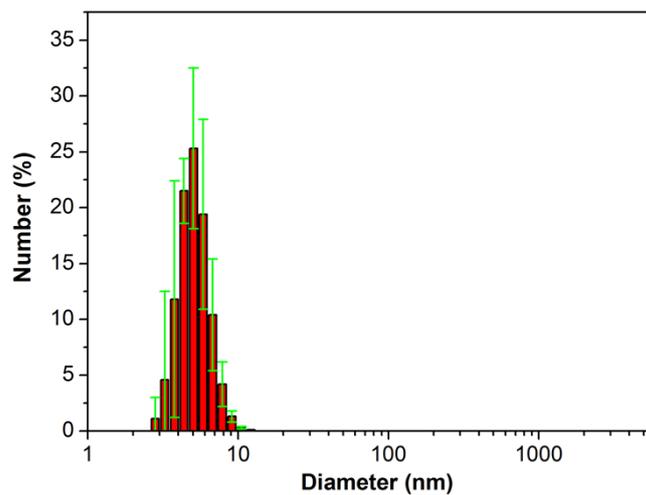


Fig. S5 DLS plot of MV-SH. The concentration was  $1.5 \text{ mg mL}^{-1}$ .

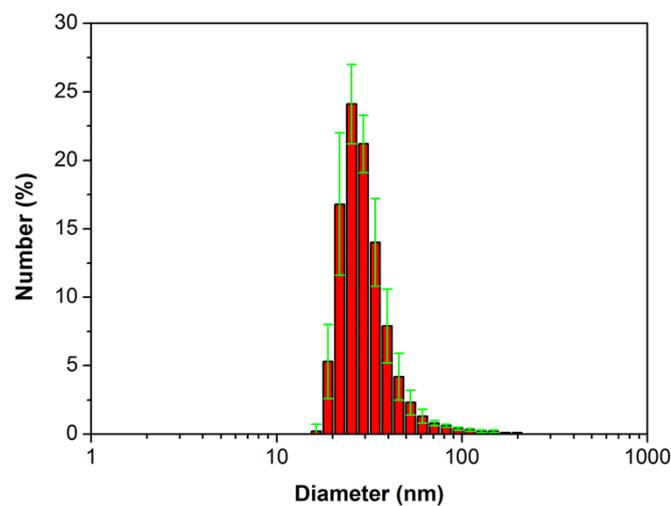


Fig. S6 DLS plot of WP5. The concentration was  $1.5 \text{ mg mL}^{-1}$ .

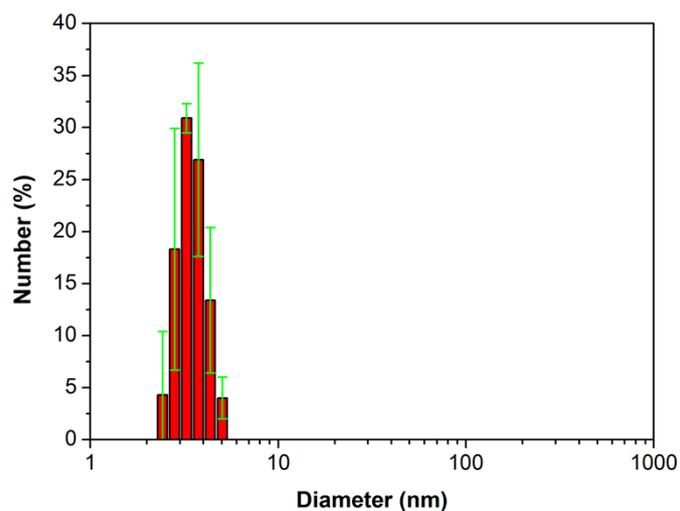


Fig. S7 DLS plot of WP5@MV-SH. The concentration was  $1.5 \text{ mg mL}^{-1}$ .

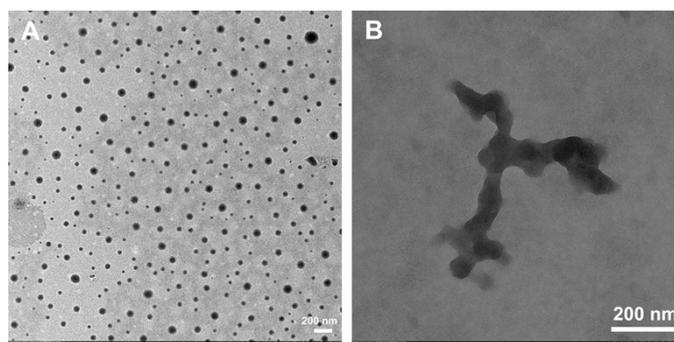


Fig. S8 Representative TEM images of the supramolecular micelles at pH 7.4 (A) and pH 6.5 (B). The scale bars are 200 nm.

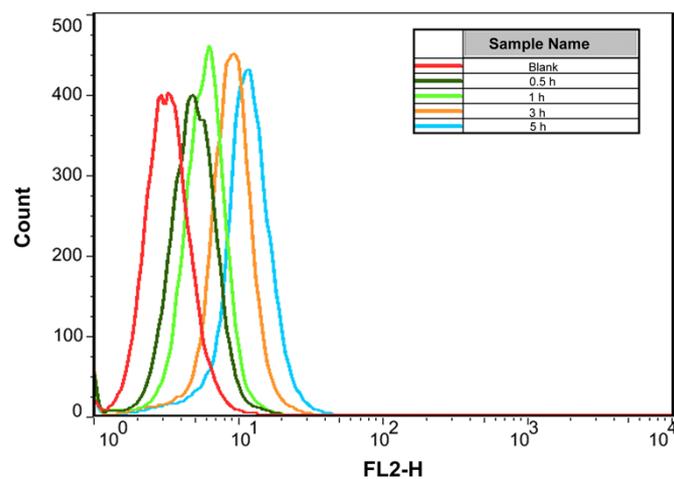


Fig. S9 Flow cytometric profiles of HepG2 cells incubated with prodrug micelles ( $10 \mu\text{g mL}^{-1}$ ) for 0.5 h, 1 h, 3 h or 5 h.

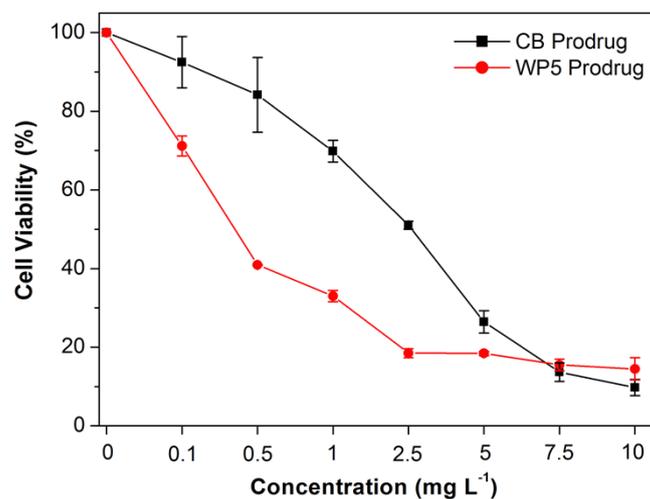


Fig. S10 Cell viability of HepG2 cells incubated with various concentrations of PEG-Np@ CB[8]@MV-DOX and WP5@MV-DOX prodrug for 48 h.

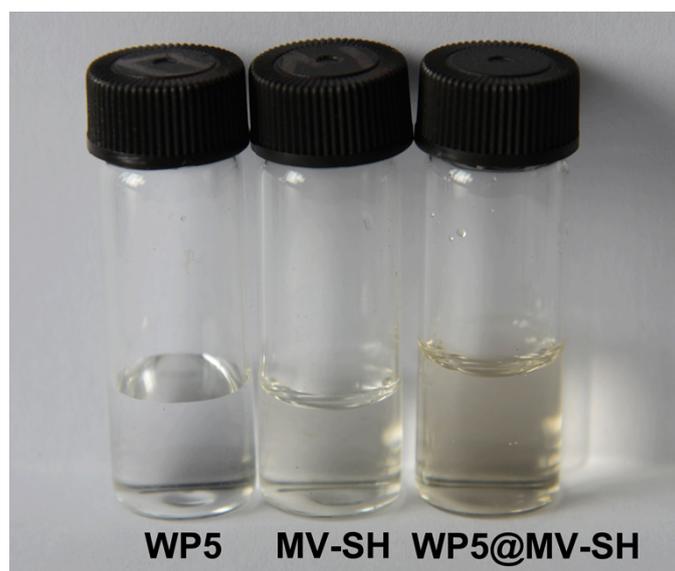


Fig. S11 A digital photo of WP5, MV-SH and WP5@MV-SH aqueous solutions.

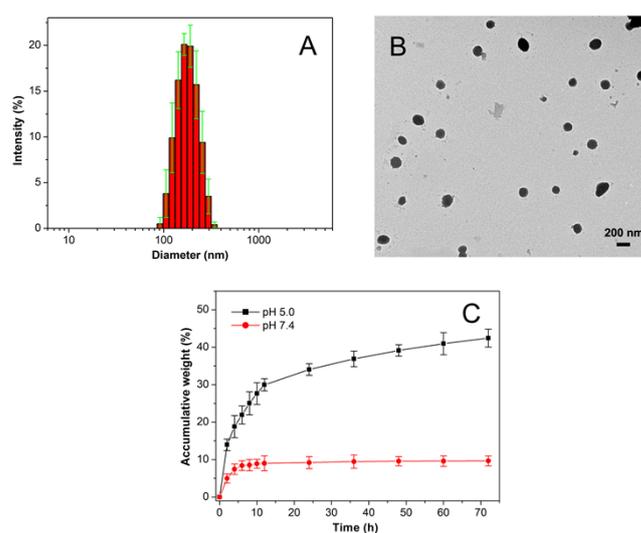


Fig. S12 DLS plot (A) and representative TEM image (B) of the PEG-Np@CB[8]@MV-DOX supramolecular micelles; *in vitro* release of DOX from the PEG-Np@CB[8]@MV-DOX supramolecular prodrug micelles in PBS under different pH conditions (C). The concentration of supramolecular micelles for DLS measurements was  $4 \text{ mg mL}^{-1}$ .<sup>2</sup>

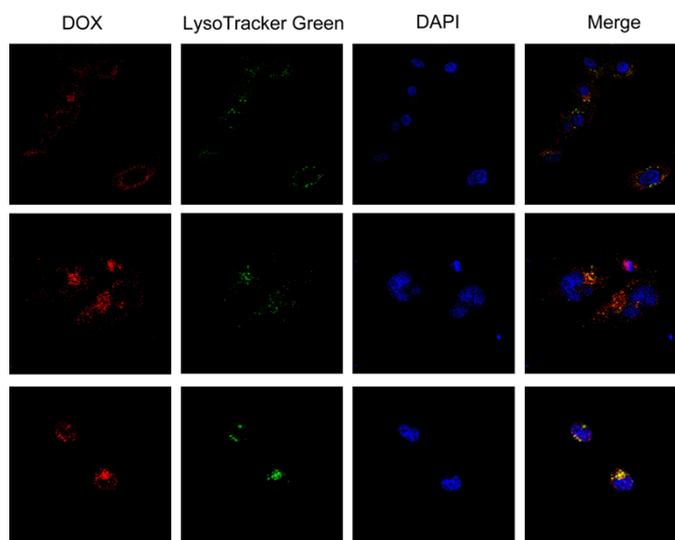


Fig. S13 CLSM images of HepG2 incubated with the prodrug ( $10 \mu\text{g mL}^{-1}$ ). From left to right: DOX (red), LysoTracker Green (Green), DAPI (blue), and a merge of the three images. Upper 0.5 h; Middle 3 h; Bottom 5 h.

#### Reference

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