Supramolecular nanoparticles constructed from pillar[5]arene-based host–guest complexation with enhanced aggregation-induced emission for imaging-guided drug delivery

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Electronic Supplementary Information (21 pages)

1. Materials and methods

Tetrakis(triphenylphosphine)palladium(0), 4-pyridineboronic acid, potassium carbonate, 4-phenylpyridine, and other reagents were commercially available and used as received. Solvents were either employed as purchased or dried according to procedures described in the literature. Compound **1**[S1] was synthesized according to literature procedures. NMR spectra were recorded on a Bruker Avance DMX 400 spectrophotometer using the deuterated solvent as the lock and the residual solvent and TMS as the internal reference. Mass spectra were obtained on a Bruker Esquire 3000 plus mass spectrometer (Bruker-Franzen Analytik GmbH Bremen, Germany) equipped with an ESI interface and an ion trap analyzer. Transmission electron microscopy (TEM) investigations were carried out on a HT-7700 instrument. Isothermal titration calorimetry (ITC) experiments were measured on a Microcal VP-ITC calorimeter. Fluorescence lifetime measurements were carried on a transient fluorescence spectrometer (Edinburgh Instruments FLS920), and the samples were excited by a 405 nm picosecond pulsed laser at a repetition frequency of 2 MHz. UV-vis spectra were taken on a Shimadzu UV-2550 UV-vis spectrophotometer. The fluorescence experiments were conducted on a RF-5301 spectrofluorophotometer (Shimadzu Corporation, Japan).

Fabrications of SNPs and SNPs@DOX. Host−guest complex **H⊃G** was firstly prepared by mixing an equivalent amount of **H** (8.16 mg) and **G** (4.09 mg) in acetone (3 mL). The solution was injected into water (50 mL) under sonication, and the obtained solution was stirred at room temperature over night to evaporate the organic solvent. For the preparation of **SNPs@DOX**, the acetone solution containing **H⊃G** and DOX was injected into water (50 mL) under sonication, and the final solution was stirred at room temperature over night to evaporate the organic solvent. The free DOX was eliminated by PD-10 column, and the drug loading content was calculated by the following equation: Drug loading content (%) = (m_{loaded}/m_{toal})*100%. Where the m_{loaded} is the mass of the encapsulated DOX in **SNPs@DOX** and m_{total} is the total mass of **SNPs@DOX**, respectively.

ITC Measurement. The binding affinity and other parameters of host–guest complex

H′⊃M were determined by ITC. Microcalorimetric titration of **H′** with **M** was conducted in water at 298.15 K. The solution of the guest molecule was put into a syringe and the solution of the host molecule was located in the cell. The solution containing **M** (2.00 mM) was injected drop by drop (10 μL per injection, 28 drops) into an **H′** solution (0.100 mM). Net reaction heat was obtained from the integration of the calorimetric traces. The data was fitted by using a single site binding model.

Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) Studies. The nanostructures of **SNPs** and **SNPs@DOX** were revealed using TEM. TEM samples were prepared by drop-coating the solution onto a carbon-coated copper grid. TEM experiments were performed on an HT-7700 instrument. The corresponding solution was left to stand overnight and the insoluble precipitate was eliminated by using a microporous membrane before DLS tests. Dynamic light scattering (DLS) measurements were carried out using a 200 mW polarized laser source Nd:YAG (λ = 532 nm). The polarized scattered light was collected at 90° in a self-beating mode with a Hamamatsu R942/02 photomultiplier. The signals were sent to a Malvern 4700 submicrometer particle analyzer system.

Cell Culture. HeLa cells were cultured in Eagle's MEM (EMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. U87MG cells were incubated in Minimum Essential Medium (MEM) containing FBS (10%) and penicillin/streptomycin (1%). HEK293 and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. A549 cells were cultured in Ham's F12K medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were harvested from cell culture medium by incubating in trypsin solution for 5 min. The cells were centrifuged, and the supernatant was discarded. A 3 mL portion of serum-supplemented medium was added to neutralize any residual trypsin. The cells were resuspended in serum-supplemented culture medium at a concentration of 1×10^4 cells/mL. Cells were cultured at 37 °C and 5% CO_2.

Evaluation of Cytotoxicity. The cytotoxicity of **SNPs**, DOX, or **SNPs@DOX** against various cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays in a 96-well cell culture plate. All solutions were sterilized by filtration with a 0.22 μm filter before tests. The cells were seeded at a density of 1×10^4 cells/well in a 96-well plate, and incubated for 24 h for attachment. Cells were then incubated with **SNPs**, DOX, or **SNPs@DOX** at various concentrations for 24 h. After washing the cells with PBS buffer, 20 μL of a MTT solution (5 mg/mL) was added to each well. After 4 h of incubation at 37 °C, the MTT solution was removed, and the insoluble formazan crystals that formed were dissolved in 100 μL of dimethylsulfoxide (DMSO). The absorbance of the formazan product was measured at 570 nm using a spectrophotometer (Bio-Rad Model 680). Untreated cells in media were used as a control. All experiments were carried out with five replicates.

Confocal Fluorescence Imaging. CLSM was utilized to study the cellular internalization of **SNPs**. For confocal imaging, the tested cells were cultured in the chambers at a density of 5×10^5 per mL for 24 h. U87MG cells were treated with **SNPs** in the culture medium at 37 °C for 4 h. After being washed three times with PBS, the cells were stained with LysoRed (200 nM) for 30 min. After being washed three times with PBS, the cells were fixed with fresh 4.0% formaldehyde at room temperature for 15 min. The cells were washed with PBS for two times and stained with DAPI for 30 min. The cells were washed with PBS for two times and the images were taken using a Zeiss LSM 780 confocal laser scanning microscope.

For the study of imaging-guided drug delivery, U87MG cells were treated with **SNPs@DOX** in the culture medium at 37 °C for 1 h, 4 h, and 8 h, respectively. The culture medium was removed and the cells were washed by PBS for three time. Then the cells were fixed with fresh 4.0% formaldehyde at room temperature for 15 min. The cells were washed with PBS for two times and stained with DAPI for 30 min. The cells were washed with PBS for two times and the images were taken using a Zeiss LSM 780 confocal laser scanning microscope.

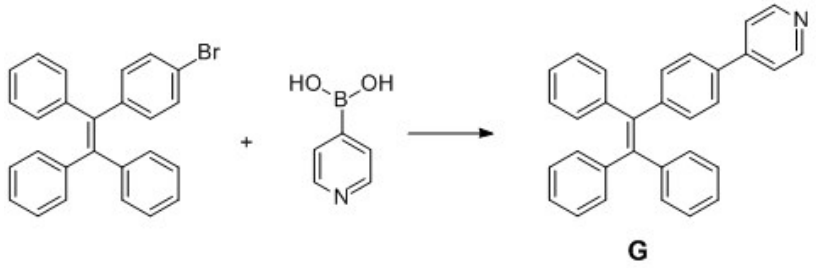
Photostability Studies of SNPs and LysoRed. Continuous scanning by confocal

microscope was used to quantitatively investigate the photostability of **SNPs** and LysoRed. With the help of a power meter, excitation power from 405 and 560 nm channels of the microscope were unified (65 μW) and used to irradiate the **SNPs** and LysoRed stained cells. The initial intensity referred to the first scan of **SNPs** and LysoRed stained cells was normalized, and the percentage of fluorescence signal loss was calculated.

Determination of the Percentage of Apoptotic Cells at Different Stages. U87MG cells were seeded in 6-well cell culture plates (5.0×10^5 cells/well). After 12 h incubation, the medium was replaced by fresh growth media containing free DOX (100 nM) or **SNPs@DOX** (the concentration of DOX was 1.00 μM). The cells were incubated for 24 h and harvested with EDTA-free trypsin (0.25%). Then the cells were carefully washed by PBS and stained by annexin-V FITC and propidium iodide (PI) according to the manufacturer's protocol. Flow cytometry was performed and data were analyzed. The cells without any treatment were utilized as a control.

Analyses of the Endocytotic Pathways. The evaluation of endocytotic pathways was performed as follows. U87MG cells were seeded at a density of 5.0×10^5 cells/well in 6-well cell culture plates. The cells were left to grow for 24 h in culture medium containing 10% FBS at 37 °C in 5% CO_2 atmosphere. For the inhibition of energy-dependent endocytosis, U87MG cells were cultured at 4 ^oC in the presence of **SNPs** for 3 h. U87MG cells were treated with different inhibitors including chlorpromazine (30.0 μM), genistein (0.2 mM), or amiloride (1.0 mM) in serum free medium for 1 h at 37 °C, respectively. Then, **SNPs** were further added to the medium for another 3 h incubation. Subsequently, the medium was removed and the cells were washed 3 times using PBS. HepG2 cells treated with **SNPs** in the absence of inhibitors at 37 °C for 3 h was used as a control. Percent internalization was normalized to the control group in the absence of inhibitors. All experiments were carried out with four replicates.

*2. Synthesis of **G**, **M** and **H***



Scheme S1. Synthetic route of **G**.

Synthesis of G: 4-Pyridineboronic acid (2.46 g, 20.0 mmol), tetrakis(triphenylphosphine)palladium(0) (115 mg, 0.100 mmol), K_2CO_3 (6.61 g, 48.0 mmol) and chemical **1** (2.45 g, 6.00 mmol) were added into the mixture of toluene, enthol and water (100 mL, toluene/CH_3CH_2OH/H_2O = 7/2/1, *v*/*v*/*v*). The solution was heated under nitrogen at reflux for 24 h. The organic solvent was removed and the water layer was extracted with dichloromethane (3 × 100 mL). The combined organic phase was washed with water (3 × 50 mL) and dried over anhydrous Na_2SO_4. After filtration and evaporation, the residue was purified by column chromatography on silica gel (dichloromethane/ethyl acetate, 5:1 *v*/*v*) to provide **G** as a light yellow solid (1.78 g, 73%). The ^{1}H NMR spectrum of **G** is shown in Fig. S1. ^{1}H NMR (400 MHz, $CDCl_3$, room temperature) δ (ppm): 8.62 (s, 2H), 7.44 (d, J = 4 Hz, 2H), 7.38 (d, J = 4 Hz, 2H), 7.17−7.05 (m, 17H). The ^{13}C NMR spectrum of **2** is shown in Fig. S2. ^{13}C NMR (100 MHz, $CDCl_3$, room temperature) δ (ppm): 150.16, 144.87, 143.52, 143.47, 132.13, 131.37, 131.35, 131.32, 127.87, 127.83, 127.72, 127.67, 126.74, 126.67, 126.64, 126.18, 121.31.

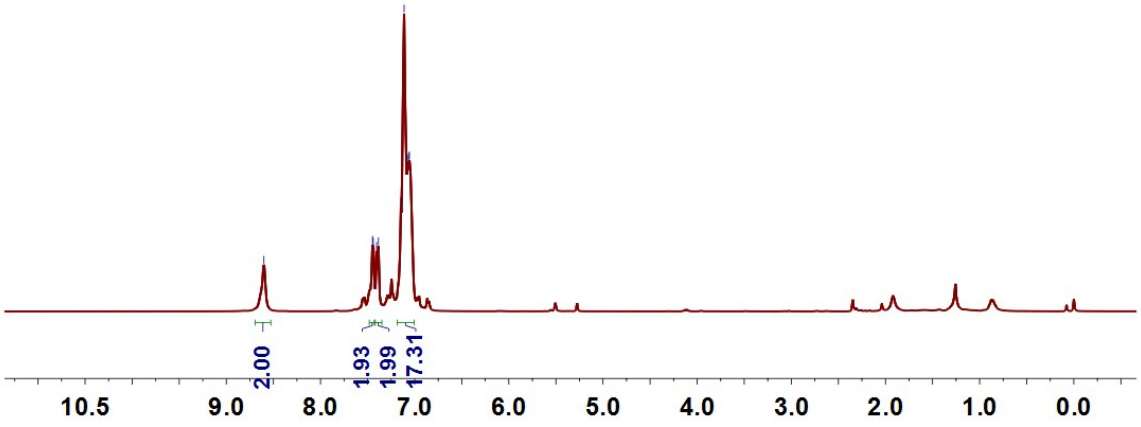


Fig. S1 ^{1}H NMR spectrum (400 MHz, $CDCl_3$, room temperature) of **G**.

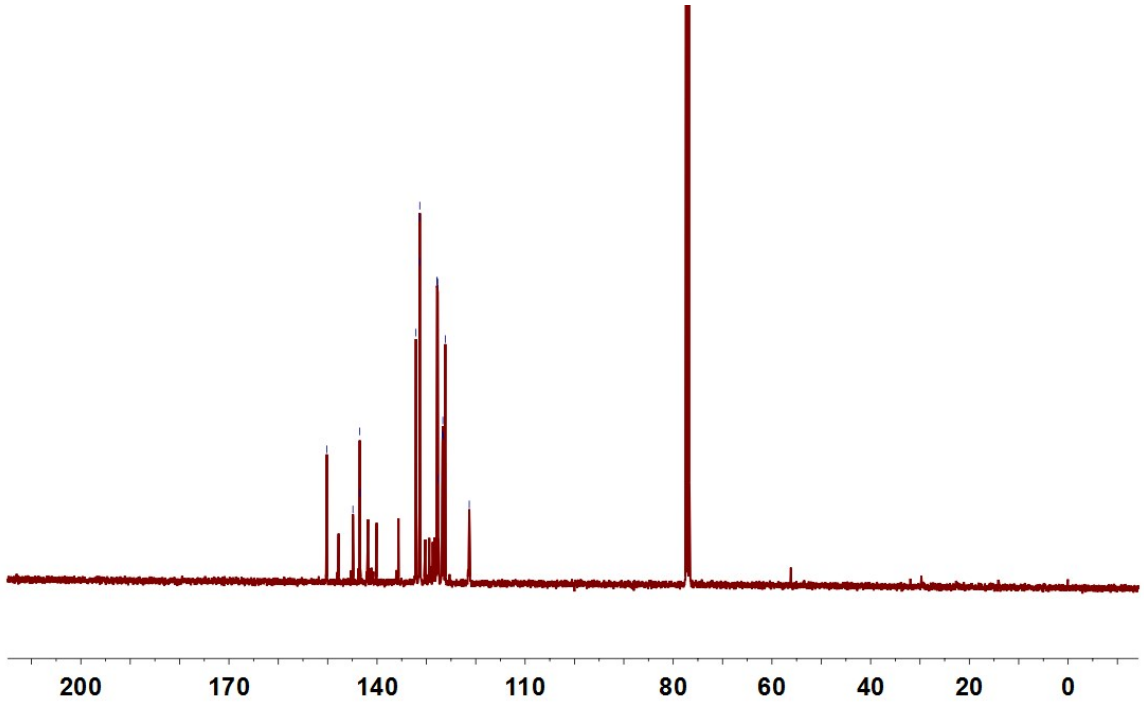
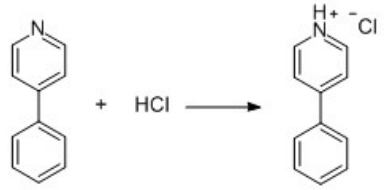


Fig. S2 ^{13}C NMR spectrum (100 MHz, $CDCl_3$, room temperature) of **G**.



Scheme S2. Synthetic route of **M**.

Synthesis of M: 1 mL of hydrochloric acid (37%) was added to a solution of 4-phenylpyridine (1.55 g, 10.0 mmol) in acetonitrile (30 mL). The mixture was stirred at room temperature for 8 h. Then the solution was evaporated under vacuum to yield M as a pale solid (1.90 g, 100%). The proton NMR spectrum of **M** is shown in Fig. S3. ^{1}H NMR (400 MHz, CD_3CN, room temperature) δ (ppm): 9.09 (d, J = 4 Hz, 2H), 8.50 (d, J = 4 Hz, 2H), 8.06 (d, J = 4 Hz, 2H), 7.71–7.68 (m, 3H). The ^{13}C NMR spectrum of M is shown in Fig. S4. ^{13}C NMR (100 MHz, D_2O, room temperature) δ (ppm): 155.34, 143.50, 133.09, 132.10, 129.59, 127.70, 124.48.

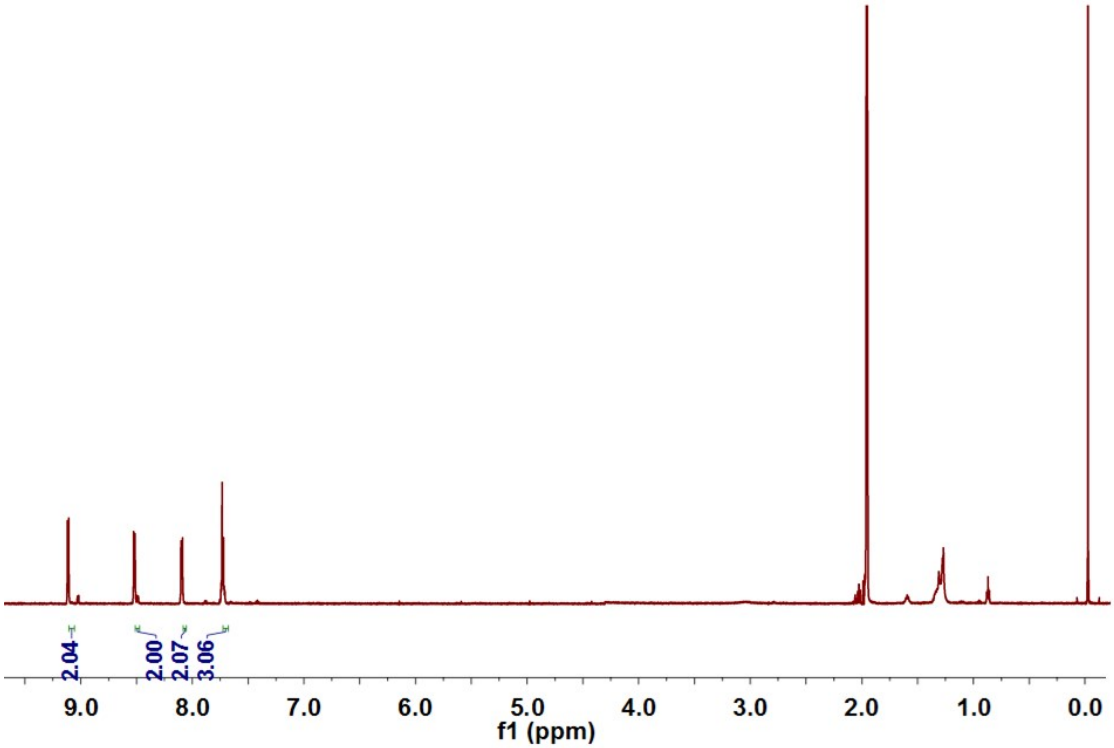


Fig. S3 ^{1}H NMR spectrum (400 MHz, CD_3CN, room temperature) of **M**.

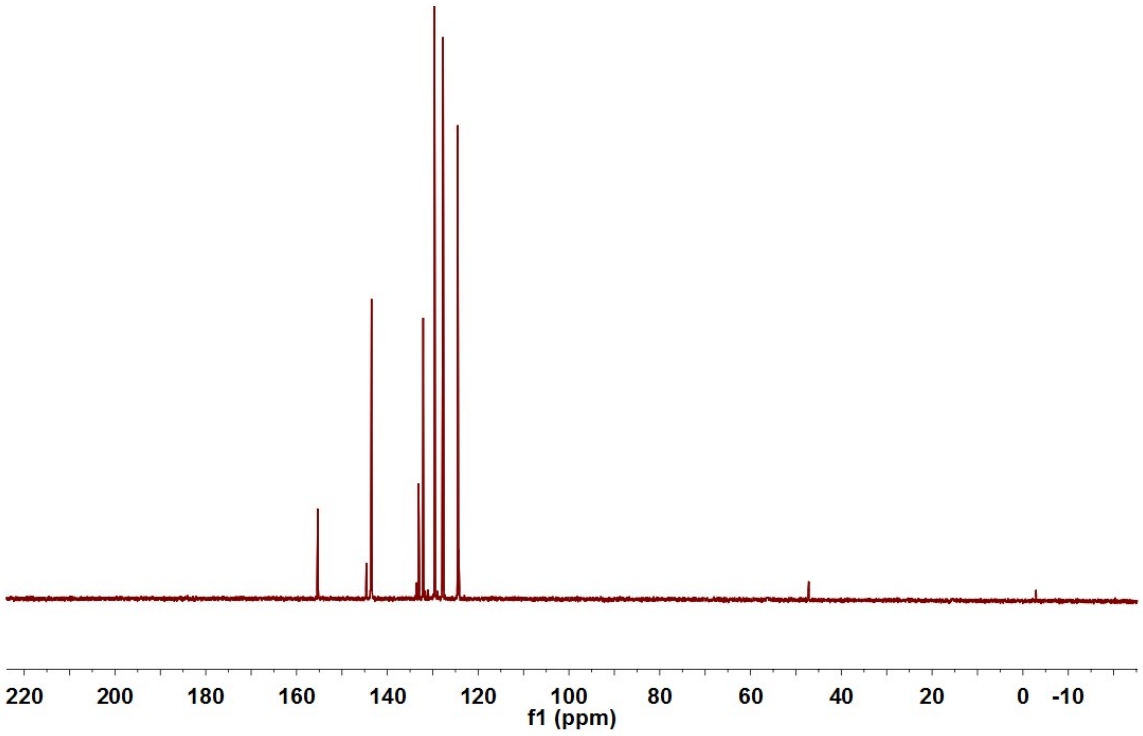
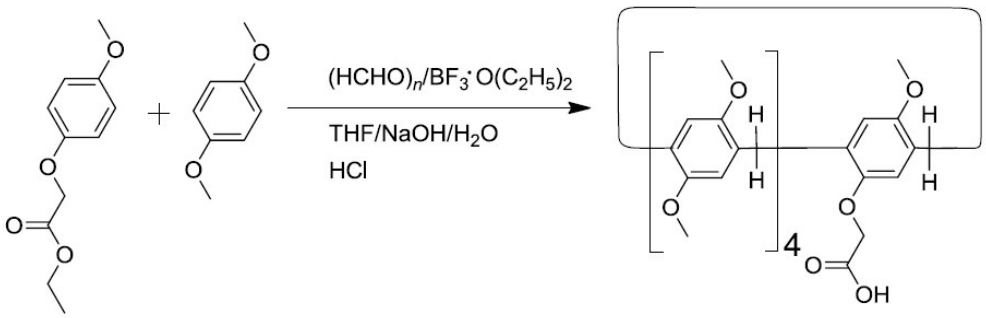


Fig. S4 ^{13}C NMR spectrum (100 MHz, D_2O, room temperature) of **M**.



Scheme S3. Synthetic route of **H**.

Synthesis of H: A mixture of 1,4-dimethoxybenzene (12.4 g, 90.0 mmol), ethyl-4-methoxy phenoxy acetate (2.10 g, 10.0 mmol) and paraformaldehyde (3.10 g, 100 mmol) were added into 1,2-dichloroethane (500 mL), and the mixture was stirred at room temperature for 30 min. Then $BF_3{\cdot}O(C_2H_5)_2$ (6.25 mL, 50.0 mmol) was added and the mixture was stirred for another 2 hours. After filtration, the solvent was removed in vacuo. Then the resultant residue was dissolved in CH_2Cl_2 (200 mL) and washed with water (3 × 100 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated

to afford the yellow solid, which was used without further purification. A solution of the yellow solid and 40% aqueous NaOH (15.0 mL) in THF (50 mL) was stirred at 60 °C for 8 hours. After cooling, the solution was treated with 50 mL of 4 M HCl (aqueous). The mixture was concentrated, the residue was purified by column chromatography to give H (1.42 g, 18%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$, room temperature) δ (ppm): 6.81–6.65 (m, 10H), 4.69 (s, 2H), 4.17 (s, 2H), 3.76–3.57 (m, 35H). ^{13}C NMR (100 MHz, $CDCl_3$, room temperature) δ (ppm): 151.11, 150.92, 150.88, 150.76, 150.49, 150.35, 148.47, 128.72, 128.59, 128.39, 128.26, 128.14, 127.64, 114.61, 114.56, 114.44, 114.19, 114.15, 114.06, 114.01, 113.89, 113.71, 113.55, 113.36, 56.34, 56.00, 55.90, 55.85, 55.81, 55.73, 55.67, 55.59, 55.51, 53.05, 29.71, 29.58, 29.53, 29.45, 29.42. For the preparation of the deprotonated host **H′**, equivalent amount of **H** and NaOH were added into the mixture of acetone and H_2O (25 mL, acetone/H_2O = 4/1, *v*/*v*) and stirred at room temperature for 4 h. The solvent was removed under reduced pressure to quantitatively give the deprotonated host as a white solid.

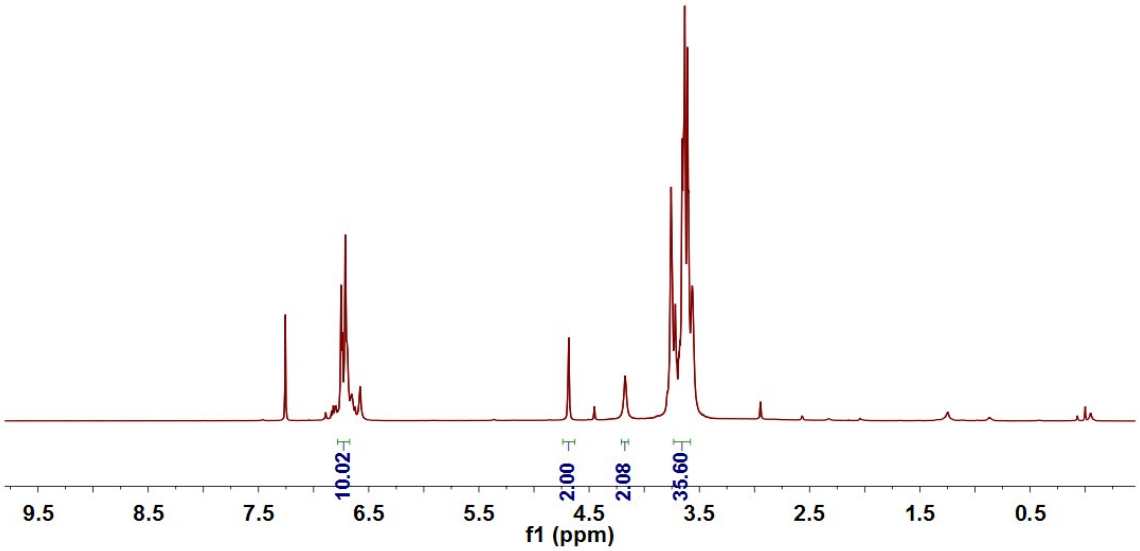


Fig. S5 ^{1}H NMR spectrum (400 MHz, $CDCl_3$, room temperature) of **H**.

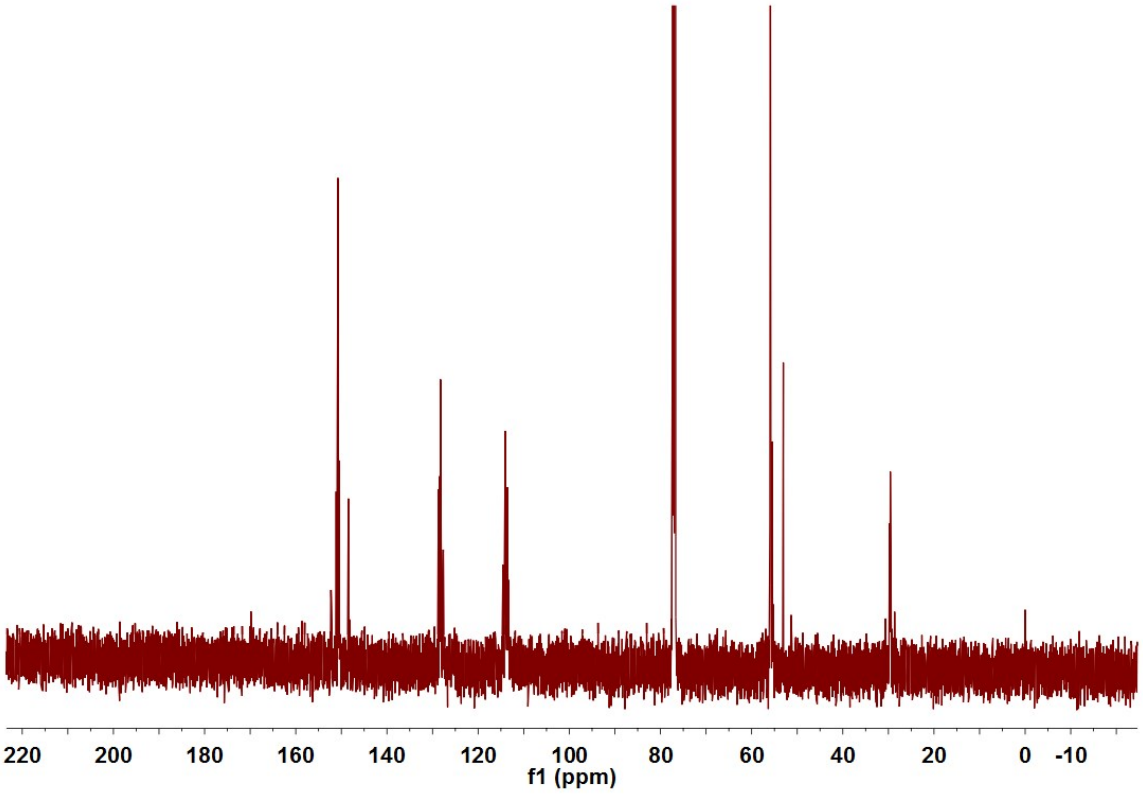


Fig. S6 ^{13}C NMR spectrum (100 MHz, $CDCl_3$, room temperature) of **H**.

*3. Preparation of **SNPs***

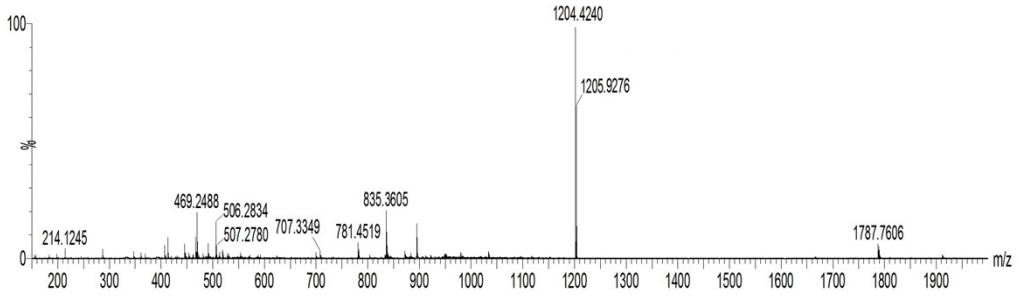


Fig. 7 Electrospray ionization mass spectrum of **H⊃G**. Assignment of the main peak: *m*/*z* 1204.4 [**H⊃G** + H]$^+$.

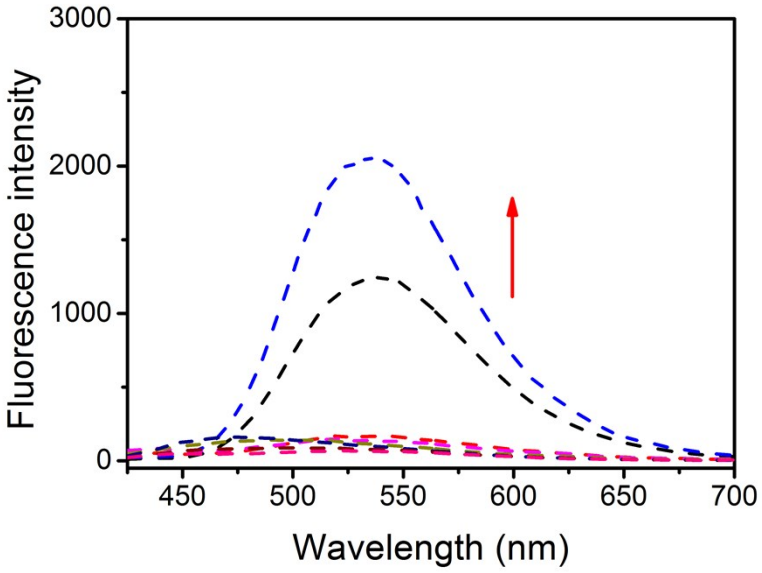


Fig. S8 Fluorescence spectra of **H⊃G** in mixtures of THF and water with different f_w values.

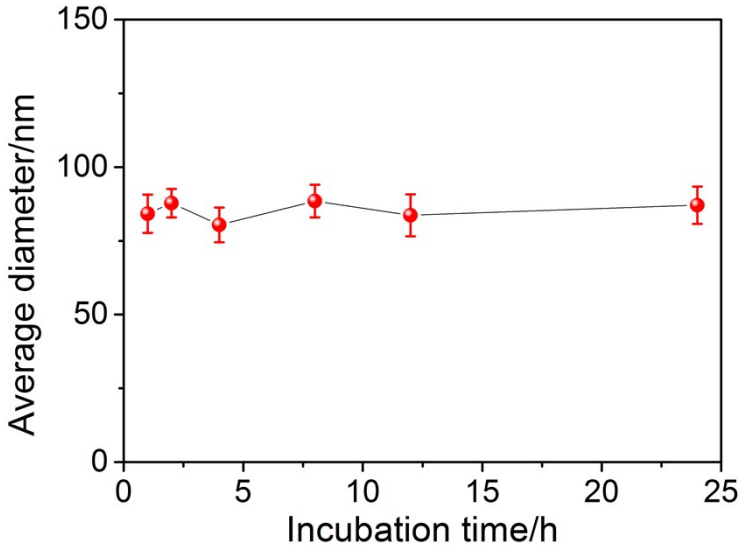


Fig. S9 The hydrodynamic diameter variations of **SNPs** during 24 h of dispersion in PBS.

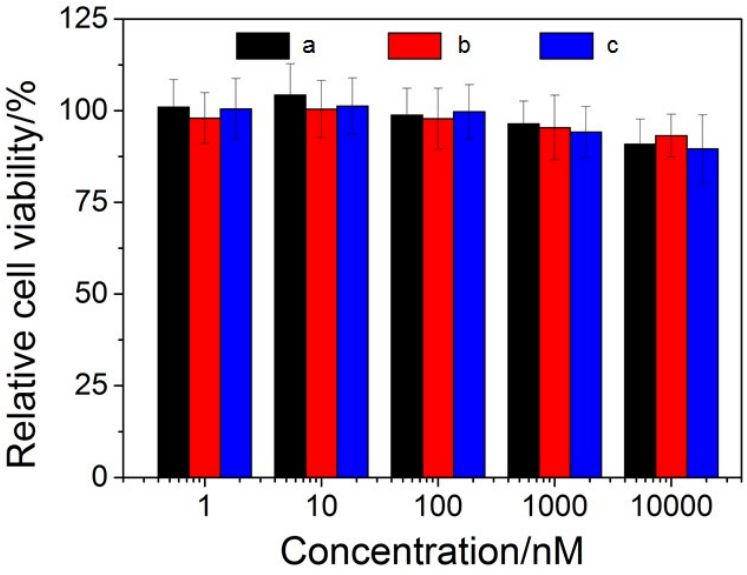


Fig. S10 Relative cell viability of (a) **H**, (b) **G**, (c) **SNPs** against U87MG cells at different concentrations after 24 h incubation.

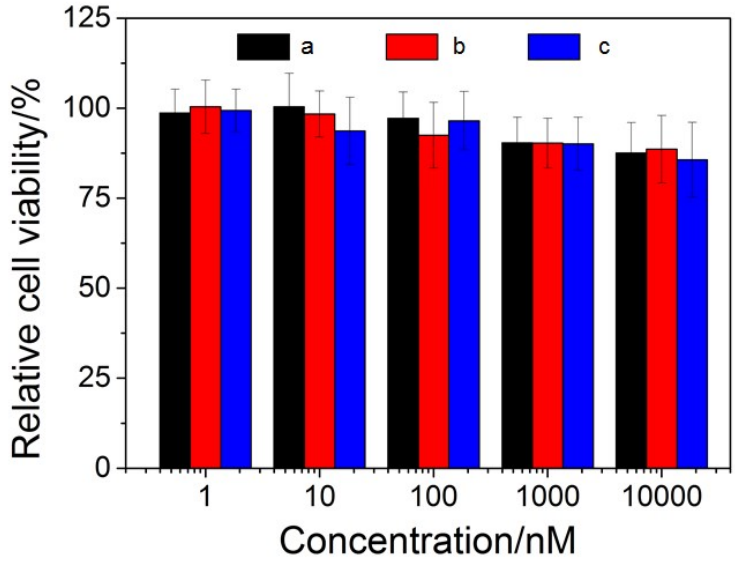


Fig. S11 Relative cell viability of (a) **H**, (b) **G**, (c) **SNPs** against HEK293 cells at different concentrations after 24 h incubation.

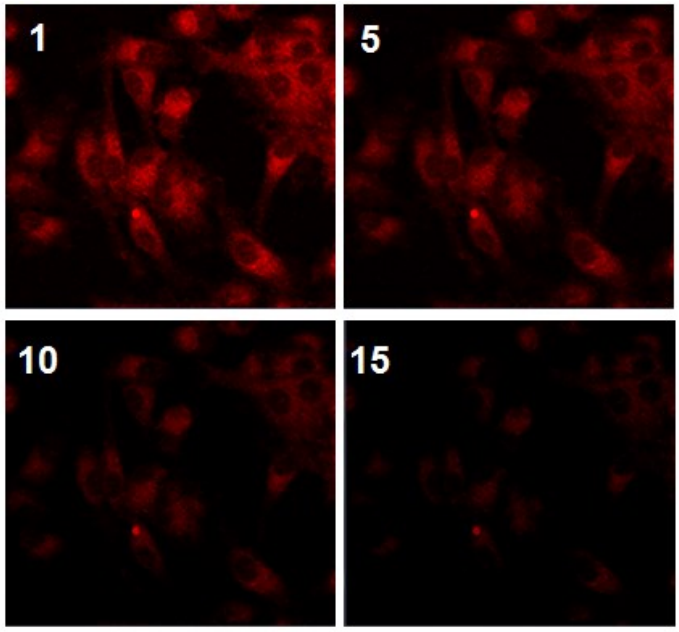


Fig. S12 CLSM images of U87MG cells stained with LysoRed with increasing number of scans (the number of scans shown in upper left corner).

4. Preparation and in vitro evaluation of ***SNPs@DOX***

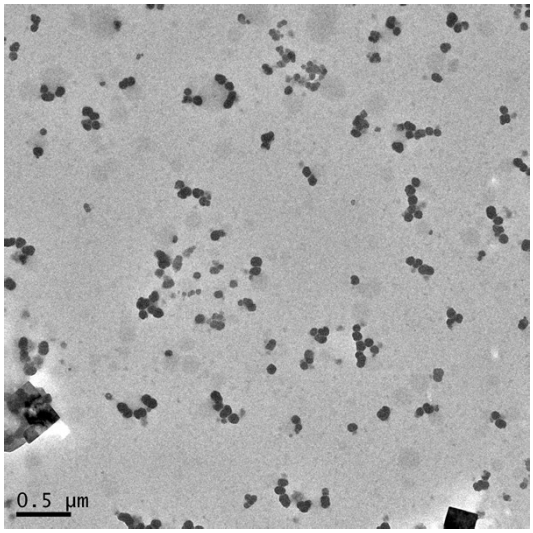


Fig. S13 TEM image of **SNPs@DOX**.

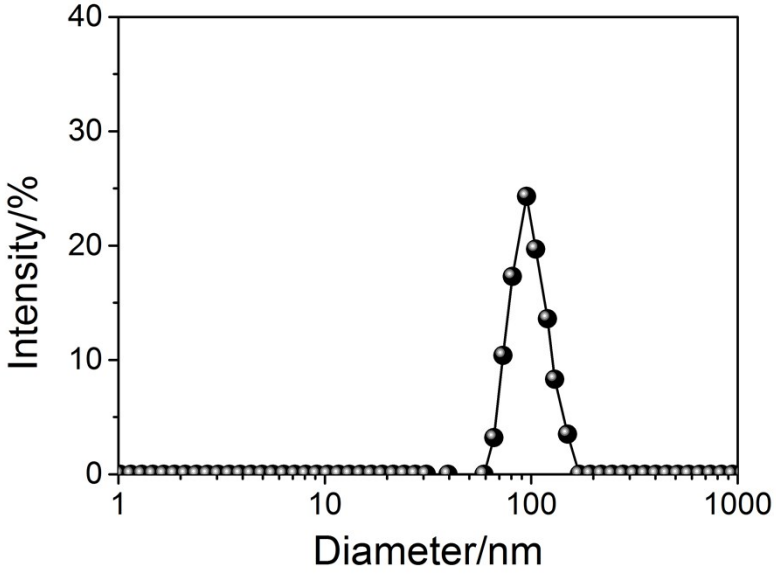


Fig. S14 DLS result of **SNPs@DOX**.

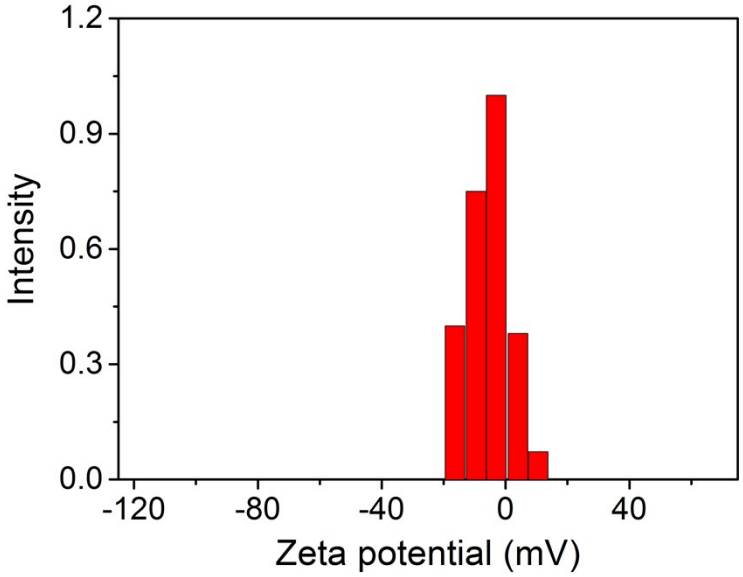


Fig. S15 Zeta potential of **SNPs**.

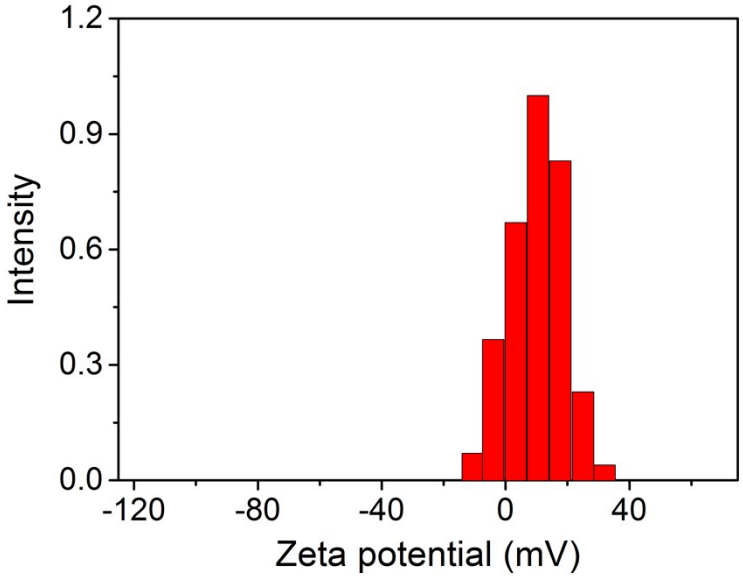


Fig. S16 Zeta potential of **SNPs@DOX**.

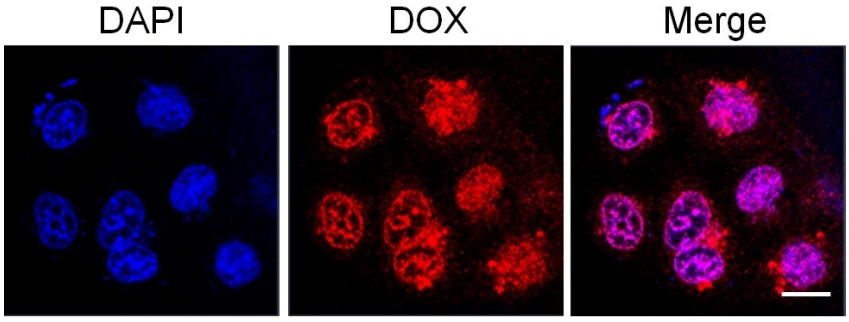


Fig. S17 CLSM images of U87MG cells incubated with DOX for one hour.

Compared with **SNPS@DOX**, free DOX could easily diffuse into U87MG cells and translocate into nucleus. As shown in Fig. S17, the red signal from DOX well overlapped with blue fluorescence from DAPI after 1 h incubation, which was quite different from that of **SNPS@DOX**.

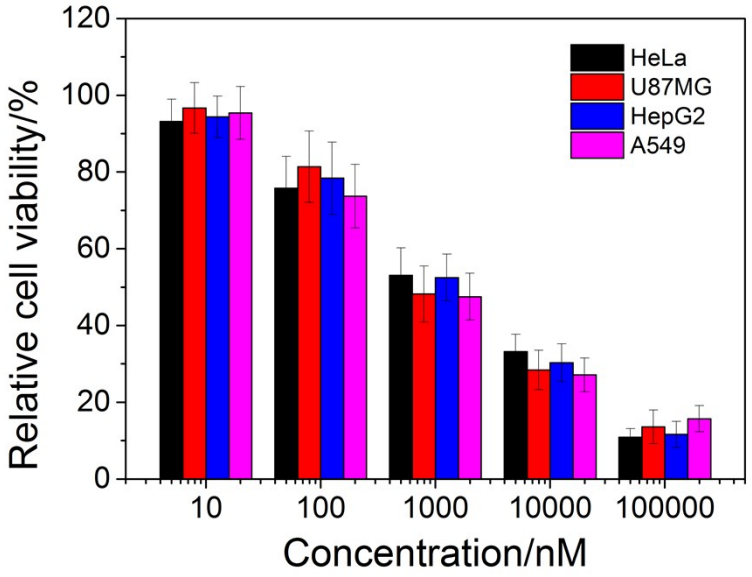


Fig. S18 Relative cell viability of DOX against various cell lines at different concentrations after 24 h incubation.

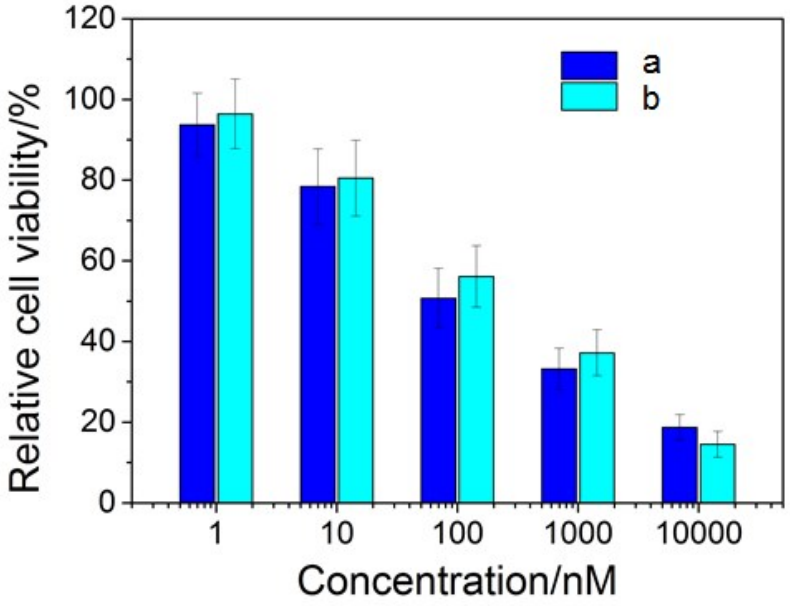


Fig. S19 Relative cell viability of free (a) PTX and (b) **SNPs@PTX** against A549 cells at different concentrations after 24 h incubation.

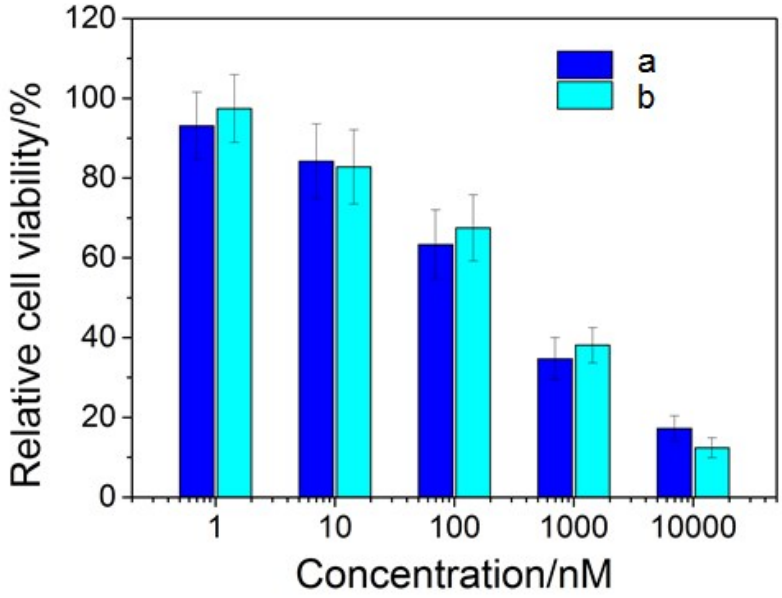


Fig. S20 Relative cell viability of free (a) PTX and (b) **SNPs@PTX** against HeLa cells at different concentrations after 24 h incubation.

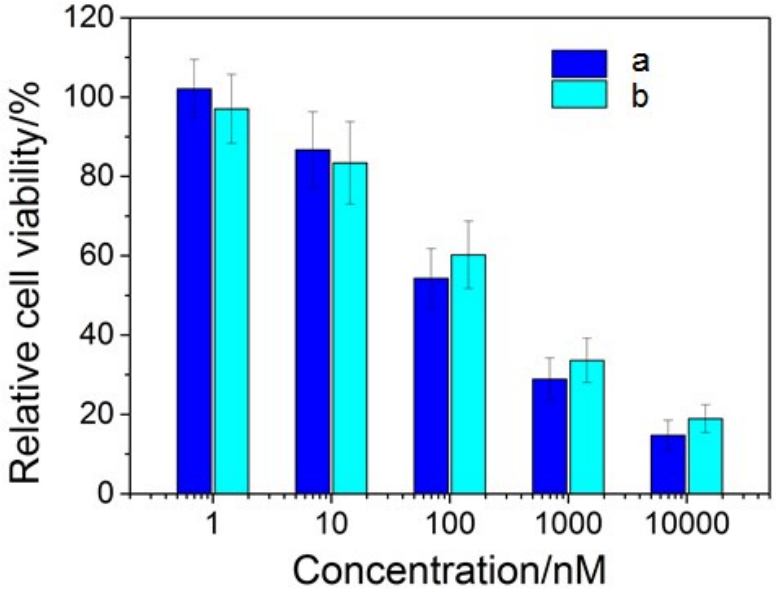


Fig. S21 Relative cell viability of free (a) PTX and (b) **SNPs@PTX** against HepG2 cells at different concentrations after 24 h incubation.

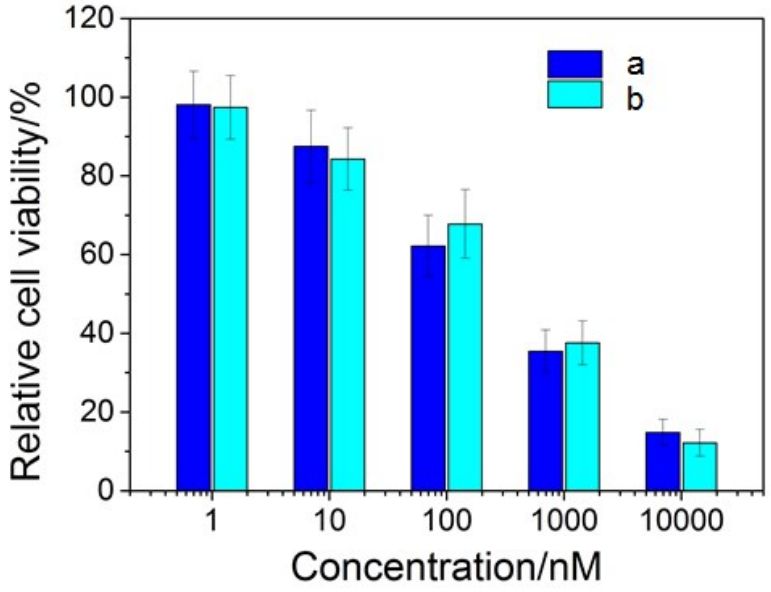


Fig. S22 Relative cell viability of free (a) PTX and (b) **SNPs@PTX** against U87MG cells at different concentrations after 24 h incubation.

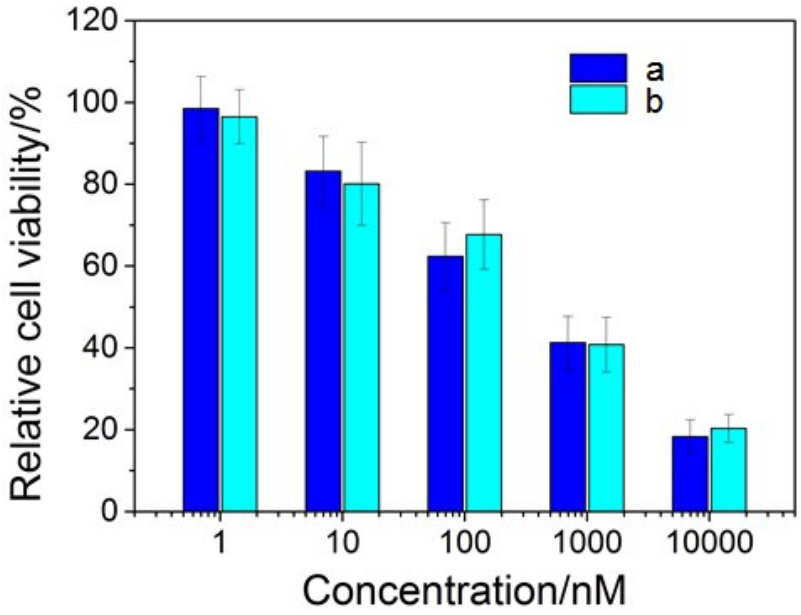


Fig. S23 Relative cell viability of free (a) CPT and (b) **SNPs@CPT** against A549 cells at different concentrations after 24 h incubation.

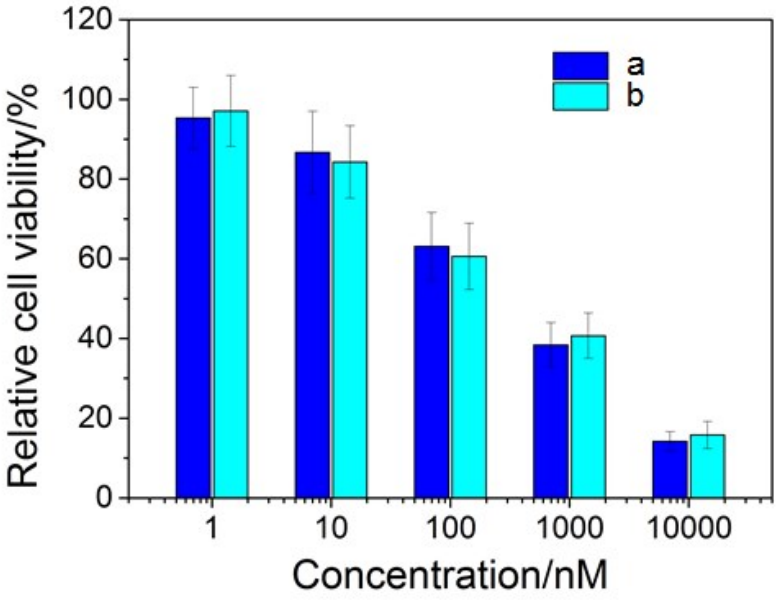


Fig. S24 Relative cell viability of free (a) CPT and (b) **SNPs@CPT** against HeLa cells at different concentrations after 24 h incubation.

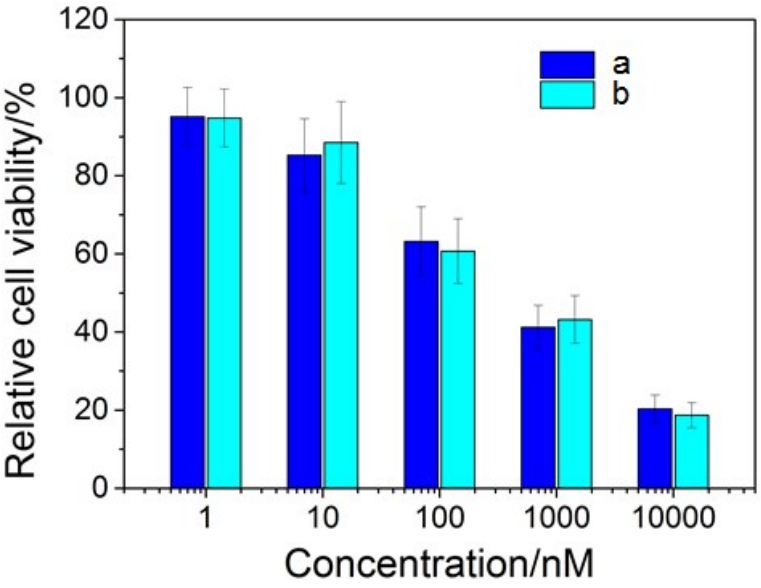


Fig. S25 Relative cell viability of free (a) CPT and (b) **SNPs@CPT** against HepG2 cells at different concentrations after 24 h incubation.

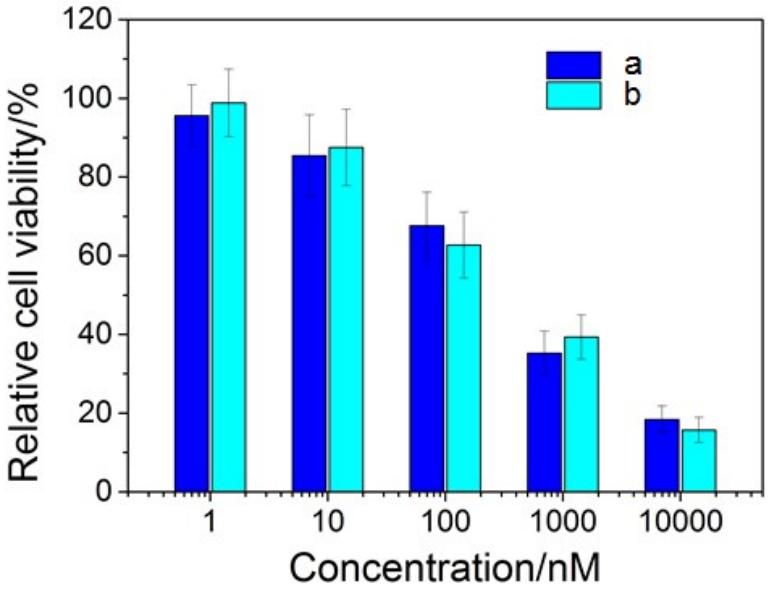


Fig. S26 Relative cell viability of free (a) CPT and (b) **SNPs@CPT** against U87MG cells at different concentrations after 24 h incubation.

5. References

S1 Duan, X.-F.; Zeng, J.; Lü, J.-W.; Zhang, Z.-B. *J. Org. Chem*. **2006**, *71*, 9873–9876.