Electronic Supplementary Material (ESI) for Chemical Communications

Supplementary information

1. Experimental section

1.1. *Materials and Measurements*

10-Hydroxycamptothecin (HCPT) was purchased from Knowshine (Shanghai, China), and ethanol from Sinopharm Chemical Reagent Co. (China). Fetal bovine serum (FBS), Roswell Park Memorial Institute-1640 (RPMI-1640) medium and Penicillin-streptomycin solution were purchased from Invitrogen (San Diego, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (Milwaukee, WI). Distilled water was obtained from a Milli-Q Biocel water purification system (18.2 MΩ·cm resistivity) (Millipore Corporation, Breford, USA). A human nasopharyngeal epidermal carcinoma cell line (KB cell) was provided by American Type Culture Collection (ATCC). Unless otherwise noted, all the chemicals were obtained from commercial suppliers and used without further purification. UV-vis absorption spectra were obtained on a Perkin-Elmer Lambda 750 UV/vis/NIR absorbance spectroscopy. The fluorescence spectra were obtained from a FluoroMax 4 (Horiba Jobin Yvon) spectrofluorimeter. Scanning electron microscopic (SEM) images were obtained on a FEI Quanta 200 FEG field emission scanning electron microscope operated at an accelerating voltage of 30 kV. Transmission electron microscopy (TEM) images were taken from FEI Tecnai G2 F20 S-TWIN operated at an accelerating voltage of 200 kV. Dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano ZS (Malvern Instruments,

Malvern, U.K.) with a 633 nm He-Ne laser.

1.2. Synthesis of Poly(maleic anhydride-alt-1-octadecene)-poly(ethylene glycol) (C18PMH-PEG)

C18PMH-PEG was synthesized following a literature procedure. Briefly, 10 mg (1 eq) Poly(maleic anhydride-alt-1-octadecene) (C18PMH, Sigma Aldrich) and 143 mg (1 eq) poly(ethylene glycol) methyl ether (mPEG-NH_2, 5 KDa) were dissolved in 5 mL dichloromethane with 6 μL triethylamine (TEA, Sinopharm Chemical Reagent Co.) and 11 mg 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Fluka) added. After 24 h of stirring, the dichloromethane solvent was blowed dry by N_2. The leftover solid was dissolved in water, forming a transparent clear solution, which was dialyzed against distilled water for 2 days in a dialysis bag with molecular weight cut-off (MWCO) of 14 KDa to remove unreacted mPEG-NH_2. After lyophilization, the final product in a white solid was stored at 20 °C for future use. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.70–3.38 (m, br, CH_2 of mPEG), 1.43–1.10 (m, CH_2 of C18PMH), 0.88 (m, br, CH_3 of C18PMH).

1.3. Preparation and PEGylated of HCPT Nanocrystals (Nanorods and Nanospheres)

The nanorods (NRs) synthesis was synthesized by modifying a previously published protocol. In detail, 300 μL of 1×10^{-3} M HCPT/ethanol solution was poured into 5 mL of aqueous solution at room temperature under rapid stirring and the NRs were obtained by moderate sonication. HCPT nanospheres (NSs) were

prepared by a solvent exchanging method. Briefly, 350 μL of 1 × 10^{-3} M HCPT/ethanol solution was poured into 5 mL of aqueous solution at 50 °C under vigorous stirring at 1000 rpm. After mixing for 5 min, the sample stabilized for 48 hours. For PEGylated, make sure 1 mg C18PMH-PEG polymer equably dispersed in 10 mL distilled water. Then add 300 μL solutions to 5 mL stock solution of HCPT NRs or HCPT NSs rapidly. The mixture was then ultrasonic treatment for 5 min. The obtained particles were stored at 4 °C.

1.4. Nanocrystals Characterization

The zeta potential and size of the nanocrystals (NCs) suspensions were measured by DLS at 25 °C. The mean values of each sample from at least 20 measurements were obtained. The morphology and particle size were further characterized by SEM and TEM. Samples for SEM were prepared by dropping a few nanocrystal suspensions onto a silicon substrates, then the solvent was to evaporate, 2 nm layer of Au was deposited on samples and then imaged by SEM. For TEM analysis, a few drops of the sample suspension was dropped on a carbon-coated copper TEM grid and dried in air before observation.

1.5. HCPT Release Assay from PEGylated HCPT NCs

PEGylated HCPT NCs suspensions (2 mL) were added into a dialysis bags (MWCO 5000) and immersed into 50 mL of phosphate buffered saline (PBS) at 37 °C with stirring for drug release. Aliquots of 2 mL were at a predetermined time

withdrawn from the solution to containers. The solution volume was kept constant by adding 2 mL fresh PBS after each sampling. The amount of HCPT released was determined by using fluorescence measurement (excitation at 382 nm). The drug release assay was carried out three times and the average value of the three measurements was taken.

1.6. Cell Culture

KB cells were grown in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were routinely passaged by treatment with trypsin (0.05%)/EDTA.

1.7. Confocal Imaging of Cells

Confocal imaging of cells was performed using a Leica laser scanning confocal microscope. Imaging of HCPT was carried out under 405 nm laser excitation and emission was collected in the range of 500 nm to 600 nm. KB cells were incubated with PEGylated HCPT NRs and HCPT NSs ([HCPT] = 12 μM, the concentration of the both shaped particles was determined by UV-vis measurement at 382 nm from a mixture of ethanol and HCPT NCs suspensions (v/v = 9:1)) for 1, 2, 4, and 8 h before Confocal imaging. All cells were washed twice with PBS buffer before confocal imaging.

1.8. Cellular Uptake Measured by Fluorescence Measurement

KB cells were seeded in 24-well plates (5×10^6 mL/well). The plates were then incubated at 37 °C for 24 h in a humidified atmosphere containing 5% CO_2. The cells were then incubated with equivalent concentrations of PEGylated HCPT NRs and HCPT NSs. The drug-treated cells were incubated for pre-determined time at 37 °C, and then washed twice with cold PBS, and digested by trypsin (0.05%)/EDTA treatment. The suspensions were centrifuged at 1000 rpm and 4 °C for 4 min. The supernatants were discarded and the cell pellets were washed with PBS to remove the background fluorescence in the medium. After two cycles of washing and centrifugation, cells were resuspended with 2 mL PBS and disrupted by vigorous sonication. The amount of HCPT in sonicated mixture was analyzed by using fluorescence measurement (excitation at 382 nm). Blank cells sample without drug nanocrystals add was measured to determine the cells auto-fluorescence level, which was subtracted from the fluorescence intensities of injected samples during the concentration calculation.

1.9. Cytotoxicity Assays

The *in vitro* cytotoxicity was measured using a standard MTT assay. KB cells were seeded into 96-well plates (100 µL/well). The plates were then incubated at 37 °C for 24 h in a humidified atmosphere containing 5% CO_2. Cells were then treated with various concentrations of modified HCPT NRs or HCPT NSs. The drug-treated cells were incubated at 37 °C for pre-determined time in a humidified atmosphere

containing 5% CO_2. The cells were then treated with 20 μL of MTT solution (5 mg/mL in PBS) and incubated for 5 h. The medium was removed and the cells were lysed by adding 150 μL of DMSO, the cell viabilities were then measured by MTT assay and the relative cell survival percentages compared to the drug-free control were plotted against the drug concentration in logarithmic scale.

1.10. Tumor Models

4T1 murine breast cancer cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin. BALB/c mice were obtained from Nanjing Peng Sheng Biological Technology Co, Ltd and performed under protocols approved by Soochow University Laboratory Animal Center. The 4T1 tumor models were generated by subcutaneous injection of 2×10^6 cells in 60 μL PBS into the right shoulder of female BALB/c mice. The mice were used for treatment when the tumor volume reached 50-100 mm^3 (~6 days after tumor inoculation).

1.11. Blood Circulation

Blood circulation was measured by drawing ~10 μL blood from the tail vein of BALB/c mice post injection of two shaped PEGylated HCPT NCs. Each blood sample was dissolved in 1 mL of lysis buffer (1% SDS, 1% Triton X-100, 40 mM Tris Acetate). The concentration of HCPT NSs and HCPT NRs in the blood was determined by the fluorescence spectrum of each solubilized blood sample using a FluoroMax 4 fluorometer. A series of dilutions of the PEGylated HCPT NSs or

PEGylated HCPT NRs suspensions were measured to obtain a standard calibration curve. Blank whole blood sample without PEGylated HCPT NCs injection was measured to determine the blood auto-fluorescence level, which was subtracted from the fluorescence intensities of injected samples during the concentration calculation.

1.12. Biodistribution Measurement

For biodistribution study, 4T1 tumor-bearing mice (tumor size ~100 mm^3) were sacrificed at 6 and 24 h post injection of two shaped PEGylated HCPT NCs. The organs/tissues were weighed and homogenized in the lysis buffer (the same as the above used in the blood circulation experiment) with a PowerGen ho mogenizer (Fisher Scientific). Clear homogeneous tissue solutions were obtained and diluted 10-100 times to avoid significant light scattering and self-quenching during fluorescence measurement. The fluorescence intensities of both standard samples and real tissue samples were all adjusted to be in the linear range by appropriate dilution. The sample was measured in triplicate to ensure reproducibility and measurement accuracy. The biodistribution of two shaped PEGylated HCPT NCs in various organs of the mice was then calculated and plotted in units of % ID/g.

1.13. In Vivo Therapeutic Efficacy

For the treatment, tumor bearing mice were divided into three groups (n = 6) in a way to minimize weight and tumor size differences among the groups: (1) PBS vehicle (the control group), (2) HCPT excipient at 10 mg/kg, (3) PEGylated HCPT

NSs at 10 mg HCPT/kg, and (4) PEGylated HCPT NRs at 10 mg HCPT/kg. When the tumor volume reached 50-100 mm^3, therapy was continued once per week through tail vein injection for two weeks (at day 0, and 7). The tumor sizes were measured by a caliper every the other day and tumor volumes were calculated as $\mathbf{a} \times \mathbf{b}^2/2$. Where **a** was the largest and **b** the smallest diameter. Relative tumor volumes were calculated as V/V_0 (V_0 was the tumor volume when the treatment was initiated). Body weights of animals were also daily monitored. Mice were weighed with the relative body weights normalized to their initial weights.

2. Supporting Figures

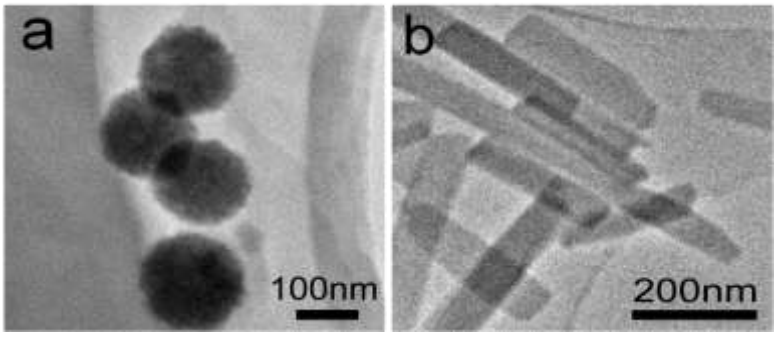


Fig. S1 TEM images of HCPT NSs (a) and NRs (b).

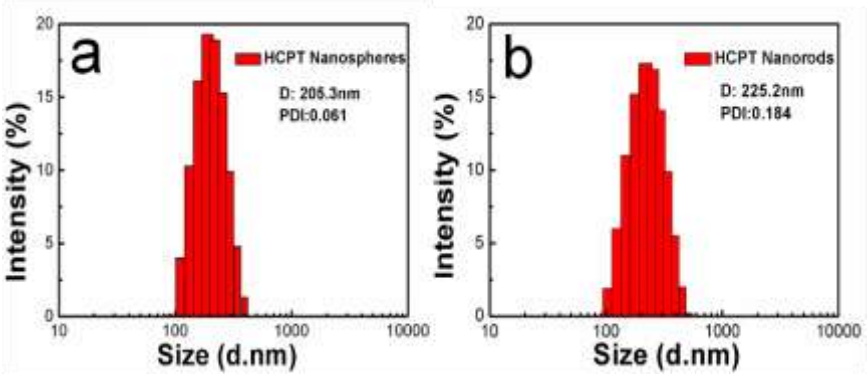
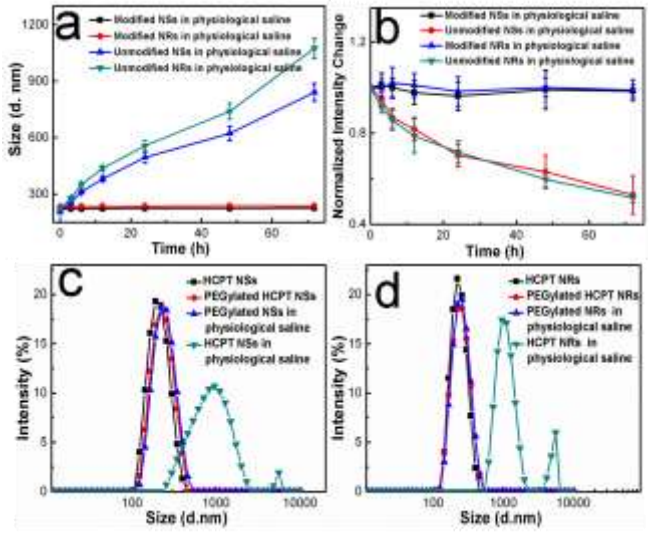


Fig. S2 Size distribution of the HCPT NSs (a) and NRs (b) by DLS measurements at 25 °C in water.



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Sample	Hydrodynamic diameter[a] (nm)	PDI[a]	Drug loading content (%)
PEGylated HCPT NSs	220.8	0.104	91.9
PEGylated HCPT NRs	232.1	0.137	93.3

[a] Size and PDI of PEGylated HCPT NSs and PEGylated HCPT NRs were determined by DLS.
[b] Determined by HPLC.

Fig. S3 (a) Stability studies of modified HCPT NSs, modified HCPT NRs, unmodified HCPT NSs and unmodified HCPT NRs in physiological saline. (b) Fluorescence comparison of the HCPT NSs, modified NSs, HCPT NRs, and modified NRs in physiological saline. (c), (d) Size distribution and the stability in physiological saline of the PEGylated HCPT NSs and NRs by DLS measurements at 25 °C; (e) Drug loading content for HCPT with PEGylated HCPT NSs and NRs.

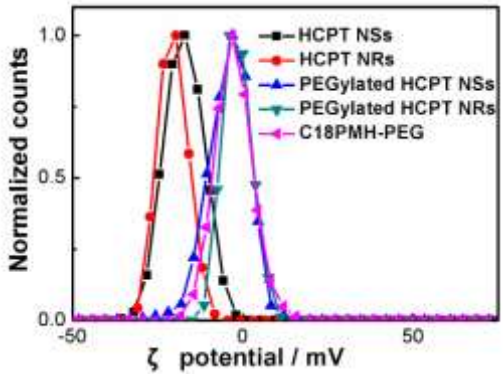

Fig. S4 Surface charge of HCPT NRs, HCPT NSs, PEGylated HCPT NRs, PEGylated HCPT NSs and C18PMH-PEG (dispersed in water). We found that the mean ζ potential were -20.10 ± 0.46 mV, -16.87 ± 0.59 mV, -3.83 ± 0.33 mV, -4.73 ± 0.44 mV, and -2.79 ± 0.18 mV for the HCPT NRs, HCPT NSs, PEGylated HCPT NRs, PEGylated HCPT NSs and C18PMH-PEG respectively.

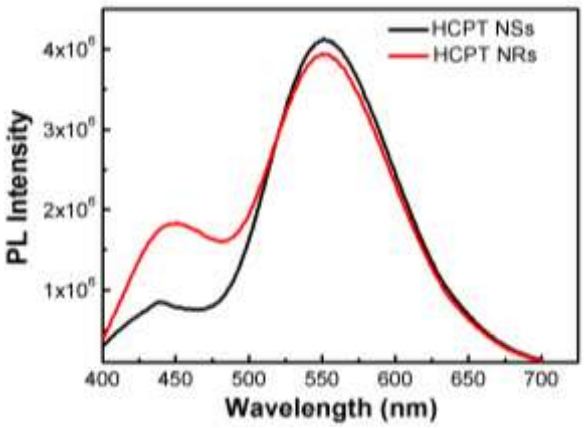

Fig. S5 Fluorescence spectra of HCPT NSs and NRs solutions with the same HCPT concentrations. The results showed that fluorescence intensity of NSs and NRs is roughly the same at same concentrations.

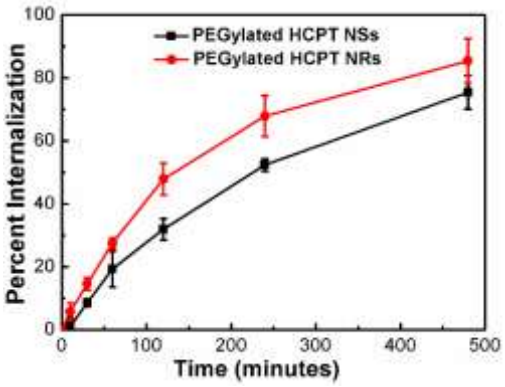

Fig. S6 Internalization profile of PEGylated HCPT nanocrystals with KB cells over a 8 h incubation period at 37 °C.

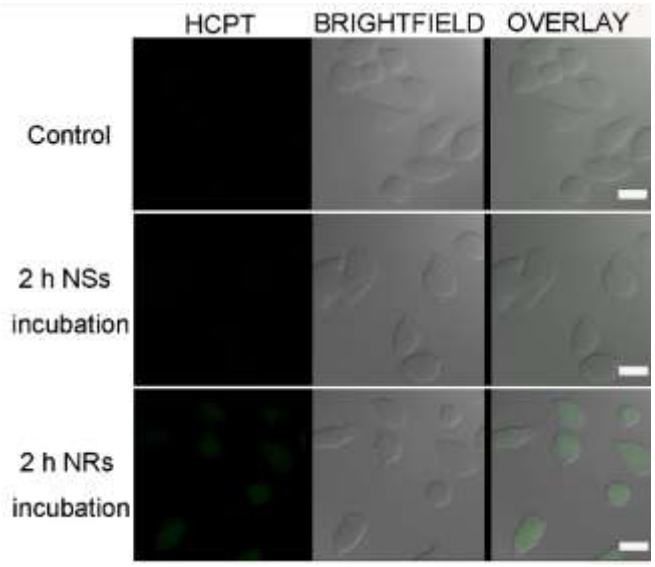

Fig. S7 CLSM images of KB cells incubated with PEGylated HCPT NSs ([HCPT] = 12 μM) and PEGylated HCPT NRs ([HCPT] = 12 μM) for 2 h at 4 °C. All images were taken under identical instrumental conditions and presented at the same intensity scale. (Scale bar: 20 μm)

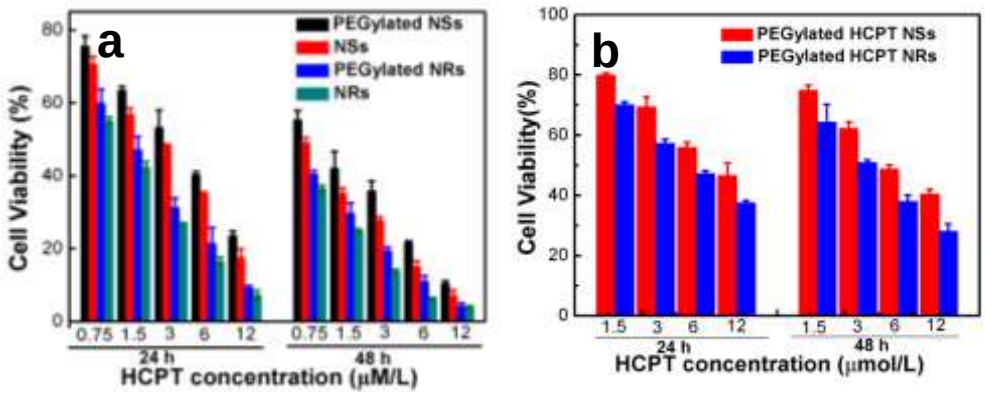

Fig. S8 (a) Concentration-dependent and time-dependent cell survival data of KB cells treated with PEGylated HCPT NSs, HCPT NSs, PEGylated HCPT NRs and HCPT NRs. Viability was measured using an MTT assay. Error bars were based on standard deviations of three parallel samples at each data point. (b) Concentration-dependent cell survival data of KB cells treated with PEGylated HCPT NSs or PEGylated HCPT NRs. Cells were incubated with a series of concentrations of PEGylated HCPT NSs and PEGylated HCPT NRs for 8 h followed by a 16 or 40 h post-incubation in drug-free media. Error bars were based on standard deviations of three parallel samples at each data point.

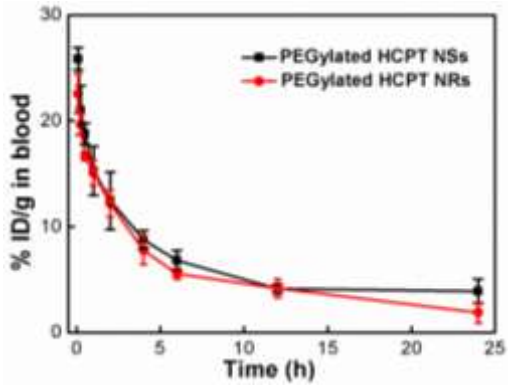

Fig. S9 The blood circulation curve of PEGylated HCPT NSs and NRs determined by measuring HCPT fluorescence in the blood at different time points post-injection. The unit was a percentage of injected dose per gram tissue (% ID/g).

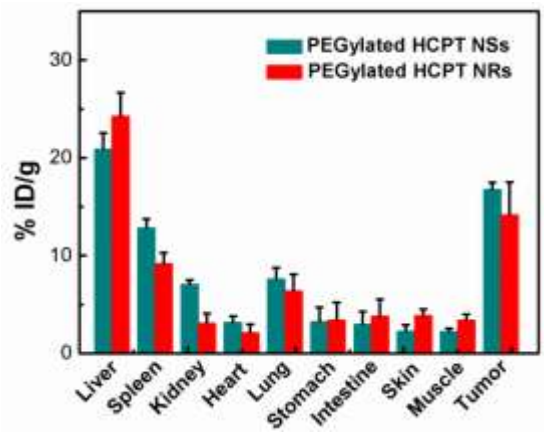

Fig. S10 Biodistribution of PEGylated HCPT NSs and NRs in tumor-bearing mice at 6 h post-injection. The drug concentration was measured by the HCPT fluorescence from diluted tissue lysates. The HCPT NRs levels in organs are presented in the unit of the percentage of injected dose per gram tissue (% ID/g). Error bars were based on standard deviations of three mice per group.