Electronic Supplementary Information

A fluorescence and photoactivity dual-activatable prodrug with self-synergistic magnification of anticancer effect

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Experimental

Materials

4-Nitrophenyl chloroformate was provided by J&K Chemical. 10-Hydroxycamptothecin (HCPT) was customized from Energy Chemical. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) was obtained from Laysan Bio, Inc. (Arab, AL). All the other starting materials were purchased from Sigma-Aldrich. The chemicals and reagents were used as received. 4-Dimethylamino-2'-hydroxychalcone (HCA) was synthesized according to the previous literature.[1]

Characterization

^{1}H NMR and ^{13}C NMR in $CDCl_3$ were measured on a Bruker ARX 400. High resolution mass spectra (HRMS) were obtained by an Agilent 6520 Q-TOF. HPLC was carried out at a LUMTECH HPLC (Germany) system using a C_{18} RP column

with MeOH (0.1% of TFA) and water (0.1% of TFA) as the eluents. UV-vis absorption spectra were recorded on a Shimadzu UV-1700 spectrometer. Photoluminescence (PL) spectra were recorded on a Perkin-Elmer LS 55 spectrofluorometer. To measure the size of the nanoparticles, dynamic light scattering (DLS, Malvern Zetasizer Nano ZS90) tests were carried out at room temperature. Transmission electron microscopy (JEM-2010F, JEOL, Japan) was employed to study the morphology of the samples. MTT absorbance was obtained with a Bio-rad iMark microplate reader. Cell imaging studies were performed by confocal laser scanning microscopy (CLSM, Zeiss LSM 710, Jena, Germany).

Synthesis of HCA -SS-OH

To a solution of mono-*O*-DMTr-2-hydroxyethyl disulfide (1 g, 2.19 mmol) in 10 mL of dichloromethane, DIPEA (850 mg, 6.58 mmol) was added at 0 ^{o}C. 4-Nitrophenyl chloroformate (1.1 g, 5.46 mmol) in 10 mL dichloromethane was then added. The reaction mixture was stirred at room temperature for 3 h. At this point, the reaction mixture was concentrated under reduced pressure. To another solution of HCA (200 mg, 0.75 mmol) in 10 mL anhydrous DMF, NaH (36 mg, 1.5 mmol) was added at 0 ^{o}C and then stirred at room temperature for 3 h. After that, the first reaction mixture dissolved in anhydrous DMF was dropped into the solution of HCA at 0 ^{o}C. The reaction mixture was stirred at room temperature for 12 h. The reaction mixture was washed with saturated aqueous NaCl solution for 3 times and the desired product was extracted into dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and purified *via* silica gel column chromatography. After removal of the

volatiles and drying in vacuo, compound HCA-SS-*O*-DMTr (240 mg, 42.7% yield) was obtained. Then intermediate HCA-SS-*O*-DMTr (240 mg, 0.32 mmol) was stirred at room temperature in a 4:1 mixture of acetic acid and dichloromethane (5 mL total volume) for 24 h. Then saturated aqueous $NaHCO_3$ was dropped into the reaction mixture. The organic product was extracted into ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4. The crude compound was purified by flash chromatography. Collecting the appropriate fractions and removal of the volatiles afforded 50.0 mg (35.0% yield) of HCA-SS-OH. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.70 (d, J = 7.6 Hz, 1H), 7.58 – 7.50 (m, 2H), 7.48 (d, J = 8.8 Hz, 2H), 7.37 (t, J = 7.5 Hz, 1H), 7.25 (d, J = 8.2 Hz, 1H), 6.99 (d, J = 15.7 Hz, 1H), 6.67 (d, J = 8.8 Hz, 2H), 4.44 (t, J = 6.7 Hz, 2H), 3.85 (t, J = 6.0 Hz, 2H), 3.04 (s, 6H), 2.92 (t, J = 6.7 Hz, 2H), 2.85 (t, J = 6.0 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 191.49, 153.25, 152.28, 148.80, 147.41, 132.66, 132.12, 130.70, 129.97, 126.40, 122.93, 122.09, 119.95, 111.83, 66.52, 60.42, 41.41, 40.14, 36.56. HRMS (ESI$^+$): calcd. for [$C_{22}H_{25}NO_5S_2$+H^+] 448.1247, found 448.1248.

Synthesis of HCA-SS-HCPT

To a solution of HCA-SS-OH (50 mg, 0.11 mmol) in 5 mL dichloromethane, DIPEA (145 mg, 1.12 mmol) and 4-nitrophenyl chloroformate (45 mg, 0.22 mmol) were added at 0 ^{o}C. The reaction mixture was then stirred at room temperature overnight. Then the reaction mixture was concentrated under reduced pressure. The resulting crude intermediate was dissolved in 3.0 mL anhydrous DMF and chilled to 0 ^{o}C. HCPT (60.0 mg, 0.16 mmol) was then dissolved in 3.0 mL anhydrous DMF and

dropped into the solution. This was followed by TEA (300 mg, 3 mmol) addition. While maintaining the temperature, the reaction mixture was allowed to stir at room temperature for 24 h. The compound was extracted into dichloromethane. The organic layer was dried over sodium sulfate and concentrated in vacuo. The resulting crude compound was purified by HPLC. The final product was lyophilized in vacuo to afford 20.0 mg (21% yield) of HCA-SS-HCPT. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.31 (s, 1H), 8.21 (s, 1H), 7.77 (d, J = 2.4 Hz, 1H), 7.71 (d, J = 7.6 Hz, 1H), 7.66 (s, 1H), 7.64 (dd, J = 9.2, 2.5 Hz, 1H), 7.52 (dd, J = 15.5, 4.8 Hz, 2H), 7.44 (d, J = 8.8 Hz, 2H), 7.36 (t, J = 7.4 Hz, 1H), 7.24 (d, J = 8.1 Hz, 1H), 6.98 (d, J = 15.7 Hz, 1H), 6.65 (d, J = 8.6 Hz, 2H), 5.34 – 5.27 (m, 2H), 5.25 (s, 2H), 4.56 (t, J = 6.4 Hz, 2H), 4.47 (t, J = 6.8 Hz, 2H), 3.06 (t, J = 6.4 Hz, 2H), 3.02 (s, 6H), 2.98 (t, J = 6.8 Hz, 2H), 1.89 (tt, J = 14.3, 7.1 Hz, 2H), 1.04 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 190.93, 173.86, 157.56, 153.23, 153.07, 152.60, 152.09, 150.10, 149.75, 148.85, 146.89, 146.15, 132.64, 132.08, 131.40, 130.90, 130.55, 129.94, 129.24, 128.40, 126.40, 125.22, 122.94, 119.99, 118.86, 118.27, 111.86, 98.13, 72.76, 66.64, 66.54, 66.33, 50.03, 40.17, 37.01, 36.64, 31.64, 30.95, 7.84. HRMS (ESI^+): calcd. for [$C_{43}H_{39}N_3O_{11}S_2$+H^+] 838.2099, found 838.2109.

Preparation of HCA-SS-HCPT nanoparticles (NPs)

HCA-SS-HCPT (1 mg) and DSPE-PEG_{2000} (2 mg) were dissolved in 1 mL of tetrahydrofuran (THF). Accompanied by sonication with a microtip probe sonicator (12 W output, XL2000, Misonix Incorporated, NY), the above THF solution was added into 9 mL of water, which was followed by further sonication for 120 s. Then

the mixture was violently stirred in fume hood overnight at room temperature to evaporate residue THF. The HCA-SS-HCPT NP suspension was carried out for ultrafiltration (molecule weight cutoff 100 kDa), which was subsequently purified by a 450 nm syringe driven filter. Moreover, the HCA-SS-OH NPs were prepared following the same procedure as that for the fabrication of HCA-SS-HCPT NPs.

Cell culture

HeLa human cervical cancer cells, PC-3 human prostate cancer cells, HepG2 human liver cancer cells and Caco-2 human colon cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 ^{o}C in a humidified environment containing 5% CO_2, respectively. Before experiments, the cells were pre-cultured until confluence was reached.

Cell imaging

HeLa, PC-3, HepG2, and Caco-2 cancer cells were seeded in confocal imaging chambers at a density of 1×10^5 cells. After 12 h, the cells were incubated with HCA-SS-HCPT NPs (10 μM based on HCA-SS-HCPT molecule) in serum-free cell culture medium at 37 ^{o}C for 3 h. Subsequently, the NP-treated cells were washed three times with 1 × PBS buffer, which was followed by imaging with CLSM upon excitation at 405 nm and a collection of fluorescent signal at 560 ± 50 nm.

Intracellular detection of ROS

The ROS production of the HCA-SS-HCPT NPs in HeLa cancer cells upon exposure to white light (400-700 nm) was assessed using DCFDA as the indicator.

After incubation with HCA-SS-HCPT NPs (10 μM based on HCA-SS-HCPT) at 37 ^{o}C for 3 h in the dark, HeLa cancer cells were incubated with 20 μM of DCFDA for 30 min. Subsequently, the cells were washed with 1 × PBS and exposed to white light irradiation (0.25 W cm^{-2}) for 3 min, followed by imaging with CLSM. For DCF detection: excitation at 488 nm and signal collection at 530 ± 20 nm. Alternatively, before treatment with HCA-SS-HCPT NPs, the cancer cells were pre-treated with *N*-acetylcysteine (NAC) (1 mM) for 2 h. In addition, the NP-treated cells without light irradiation and the cells with only pure light irradiation were used as the controls.

Study on synergistic anticancer activity *in vitro*

The amplification of antitumor efficacy of HCA-SS-HCPT NPs with light irradiation was studied by MTT assay. HeLa, PC-3, HepG2, and Caco-2 cancer cells were incubated with different concentrations of HCA-SS-HCPT NPs or HCA-SS-OH NPs (based on HCA-SS-HCPT or HCA-SS-OH) at 37 ^{o}C, respectively, which were then irradiated with white light (0.25 W cm^{-2}, 3 min) at 8 h post addition of the NPs. Then it was followed by standard MTT test at 24 h. In brief, at 24 h post add of the NPs, the cells in 96-well plate were washed with 1 × PBS and 100 μL of freshly prepared MTT in cell culture medium (0.5 mg mL^{-1}) was added to each well. After 4 h incubation, the MTT solution was removed cautiously and then 100 μL of DMSO was added into each well. The plate was shaken for 10 min and then the absorbance of MTT at 490 nm was measured by a microplate reader. In addition, the NP-treated cancer cells without light irradiation were used as the control.

Animals and tumor-bearing mouse model

All animal studies were performed in compliance with the guidelines set by Tianjin Committee of Use and Care of Laboratory Animals, and the overall project protocols were approved by the Animal Ethics Committee of Nankai University. Six-week-old male BALB/c nude mice were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). To establish the xenograft PC-3 tumor-bearing mouse model, 1×10^7 PC-3 cancer cells in 30 μL of cell culture medium were injected subcutaneously into the right axillary space of the nude mouse. The mice with tumor volumes at around 80-120 mm^3 were used subsequently.

***In vivo* theranostic studies and self-synergistic anticancer effect**

The xenograft PC-3 tumor-bearing mice were intravenously administrated with 200 μL of HCA-SS-HCPT NP suspension (2.5 mg kg^{-1} based on HCPT). Afterward, the mice were sacrificed at designated time intervals (n = 3 mice for each time point), and then the tumors were isolated for fluorescence imaging using a *in vivo* Maestro small-animal fluorescence imaging instrument. Furthermore, at 5 h post-injection, the tumors were fixed in 4% paraformaldehyde for 2 h, incubated in 20% sucrose/PBS overnight and embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek). Sections (6 μm) were stained by 4',6-diamidino-2-phenylindole (DAPI) and then imaged by CLSM.

For antitumor study, the PC-3 tumor-bearing mice were randomly divided into 6 groups (6 mice per group): 1) "Saline", 2) "free HCPT", 3) "HCA-SS-OH NPs without light", 4) "HCA-SS-OH NPs with light", 5) " HCA-SS-HCPT NPs without

light", and 6) "HCA-SS-HCPT NPs with light". For the treatments of "Saline", “free HCPT”, “HCA-SS-OH NPs without light”, and “HCA-SS-HCPT NPs without light”, on day 0, saline, free HCPT (2.5 mg kg^{-1}), HCA-SS-OH NPs (1.8 mg kg^{-1} based on HCA), and HCA-SS-HCPT NPs (2.5 mg kg^{-1} based on HCPT and 1.8 mg kg^{-1} based on HCA) were intravenously injected into the tumor-bearing mice, respectively, followed by tumor volume monitoring of each mouse over time. For the treatments of “HCA-SS-OH NPs with light”, and “HCA-SS-HCPT NPs with light”, after intravenous administration of HCA-SS-OH NPs and HCA-SS-HCPT NPs, respectively, for 5 h, the tumor tissues of mice in these two cohorts were irradiated with white light (0.3 W cm^{-2}) for 10 min. The weight and tumor volume of mice in all the groups were measured every other day for 16 days. Tumor size was measured by a caliper and tumor volume was calculated using the following formula: $V = W^2 \times L/2$, where W and L were the shortest and longest diameters of tumors, respectively. Relative tumor size was calculated as V/V_0 (V_0 was the initial tumor volume).

Histological and immunohistochemical analysis

On day 16 after treatment, the tumour tissues and the main organs (spleen and kidney) of the mice in different groups were collected, sliced and stained. The fluorescent PCNA staining was conducted following common immunohistochemical steps. The fluorescent TUNEL staining was conducted following manual instruction of Dead End fluorometric TUNEL system kit (Promega, USA). For hematoxylin and eosin (H&E) staining, the tissues of the mice were fixed in 4% formalin solution, processed into paraffin, and sectioned at 7 μm thickness. The slices were examined

with a digital microscope (Leica QWin) and evaluated by three independent pathologists who were blinded to the project.

Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). Statistical comparisons were made by ANOVA analysis and two-sample Student's *t*-test. *P* value < 0.05 was considered statistically significant.

Reference

1. Z. Song, R. T. K. Kwok, E. Zhao, Z. He, Y. Hong, J. W. Y. Lam, B. Liu and B. Z. Tang, *ACS Appl. Mater. Interfaces*, 2014, **6**, 17245.

Figures

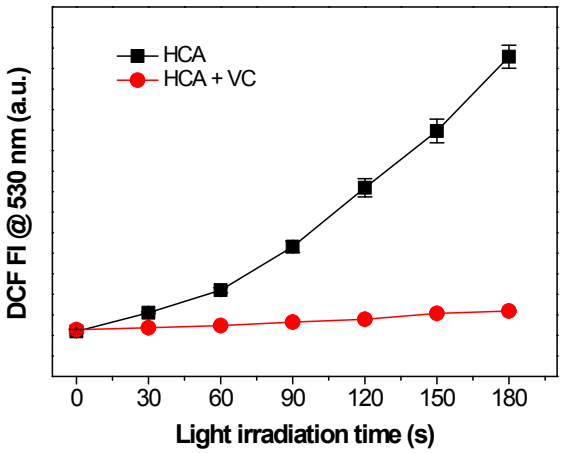

Fig. S1 Fluorescence intensity (FI) of DCF at 530 nm as a function of light irradiation time of HCA in aqueous solution with and without addition of vitamin C (VC).

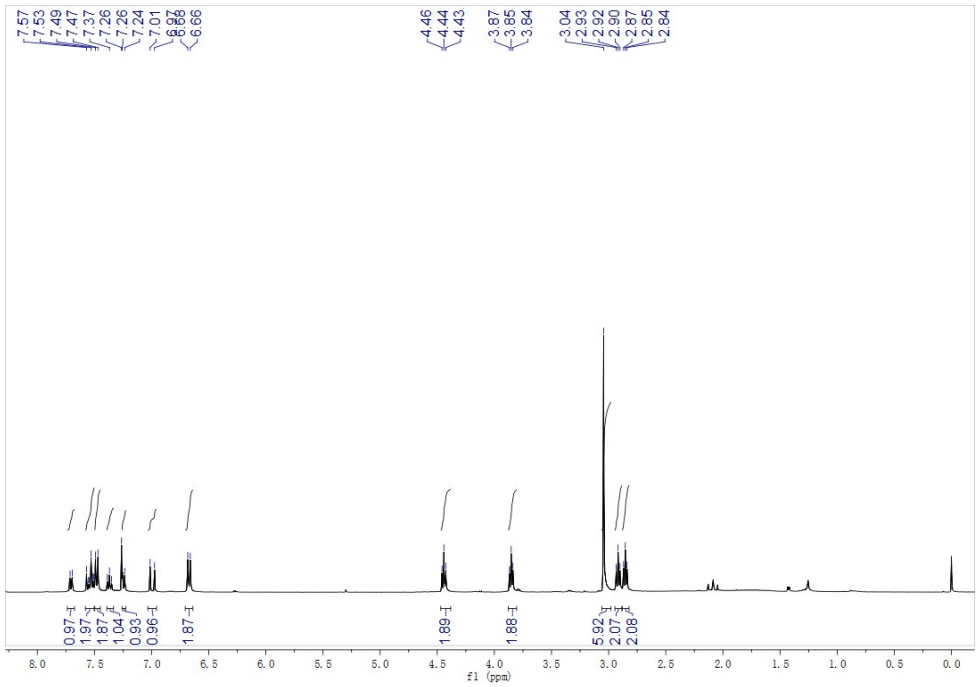

Fig. S2 ^{1}H NMR spectrum of HCA-SS-OH in $CDCl_3$.

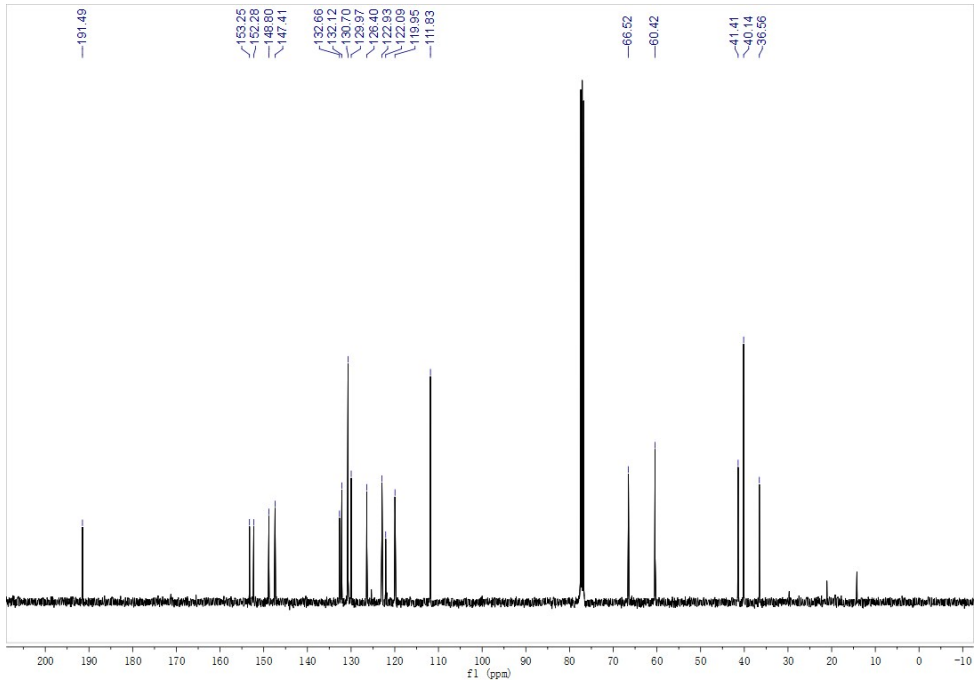

Fig. S3 ^{13}C NMR spectrum of HCA-SS-OH in $CDCl_3$.

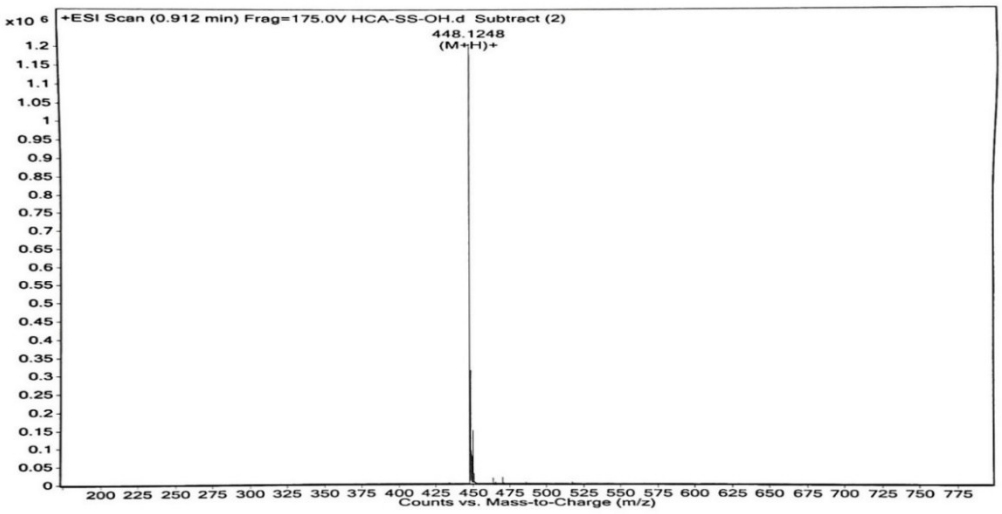

Fig. S4 HRMS spectrum of HCA-SS-OH.

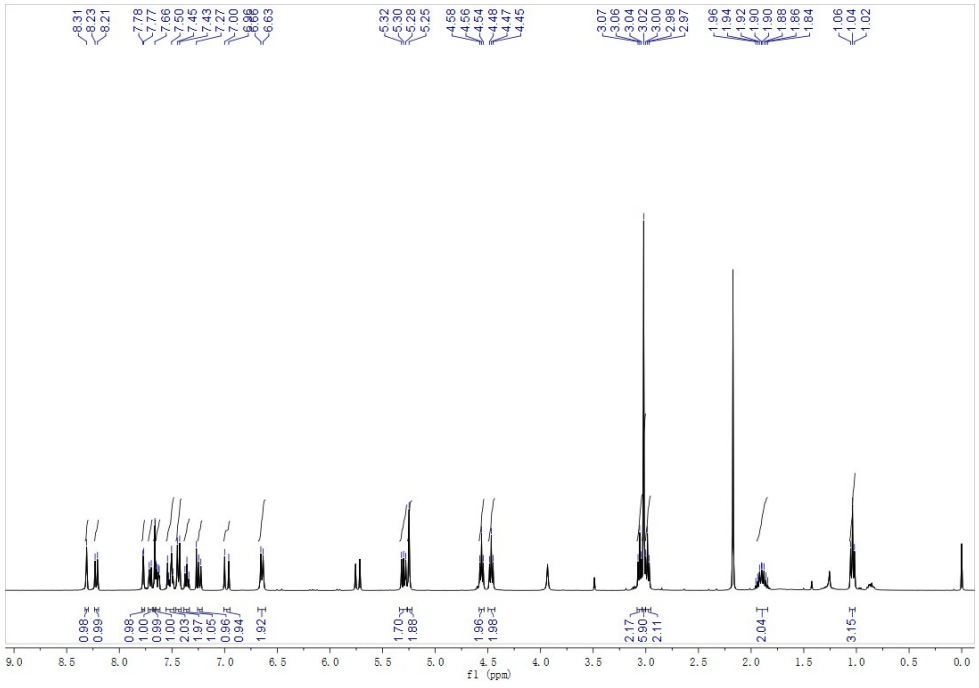

Fig. S5 ^{1}H NMR spectrum of HCA-SS-HCPT in $CDCl_3$.

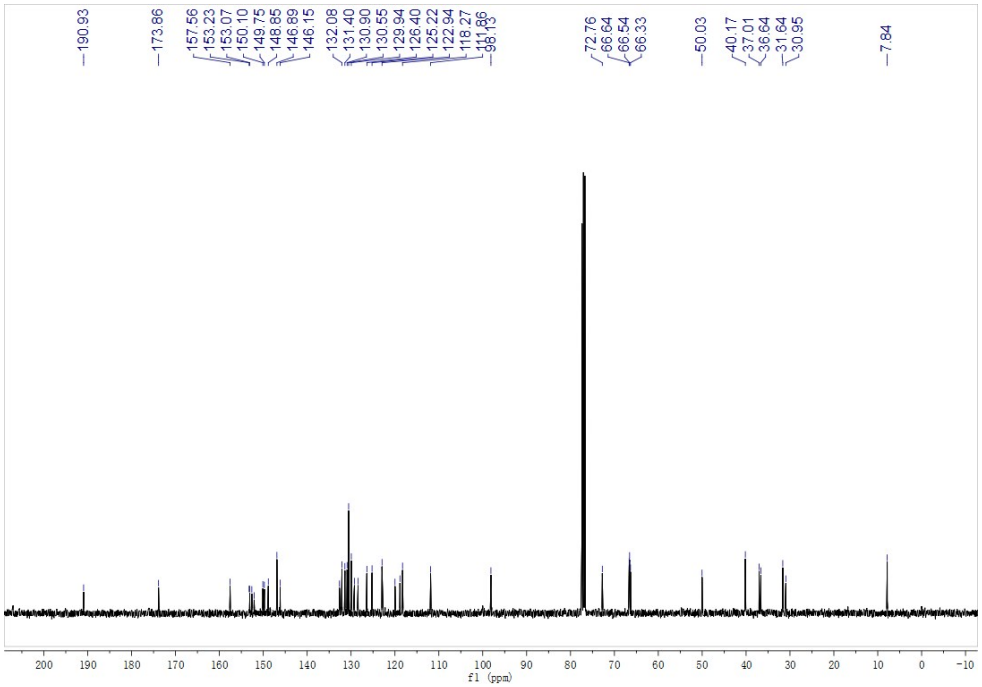

Fig. S6 ^{13}C NMR spectrum of HCA-SS-HCPT in $CDCl_3$.

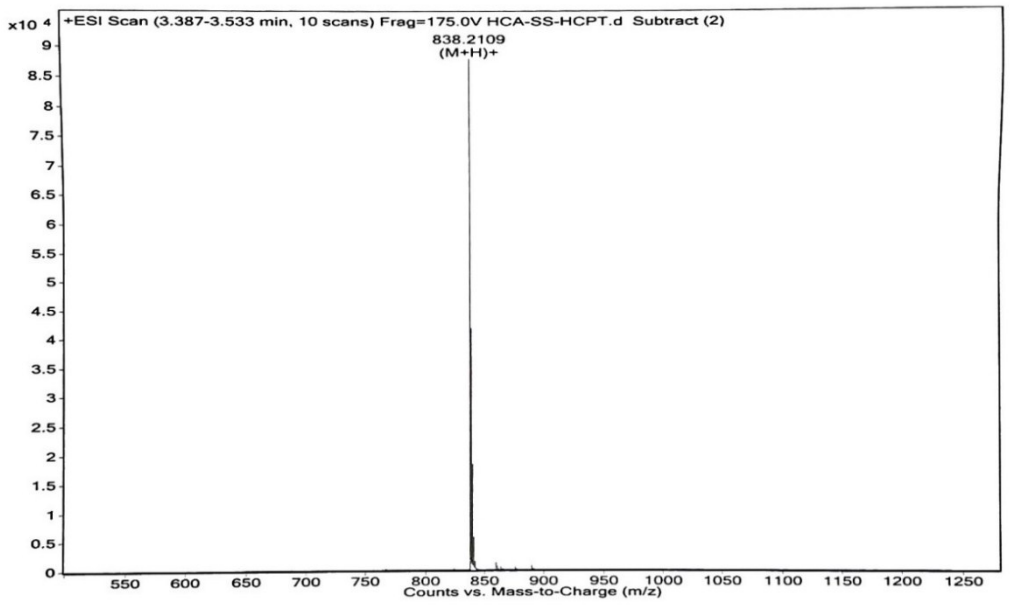

Fig. S7 HRMS spectrum of HCA-SS-HCPT.

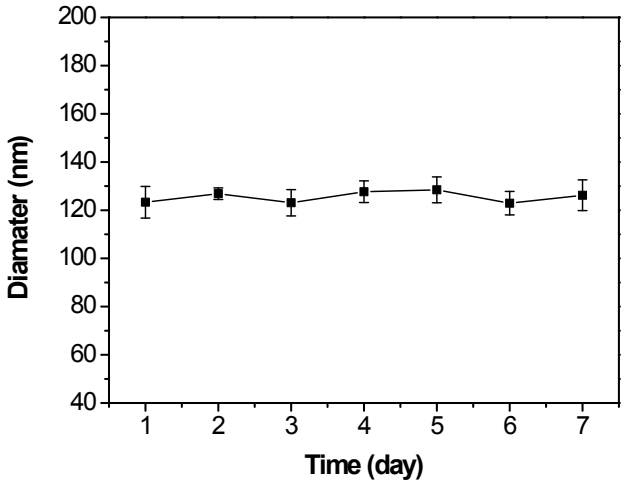

Fig. S8 The size change of HCA-SS-HCPT NPs in aqueous solution within 7 days measured by DLS.

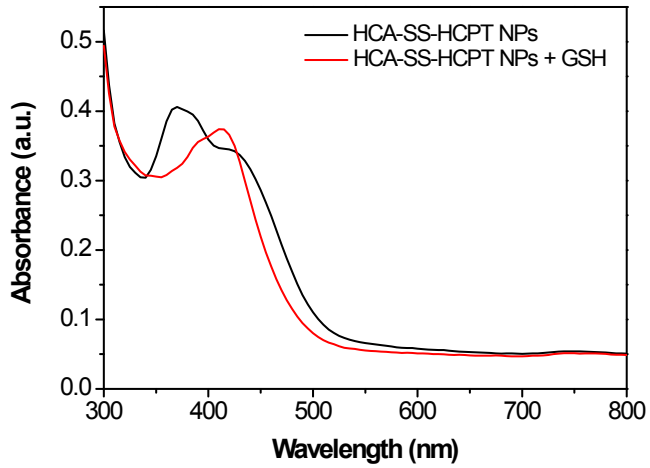

Fig. S9 UV-vis absorption spectra of HCA-SS-HCPT NPs in PBS (pH = 7.4) in the presence and absence of GSH (1.0 mM).

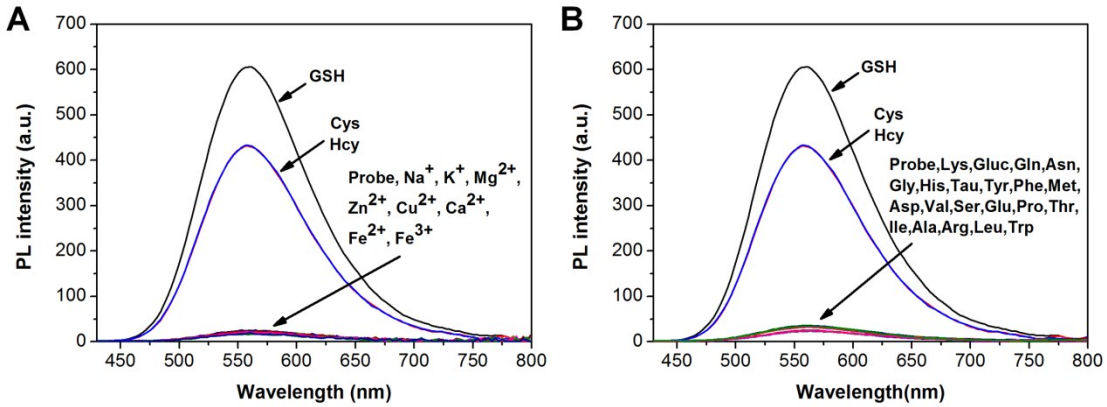

Fig. S10 (A) PL spectra of HCA-SS-HCPT NPs in the absence and presence of various biomolecules (1 mM) including GSH, Cys, Hcy and various metal cations. (B) PL spectra of HCA-SS-HCPT NPs in the absence and presence of various biomolecules including GSH, Cys, Hcy (1 mM) and other amino acids (5 mM). Excitation at 405 nm. [HCA-SS-HCPT NPs] = 10 μM based on HCA-SS-HCPT.

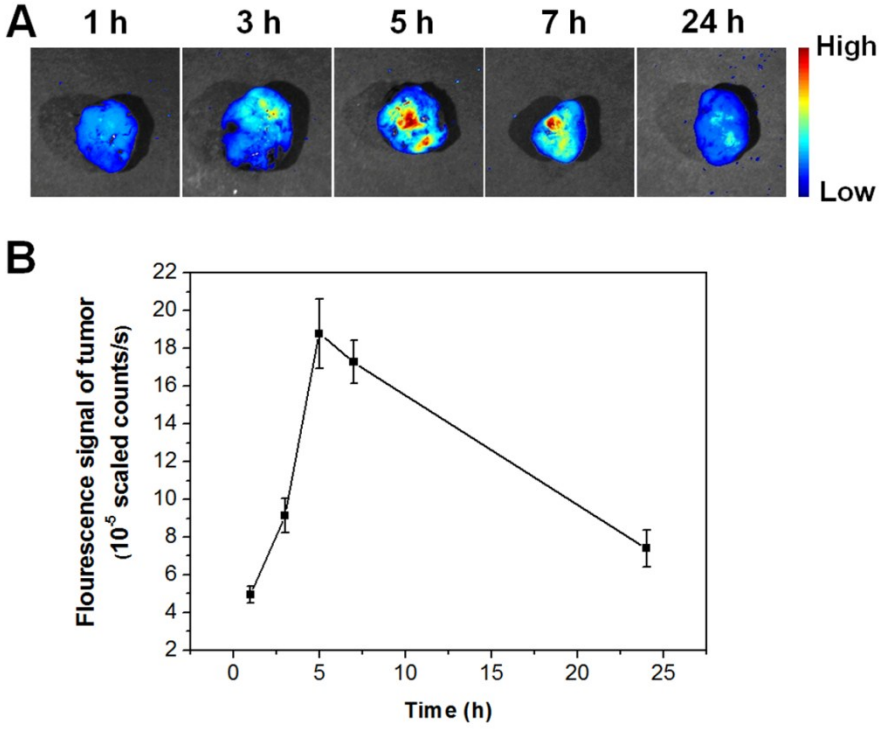

Fig. S11 (A) Fluorescence imaging of tumor tissues from mice intravenously injected with HCA-S-HCPT NPs. The tumors were excised at designated time intervals post

NP injection. (B) Semi-quantitative analysis of the result in (A). The data are presented as mean ± s.d. (n = 3).

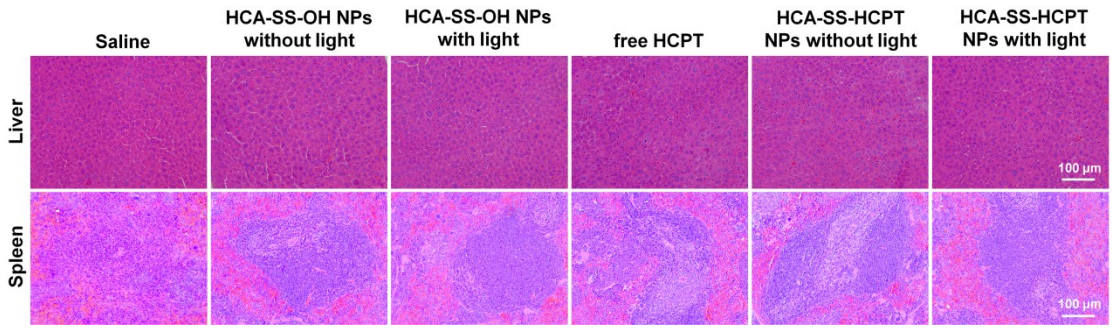

Fig. S12 H&E staining for spleen and liver organs from mice received different treatments indicated. The organs were excised on day 16 after treatments.