Supporting Information

Self-assembly of intelligent nanoplatform for endogenous H_2S-triggered multimodal cascade therapy of colon cancer

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GENERAL

Materials and Reagents. All chemical reagents were used as supplied without further purification unless otherwise specified. Chlorin e6 (Ce6) was purchased from Frontier Scientific, Inc. (Salt Lake City, UT, USA). Cupric chloride ($CuCl_2$) was abtained from Chengdu Kelon Chemical Reagent Factory (Chengdu, China). Sodium hydrosulfide hydrate (NaHS) was purchased from Energy Chemical (Shanghai, China), Cell Counting Kit-8 (CCK-8) was purchased from Chongqing Baoguang Biotechnology Co., Ltd. (Chongqing, China). 4,6-diamidino-2-phenylindole (DAPI) was purchased from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Singlet oxygen sens or green (SOSG) was purchased from Thermo Fishcer Scientific (Waltham, MA, USA). Chlorpromazine (Cp) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd (Shanghai, China). Banoxantrone dihydrochloride (AQ4N), methyl-β-cyclodextrin (M-β-CD), Ivermectin and genistein were purchased from Bide Pharmatech Ltd. (Shanghai, China).

Equipments. The morphology of nanoparticles was observed by scanning electron microscopy (SEM, Hitachi regulus8100, Japan) and transmission electron microscope (TEM, Talos F200S, Holland). Elemental analysis by X-ray photoelectron spectrometer (XPS, ThermoFischer, ESCALAB 250Xi, USA). Hydrodynamic sizes and zeta potentials were measured by multi angle particle size and high sensitive zeta potential analyzer (Omni, USA). Ultraviolet-visible light spectrophotometry (UV-Vis, Agilent Cary60, USA) and Fluorescence Spectrometer (Hitachi) was used to demonstrate the assembly behavior of Ce6 and nanomaterials.

EXPERIMENTAL SECTION

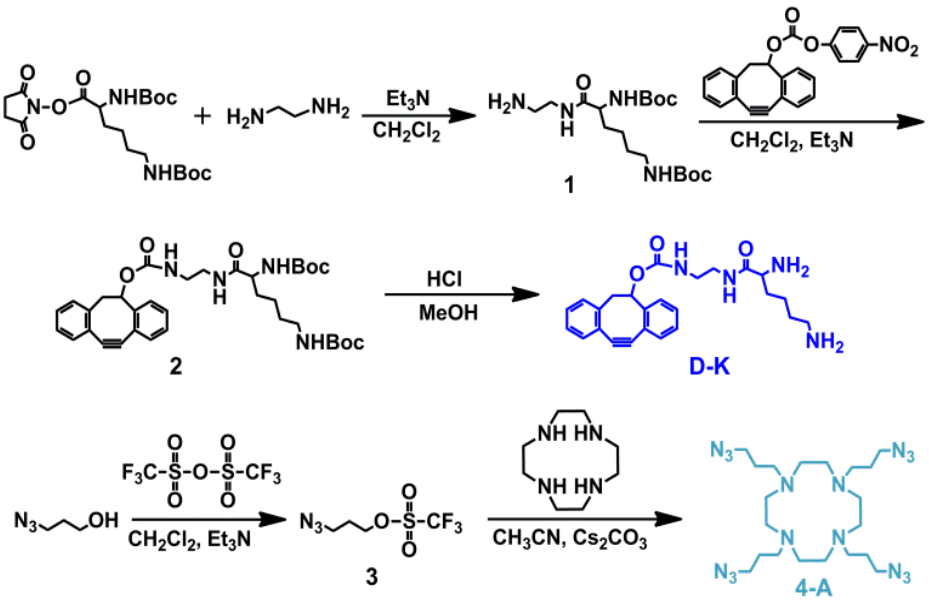

Figure S1. The synthetic route of D-K and 4-A.

Synthesis of Compound 1

Ethylenediamine (670 mg, 11.16 mmol) was added to a solution of N, N'-Di-Boc-L-lysine (500 mg, 1.13 mmol) in DCM (40 mL). After stirring overnight at room temperature, the solvent was

evaporated and the residue was purified by column chromatography with dichloromethane/methanol (v/v = 20:1) as eluent to afford compound **1** as a white solid.

Synthesis of Compound 2

Compound **1** (201 mg, 0.52 mmol) was added to a solution of 4-nitrophenyl chloroformate-substituted DIBO (100 mg, 0.26 mmol) in 10 mL DCM, then trimethylamine (56 μL, 0.40 mmol) was added. The reaction mixture was stirred overnight at room temperature and the solvent was removed in vacuum. The residue was dissolved in EtOAc, washed with water (2 x 50 mL), filtration and solvent removal to afford the crude product, which was further purified by column chromatography with petroleum ether/ethyl acetate (v/v = 1:1) as eluent to get compound **2** as white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.2-7.6 (m, 8H), δ 6.61 (t, 1H), δ 5.75 (t, 1H), δ 5.46 (t, 1H), δ 5.19 (t, 1H), δ 4.63 (t, 1H), δ 3.98 (t, 1H), δ 3.2-3.6 (m, 4H), δ 3.06 (m, 2H), δ 2.86 (m, 2H), δ 1.73 (m, 4H), δ 1.42 (m, 18H), δ 1.31 (m, 2H).^{13}C-NMR (100 MHz, $CDCl_3$): δ173.2, 156.5, 156.4, 156.1, 152.1 ,151.1, 130.0, 128.1, 127.2, 126.3, 126.0, 123.9, 121.3, 113.0, 110.0, 80.3, 79.3, 54.9, 46.2, 41.1, 39.8, 38.7, 29.7, 28.5, 28.3, 22.6. LC-MS (m/z): $[M+H]^+$, calcd for 635.77, found: 635.60.

Synthesis of Compound D-K

The compound **2** was dissolved with MeOH contain 2 M HCl and stirred for 2 h at room temperature to remove Boc to get the final product **D-K** with a yield of 56.3%. ^{1}H-NMR (400 MHz, D_2O): chemical shifts (δ) in ppm: δ 8.03 (s, 2H), δ 7.71 (m, 2H), δ 7.55 (m, 6H), δ 5.44 (t, 1H), δ 5.11 (s, 4H), δ 3.68 (m, 1H), δ 3.38-3.45 (m, 4H), δ 2.99 (d, 2H), δ 2.88 (t, 2H), δ 1.83

(dd, 2H), δ 1.52 (m, 2H), δ 1.37 (m, 2H). ^{13}C-NMR (100 MHz, D_2O): 169.52, 169.19, 155.75, 152.43, 151.32, 130.67, 128.96, 127.78, 126.55, 126.22, 124.41, 123.29, 120.71, 94.18, 75.92, 52.46, 38.72, 38.66, 38.02, 36.96, 30.58, 26.71, 21.59. LC-MS (m/z): $[M+H]^+$, calcd 435.53, found 435.46.

Synthesis of Compound 4-A

To a solution of tetraazacyclododecane (115 mg, 0.67 mmol) in CH_3CN (20 mL) was added Cs_2CO_3 (977 mg, 3 mmol). Compound **3** (699 mg, 3 mmol) in anhydrous CH_3CN was added slowly. The reaction mixture was stirred at room temperature for 8 h, filtered and the solvent was removed under reduced pressure. The residue was diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$. The organic phase was collected and dried over Na_2SO_4. After filtered, the solvent was removed in vacuo and the residue was purified via flash chromatography on silica gel with methanol/dichloromethane (V/V = 1:10) to get compound **4-A**. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.46-3.69 (t, 8H), δ 2.49-3.08 (m, 16H), δ 1.88-2.30 (m, 8H), δ 1.60-1.83 (m, 8H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 51.00, 49.15, 47.76, 21.86. FT-MS (m/z): $[M + H]^+$, calcd 504.36, found 504.76.

Preparation of 4-A/Cu

To prepare 4-A/Cu. 4-A (10 mg, 0.02 mmol) was dissolved in deionized water. Then the solution of $CuCl_2$ (2.9 mg, 0.02 mmol) was slowly added dropwise. The mixed solution was stirred at room temperature overnight.

Singlet oxygen ($^1O_2$) detection by SOSG

To measure $^1O_2$ generation efficiency, C+A, NP, NP-Cu nanoparticles with or without NaHS (100 μM) were incubated in water. After incubation, SOSG (2 μM) was added to each sample and the fluorescence intensity was detected at 530 nm after irradiation at different time points (660 nm, 100 mW cm^{-2}).

Cell culture

Human colon cancer HCT116 cells were cultured in IMDM medium. Human hepatoellular carcinomas HepG2 cells and Human cervical cancer Hela cells were cultured in DMEM medium. Each medium contains 10% fetal bovine serum and 1% penicillin/streptomycin.

Cellular uptake

HCT116 cells were seeded in 24-well plates over glass coverslips and incubated for 12 h at 37 ˚C under 5% CO_2. 200 μL fresh medium containing C+A, NP or NP-Cu (35 μg mL^{-1} for Ce6 and 26 μg mL^{-1} for AQ4N) was then added. After 4 h incubation, the cells were fixed with 4% paraformaldehyde, stained with DAPI and imaged with Laser scanning confocal microscopy (Leica TCS SP8, Germany).

To verify the activation of NP-Cu in different cells, HCT116, HepG2 and Hela cells were used to observe the endocytosis and activation of NP-Cu in cells. And quantitative analysis by flow cytometry (CytoFLEX, China).

Endocytosis mechanism

HCT116 cells (density of 1×10^4 cells/well) were seeded in 96-well plates and incubated 12 h at 37 ˚C under 5% CO_2. The cells were incubated with different endocytosis inhibitor (10 mM NaN_3, 10 mM M-β-CD, 100 μM Cp or 200 μM genistein) or placed at 4 ˚C for 1 h, and then NP-Cu was added and incubated at 37 ˚C or 4 ˚C for another 4 h. After that cells were trypsinsized and collected for flow cytometry analysis.

Determination of intercellular ROS

DCFH-DA as a fluorescent probe for ROS in cells. HCT116 cells were seeded in 96-well plates and incubated overnight. Then the medium was replaced with fresh medium containing C+A, NP or NP-Cu. After 6 h incubation, flow cytometry was used to detect the fluorescence intensity of DCF.

In vitro cytotoxicity

HCT116 cells (density of 1×10^4 cells/well) were seeded in 96-well plates and incubated for 12 h at 37 ˚C under 5% CO_2. Then the medium was replaced with fresh medium containing free Ce6, AQ4N, C+A, NP or NP-Cu with the equivalent of Ce6 (0.5μg mL^{-1}) and AQ4N (0.375μg mL^{-1}). After 12 h incubation, the cells were illuminated with laser (660 nm, 100 mW cm^{-2}, 1 min; 808 nm, 1.5 W cm^{-2}, 10 min) for PDT and PTT. The cell viability was measured by CCK-8 at 450 nm.

Animal Model

All animal experiments were carried out in compliance with the requirements of the National Act on the Use of Experimental Animals (People's Republic of China) and were approved by the

Experimental Animal Ethical Committee of Chongqing University Cancer Hospital. Female BALB/c-nude mice (4-6 weeks) were supplied by the Animal Center of Chongqing Medical University (Chongqing, China).

Biodistribution

HCT116 tumor-bearing mice were injected intravenously with C+A, NP-Cu (4 mg kg^{-1} for Ce6, 3 mg kg^{-1} for AQ4N). The mice were imaged at different time points (0.5, 4, 6, 8, 12, 24 h) postinjection by small animal imaging system (IVIS Lumina III, USA). 24 h after administration, the mice were sacrificed, major organs (heart, liver, spleen, lung and kidney) and tumors were imaged and analyzed by an IVIS lumina imaging system.

The therapeutic efficiency of NP-Cu *In Vivo*

For synergistic anti-tumor therapy studies, HCT116 cells (2×10^6) were injected subcutaneously into female BALB/c-nude mice. When the tumor volume grew to approximately 50 mm^3, the mice were randomly divided into 5 groups (n=5): (1) PBS group, (2) Ce6 with 660 nm laser group (L+), (3) C+A with 660 nm laser group (L+), (4) NP-Cu with 660 nm laser group (L+), (5) NP-Cu with 660 nm and 808nm laser group (L++). 200 μL of different samples with equal amount of Ce6 (2 mg kg^{-1}) and AQ4N (1.5 mg kg^{-1}) were intravenously injected into the mice every three days for a total of three times. The tumor region of mice was irradiated by 660 nm laser (200 mW cm^{-2}, 10 min) or 808 nm laser (3 W cm^{-2}, 10 min) at 12 h post each injection. The tumor volumes and weight were measured every two days for 16 days. The tumor volumes value was calculated as the following equation: tumor volumes = length × $width^2$ × 0.5. At the end of

experiment, the tumor was excised and weighed. For histological examination, tumor tissues and major organs (heart, liver, spleen, lung and kidney) were collected for hematoxylin and eosin (H&E) staining.

Statistical analysis

Experimental data were presented as the mean ± standard deviation (SD) of at least three independent experiments. Analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA). Statistical analysis was conducted using one-way ANOVA or Student's t-test. Statistical significance is indicated as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

SUPPLEMENTARY FIGURES

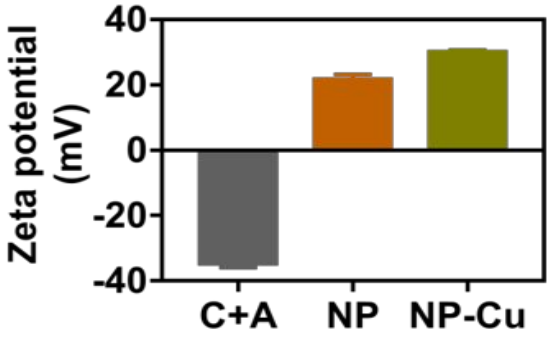

Figure S2. Zeta potential of different formulations.

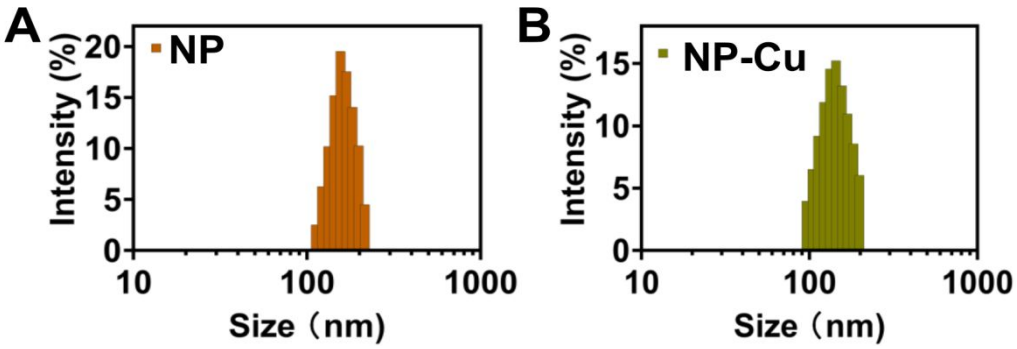

Figure S3. Size distribution of (A) NP and (B) NP-Cu measured by dynamic light scattering (DLS).

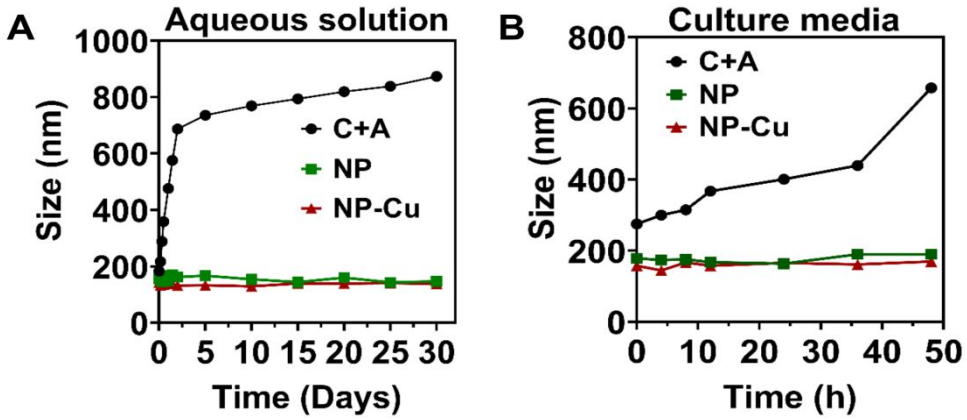

Figure S4. (A) Long-term storage stability of various nanoparticles in aqueous solution. The results indicated that the assembled NP and NP-Cu nanoparticles exhibited good stability in aqueous solution for at least one month. (B) Serum stability of the assembled nanoparticles in cell medium containing 10% (w/w) fetal bovine serum (FBS). All nanoparticles exhibited good stability in the cell culture medium with minimal protein adsorption for 48 h.

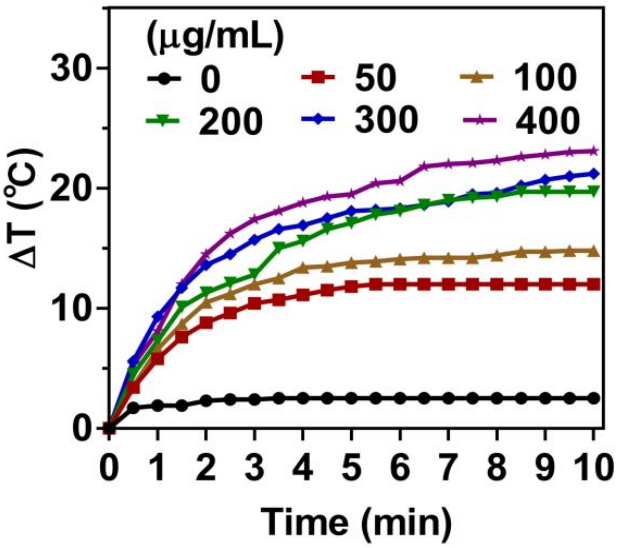

Figure S5. Photothermal performance of different concentration of NP-Cu in the presence of NaHS under a continuous irradiation of 808 nm laser (1.5 W cm^{-2}).

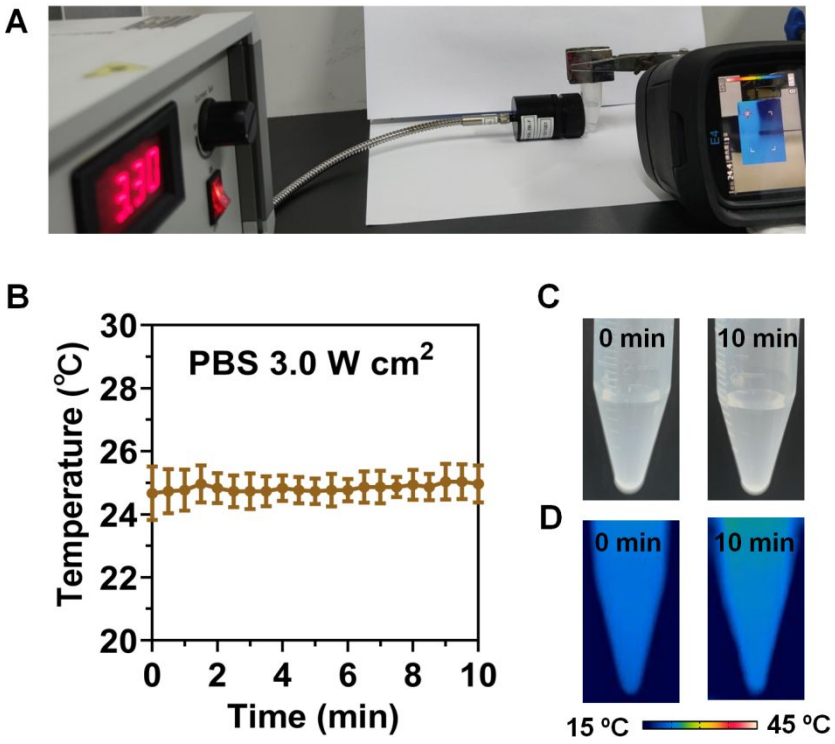

Figure S6. Photothermal performance of PBS solution under a continuous irradiation of 808 nm laser (3 W cm^{-2}). (A) Picture of experimental device. (B) Temperature change as a function of irradiation time. (C) Photos and (D) photothermal images of PBS solution before and after 10 min continuous irradiation of 808 nm laser (3 W cm^{-2}).

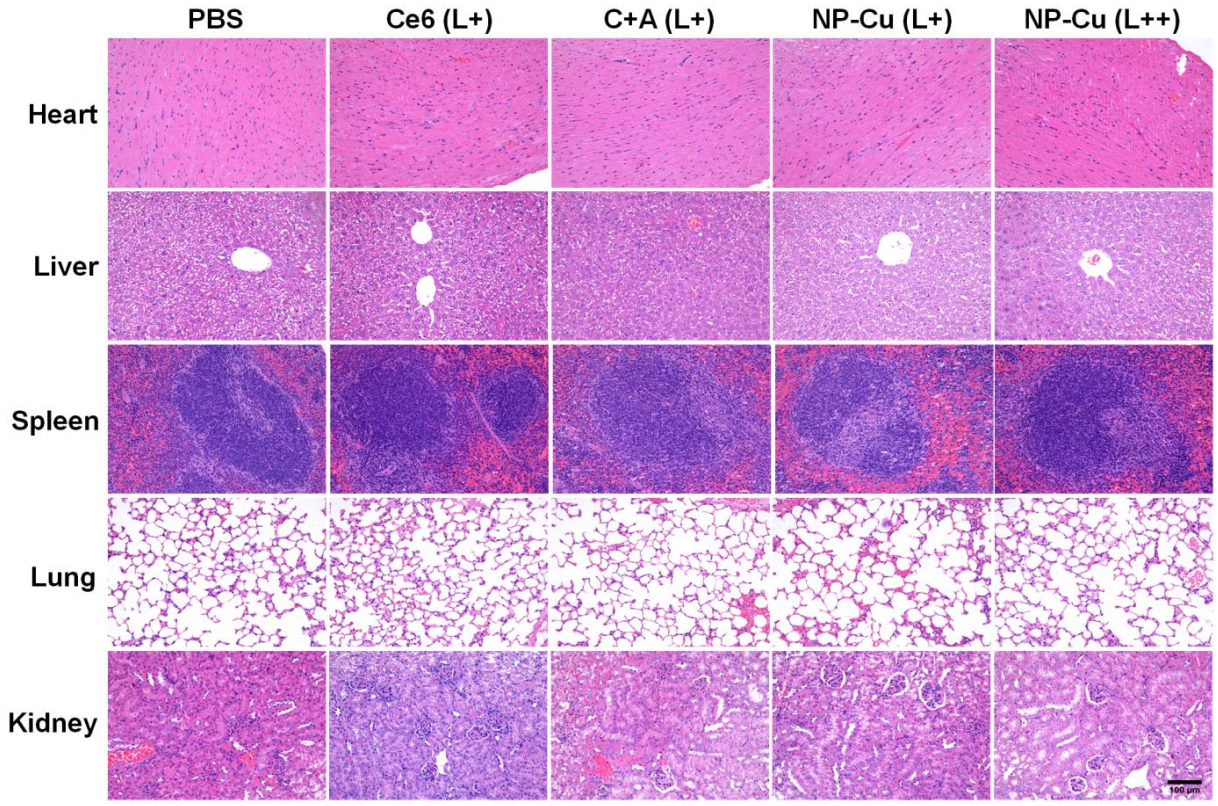

Figure S7. H&E staining of major organs after different treatments. Scale bars: 100 μm.