Supporting Information

A cancer cell targeted photosensitizer and therapeutic protein co-delivery nanoplatform based on metal-organic framework for enhanced synergistic photodynamic and protein therapy

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Experiment Section

1 Materials. 1,3-Diphenylisobenzofuran (DPBF), 2′,7′-dichlorodihydr fluorescein diacetate (DCFH-DA), Calcein AM, pidiuprom iodide (PI) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich Inc. (U.S.A.). 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased for J&K Chemical Ltd. Cell Counting Kit (CCK-8) was brought from Dojindo Laboratories (Kumamoto, Japan). Annexin V-FITC/PI apoptosis detection kit was purchased from Life Technologies, Inc. Cell culture products (Gibco) were obtained from Thermo Fisher Scientific (U.S.A.). All other chemicals with analytical reagent grade were provided by Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Ultrapure water used for solution preparation obtained from a Millipore NanoPure water filtration system (18.2 M·Ω resistivity at room temperature).

2 The Characterization of Ce6/Cyt c@ZIF-8/HA. The morphology of the ZIF-8 and Ce6/Cyt c@ZIF-8/HA was characterized by transmission electron microscopy (TEM) (JEM-200CX, JEOL, Japan). Dynamic light scattering (DLS) and polydispersity index (PDI) for particle size and size distribution of nanoparticles were tested on Zeta Sizer (NanoZS, Malvern, U.K.) The specific crystal structure of ZIF-8, Ce6/Cyt c@ZIF-8 and Ce6/Cyt c@ZIF-8/HA were verified using the XRD (XRD, Ultima IV, RIGAKU) equipped with Cu Kα radiation within the 2θ range of 5°-60°. The Fourier transform infrared (FT-IR) spectra of Cyt C, ZIF-8 and Ce6/Cyt c@ZIF-8/HA were tested on a FT-IR Spectrophotometer (Spectrum Two, Perkin Elmer). Fluorescence spectra of Ce6 or nanoparticles were obtained on an Agilent Cary Eclipse fluorescence spectrophotometer. The absorption spectra of nanoparticles and CCK-8 assay were measured on a microplate reader (Spectra 206 Max M5, Molecular Devices).

3 Cellular Experiments:

3.1 Cell Culture and Cellular Uptake Investigation. Human cervical carcinoma cells (HeLa cells), SMMC7721 cells, and HL-7702 cells were cultured under standard conditions (DMEM medium containing 10% heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 U/mL)). L929 cells was cultured in MEM medium containing 10% FBS, penicillin (100 U/mL) and streptomycin (100 U/mL). The four cell lines were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. To qualitatively image the cellular uptake, 1×10^5 HeLa cells were seeded on 35 mm diameter glass-bottom Petri dish for 24 h. Then, the cells were incubated with 40 μg/mL of Ce6/Cyt c@ZIF-8/HA for predefined time (1 h, 2 h and 4 h). Afterwards, washing with PBS twice and fixed with paraformaldehyde (4%) for 15 min. Finally, the cells were washed with PBS and imaged on a confocal fluorescence microscope (Carl Zeiss, LSM 780, Germany) with 633 nm excitation for Ce6. To verify the targeting ability of HA, the HeLa cells were pre-blocked with free HA (10 mg/mL) for 2 h before incubation with Ce6/Cyt c@ZIF-8/HA. To quantitatively assess cellular uptake, HeLa cells were seed on the six-wells plate at a density of 2×10^5 cells for 24 h, then the Ce6/Cyt c@ZIF-8/HA NPs were added and incubated for predefined time. After rinse with PBS twice, the cells were treated with trypsin and then analyzed by flow cytometry (BD FACSverse).

To further verify the delivery efficiency of Cyt c, the Cyt c was labeled with FITC according to previous reported method. The Cyt c was replaced with Cyt c-FITC to prepared Ce6/Cyt c@ZIF-8/HA, and then used for fluorescence imaging (the excitation wavelengths for Ce6 and FITC were 633 nm and 488 nm, respectively).

3.2 Intracellular ROS Generation Analysis. DCFH-DA was used as the ROS fluorescence probe for imaging intracellular ROS generation of nanoparticles. HeLa cells (1×10^4) were seeded on 96-

wells plate overnight, after being rinsed with cold PBS, two groups of cells were treated with culture medium containing Cyt c, Ce6, Ce6/ZIF-8/HA, Ce6/Cyt c@ZIF-8/HA for 4 h, respectively. Then, DCFH-DA solution (40 μM) was co-incubated with the cells for 30 min. One group of cells was rinsed with PBS and then exposed to 670 nm laser (50 mW/cm^2) for 2 min, the other group was kept under dark. The cells were also blocked by free HA to verify the HA targeting ability as above described procedures. Subsequently, the cells were imaged on a fluorescence microscope (excitation wavelength at 488 nm).

3.3 Cytotoxicity Assay. The *in vitro* cytotoxicity of Ce6, Cyt c and different nanoparticles was evaluated using the cell counting kit (CCK8) assay following the manufacturer's instructions. First, the cytotoxicity of ZIF-8/HA, without drug embedding, was tested in different types of cells, including two cancer cells (HeLa cells and SMMC7721 cells) and two normal cells (L929 cells and HL-7702 cells). These cells at a density of 1×10^4 cells per well were seeded on 96-wells plate for 24 h, respectively. Different concentrations of ZIF-8/HA (0, 5, 10, 20, 40, 60 μg/mL) in culture medium were added and incubated for 24 h. After washing with PBS thrice, 10 μL of CCK-8 solution mixed with 90 μL of culture medium was added into the plate and further incubation for 30 min 37 °C. The absorbance intensity at 450 nm was measured at a microplate reader for calculating the cytotoxicity. The relative cell viabilities (%) were calculated and expressed as the means ± SD of five samples as: cell viability (%) = OD (sample)/OD (control) × 100%, where the sample group was that treated with nanoparticles, while control group was that treated with just culture medium.

To tested the protein and photodynamic combination therapy, the HeLa cells were treated with increasing concentration of Ce6, proteins or nanoparticles (total dose of Ce6 from 0 to 1.6 μg/mL)

for 4 h and exposed without or with 670 nm laser (100 mW/cm^2) for 2 min. After incubation for another 24 h, the relative cell viabilities were tested and calculated using CCK-8 assay as descripted above.

Live/dead cell staining assay which uses Calcein AM staining for live cells and propidium iodide (PI) staining for dead cells were used to directly visualize the therapeutic efficacy. The HeLa cells were seeded on 96-wells plate for 24 h, then washed with PBS and incubated with nanoparticles or proteins (at dose of Ce6 at 1.6 μg/mL). The cells were exposed without or with 670 nm laser (100 mW/cm^2) for 2 min. After incubation for another 12 h, the cells were incubated with calcein AM (5 μM) and PI solution (50 μM) for 20 min staining, finally, it was observed by fluorescence microscope with 488 nm excitation.

Cell apoptosis assay against HeLa cells was also tested using Annexin V-FITC/PI Apoptosis Detection Kit by flow cytometry (BioLegend, USA). The HeLa cells (2×10^5) were seeded on 12-wells plate for 24 h, followed by washing with cold PBS twice and the cells were treated with Ce6, Cyt c or nanoparticles (at dose of Ce6 at 1.6 μg/mL) for 4 h, and then irradiated without or with 670 nm laser. After 12 h incubation, the cells were treated with Annexin V-FITC/PI reagent according to the manufacturer's protocol. Finally, the cells were detached by trypsin and analyzed by flow cytometry.

4 Hemolysis Test. The red blood cells (RBCs) extracted from the plasma of SD rats were used for evaluating the hemolysis ratio of Ce6/Cyt c@ZIF-8/HA. RBCs were washed and diluted using physiological saline (NaCl, 0.9%) following reported method.[1] Then, 0.2 mL of a diluted RBC suspension was mixed with 0.8 mL ultrapure water (as positive control), physiological saline (as negative control) and Ce6/Cyt c@ZIF-8/HA solution (0 to 100 μg/mL). After keeping static

conditions for 2 h, the mixtures were centrifuged at 2000 rpm for 10 min. Thereafter, they were photographed and the absorbance values at 540 nm of supernatants were measured. The hemolysis ratios of Ce6/Cyt c@ZIF-8/HA were calculated and expressed as the means ± SD of three samples: Hemolysis (%) = (OD (sample)-OD (negative)) / [OD (positive)-OD (negative)] × 100%, where OD (sample) was the absorbance at 540 nm of sample group treated with nanoparticles, while OD (negative) and OD (positive) were that of corresponding control groups.

5 *In Vivo* Experiments:

5.1 *In Vivo* Optical Imaging and Tissue Distributions. All animal experiments were operated strictly according the guidelines approved by the Animal Ethics Committee of Mengchao Hepatobiliary Hospital of Fujian Medical University. For caner model establishment, 6-8 weeks old female BALB/c mice (obtained from Wushi Laboratory Animal Co. Ltd., China) were acclimatized 1 week and then injected subcutaneously with 5×10^6 HeLa cells in the right flank of hind leg region. After the tumor volume reaching about 80~100 mm^3, the mice were used for imaging and therapeutic experiments. For fluorescence imaging, the HeLa tumor-bearing mice were intravenously injected with 100 μL of Ce6/Cyt c@ZIF-8/HA (1 mg/mL, dispersed in saline solution) and then imaged on an in vivo imaging system (IVIS, Perkin Elmer) with 633 nm excitation at different time points. After 24 h, the mouse was executed. The tumor, kidney, lung, spleen, heart and liver were dissected and imaged to examine the targeting distribution ability of Ce6/Cyt c@ZIF-8/HA.

5.2 *In Vivo* Anticancer Efficacy Assessment. HeLa tumor-bearing mice were allocated into 6 groups (5 mice per group) randomly: (a) PBS; (b) Cyt c; (c) Ce6 with laser; (d) Ce6@ZIF-8/HA with laser; (e) Ce6/Cyt c@ZIF-8/HA; (f) Ce6/Cyt c@ZIF-8/HA with laser. For therapeutic

evaluation, 100 μL of PBS or solution of Cyt c (100 μg/mL), Ce6 (10 μg/mL), Ce6@ZIF-8/HA (1 mg/mL) and Ce6/Cyt c@ZIF-8/HA (1 mg/mL) were intravenously injected *via* the tail vein into mice at day 0 and day 4. After 12 h, the tumor sites were exposed with or without 670 nm laser at 200 mW/cm^2 for 5 min. The tumor dimensions were measured using an electronic caliper every 2 days during treatment. The tumor volume was calculated as follows: volume= length×(width)^2/2. After 16 days of treatment, all mice were sacrificed and their tumors were excised and then weighed. Meanwhile, the dissected tumor and major organs (heart, liver, spleen, kidney, and lung) were rinsed with PBS, soaked in formalin, embedded in paraffin, and then sliced for staining with hematoxylin and eosin (H&E) and examined microscopically for histological analysis. The tumor slices were also analyzed by Ki67 immunohistochemistry for proliferation and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining for cell death refer to the manufacturer's method.

To determine the biosafety of Ce6/Cyt c@ZIF-8/HA, the nanoparticles (1 mg/mL) were injected intravenously into the female BALB/c mice *via* the tail vein at 0 and 4 day, and the mice treated with PBS were used as control. At 0- and 14-days post-injection, the blood samples were collected from rat eyes and then blood chemistry tests and routine blood analysis were analyzed using an automated analyzer (CX5, Beckman, USA).

The wight of each mouse was also measured every other day during treatment for evaluating the biocompatibility of the nanoparticles.

6 Statistical Analysis. Data were presented as the mean result ± standard deviation (SD). The statistical analysis was determined using ANOVA or the two-tail paired Student's t test, where *p < 0.05, **p <0.01 and ***p < 0.001. When p < 0.05, there are statistical significance between the

compared groups.

(1) Wu, M.; Wu, L.; Li, J.; Zhang, D.; Lan, S.; Zhang, X.; Lin, X.; Liu, G.; Liu, X.; Liu, J. Self-Luminescing Theranostic Nanoreactors with Intraparticle Relayed Energy Transfer for Tumor Microenvironment Activated Imaging and Photodynamic Therapy. *Theranostics* **2019**, *9* (1), 20–33. https://doi.org/10.7150/thno.28857.

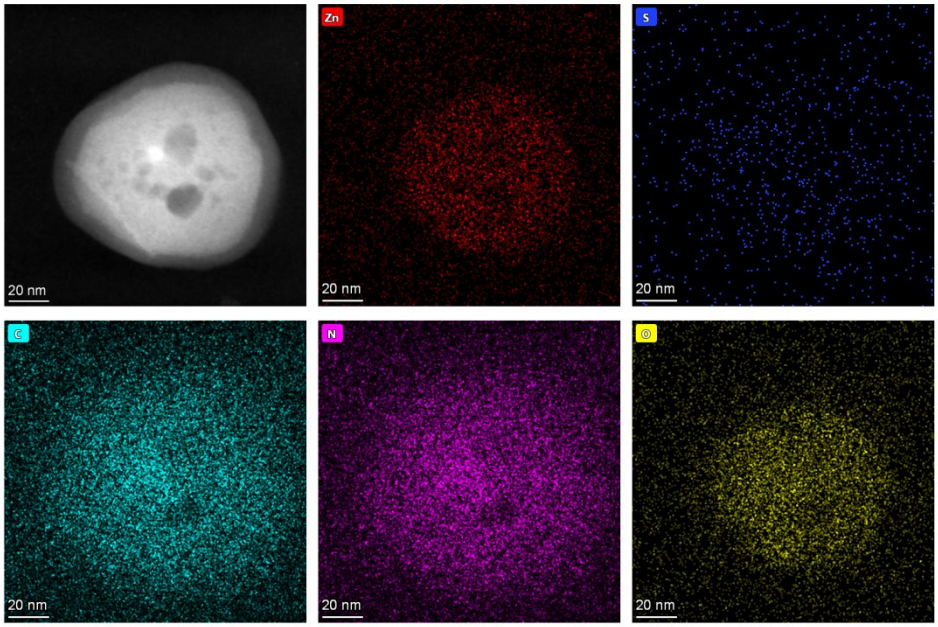

Figure S1. Dark-field TEM image of Ce6/Cyt c@ZIF-8/HA NPs and corresponding TEM elemental mappings of the Zn-K edge, S-K edge, C-K edge, N-K edge, and O-K edge signals.

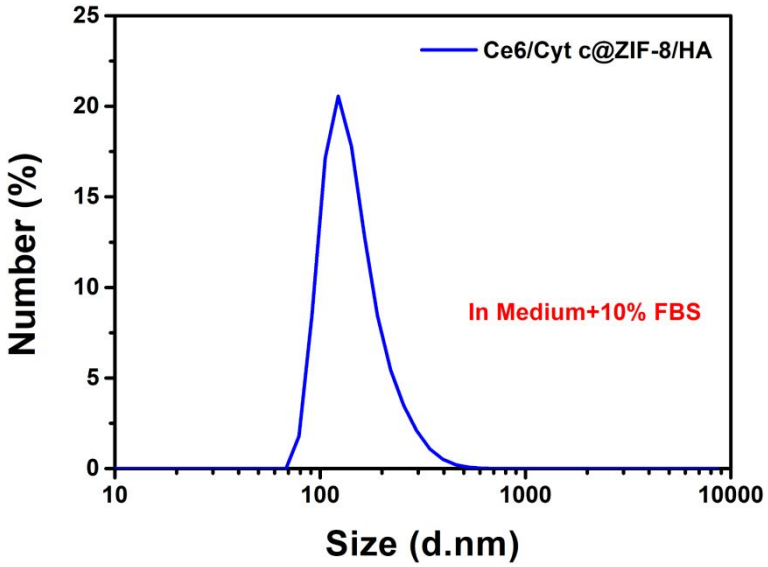

Figure S2. Dynamic light scattering result of the Ce6/Cyt c@ZIF-8/HA NPs in 10% FBS DMEM medium.

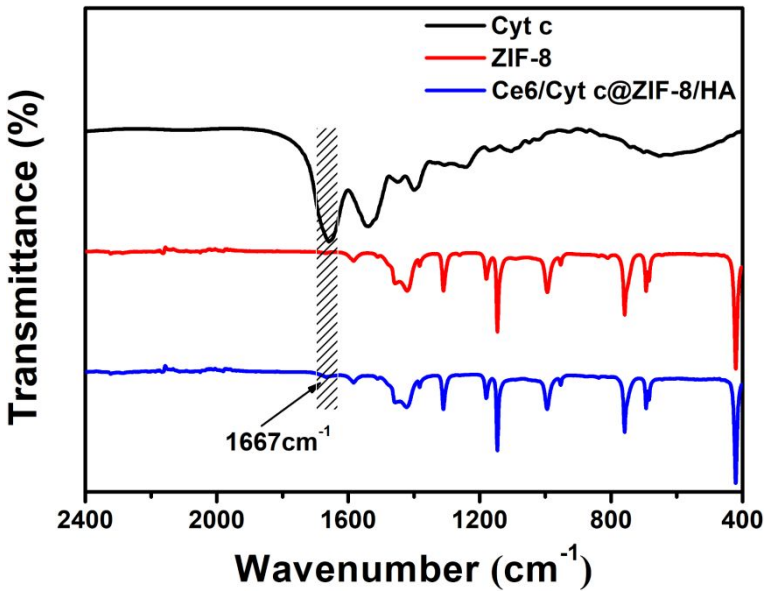

Figure S3. FTIR spectra of Cyt c, ZIF-8 and Cyt c/Ce6@ZIF-8 NPs.

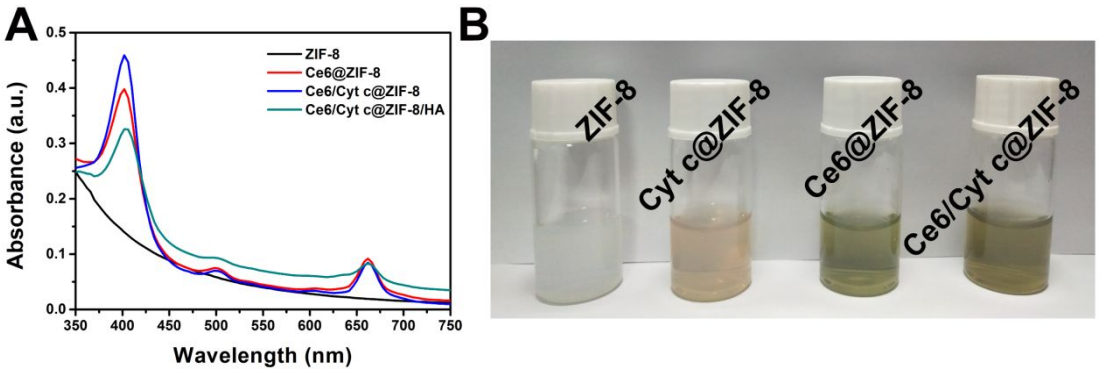

Figure S4. The absorption spectra (A) and corresponding photograph of ZIF-8 NPs, Ce6/Cyt c@ZIF-8 NPs and Ce6/Cyt c@ZIF-8/HA NPs.

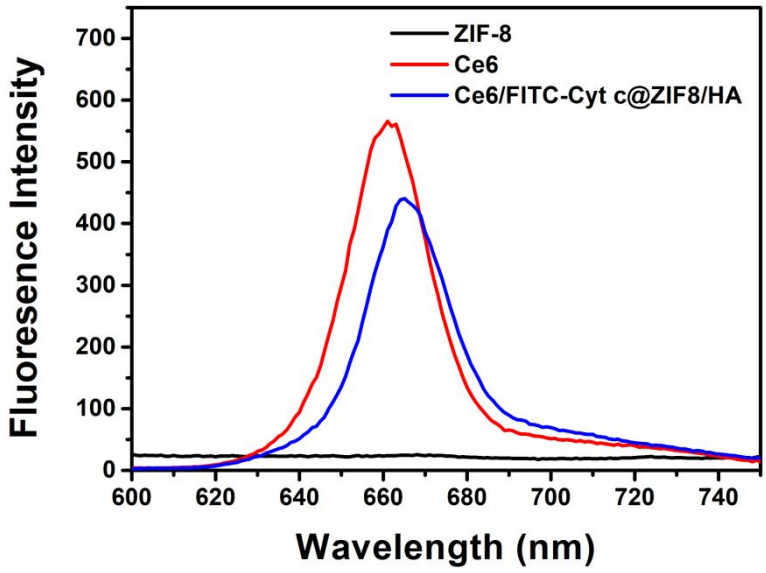

Figure S5. Fluorescence spectra of ZIF-8, Ce6 and Ce6/Cyt c@ZIF-8/HA NPs (excitation at 404 nm for Ce6).

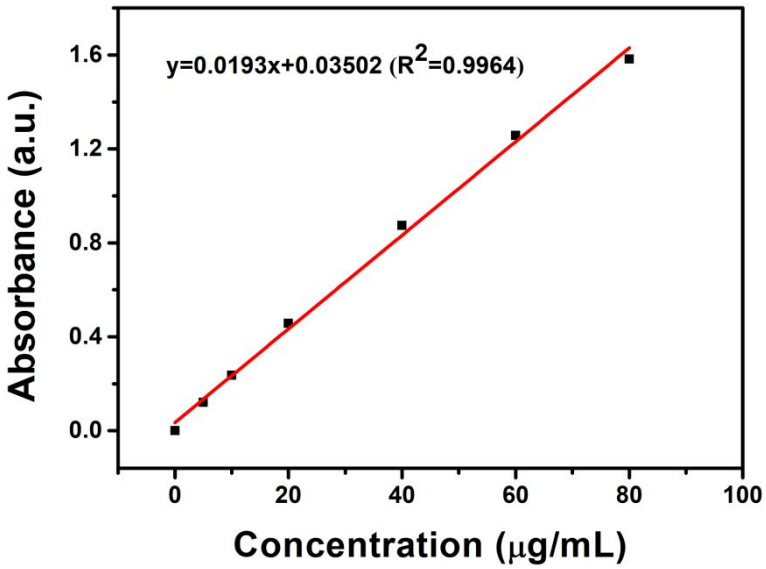

Figure S6. Standard linear calibration curves of Ce6 (absorption at 656 nm).

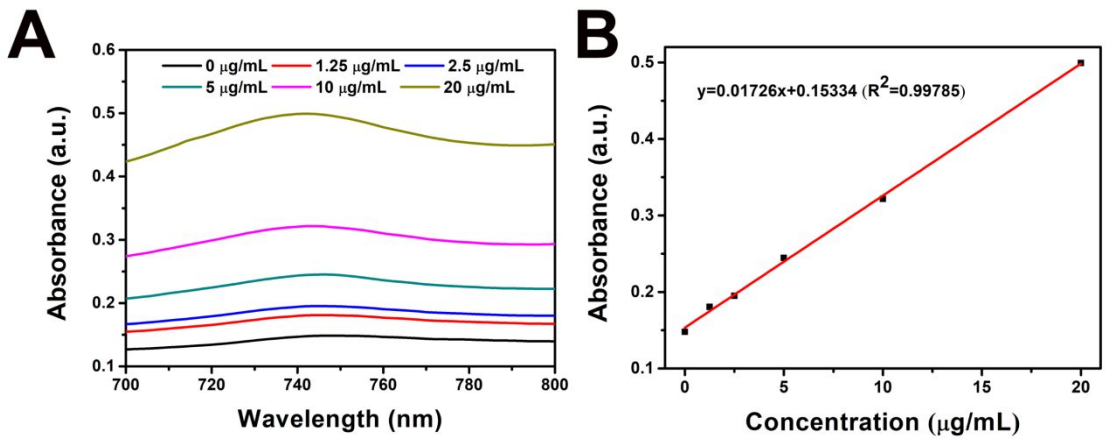

Figure S7. (A) The absorbance spectra of ABTS with different concentrations of Cyt c (H_2O_2: 0.2 mM and ABTS: 0.5 mg/mL). (B) The absorbance at 742 nm versus different concentration of Cyt c.

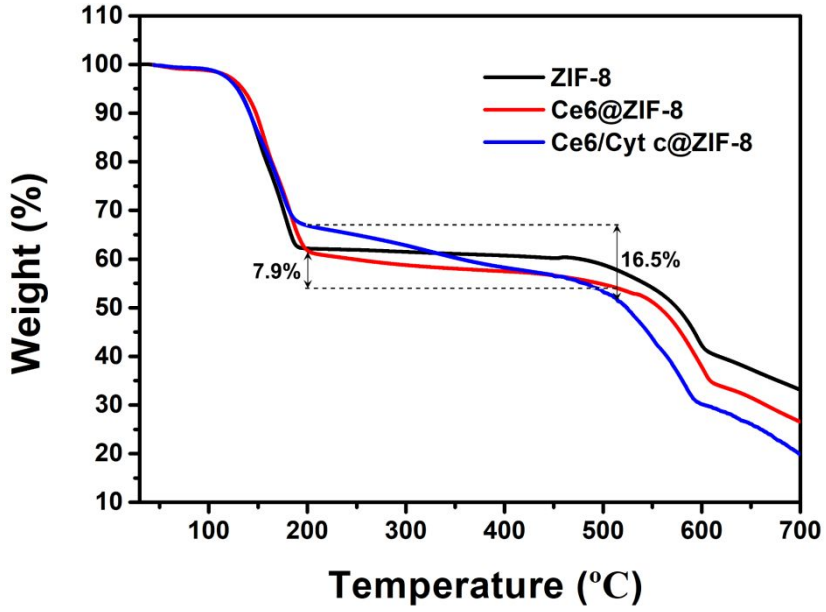

Figure S8. The thermogravimetric analysis (TGA) of ZIF-8, Ce6@ZIF-8 and Ce6/Cyt c@ZIF-8. The second-stage decomposition of the Ce6@ZIF-8 NPs or Cyt c/Ce6@ZIF-8 NPs composite in air starts from 200 °C and finishes around 510 °C, being much lower than that of the pure ZIF-8 crystal. About 8% (for Ce6@ZIF-8 NPs) or 16.5% (for Cyt c/Ce6@ZIF-8 NPs) of weight loss occurs during this stage, which is attributed to the decomposition of the Ce6 or protein/Ce6 complex.

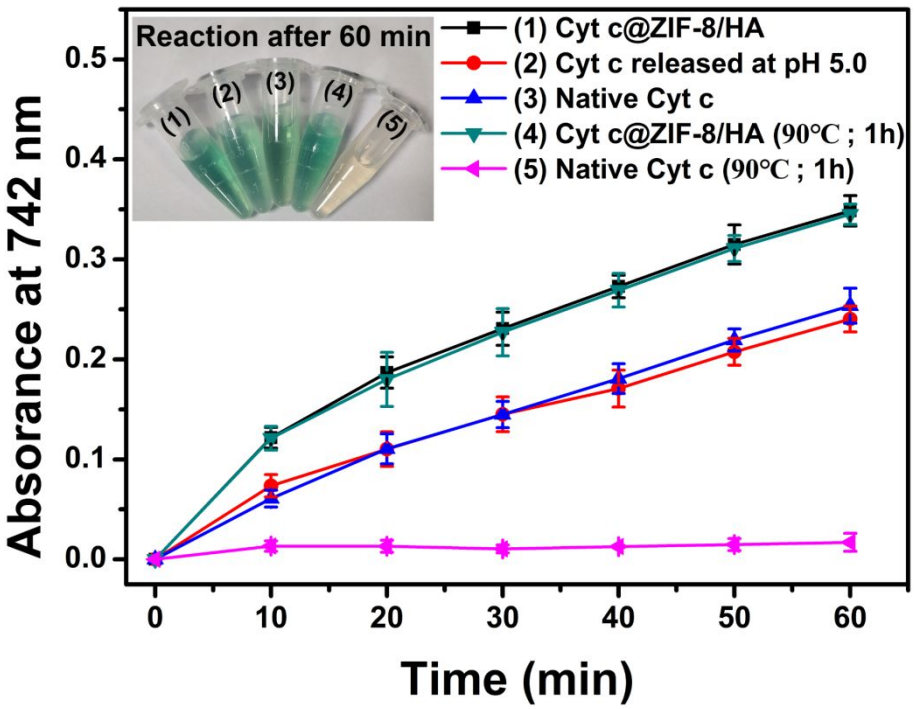

Figure S9. The thermal stability of encapsulation Cyt c in ZIF-8 was tested by the catalyzed oxidization of colorless ABTS for different time.

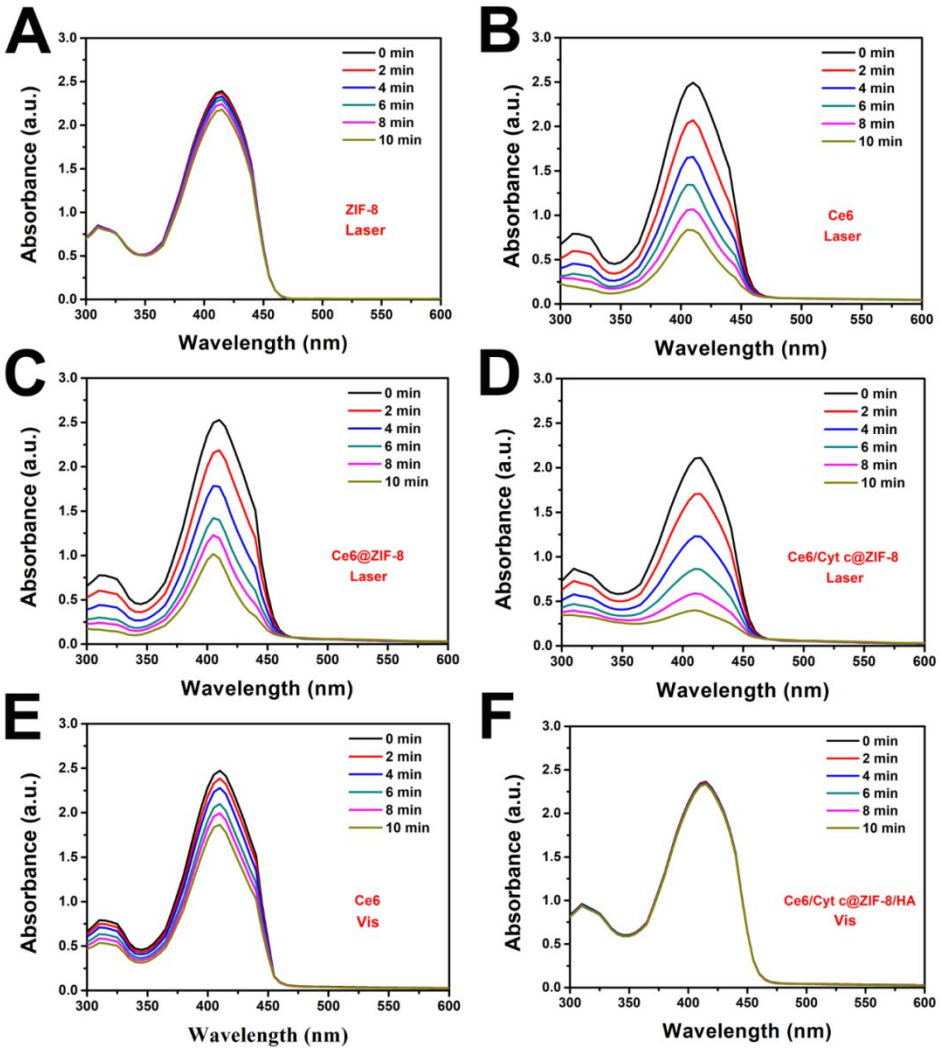

Figure S10. The absorbance spectra change of DPBF with ZIF-8 (A), Ce6 (B), Ce6@ZIF-8 NPs (C) and Ce6/Cyt c@ZIF-8 (D) upon laser irradiation, Ce6 (E) and Ce6/Cyt c@ZIF-8/HA NPs (F) under room light for different times.

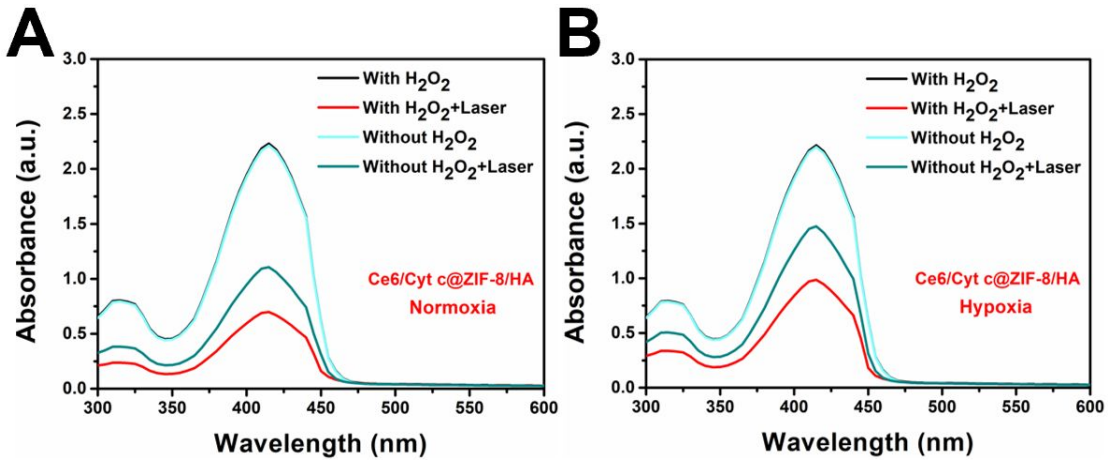

Figure S11. The absorbance spectra change of DPBF with Ce6/Cyt c@ZIF-8/HA NPs with or without H_2O_2 under (A) normoxia or hypoxia (B) conditions before and after laser irradiation for 6 min.

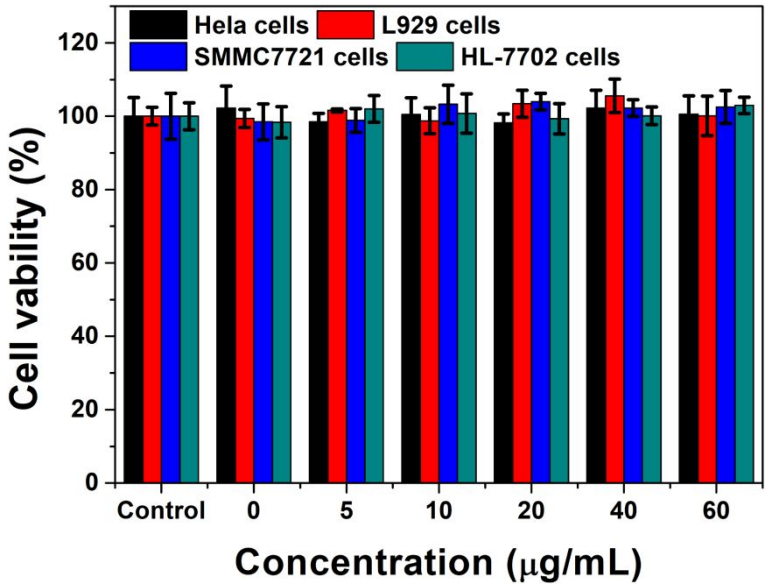

Figure S12. Cell viability of HeLa cells, L929 cellls, SMMX7721 cells and HL-7702 cells treated with ZIF-8/HA NPs for 24 h.

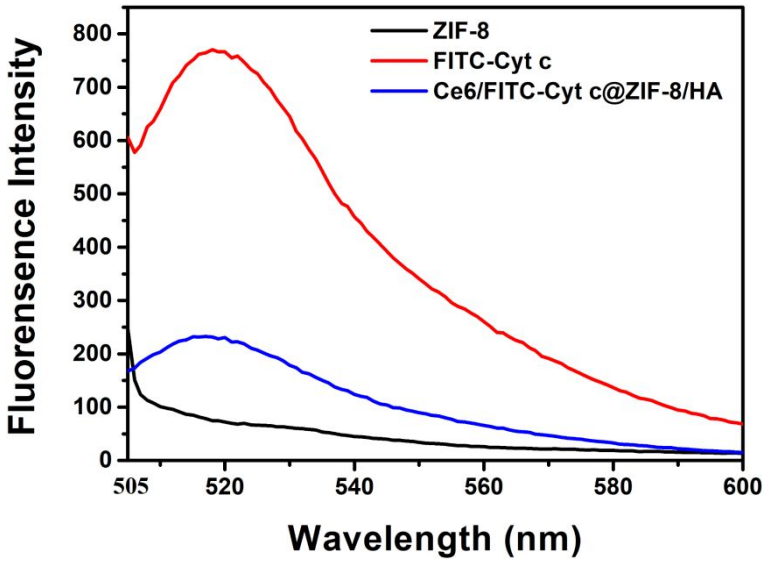

Figure S13. Fluorescence spectra of ZIF-8, FITC-Cyt c and Ce6/FITC-Cyt c@ZIF-8/HA NPs (excitation at 488 nm for FITC).

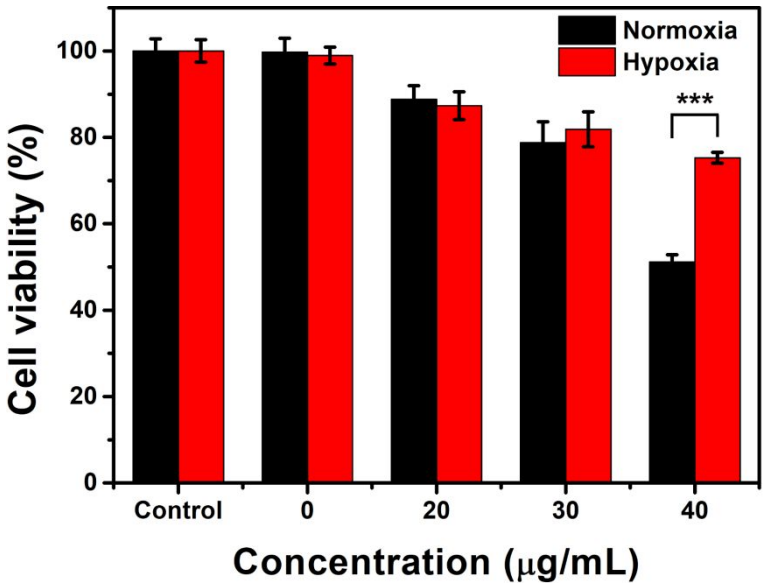

Figure S14. Cell viability of HeLa cells treated with Ce6/Cyt c@ZIF-8/HA NPs under normoxia or hypoxia conditions.

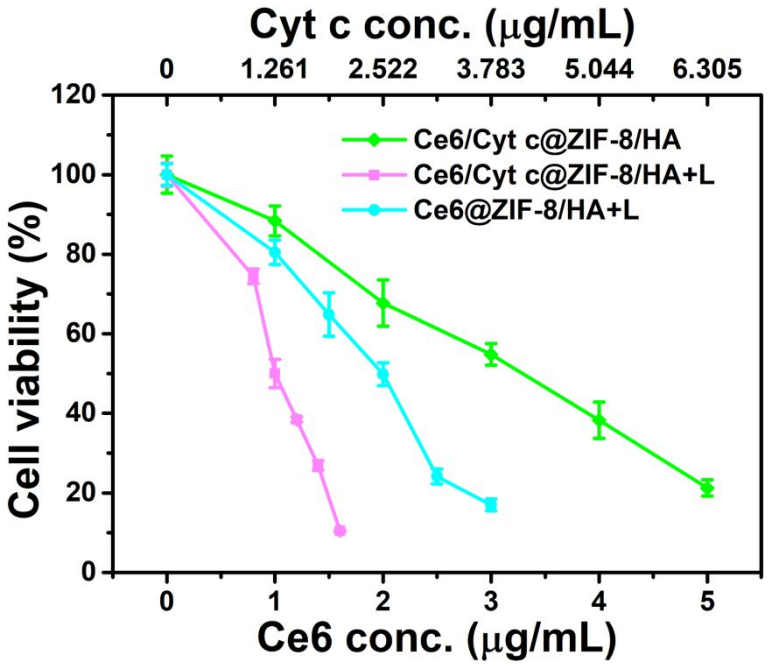

Figure S15. Cytotoxicity assays of Ce6/Cyt c@ZIF-8/HA NPs, Ce6/Cyt c@ZIF-8/HA NPs plus laser and Ce6 @ZIF-8/HA NPs plus laser over 24 h in HeLa cells.

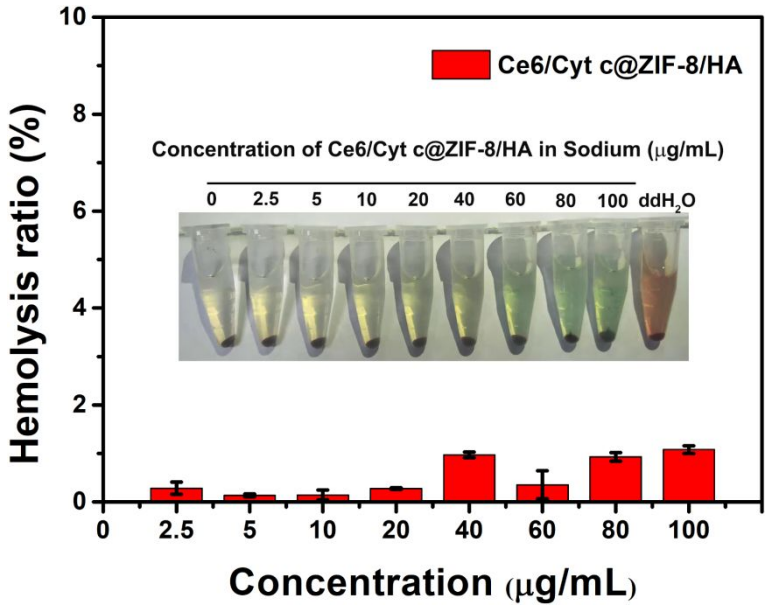

Figure S16. The hemolysis ratio of Ce6/Cyt c@ZIF-8/HA NPs at different concentration.

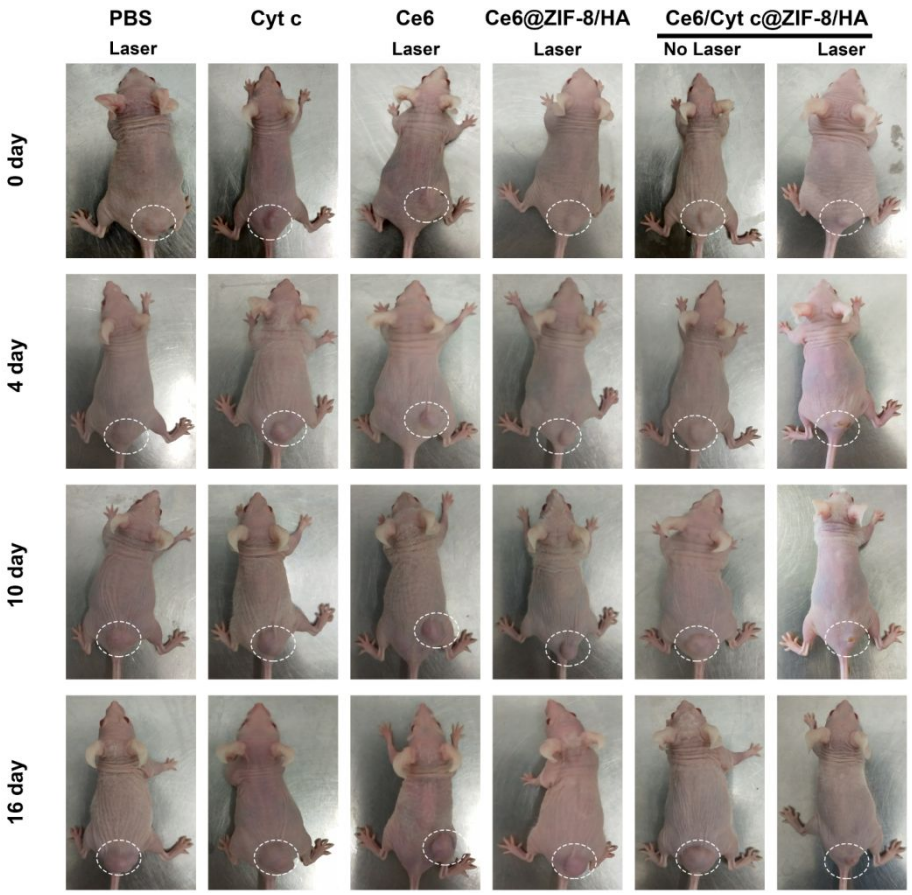

Figure S17. Photographs of the tumor-bearing mice treated with various conditions (PBS, Cyt c, Ce6 plus laser, Ce6@ZIF-8/HA NPs plus laser, Ce6/Cyt c@ZIF-8/HA NPs, Ce6/Cyt c@ZIF-8/HA NPs plus laser) at different time points.

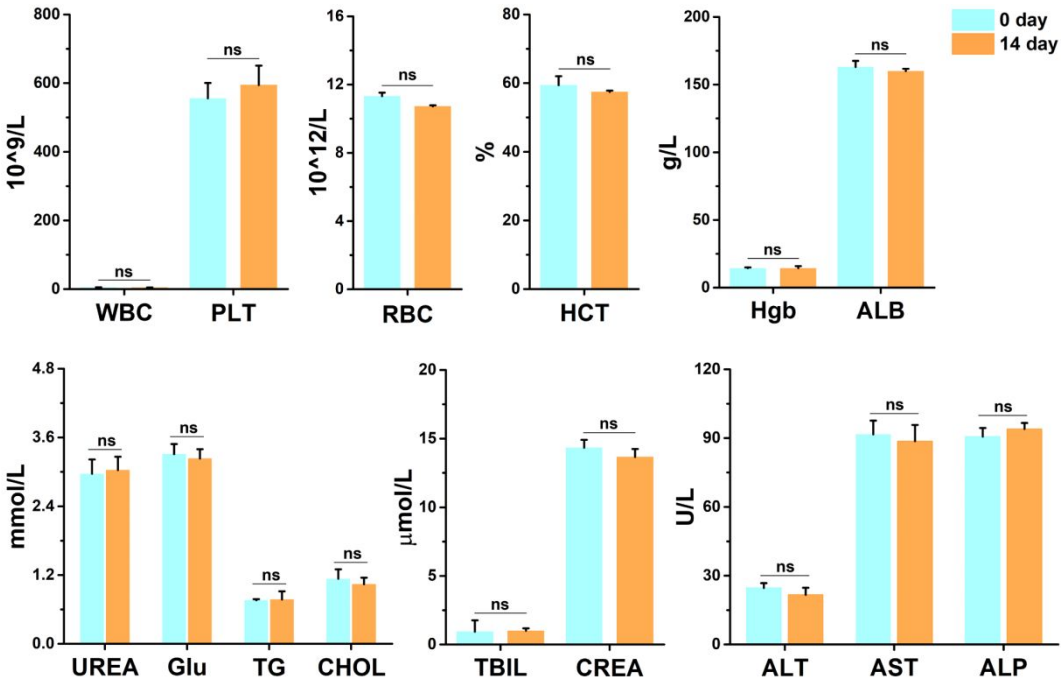

Figure S18. The blood biochemical analysis of health mice intravenously injected with PBS (marked as 0 day in the figure, as control) and Ce6/Cyt c@ZIF-8/HA NPs (marked as14 day in the figure) at 0 day and 14 day. Routine blood tests: white blood cell count (WBC), platelet (PLT), red blood cells (RBC), hematocrit (HCT), hemoglobin (Hgb), albumin (ALB). Kidney function: Urea (UREA), serum creatinine (CREA). Metabolic index: glucose (Glu), triglyceride (TG), total cholesterol (CHOL). Liver function: total bilirubin (TBIL), alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP). (n=3)