Supporting Information

H_2O_2 Self-Supplying Nanoparticle for Chemodynamic Therapy Synergetic Photodynamic Therapy to Augment cGAS/STING Activation

Ai-Hong Zhang[1], Wei-Chuang Kong[1], Xiao-Lei Zhang[1], Ya-Li Meng[1], Zhen-Hui Xin[1], Xiao-Juan Jia[2], Xu-Ying Liu[1,*], Yan-Fei Kang[1,*]

[1] College of Laboratory Medicine, Institute of Pathogen Biology and Immunology, Hebei Key Laboratory of Neuropharmacology, Hebei Key Laboratory of Quality & Safety Analysis-Testing for Agro-Products and Food and Zhang Jiakou Key Laboratory of Organic Light Functional Materials, Hebei North University, Zhangjiakou, 075000, Hebei Province, China.

[2] CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China.

*Corresponding author, E-mail addresses: liuxuying0045@163.com (X.-Y. Liu); kangyanfei172@163.com (Y.-F. Kang).

Contents

1. Experimental section

1.1 Materials

Polyvinylpyrrolidone (PVP, average MW 40000), hydrogen peroxide (H_2O_2, 30%), copper chloride dihydrate ($CuCl_2$•$2H_2O$), sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), potassium permanganate ($KMnO_4$), dimethyl sulfoxide (DMSO) were acquired from Sino pharm Group Chemical Reagent (China). 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB), 3, 3′, 5, 5′-tetrame thylbenzidine (TMB), 9, 10-anthracenediylbis (methylene) dimalonic acid (ABDA), 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT), chlorine e6 (Ce6) and reduced glutathione (GSH) were obtained from Aladdin Biochemical Technology (Shanghai, China). Hoechst 33342, 2′, 7′-dichloro fluorescin diacetate (DCFH-DA), propidium iodide (Calcein AM/PI) were purchased from Beyotime (Shanghai, China). Mouse IFN-β ELISA Kit, Mouse TNF-α ELISA Kit and Mouse IL-6 ELISA Kit were obtained from Shanghai Enzyme-linked Biotechnology. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and penicillin–streptomycin were bought from Thermo-Fisher Biochemical Products (Beijing, China).

1.2 Instruments

Transmission electron micro scopy (TEM) was performed on a H-7650 electron microscope (Japan). Dynamic light scattering (DLS) was accomplished with a dynamic laser light scatterer (Wyatt, USA). HITACHI F-7000 fluorescence spectrometer was used for analysis of fluorescence maps (Japan). FV1200 laser confocal microscope was used for fluorescence imaging analysis (Japan). MTT assay was measured at 570 nm

by using a microplate reader (Thermo, USA). Confocal laser scanning microscope (CLSM) (CarlZeiss LSM900, Germany) and Fluorescence Microscope (Nikon, Japan) were employed to image cell biological function. Flow cytometry was performed on FACS Calibur erse (Becton Dickinson, USA). The protein quantitative results were obtained from the Image J software (NIH, USA).

1.3 Synthesis of CPN

The synthesis of CPN was referred to previously reported methods [1, 2]. PVP (0.5 g) was dissolved in an aqueous solution containing $CuCl_2$•$2H_2O$ (5 mL, 0.01 M). Then, NaOH (5 mL, 0.02 M) and H_2O_2 (100 μL) were added to the above mixture sequentially. After stirring for 0.5 h, the samples were obtained by centrifugation and washed for three times at 14800 rpm/min.

1.4 Colorimetric Assay of Peroxo Groups

To confirm the presence of peroxo groups in the nanoparticles, the $KMnO_4$ solution was diluted by 0.1 M H_2SO_4 to 40 μg mL^{-1}. Then CPN, H_2O_2 and the as-synthetic Ce6-CPN@CDC was added into the above mixture, respectively. Afterwards, the UV-Vis spectra were measured from 430 to 650 nm.

1.5 Measurement of ·OH Generation

3, 3′, 5, 5′-tetramethyl benzidine (TMB) was used as ·OH generation indicator. 1 mL of CPN was mixed with PBS solution (pH 5.5, 7.4) to make the total volume of 2 mL. CPN was incubated with TMB solution (concentration of 50 μg mL^{-1}), and then UV-Vis spectrophotometer was used to record the absorbance of oxTMB at 652 nm. Similarly, the generation of •OH by Ce6-CPN@CDC could also be evaluated under

similar conditions.

1.6 Cell Culture

293T cell, Mouse breast cancer cell line 4T1 and MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC). They were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO_2 in a humidified incubator.

1.7 ROS Observations in Vitro

DCFH-DA was utilized to detect the generation of ROS, which could be oxidized into fluorescent DCF by cellular ROS. MDA-MB-231 cells were uniformly inoculated in laser confocal petri dishes and cultured in a cell incubator at 37℃ for 24 h. After treating the cells with different nanoparticles for 4 h, the cells were irradiated with a 660 nm laser (60 mW cm^{-2}) for 10 min or not. Then, fresh medium containing DCFH-DA (20 μM) was substituted for the old medium, followed by incubation for 30 min. Eventually, the intensity of DCF fluorescence was immediately observed by CLSM.

1.8 Live/dead Cell Assay

MDA-MB-231 cells were uniformly inoculated in laser confocal petri dishes and cultured in a cell incubator at 37℃ for 24 h. After treating the cells with different nanoparticles for 24 h, the original medium was discarded. Calcein AM and PI staining solution were added to each petri dish. After 30 min of dark staining at 37℃, laser confocal microscopy was used to observe the staining of living cells and dead cells under different nanoparticles.

1.9 Apoptosis Assay

MDA-MB-231 cells were inoculated in 6-well plates and cultured in a cell incubator at 37°C for 24 h. The original medium was discarded. Nanoparticle treatments were performed as previously described. The suspended cells in the original medium were collected and the adherent cells were digested into single cell suspension with trypsin. Centrifugation was conducted at 1080 rpm for 7 min and the supernatant was discarded. The cells were washed twice with PBS and centrifuged for collection. Annexin V Apoptosis Detection Kit I was used for flow cytometry.

1.10 Hemolysis Assay

After collecting venous blood from mice, the red blood cells (RBCs) were obtained by centrifugation (3000 rpm, 15 min) and the supernatant was discarded. Ce6-CPN@CDC at various concentrations was mixed with the 2% suspension of RBCs. Negative and positive controls were respectively obtained by incubating RBCs with PBS and deionized water. Incubation of the samples at 37°C for 4 h followed by centrifugation was performed. A 542 nm absorbance measurement was conducted on the sample supernatant. Under the same conditions, the hemolysis of Ce6@CDC was calculated. Hemolysis was calculated from Equation (1):

$$\text{Hemolysis}\ (\%) = \frac{\text{OD (test) - OD (PBS)}}{\text{OD (ddH2O) - OD (PBS)}} \times 100\ \% \quad , \quad (1)$$

1.11 CRT Expression in MDA-MB-231 Cells

MDA-MB-231 cells were inoculated in laser confocal culture dish and incubated for 24 h. It was replaced with a 1 mL new medium containing Ce6-CPN@CDC and Ce6@CDC. After 24 h of nanoparticle treatment, the original medium was discarded.

Normal temperature TBS was slowly added to the cells and washed twice, 5 s each time. Freshly prepared CRT primary antibody working solution (200 μL) was added to the sample and incubated at 4℃ overnight. The primary antibody working solution was removed and washed once (5 min) with TBS-T buffer solution, followed by three times (5 min) with TBS buffer solution. Cy3-labeled secondary antibody working solution (1:500) was added to the samples and incubated at 37℃ for 1 h. The secondary antibody working fluid was removed and washed once (5 min) with TBS-T buffer, followed by three times (5 min each) with TBS buffer. Hoechst 33342 was added to each petri dish and stained for 5 min at room temperature. Immunofluorescence images were acquired by CLSM.

1.12 HMGB1 Release of MDA-MB-231 Cells

The expression of HMGB1 protein in MDA-MB-231 cells was imaged by immunofluorescence assay. The specific operation method was the same as the expression of detecting CRT.

1.13 ATP Release Assay

An ATP Assay Kit was used to detect the released ATP. MDA-MB-231 cells were seeded into T25 cell culture flask and incubated for 24 h. The cells were incubated in 2 mL fresh medium containing Ce6-CPN@CDC and Ce6@CDC. After centrifugation (12000 rpm, 4ºC, 5 min), the supernatant was taken and placed on ice for testing. The fluorescence intensity of ATP was assessed via a 96-well fluorescence microplate reader.

1.14 Scratching Test

MDA-MB-231 cells and 293T cells were inoculated in 6-well plates and cultured in a cell incubator at 37℃ for 24 h. The original medium was discarded. Nanoparticle treatments were performed as previously described. The initial scratch width and the scratch width after 24 h of incubation were recorded separately using an inverted fluorescence microscope.

1.15 Western Blot

MDA-MB-231 cells were seeded in six-well plates and incubated for 24 h, the original medium was discarded. After 24 h of treatment with different concentrations of nanoparticles, the cells were lysed with lysates and the protein content was collected and measured. The protein was separated by gel electrophoresis and moved to a polyvinylidene fluoride (PVDF) membrane. Then it was incubated with specific primary antibodies at 4°C overnight, followed by the corresponding secondary antibody. Finally, protein imaging analysis was performed.

1.16 In Vivo Biodistribution

Female BALB/c mice (6 weeks, 18−20 g each) were purchased from Sibeifu (Beijing) Biotechnology Co., Ltd. BALB/c mice were inoculated with 4T1 cells on both right gluteal region. When the tumor volumes were 150−200 mm^3, the mice were injected with Ce6-CPN@CDC via the tail vein. The mice were imaged using IVIS Lumina III at 0, 6, 12, 24, 36 and 48 h to show the fluorescence signals. For the biodistribution analysis, the mice were sacrificed after intravenous injection for 48 h, and the tumors and major tissues were harvested and subjected to fluorescence imaging.

1.17 ELISA Assay

The immune-related cytokines from tumors including IFN-β, TNF-α, and IL-6 were determined by ELISA kits, and the specific operation methods were carried out according to standard protocols.

2. Supporting Figures

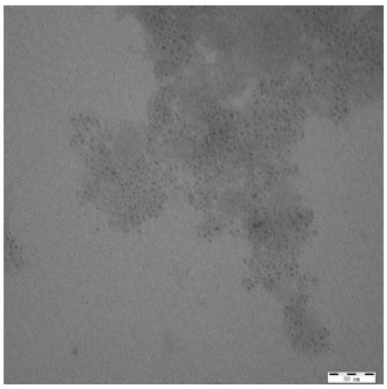

Fig. S1. TEM image of the synthesized CPN.

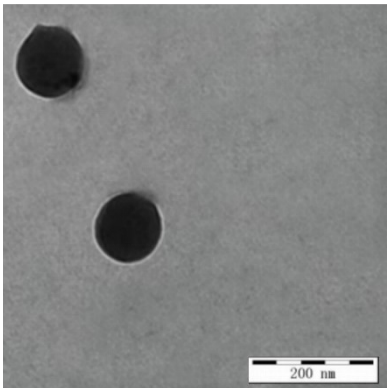

Fig. S2. TEM image of the synthesized Ce6-CPN@CDC.

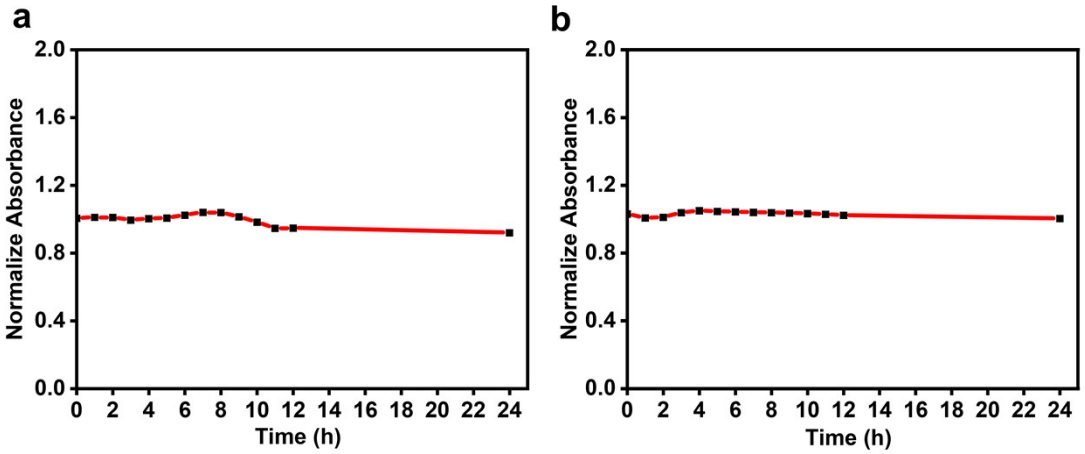

Fig. S3. Stability evaluation of nanoparticle. Absorption of Ce6-CPN@CDC in (a)

deionized water or (b) DMEM within 24 h.

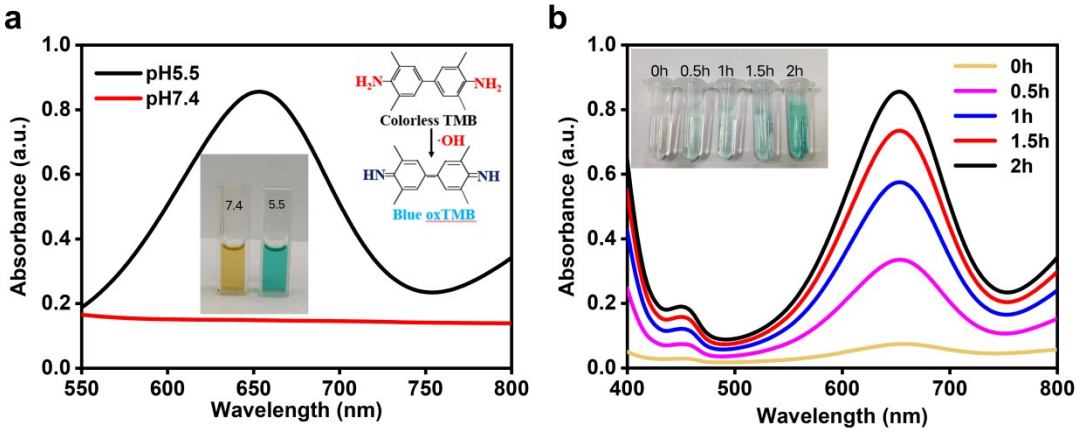

Fig. S4. (a) UV–Vis absorption spectra of oxTMB solutions treated with CPN under different pH conditions. Inset: Photos of the corresponding samples and the •OH detection reaction based on TMB. (b) The ability of CPN to generate •OH increases over time. Inset: Photos of solutions with different time before and after the addition of TMB.

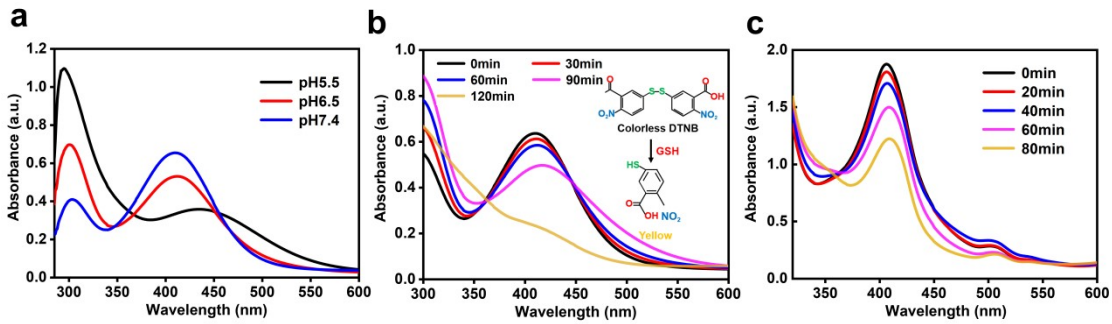

Fig. S5. (a) pH-dependent GSH depletion of CPN reacted with DTNB; (b) UV-Vis

spectra of DTNB solutions after treatment with CPN at pH 5.5 for different time. Inset: The GSH detection reaction based on DTNB. (c) Time-dependent GSH depletion of Ce6-CPN@CDC reacted with DTNB.

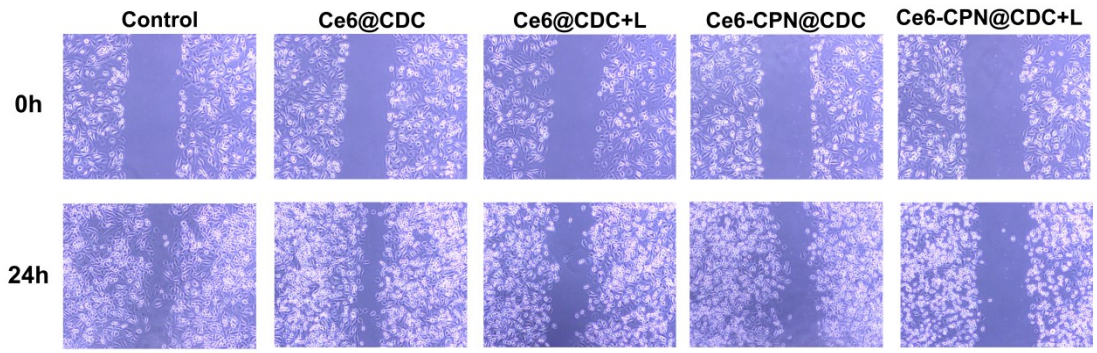

Fig. S6. Microscopy images of wound healing of MDA-MB-231 cells after treatment with PBS, Ce6@CDC and Ce6-CPN@CDC with laser or without laser for 24 h.

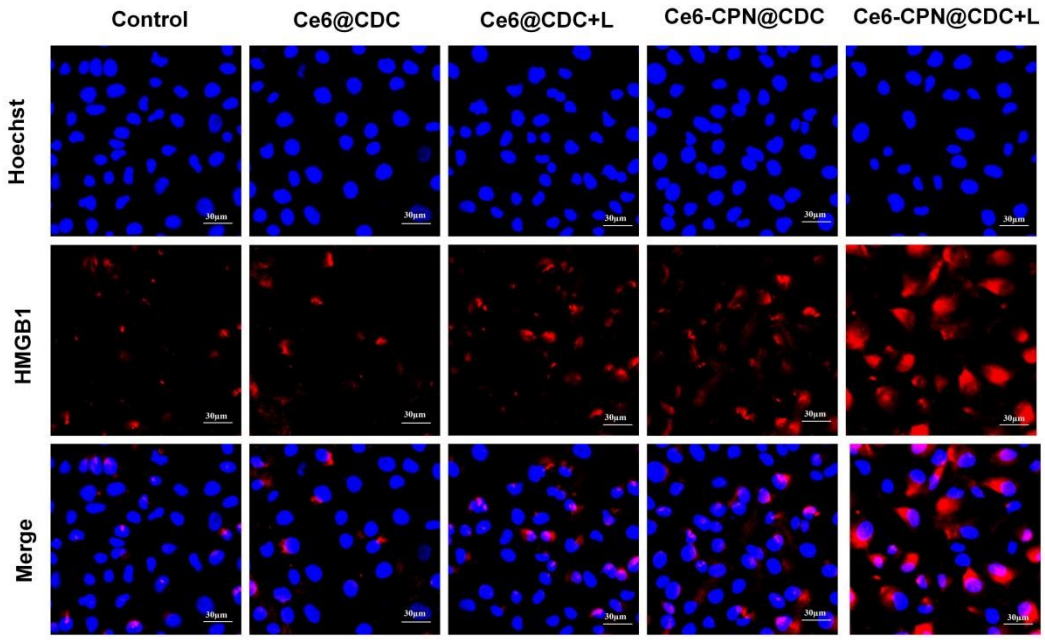

Fig. S7. CLSM images of HMGB1 expressed in MDA-MB-231 cells treated with PBS, Ce6@CDC and Ce6-CPN@CDC with laser or without laser for 24 h.

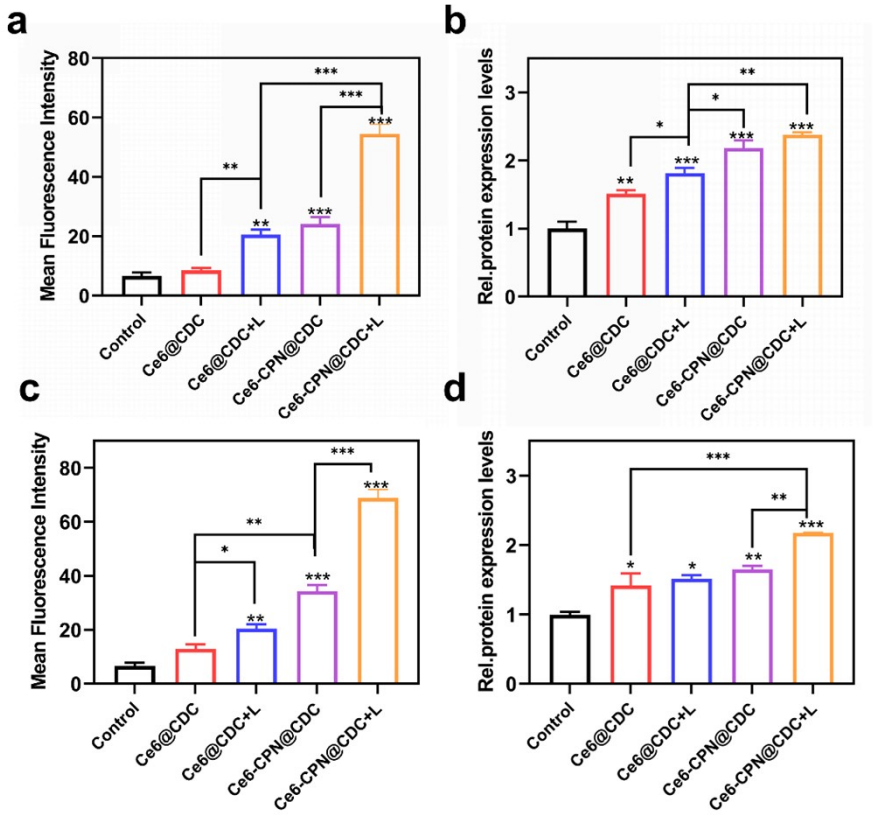

Fig. S8. (a) Quantitative analysis of the mean fluorescence intensity (MFI) of CLSM

images of CRT expressed in MDA-MB-231 cells. (b) Western blot quantification results of CRT in MDA-MB-231 cells after 24 h of treatment. (c) Quantitative analysis of the mean fluorescence intensity (MFI) of CLSM images of HMGB1 expressed in MDA-MB-231 cells. (d) Western blot quantification results of HMGB1 in MDA-MB-231 cells after 24 h of treatment. Scale bars: 30 μm. n=3, *P < 0.05, significant; **P < 0.01, moderately significant; ***P < 0.001, highly significant.

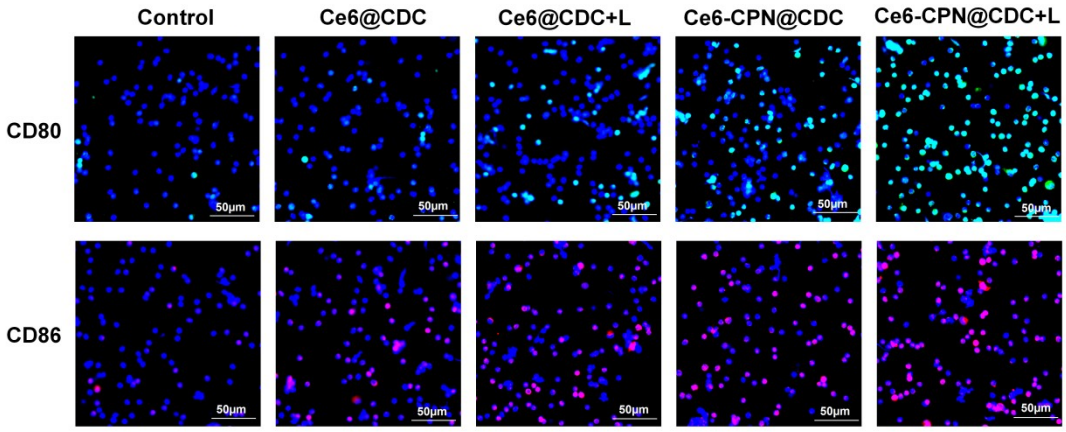

Fig. S9. The expression of CD80 and CD86 in mice was analyzed by CLSM.

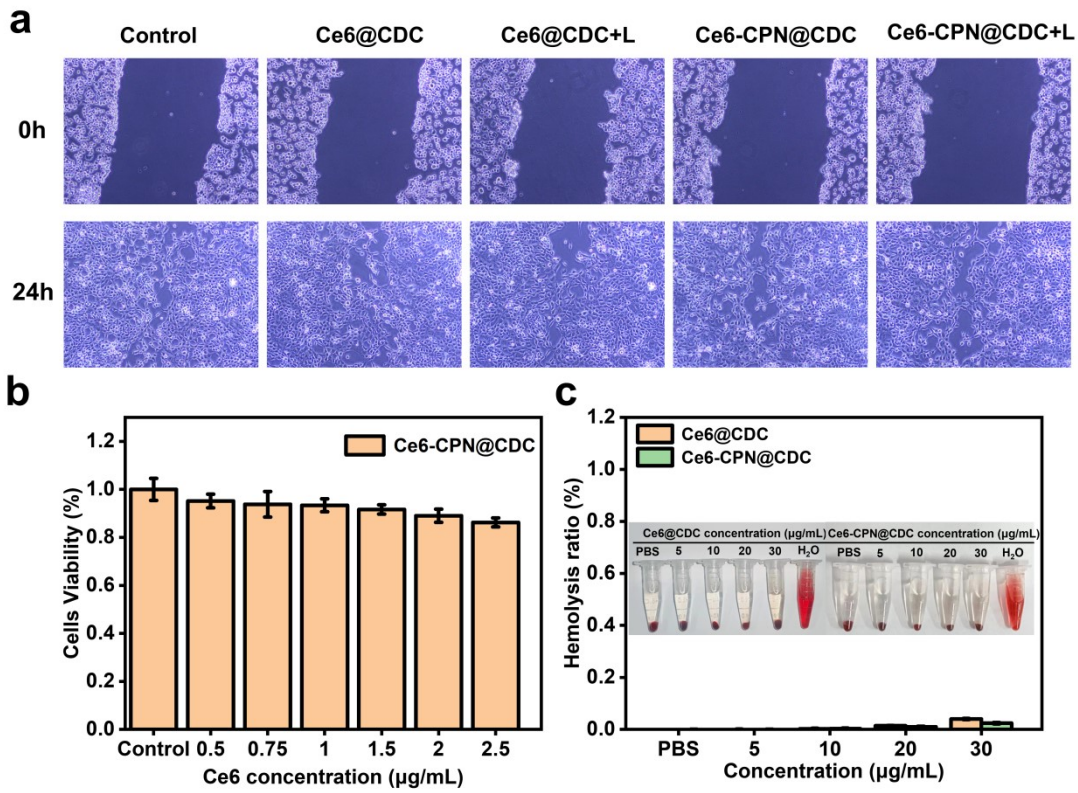

Fig. S10. (a) Scratched wound photos of 293T cells treated with PBS, Ce6@CDC and Ce6-CPN@CDC with laser or without laser. (b) The cell viabilities of 293T cells upon incubation with Ce6-CPN@CDC. (c) Hemolysis rates of RBC in water, Ce6@CDC and Ce6-CPN@CDC at various concentrations. Inset: The corresponding digital photograph.

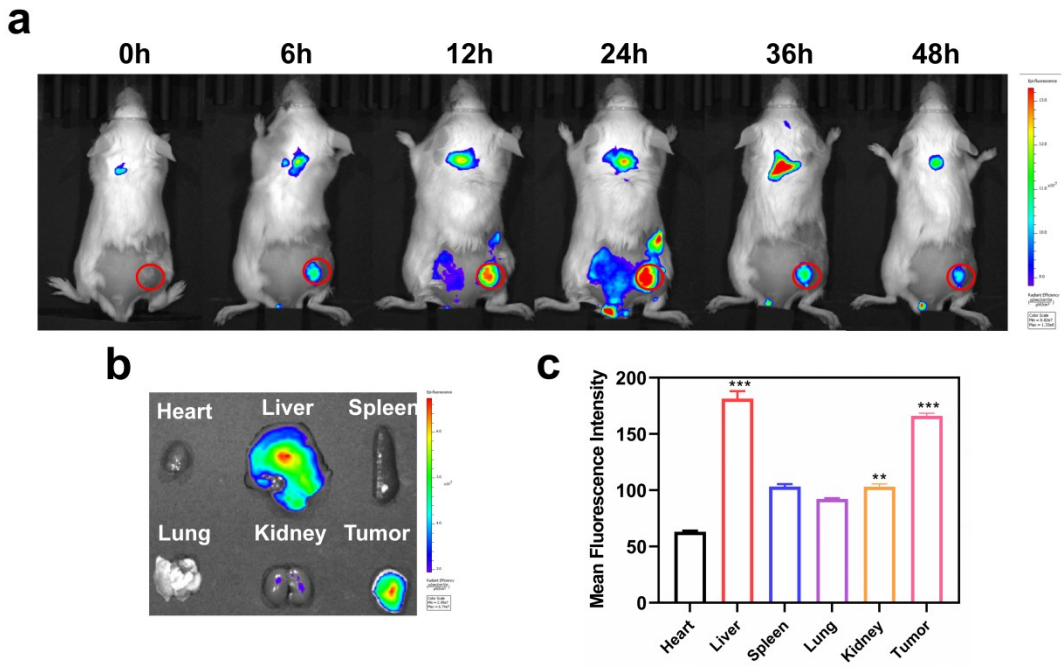

Fig. S11. (a) Fluorescence imaging of BALB/c bearing mice after intravenous injection of Ce6-CPN@CDC at 0 h, 6 h, 12 h, 24 h, 36 h and 48 h. (b) *Ex vivo* fluorescence imaging of major organs and tumor after 48 h post-injection of the Ce6-CPN@CDC. (c) Quantitative analysis of fluorescence intensity of (b).

References

1. Z. Li, C. Wang, C. Dai, R. Hu, L. Ding, W. Feng, H. Huang, Y. Wang, J. Bai and Y. Chen, *Biomaterials*, 2022, **287**, 121668.
2. L. S. Lin, T. Huang, J. Song, X. Y. Ou, Z. Wang, H. Deng, R. Tian, Y. Liu, J. F. Wang, Y. Liu, G. Yu, Z. Zhou, S. Wang, G. Niu, H. H. Yang and X. Chen, *J. Am. Chem. Soc.*, 2019, **141**, 9937-9945.