Supporting Information

Hollow Gold-Platinum Nanoshells as a Delivery Platform for Ce6: Cascading Catalysis for Enhanced Multimodal Therapy in Tumor Ablation and Antitumor Immunity

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Experimental section

Materials and instruments

Zirconium chloride ($ZrCl_4$), 2-aminoterephthalic acid (BDC-NH_2) and 3,3',5,5'-Tetramethylbenzidine (TMB) were purchased from Shanghai Macklin Biochemical Co., Ltd. 1,3-Diphenylisobenzofuran (DPBF) was purchased from Bide Pharmatech Co., Ltd. ATP assay kit was purchased from Shanghai Beyotime Biological Co., Ltd. 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) and 4',6-diamidino-2-phenylindole (DAPI) solution were obtained from Beijing Solarbio Science & Technology Co., Ltd. Annexin FITC/PI apoptosis detection kit was purchased from Yeasen Biotechnology (Shanghai) Co., Ltd. Calregulin polyclonal antibody (CRT), HMG-1 polyclonal antibody (HMGB1) and hypoxia-inducible factor-1alpha (HIF-1α) antibody (rabbit mono-IgG) was acquired from Proteintech Group Inc, USA. The secondary antibodies, IgG/Alexa Fluor®594 (goat anti-rabbit) and IgG/FITC (goat anti-rabbit) were obtained from Proteintech Group Inc, USA. Antibodies used for flow cytometry were all purchased from Elabscience Biotechnology Co., Ltd.

The morphology and size of the nanoparticles were examined by transmission electron microscopy (TEM) on JEOL JEM-2100F. Scanning electron microscopy (SEM) was conducted by EMAXevolutin X-Max80/EX-270. High-angle annular dark-field scanning transmission electron microscopic (HAADF-STEM) images and elemental mapping images were taken on JEOL JEM-2100F operated at an accelerating voltage of 200 kV. Dynamic light scattering (DLS) and zeta potential were analyzed with a Zetasizer Nano ZS from MALVERN Instruments. The UV-Vis absorption spectra were collected with a UV-Vis Spectrometer Lambda 25 from PerkinElmer Instruments. The inductively coupled plasma–mass spectrometry (ICP-MS) was collected with an ICAPPROI from Thermo Fisher instruments. The laser at 660 nm was carried on the 0660L instrument, and the laser at 1064 nm was carried on the VCL-MO instrument. The optical density (OD) values were collected using a TECAN M1000PRO microplate reader. The microscope images were collected using an Olympus FV3000 confocal laser scanning

microscope (CLSM) and a LSM 880 with Airyscan confocal laser scanning microscope (CLSM). The flow cytometry was performed using a BD FACS Caliber instrument.

Preparation of UiO-66-NH_2

The solution of $ZrCl_4$ (42 mg, 0.18 mM) in DMF (2.5 mL) was added into the solution of BDC-NH_2 (33 mg, 0.18 mM) in DMF (1.0 mL). Then, the deionized water (100 μL) and acetic acid (1.7 mL) were added and dispersed by sonication for 30 min. The final reaction solution was transferred to an autoclave and crystallized statically at 120 ℃ for 2 h. Subsequently, it was cooled down to room temperature, centrifuged (8,000 rpm, 5 min), and precipitates were collected, washed three times with DMF and ethanol, respectively, and finally dispersed in 30 mL of deionized water and reserved for use.

Preparation of UAuPt

The mixture of chloroplatinic acid (H_2PtCl_6, 12 mg) and chloroauric acid ($HAuCl_4$, 12 mg) in 1.2 mL of deionized water was added to the solution of K_2CO_3 (50 mg) in 42 mL of deionized water, followed by stirring at room temperature for 15 min. The aqueous of UiO-66-NH_2 (1.5 mg/mL,1.8 mL) and the aqueous PVP (1.0 mg/mL, 1.8 mL) were mixed and added to the above solution, followed by stirring at room temperature for 24 h. A pure formaldehyde (0.5 mL) was added, and stirring continued for 30 min. The reaction solution was collected, centrifuged, washed with deionized water for 5 times, and dispersed in deionized water for use.

Preparation of HAuPt

The dispersion of UAuPt (50 mg) in aqueous solution was added into the aqueous solution of sodium bicarbonate (10 M, 25 mL). The reaction was slowly stirred for 24 h at 70 ℃. The products were centrifuged (8,000 rpm, 3 min), washed with deionized water for 3 times, and finally dispersed in deionized water for use.

Preparation of HCP

The aqueous solution of HAuPt (2 mg/mL, 1 mL) was mixed with aqueous solution of PEG-SH (10 mg/mL, 1 mL) and stirred at room temperature for 12 h, centrifuged (10000 rpm, 5 min), washed once

with deionized water. 1 mL of deionized water was dispersed and stirred at room temperature. Subsequently, Ce6 in DMSO (200 μL, 2 mg/mL) was slowly added dropwise, followed by continuous stirring at room temperature for 12 h. Then, centrifugation was performed (10000 rpm, 5 min), and the resultant was washed twice with deionized water. Finally, 1 mL of deionized water was dispersed for further utilization.

Preparation of UiO-66-NH_2@Au

UiO-66-NH_2@Au was synthesized using methods from the literature with slight modification[1]. Configure a 21 mL solution of K_2CO_3 (1.1 mg/mL) and 1 mL $HAuCl_4$ (10 μg/mL) solution. The two solutions were mixed and stirred for 15 min at room temperature. Then 900 μL PVP solution (1 mg/mL), 1 mL UiO-66-NH_2 (1.5 mg/mL) solution and 40 μL formic acid/formaldehyde solution (*v*/*v* = 0.6‰) were added to the mixed system. Stirring was continued at room temperature for 10 min and the precipitate was collected by centrifugation (8000 rpm, 3 min). The precipitate was washed 3 times using deionized water and distributed in 1 mL of deionized water.

Ce6 loading efficency

To calculate the Ce6 loading efficiency, a standard curve was created by measuring the UV-Vis absorbance of different concentrations of standard Ce6. After the loading of Ce6 was completed, the HCP was centrifuged,and the supernatant was collected. The UV-Vis absorbance of the supernatant was then measured to determined the amount of Ce6 remaining, which was used to calculate the loading rate of Ce6.

Loading efficiency was calculated according to the equation:

$$Loading\ efficiency\ (\%) = \frac{Ce6\ (total) - Ce6\ (supernatant)}{Sample\ amount} \times 100\%$$

Where Ce6 (total), Ce6 (supernatant) and sample amount are the total amount of Ce6, the amount of Ce6 in the supernatant and the total amount of the sample of HCP, respectively.

Glucose oxidase activity in aqueous solutions

To study the glucose oxidase activity of HCP, the hydroxylamine ferric chloride method was used to detect gluconic acid produced from glucose catabolism. Solution 1: EDTA (14.6 mg) and trimethylamine hydrochloride (14.3 mg) were dissolved in 10 mL of water. Solution 2: neutral hydroxylamine (890 μL) was dissolved in 5mL of water. Solution 3: hexahydrate ferric chloride (270 mg) and trifluoroacetic acid (408 mg) were dissolved in 10 mL of water and 830 μL hydrochloric acid (1 M) was added. 5 mL of glucose solution (4.5 mg/mL) was incubated with different concentrations of HCP at 37 ℃ for 4 h, and was centrifuged. 1 mL of supernatant was taken, then 250 μL of solution 1 and 25 μL of solution 2 were added and incubated at 37 °C for 25 min. Finally, 125 μL of solution 3 was added and incubation was continued for 5 min. The UV-Vis absorption at 505 nm was measured.

Catalase activity in aqueous solutions

To study the catalase activity of UAuPt, HAuPt and HCP, each of them was incubated with H_2O_2 (1 mM) in water while ensuring a consistent concentration of Pt. The O_2 production was then monitored in real time using a dissolved oxygen meter.

Peroxidase activity in aqueous solutions

To study the peroxidase activity of HCP, the production of •OH by HCP was monitored using 3,3',5,5'-tetramethylbenzidine (TMB), which can be oxidized by •OH to blue oxidized TMB (oxTMB), exhibiting UV-Vis absorption at 370 nm and 652 nm. The UV-Vis absorption was first determined in the PBS buffer (pH 5.5) using H_2O_2 (100 μM), TMB (0.5 mM) and different concentrations of HCP co-incubated at 37°C for 30 min. Then H_2O_2 (100 μM), TMB (0.5 mM) and UAuPt, HAuPt and HCP were used for co-incubation and UV-Vis absorption were determined while ensuring the same concentration of Pt.

Properties of PDT in aqueous solutions

To study the photodynamic effect of HCP, 1,3-Diphenylisobenzofuran (DPBF) was used to monitor the $^1O_2$ produced by HCP at the solution level, and the PDT effect was shown by monitoring the change in the UV-Vis absorption intensity at 410 nm. The concentration of HCP was 100 μg/mL. The samples were

irradiated with a 660 nm laser (0.22W/cm^2) or left in the dark, and then the UV-Vis absorption spectroscopy was used to determine the absorption value of DPBF at 410 nm.

Properties of PTT in aqueous solutions

To investigate the photothermal effect of HCP, the same concentration of HCP was exposed to 1064 nm laser with different power densities, and then different concentrations of HCP were exposed to 1064 nm laser with the same power densities, and the temperature values were recorded once in each 30 s. Three photothermal cycles were then performed to calculate the photothermal conversion efficiency of HCP. Finally, a temperature thermography comparison of water, UAuPt, HAuPt, and HCP was performed.

Photothermal conversion efficiency (η) was calculated according to the equations:

$$t = -\tau_s ln\theta \quad (1)$$

Where t represents the time of the photothermal process. τ_s is the system constant of the photothermal sample. θ is the dimensionless driving force.

$$\theta = \frac{T - T_{surr}}{T_{max} - T_{surr}} \quad (2)$$

Where T is the real-time temperature of the photothermal sample. T_{surr} is the ambient temperature. T_{max} is the maximum temperature at which the photothermal sample warms up.

$$\tau_s = \frac{m_D C_D}{hS} \quad (3)$$

Where m_D and C_D are the mass (1 g) and heat capacity (4.2 J•g^{-1}) of water, respectively. h denotes the heat transfer coefficient. S is the surface area of the cuvette.

$$Q_s = \frac{m_D C_D}{\tau_{water}}(T_{max,H_2O} - T_{surr}) \quad (4)$$

Where Q_s represents the heat loss from light absorbed by the solvent and cuvette.

$$\eta = \frac{hS(T_{max,\,sample} - T_{surr}) - Q_s}{I\left(1 - 10^{-A_{1064}}\right)} \quad (5)$$

Where η represents the photothermal conversion efficiency. I is the power density (W/cm^2) of the laser used for the photothermal process. A_{1064} represents the absorbance value of the photothermal nanosystem at 1064 nm.

Cell culture

Mouse breast cancer 4T1 cells and mouse macrophage RAW264.7 cells were purchased from the School of Pharmaceutical Sciences, Southern Medical University. 4T1 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (procell), and 1% antibiotic mixture (10,000 U penicillin and 10 mg streptomycin) at 37 ℃, 5% CO_2. RAW264.7 cells were cultured in DMEM high glucose medium containing 10% fetal bovine serum (FBS) (procell), and 1% antibiotic mixture (10,000 U penicillin and 10 mg streptomycin) at 37 ℃ with 5% CO_2.

Cell uptake

To study the uptake behavior of 4T1 cells towards HCP, 4T1 cells (1×10^6 cells/dish) were inoculated in glass culture dishes and cultured for 24 h. Then, 20 μg/mL of HCP was added for further incubation at 0, 2, 4, 6 and 8 h. Subsequently, the medium was discarded and the cells were washed three times with PBS and applied to CLSM (λex = 405 nm; λ em = 650 nm).

Intracellular ROS detection

Detection of the ROS generated by HCP was preformed using the DCFH-DA kit. 4T1 cells (1×10^5 cells/well) were seeded into 12-well plates and incubated under normoxic conditions for 12 h to allow adherence. Then the medium was changed and the cells were incubated under either normoxic or hypoxic conditions for an additional 12 h. Next, HCP or Ce6 was added to the cells, which were then incubated for 6 h. Following this, the cells were washed with basal medium, and then basal medium containing DCFH-DA (10 μM) was added. After another 20 min of incubation, the cells were washed twice with basal medium. Subsequently, different protocols of treatment were performed. Fluorescence microscopy imaging of intracellular ROS levels was performed immediately after treatment (λ_{ex} = 488 nm; λ_{em} = 530 nm).

MTT assay

MTT method was used to detect the toxicity of different treatments for 4T1 cells. 4T1 cells (5×10^3 cells/well) were inoculated in 96-well plates and cultured for 24 h. Different concentrations of HCP (0, 20, 40, 60, 80, 100 μg/mL) were added and incubated for 24 h. Subsequently, the treatments were carried out in different protocols and continued to be incubated for 24 h. The medium was discarded and configured MTT solution (100 μL/well, 0.5 mg/mL) was added and incubated for 4 h. Then, the solution was discarded and DMSO (100 μL/well) was added and incubated for 10 min on a shaker. The optical density (OD) value at 490 nm was measured using an enzyme marker.

For RAW 264.7 cells, the cells (1.5×10^3 cells/well) were inoculated in 96-well plates and cultured for 24 h. Different concentrations of HCP (0, 20, 40, 60, 80, 100 μg/mL) were added and incubated for 24 h. The medium was discarded, and a configured MTT solution (100 μL/well, 0.5 mg/mL) was added. After incubation for 4 h, the solution was discarded and DMSO (100 μL/well) was added and incubated for 10 min on a shaking bed. The OD value at 490 nm was detected using an enzyme marker.

Flow cytometry apoptosis assay

To investigate the anticancer activity of HCP for 4T1 cells, 4T1 cells (1×10^5 cells/well) were inoculated into 12-well plates and cultured for 24 h. 20 μg/mL of HCP was added and incubated for 6 h. Subsequently, different protocols of treatments were performed and the incubation was continued for 12 h. Cells were collected and the Annexin V-FITC/PI apoptosis kit was applied for flow cytometry analysis.

Fluorescence imaging of live/dead cells

To investigate the anticancer activity of HCP on 4T1 cells, 4T1 cells (5×10^4 cells/well) were inoculated into 24-well plates and cultured for 24 h. 20 μg/mL of HCP was added and incubated for 6 h. Subsequently, different protocols of treatments were performed and the incubation was continued for 12 h. Cells were collected and the calcein-AM/PI cell live/dead fluorescence kit was applied for fluorescence microscopy imaging.

Immunogenic cell death in vitro

For immunofluorescence of HMGB1, 4T1 cells (1×10^5 cells/dish) were inoculated in a glass dish and incubated for 24 h, then 20 μg/mL HCP was added and incubated for 6 h, followed by different treatments, and the incubation was continued for 0.5 h. After the medium was removed, the cells were washed three times with PBS. They were then fixed using 4% paraformaldehyde. Subsequently, the cell membranes were broken with a PBS solution containing 0.1% Triton X-100 and, and non-specific binding sites were blocked by incubating the cells in a PBS solution of 5% BSA. The HMGB1 rabbit source primary antibody was added and incubated at 4 ℃ for 15 h. The fluorescent secondary antibody was then incubated at room temperature and protected from light for 3 h. Afterward, an anti-fluorescence quenching sealant containing DAPI was added. The sample was subsequently analyzed using confocal laser scanning microscopy (CLSM).

For immunofluorescence of CRT, The steps are the same as HMGB1, but membrane rupture is not required.

For ATP release, 4T1 cells (1×10^5 cells/well) were inoculated in 12-well plates and cultured for 24 h. Then 20 μg/mL HCP was added and incubated for 6 h. Next, different treatments were carried out and the incubation was continued for 2 h. The supernatant was collected and analyzed using an ATP assay kit to detect the release of ATP.

Macrophage repolarization in vitro

RAW 264.7 cells (5×10^5 cells/well) were inoculated in 6-well plates, and then the incubation was continued for 24 h using the cytokine interleukin 4 (IL-4, 20 ng/mL) to induce the polarization of RAW 264.7 cells into M2-type macrophages. The supernatants from 4T1 cells under different treatments were collected and added to RAW 264.7 for co-incubation for 24 h. After incubation, the cells were collected, treated with antibodies (CD206 and F4/80 OR CD86 and F4/80) and then analyzed by flow cytometry.

In vitro BMDCs activation

Tibias of C57BL/6 mice (7-8 weeks) were taken to extract the bone marrow cells for culture, and 20 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF) was added to the culture medium

to stimulate cell differentiation. After culture to day 7, BMDCs were collected in 6-well plates (1×10^7 cells/well). The supernatants from 4T1 cells under different treatments were collected and added to BMDCs for co-incubation for 24 h. The cells were then collected, stained with antibodies (CD11c, CD80 and CD86) and analyzed by flow cytometry.

Intracellular oxygen examination

4T1 cells (1×10^5 cells/culture dish) were inoculated into a CLSM specialized culture dish and culture for 24 h. Then continued to cultivate under hypoxic conditions for 24 h. Then 30 μM [Ru (dpp) $_3$] Cl_2 was added and incubated for 4 h. Then, the cells were incubated with 0.1 mM H_2O_2 and HCP (20 μg/mL) for 6 h. Then, we washed the cells three times with PBS and performed imaging using CLSM (λ_{ex}=488 nm; λ_{em}=620 nm).

Cell migration

We inoculated 4T1 cells (3×10^5 cells/well) into a 6-well plate and cultured for 24 h, followed by 12 h of hypoxia. Subsequently,the cells were scratched with a 10 μ L pipette tip, and washed with PBS three times, then culture medium with fetal bovine serum concentration of 1% and HCP were added. And we set time points of 0 h, 12 h, 24 h, and 36 h. Finally, we used an inverted fluorescence microscope to capture bright field images.

Hemolysis

SPF grade BALB/c mice were subjected to blood sampling by removing the eyeballs, and the blood was collected in anticoagulated tubes. The cells were collected by centrifugation at 4 ℃ (3000 rpm, 30 min), washed with appropriate amount of PBS, and 2 μL erythrocytes were incubated with different concentrations of HCP (40, 80, 120, 160, 200, 240, 280, 320 μg/mL). PBS was used as a negative control and 1% Triton as a positive control. The solutions were incubated at 37 ℃ for 1 h, centrifuged (3000 rpm, 10 min), photographed, and the supernatant was taken and the absorbance at 540 nm was measured using an enzyme marker.

Establishment of mouse models of subcutaneous breast cancer

Female Balb/c mice (3-4 weeks old) were purchased from the Animal Experiment Center of Southern Medical University. All animal experiments were used in accordance with the requirements of the Ethics Committee of Southern Medical University (SYXK2021-0041). To construct an animal breast cancer model, cultured 4T1 cells (1×10^6 cells per mouse) were injected into the subcutaneous layer of the right dorsal side near the hind limb, followed by natural growth of the mice for 7 days.

Antitumor effects in mice

When the tumor volume reached 80-100 mm^3, the mice were randomly divided into 5 groups (PBS, HCP, HCP + PTT, HCP + PDT, HCP + PTT + PDT), 5 mice in each group. The drug was administered intravenously at a drug dose of 10 mg•kg^{-1}. Mice in each group were subjected to different treatments 12 h later (660 nm laser irradiation, 0.45 W/cm^2, 5 min, and/or 1064 nm laser irradiation, 1 W/cm^2, 5 min). Changes in body weight and tumor volume of the mice were measured daily.

Abscopal effect and antitumor immunity studies in a bilateral 4T1 tumor model

To assess the immunomodulatory effects of HCP *in vivo*. The bilateral 4T1 cell mouse tumor model was constructed, and mice were randomly divided into 5 groups (PBS, HCP, HCP + PTT, HCP + PDT, HCP + PTT + PDT) of 5 mice each when the primary tumor volume reached 60 mm^3. The drug was administered by in situ injection at the primary tumor site with a drug dose of 10 mg•kg^{-1}. 6 h later, the primary tumors of mice in each group were treated differently (660 nm laser irradiation, 0.45 W/cm^2, 5 min, and/or 1064 nm laser irradiation, 1 W/cm^2, 5 min). The distant tumors were not subjected to any treatment. The body weights of the mice and the volumes of the primary and distant tumors were recorded daily for 15 days.

Another set of bilateral 4T1 cell mouse tumor models were constructed, treated consistently as described above, and after 3 days of light treatment, the mice were euthanized and biological samples including peripheral blood, tumor-draining lymph nodes, primary tumors, and distal tumors were collected for subsequent biological experimental analyses.

For tumor-draining lymph nodes, lymph nodes were clipped with scissors, mechanically ground to obtain cell homogenates and passed through a 70 μm cell strainer to obtain single-cell suspensions, then incubated with antibodies and then analyzed by flow cytometry for DC cell maturation status.

For primary tumors, the tumor tissue was incubated with antibodies and then applied to immunofluorescence to detect the proportion of macrophages of different phenotypes.

For primary and distant tumors, the tumor tissues were incubated with antibodies and then applied to immunofluorescence to detect the expression of immune T cells.

For peripheral blood, ELISA test kits were applied to analyze the expression levels of tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) in serum samples.

Hematoxylin and eosin (HE) staining of heart, liver, spleen, lung, kidney and tumor

At the end of 15 days of treatment, the mice were euthanized and the hearts, livers, spleens, lungs, kidneys, and tumors were collected by autopsy. These organs were fixed in 4% paraformaldehyde, and then the samples were stained with HE to assess pathological changes in the tissues.

Statistical analysis

OriginPro 2021 software (OriginLab, Northhampton, MA) was used to perform statistical analysis of experimental data. Statistical significance was assessed using two-sided t-tests. p-values < 0.05 were considered statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$), and n.s. represents no significance ($P > 0.05$).

Supporting Figures

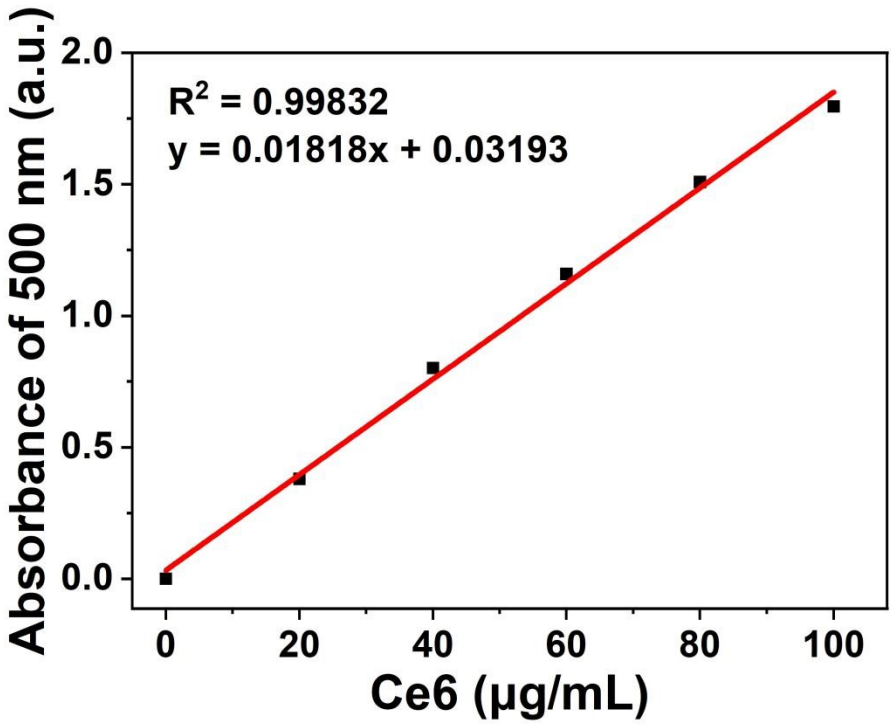

Fig.S1. The standard curve of Ce6.

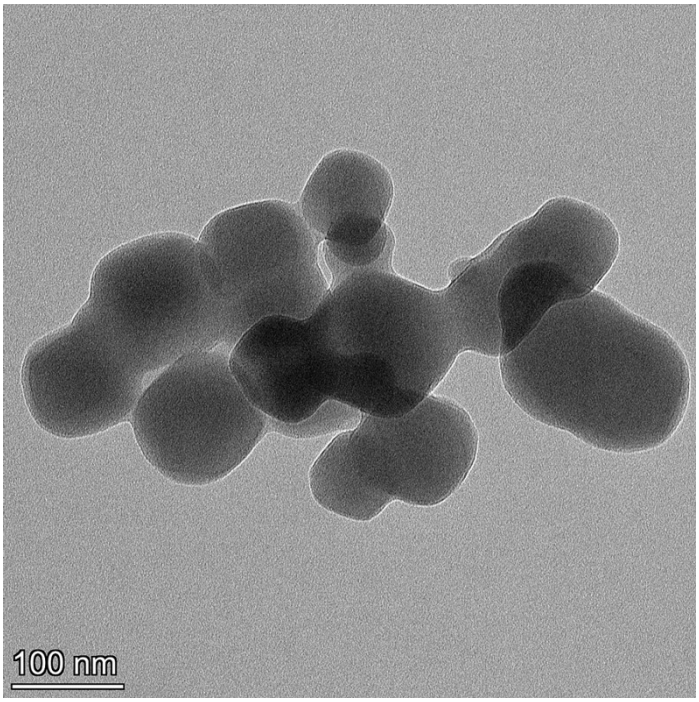

Fig.S2. The TEM of UiO-66-NH_2.

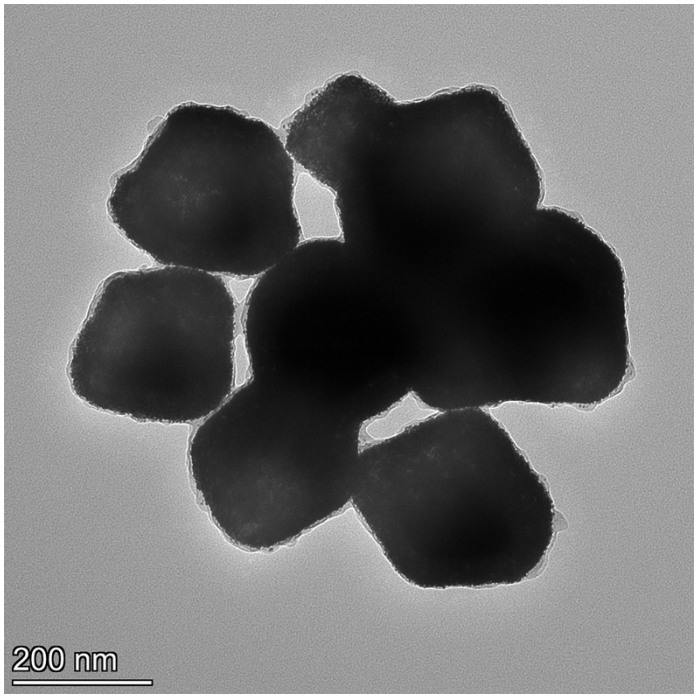

Fig.S3. The TEM of HCP.

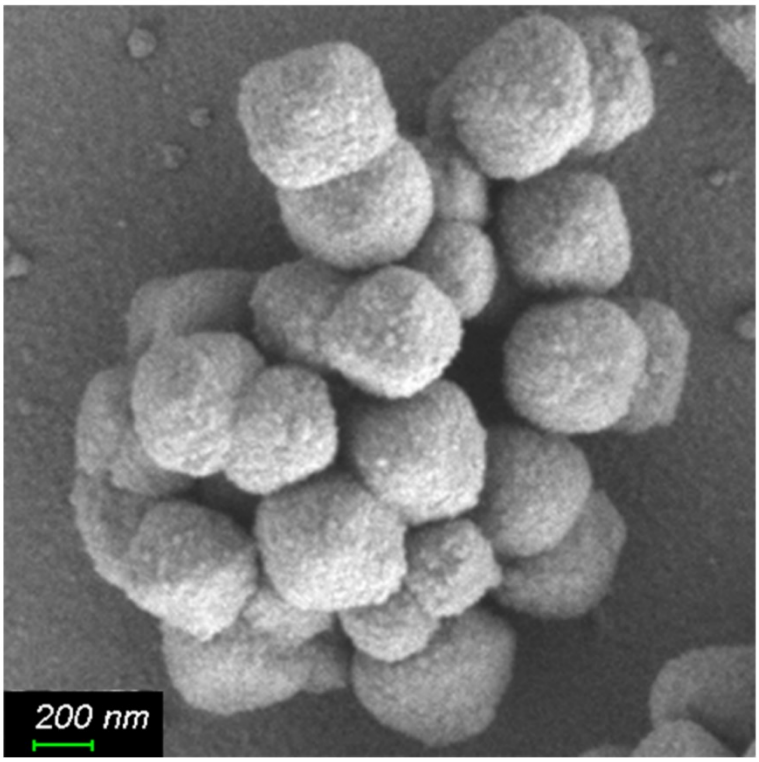

Fig.S4. The SEM of UAuPt.

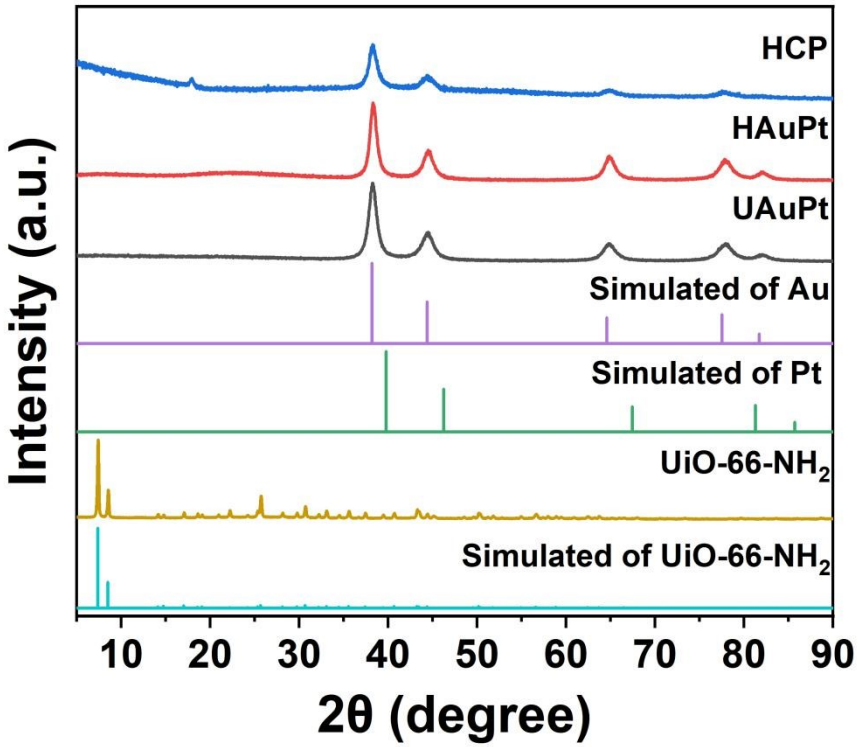

Fig.S5. The PXRD of various nanoparticles.

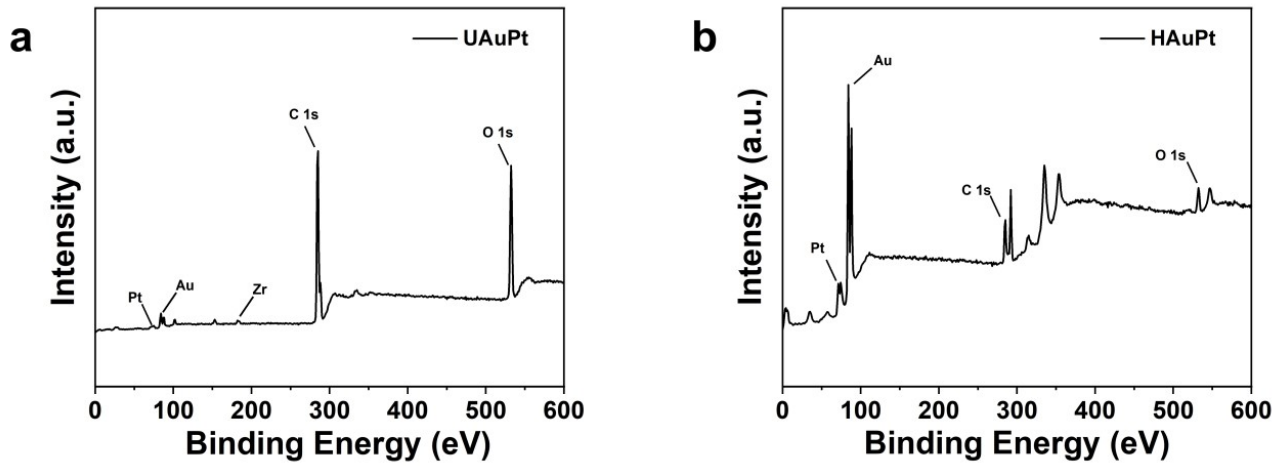

Fig.S6. The XPS of UAuPt (a) , and HAuPt (b).

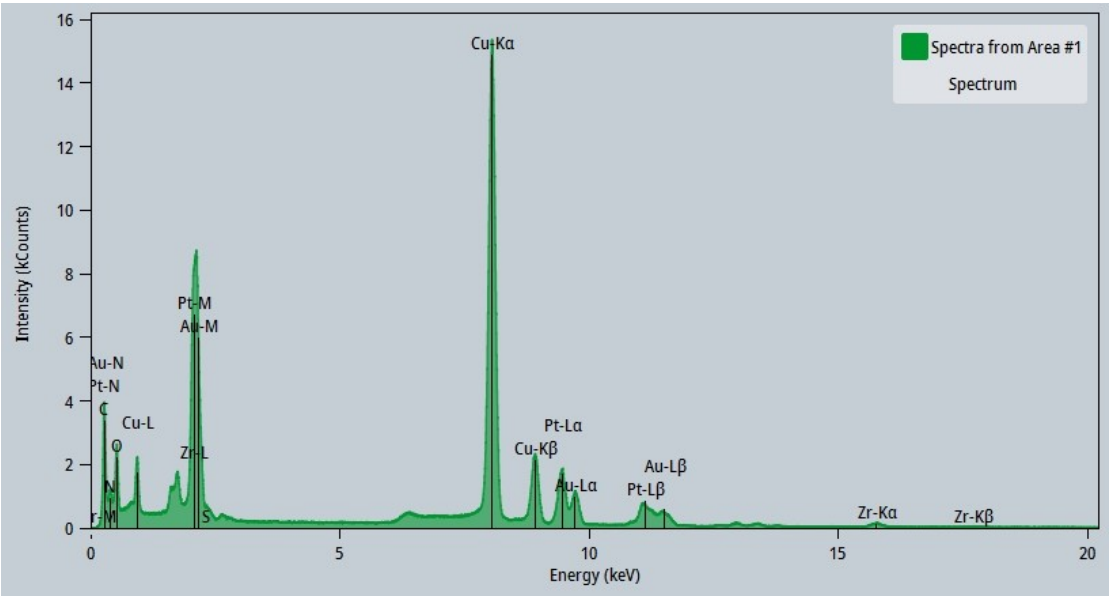

Fig.S7. The EDS of HCP.

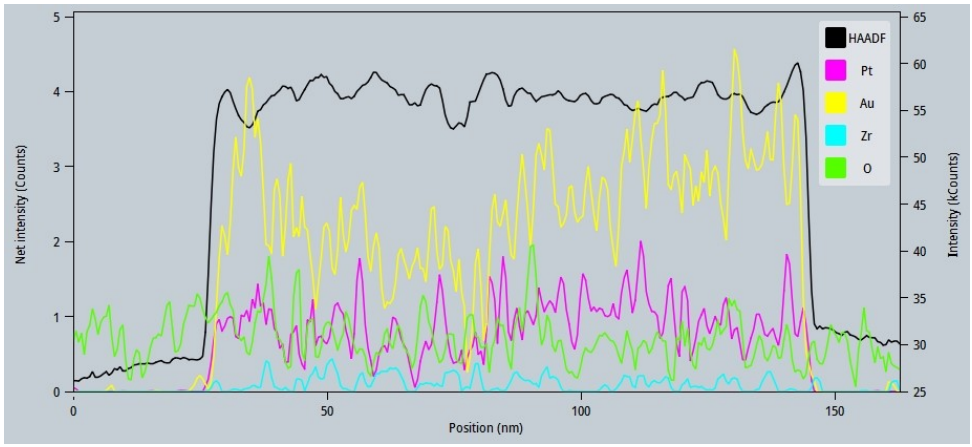

Fig.S8. The elemental line scan profile of HCP.

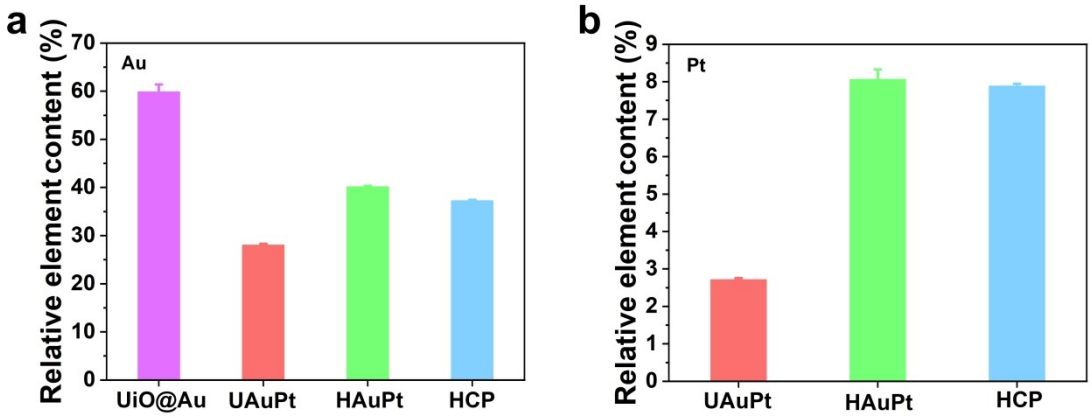

Fig.S9. The ICP-MS of relative Au content of UiO-66-NH_2@Au, UAuPt, HAuPt and HCP (a), and relative Pt content of UAuPt, HAuPt and HCP (b).

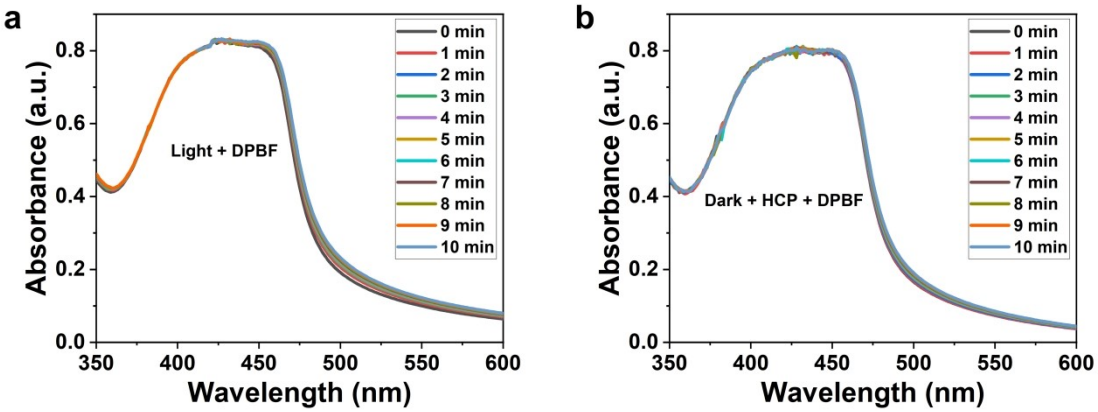

Fig.S10. The UV-Vis absorption spectrum of DPBF under 660 nm laser (0.22 W/cm^2) (a) , and DPBF incubated with HCP under dark conditions (b).

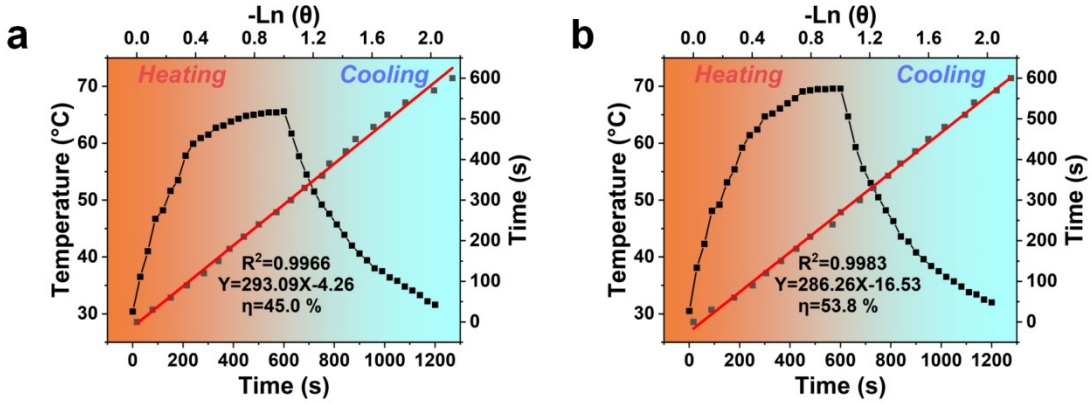

Fig.S11. The photothermal conversion efficiencies of UAuPt (a), HAuPt (b).

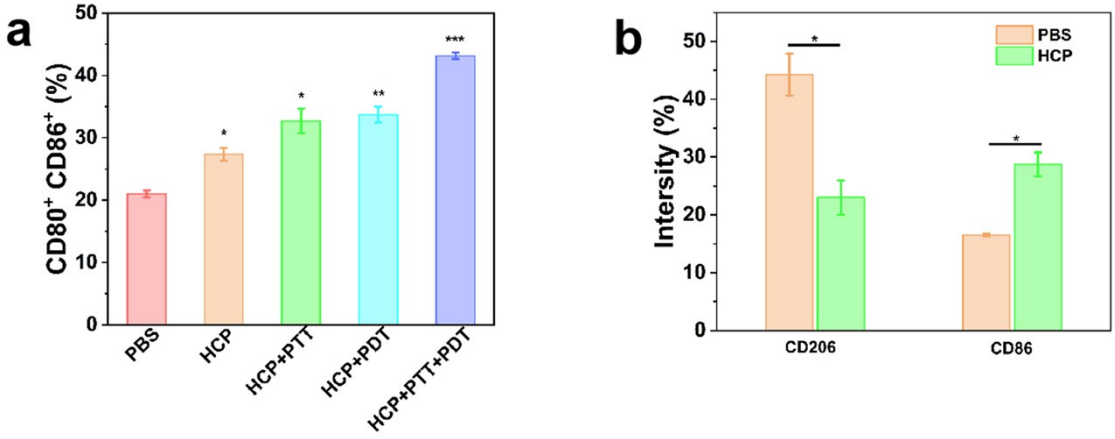

Fig.S12. Flow cytometry quantification of DC cells and RAW macrophages.

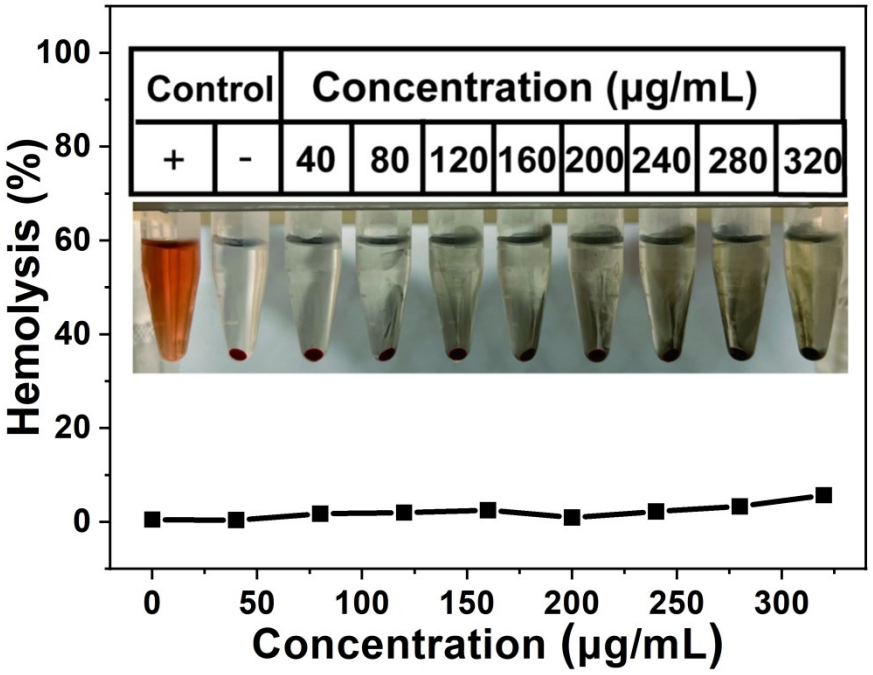

Fig.S13. The hemolysis curve of HCP.

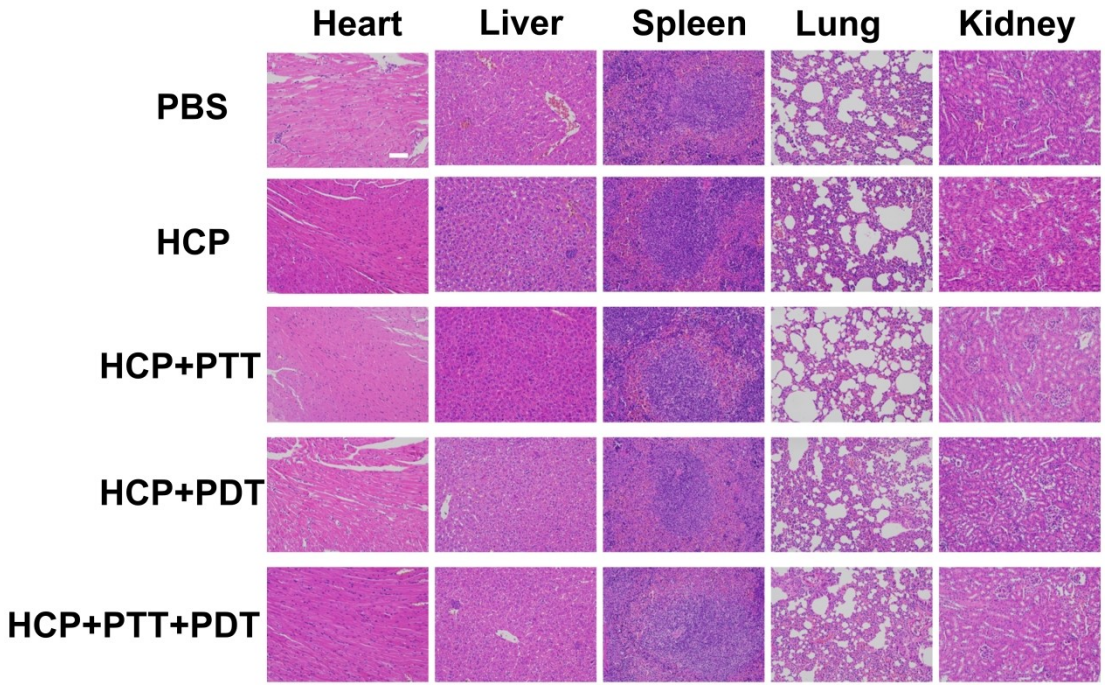

Fig.S14. The HE staining of various organs (Scale bar = 100 μm).

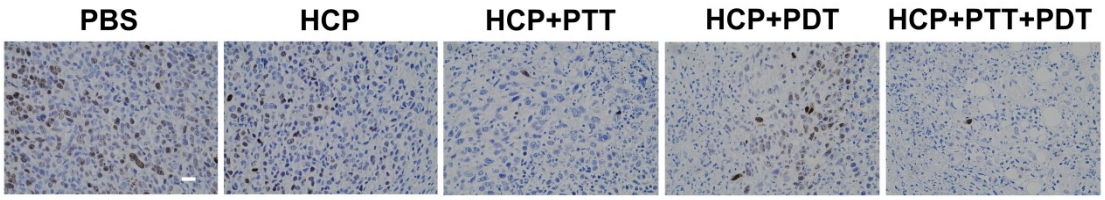

Fig.S15. The immunohistochemistry of Ki-67 in tumors (Scale bar = 50 μm).

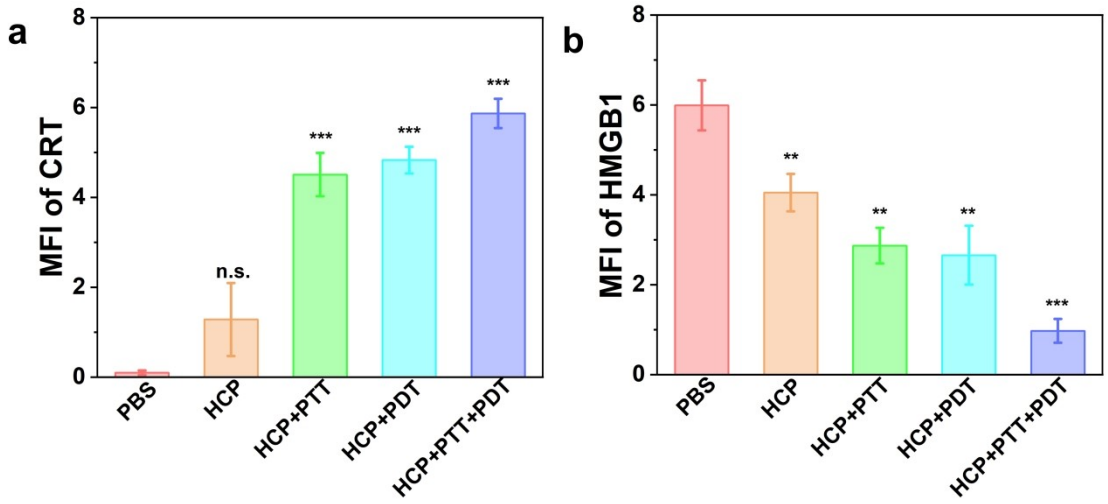

Fig.S16. The fluorescence quantitative plots of CRT (a) and HMGB1 (b) in tumor tissues of mice after different treatments.

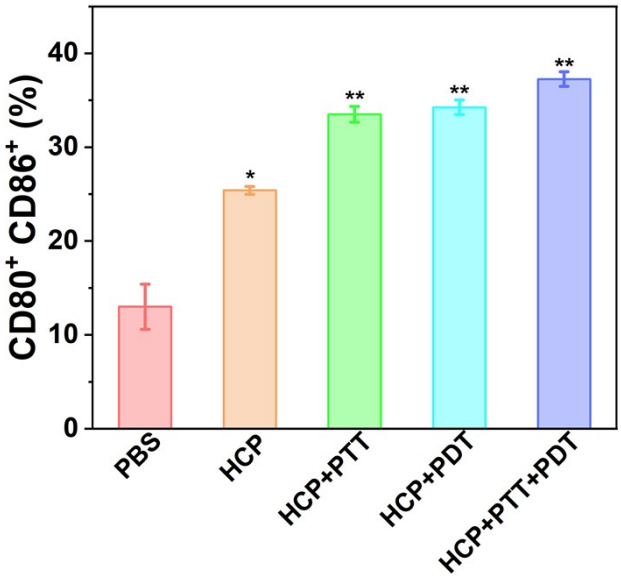

Fig.S17. The quantitative flow cytometry of DC cells in lymph nodes.

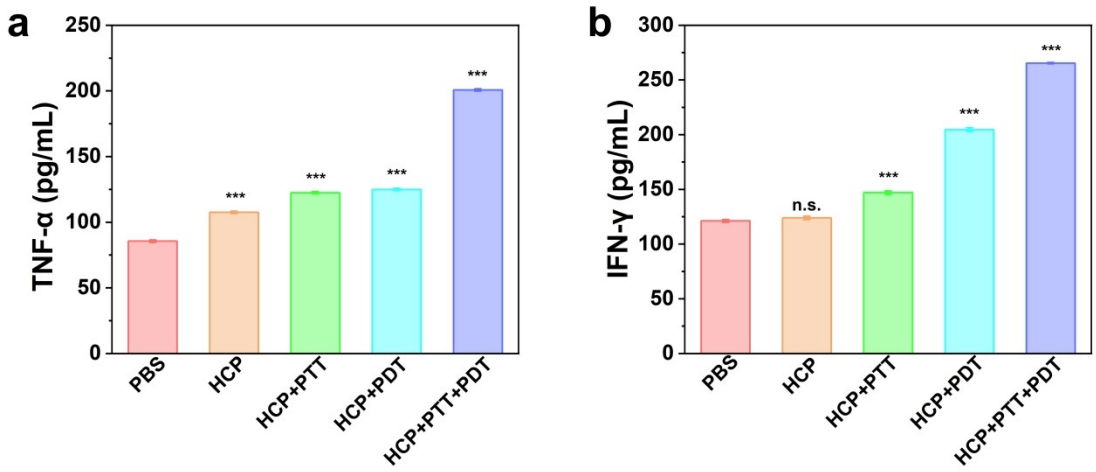

Fig.S18. The cytokine levels of TNF-α (a) , and IFN-γ (b) in serum of mice after different treatments.

Reference

1. X Qin, S He, J Wu, Y Fan, F Wang, S Zhang, S Li, L Luo, Y Ma, Y Lee, T Li, *ACS Cent. Sci,* 2020, ***6*** (2), 247-253.