Supporting Information

Cell membrane-anchoring covalent organic framework nanosheets for single-laser-triggered synergistic tumor therapy

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EXPERIMENTAL SECTION

Materials

2,5-dihydroxyterephthalaldehyde (DHa) and tetra-(p-amino-phenyl)-porphyrin (TAPP) were obtained from Changchun Third Party Pharmaceutical Technology Co., Ltd. Ethanol, NaOH, HCl and dimethylsulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd. THPC (75% aqueous solution), doxorubicin hydrochloride (Dox) and Chloroauric acid were purchased from Energy Chemical (Shanghai, China). 1,3-diphenylisobenzofuran (DPBF) and trypan blue were purchased from Tianjin Heowns Biochemical Technology Co., Ltd. Cell plasma membrane staining kit DiO and 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) and lipid peroxidation MDA assay kit was purchased from Beyotime Biotechnology. CRT polyclonal antibody, HMGB1 polyclonal antibody and CL488-conjugated goat anti-rabbit secondary antibody were purchased from BOSTER Biological Technology Co., Ltd. Antibody diluent and Hoechst 33342 were purchased from Beijing Solarbio Science & Technology Co., Ltd. pH low insertion peptide (pHLIP) were obtained from Shanghai Top-Peptide Biotechnology Co., Ltd. And the pHLIP sequence AEQNPIYWARYADWLFTTPLLLLDLALLVDADEGCT, ≧95%. Fetal bovine serum (FBS), RPMI 1640 and PBS were purchased from Biological Industries (Beit Haemek, Israel). Glass bottom dishes were purchased from Cellvis (Mountain View, CA). Antibiotics (penicillin/streptomycin) and 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl Tetrazolium Bromide (MTT) were purchased from Sigma-Aldrich, USA. 96-well plates were purchased from Hangzhou Xinyou Biotechnology Co., Ltd, China. Calcein-AM/PI double staining kit was obtained from Yeasen Biotech Co., Ltd. (Shanghai, China). The experimental water used was Mill-Q secondary ultrapure water (18.2 $M\Omega \cdot cm^{-1}$). All chemicals were of analytical grade and were used without further purification. Mouse breast cancer cell line (4T1) was purchased from Jiangsu KeyGEN BioTECH Co., Ltd. Balb/C mice (4-6 weeks old, female) were used in the experiment.

Instruments

Powder X-ray diffraction (PXRD) pattern was obtained on a Rigaku SmartLab SE X-Ray Powder Diffractometer. Transmission electron microscopy (TEM) was carried out on a JEM-100CX II electron microscope. X-ray photoelectron spectroscopy (XPS) were carried out using an Escalab 250Xi instrument (Thermo Scientific, USA). Atomic force microscope (AFM) was carried out on a Cypher VRS, which produced by Oxford Instruments (Asylum Research). Fourier infrared spectrometer (Nicolet iS50 FT-IR) was used to characterize the infrared spectrum. Zeta potential and dynamic light scattering (DLS) was performed on a Malvern Zeta Sizer Nano (Malvern Instruments). UV-vis spectroscopy was achieved with UV-1700 (Shimadzu, Japan). Fluorescence spectra were obtained using a FLS-980 Edinburgh Fluorescence Spectrometer with a Xenon lamp. The absorbance was measured in a microplate reader (Synergy 2, Biotek, USA) for the MTT assay. Confocal fluorescence imaging studies were performed using a TCS SP8 confocal laser scanning microscope (Leica, Germany). Imaging flow cytometry was accomplished on Amnis ImageStream MarkII (Merck Millipore, Seattle, WA). *In vivo* fluorescence imaging experiments were conducted using Bioluminescent Living Imager (IVIS Lumina III, USA). All pH measurements were performed with a pH3c digital pH meter (Shanghai LeiCi Device Works, Shanghai, China) equipped with a combined glass-calomel electrode.

Preparation of AuNPs

The gold nanoparticles were synthesized by THPC reduction method. In brief, 1 mL of 0.5 M NaOH was added to 45 mL of H_2O, 12.6 μL of THPC (75% aqueous solution) was added to the solution subsequently. After stirring for 5 minutes, 750 μL of 2.0 wt % chlorauric acid was quickly added, then stirred for another 15 minutes, sealed and stored at 4 °C.

Preparation of 2D COF

In accordance with the methods described in the literature, Porphyrl 2D COF was prepared from DHa (19.9 mg, 0.12 mmol) and TAPP (40.5 mg, 0.06 mmol) in 3.3 mL of mixed solution (dichlorobenzene: butanol: 6 M acetic acid = 5:5:1). After

ultrasonic for 10 min, the products were placed in a 20 mL Pyrex tube, degassed for 3 times through the freezing pump-thawing cycle, then sealed and heated at 120 °C for 3 days. The products were collected and washed with tetrahydrofuran and acetone. The synthesized COFs was mechanical exfoliation in a mortar and then treated with a 1500 W ultrasonic probe for 2 h, subsequently bathed in a 360 W ultrasonic cleaning machine for 6 h. 5000 rpm at this step were used to remove large particles. The resulting 2D COF nanosheets were collected by centrifugation (13000 rpm, 10 min) for subsequent experiments.

Preparation of COF-Au

The AuNPs were modified on the surface of COF NSs *via* gold-amino bond. In brief, 6 mL of the prepared AuNPs solution was mixed with COF NSs (1 mg) and stirred for 6 h at room temperature. And then, the mixture was centrifuged for 15000 rpm, washed with water for three times to remove the unbound AuNPs, and then dispersed in water for further use.

Preparation of COF-Au@Dox

Due to the porous characteristics of COF, Dox could be loaded into COF-Au. In brief, 1 mg Dox and 1 mg COF-Au NSs were ultrasonically mixed in 1 mL aqueous solution. After stirring for 12 h at room temperature, the solution was centrifuged for 15000 rpm and washed with water to remove the unloaded Dox.

Preparation of COF-Au-pHLIP

pHLIP was modified on the surface of COF-Au NSs *via* freezing method. 1 mg pHLIP and 1 mg COF-Au NSs were added into the mixed solution (V_{water}: V_{DMSO} = 1:1), and then frozen at -20 ℃ for 4 h. With centrifugating for 15000 rpm and washing with to remove the unattached peptide, COF-Au-pHLIP NSs were obtained.

Preparation of COF-Au-pHLIP@Dox (CApHD)

pHLIP was modified on the surface of COF-Au@Dox NSs *via* freezing method. 1 mg pHLIP and 1 mg COF-Au@Dox NSs were mixed in 1 mL of water and DMSO mixed

solution (v:v = 1:1), and then frozen at -20 ℃ for 4 h. CApHD NSs were obtained by centrifuging for 15000 rpm and washing with water to remove the unattached peptide.

Photothermal curve

0.1 mg/mL of COF or COF-Au solution were placed into quartz cuvettes respectively. With 635 nm (0.625 W/cm^2) laser irradiation, the variation of temperature was recorded per 20 s.

$^1O_2$ generating verification

1) DPBF (0.05 mM) was firstly mixed with COF (0.1 mg/mL) or COF-Au (0.1 mg/mL), respectively. After 635 nm (0.625W/cm^2) laser irradiation, the NSs were removed by centrifugation and the UV-vis spectra were measured to verify the generation of $^1O_2$. 2) DCFH-DA (0.05 mM) was firstly mixed with COF (0.1 mg/mL) or COF-Au (0.1 mg/mL), respectively. After irradiated with 635 nm laser (0.625W/cm^2) for different times (2 min, 4 min, 6 min, 8 min, 10 min), the supernate was collected by centrifugation and the fluorescence intensity was measured to verify the generation of $^1O_2$.

The loading rate and encapsulation efficiency of Dox

Fluorescent spectra of Dox were measured before and after loading into NSs. The loading rate and encapsulation efficiency of Dox were calculated *via* the equation and standard curve of Dox.

$$\text{Encapsulation efficiency} = \frac{W_1 - W_0}{W_1}$$

$$\text{Loading rate} = \frac{W_1 - W_0}{W_2 + W_1 - W_0}$$

W_0 = drug content before reaction, W_1 = Drug content in supernatant after reaction, W_2 = carrier content.

Release curve of Dox

CApHD (0.1 mg/mL) stirred under four different conditions (pH=7.4, pH = 6.5, pH = 5.6 and pH = 5.6 with laser irradiation). The leakage of Dox at different times were evaluated *via* the fluorescent spectra of Dox.

Cell culture

4T1 cells were cultured in RPMI 1640 medium containing 10% FBS and 100 U mL^{-1} of 1% antibiotics (penicillin/streptomycin) and were maintained at 37 °C in a 5% CO_2/95% air humidified incubator (SANYO).

MTT assay

1) The biocompatibility of COF-Au-pHLIP NSs were evaluated by methyl thiazolyl tetrazolium (MTT) assay. 4T1 cells were first planted into 96-well plates for 24 h, and then incubated with different concentrations of 200 μL COF-Au-pHLIP (10, 20, 50, 100, 150, 200, 250 μg/mL) at different pH (7.4, 6.5, 5.6) for another 24 h. Subsequently, 200 μL MTT (0.5 mg/mL) was added into the 96-well plate for 4 h incubation and DMSO was then used to dissolve formazan crystal. The absorbance at 490 nm was measured with the RT 6000 microplate reader. 2) The therapeutic effect of NSs *in vitro* was evaluated by MTT assay. The 4T1 cells were first planted into 96-well plates for 24 h, and then divided into 5 groups: control, only laser, COF-Au-pHLIP, COF-Au-pHLIP with laser and CApHD with laser. Each experiment was conducted under different pH conditions (pH = 7.4, 6.5 or 5.6). After incubated with 200 μL 100 μg/mL NSs for 1 h, the cells were washed with PBS and the groups needed laser were irradiated under 635 nm laser (0.625 W/cm^2) for 10 min. Subsequently, cells were incubated for another 12 h. 200 μL MTT (0.5 mg/mL) was added into 96-well plate for 4 h and then DMSO was used to dissolve formazan crystal. The absorbance at 490 nm was measured with the RT 6000 microplate reader.

Flow cytometry

4T1 cells were first cultured in cell culture dish (35 mm) for 24 h, and then incubated with 100 μg/mL COF-Au-pHLIP at different pH (7.4, 6.5, 5.6) for 1 h. After washed with PBS, cells were irradiated under 635 nm laser (0.625W/cm^2) for 10 min. Subsequently, DCFH-DA were used to evaluate the ROS generation capacity under different pH conditions with flow cytometry.

Live/dead cell staining assay.

To detect the viable and dead cells, 4T1 cells were cultured in confocal dishes overnight and divided into 15 groups. For laser irradiation groups, 635 nm laser

(0.625 W/cm^2) was utilized to irradiate the cells for 10 min. Before imaged by CLSM, the cells were stained with Calcein AM (λ_{ex}=490nm, λ_{em}=515nm) / Propidium Iodide (PI) (λ_{ex}=535nm, λ_{em}=617nm) for 15 min.

Colony formation assay

1) 4T cells were cultured in 60 mm dishes and incubated at 37 °C under 5% CO_2 in 1640 for 24 h. The cells were then subjected to 15 different treatments. After incubated with NPs for 1 h, the cells were washed three times with PBS buffer (10 mM, pH = 7.4) to remove the excess NPs that were not uptaken into the cells. Subsequently, cells were irradiated under 635 nm laser (0.625W/cm^2) for 10 min. Then the cells were incubated in fresh cell culture medium at 37 °C under 5% CO_2 in 1640 for another 5 days, before they were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet.

2) 4T1 tumor tissues were harvested, minced in RPMI-1640 medium. The obtained cells were passed through a 40-μm nylon mesh and collected by centrifugation at 800 g for 10 min, followed by lysis of RBCs. For the analysis of T cell subpopulation and antigen presenting cell, cell suspension was stained with the following antibodies: APC anti-CD3 antibody and perCP anti-CD8 antibody; APC anti-CD86 antibody.

Lipid peroxidation assay

4T1 cells were first incubated in cell culture dish (35mm) for 24 h, and then incubated with 100 μg/mL COF-Au-pHLIP at different pH (7.4, 6.5, 5.6) for 1 h. After washed with PBS, cells were irradiated under 635 nm laser (0.625W/cm^2) for 10 min. Subsequently, cells were treated according to the established method and the contents of MDA were measured by a microplate reader.

CLSM imaging

1) The membrane-targeting observation. 4T1 cells were first cultured in glass-bottom dishes (20 mm) for 24 h, and then incubated with 100 μg/mL COF-Au-pHLIP at different pH (7.4, 6.5, 5.6) for 1 h. After washed with PBS, cell plasma membrane staining kit DiO was stained according to the established method. Subsequently, the free dye was removed and the membrane targeting ability was observed by CLSM. 2)

Evaluation of ROS generation capacity. 4T1 cells were first cultured in glass-bottom dishes (20 mm) for 24 h, and then incubated with 100 μg/mL COF-Au-pHLIP at different pH (7.4, 6.5, 5.6) for 1 h. After washed with PBS for three times, the DCFH-DA was stained subsequently according to the established method. After irradiation under 635 nm laser (0.625 W/cm^2) for 10 min, the free dye was removed and the ROS generation capacity was observed by CLSM. 3) The controlled release of Dox. 4T1 cells were first cultured in glass-bottom dishes (20 mm) for 24 h, and then incubated with 100 μg/mL COF-Au-pHLIP at different pH (7.4, 6.5, 5.6) for 1 h. After washed with PBS, the group of pH 5.6 was irradiated under 635 nm laser (0.625 W/cm^2) for 10 min. The controlled release of Dox was observed by CSLM. 4) Immunofluorescent staining was performed for the observation of CRT and HMGB1 expression. 4T1 cells were first cultured in glass-bottom dishes (20 mm) for 24 h, then incubated with 100 μg/mL of COF-Au-pHLIP at different pH (7.4, 6.5, 5.6) for 1 h. The cells were irradiated under 635 nm laser (0.625 W/cm^2) for 10 min. After washed with PBS for three times, the cells were fixed and permeated before treated with blocking reagent. Subsequently, the cells were incubated with CRT or HMGB1 related primary antibody, secondary antibody and Hoechst 33342. The ICD behavior was observed by CSLM. 5) The therapeutic effect evaluation *in vitro*. 4T1 cells were first cultured in glass-bottom dishes (20 mm) for 24 h, and then divided into 5 groups (control, only laser, COF-Au-pHLIP, COF-Au-pHLIP with laser and CApHD with laser). Cells were incubated with 100 μg/mL NSs at different pH (7.4, 6.5, 5.6) for 1 h. After washed with PBS, the groups needed laser were irradiated under 635 nm laser (0.625 W/cm^2) for 10 min. Calcein-AM/PI assay kits was stained to evaluate the therapeutic effect *in vitro*.

Trypan blue staining

Trypan blue was diluted with PBS buffer into 0.4% staining solution, filtered and refrigerated for further used. 4T1 cells were first cultured in cell culture dish (35 mm) for 24 h, and then incubated with 100 μg/mL COF-Au-pHLIP at different pH (7.4, 6.5, 5.6) for 1 h. After washed with PBS, the trypan blue was subsequently stained for 5 min. The staining was observed under a microscope.

Tumor model establishment

All animal experiments were conducted and obeyed the Principles of Laboratory Animal Care (People's Republic of China). Balb/C female mice (4-6 weeks old) were fed with normal conditions of 12 h light and dark cycles and given access to food and water ad libitum. To establishing the tumor models, 4T1 cells (approximately 1×10^6) were dispersed in 80 μL PBS and injected subcutaneously into the flanks of the Balb/C mice.

***In vivo* fluorescent imaging**

To investigate the biological distribution of NSs *in vivo*, the NIR dye IR808 labeled NSs were employed for fluorescent imaging. Balb/c mice were intravenously injected with 0.2 mg of COF-Au-pHLIP@IR808 or COF-Au@IR808 dispersed in 100 μL saline. Then the fluorescent imaging was observed at different times (1 h, 2 h, 3 h, 6 h, 9 h, 12 h, 24 h, 48 h, 72 h) after injection.

***In vivo* photothermal imaging**

To further investigate the *in vivo* tumor targeting effect of the NSs, photothermal imaging was performed. Balb/c mice were intravenously injected with 0.2 mg of COF-Au-pHLIP or COF-Au dispersed in 100 μL saline. Then the photothermal imaging was observed at different times (1 h, 2 h, 3 h, 6 h, 9 h, 12 h, 24 h, 48 h, 72 h) after injection.

***In vivo* therapeutic effect**

4T1 tumor-bearing mice were randomly divided into five groups: control, laser, COF-Au-pHLIP, COF-Au-pHLIP with laser and CApHD with laser. When tumor volumes have grown to about 50 mm^3, the mice were intravenously injectied with PBS or corresponding NSs. After injection for 24 h, the tumor need laser were placed under 635 nm laser (0.625 W/cm^2) irradiation for 10 min. The body weight and volume of tumors were recorded during the two-weeks treatment course. After two-weeks treatment course, the tumors were removed for photographing and weighing.

The calculation formula of tumor volume: $V = (\text{tumor width})^2 \times (\text{tumor length})/2$

Histopathological analysis

Tumors and major organs (heart, liver, spleen, lung, and kidney) of Balb/c mice were removed for section staining after treatment course. The damage of tumor was evaluated *via* H&E staining, Ki67 immumohistochemical staining and CD3 immunofluorescent staining. Biosecurity was proved *via* H&E staining of primary organs tissues.

Supporting Figures

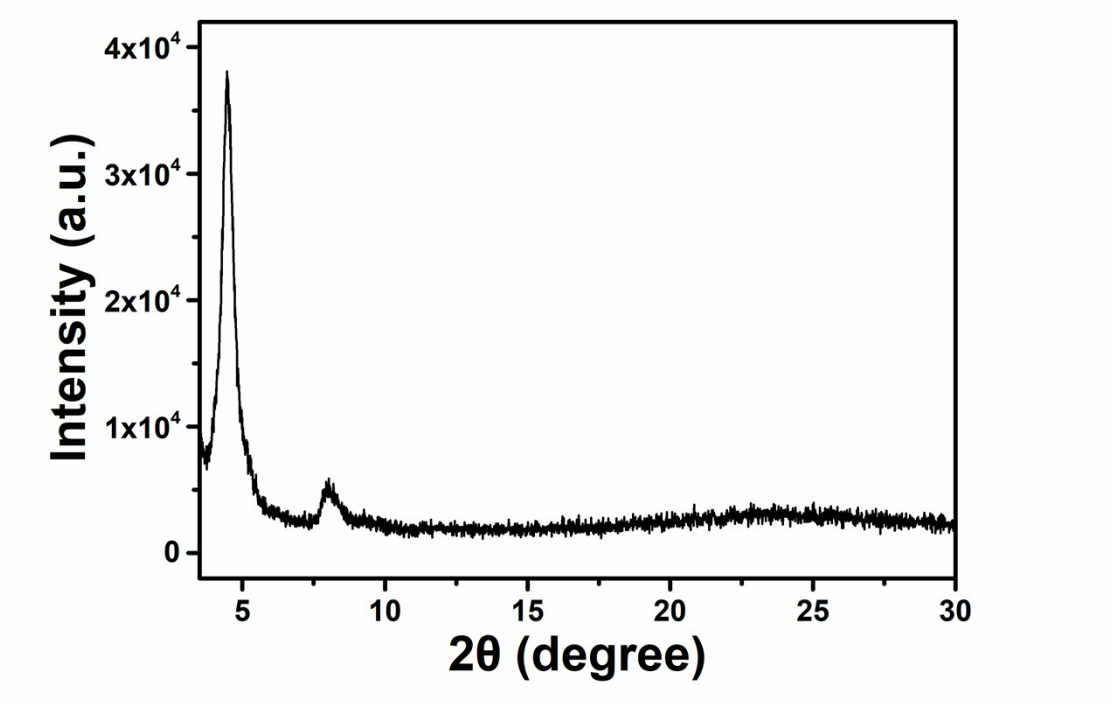

Figure S1. PXRD pattern of 2D COF NSs.

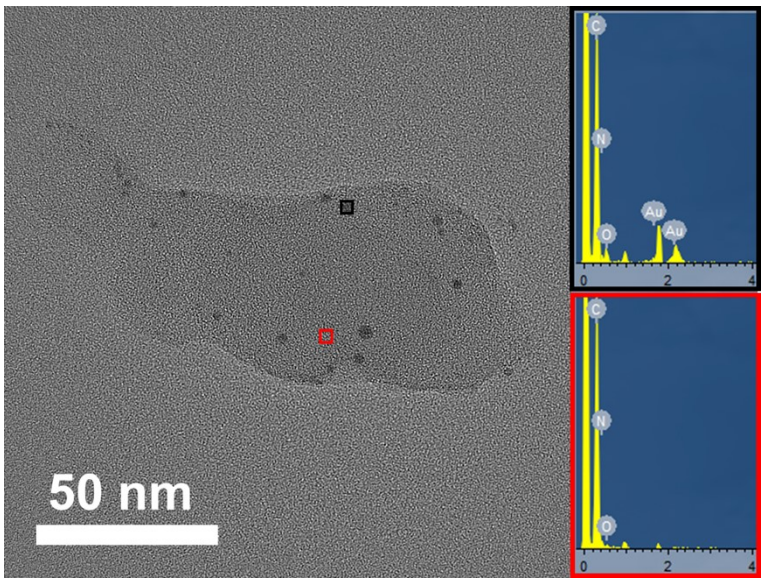

Figure S2. TEM image and EDS analysis of COF-Au NSs. Black box with AuNPs and red box without AuNPs.

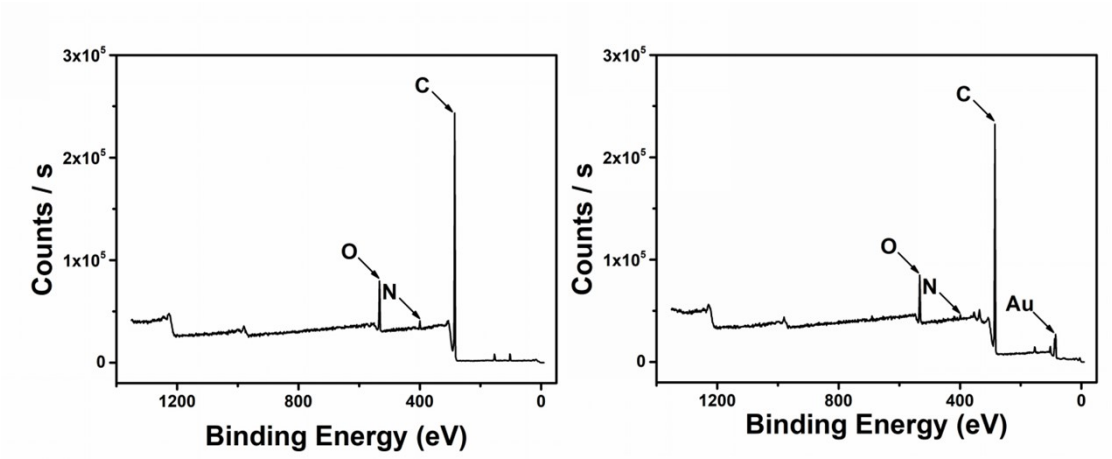

Figure S3. XPS of COF NSs (left) and COF-Au NSs (right).

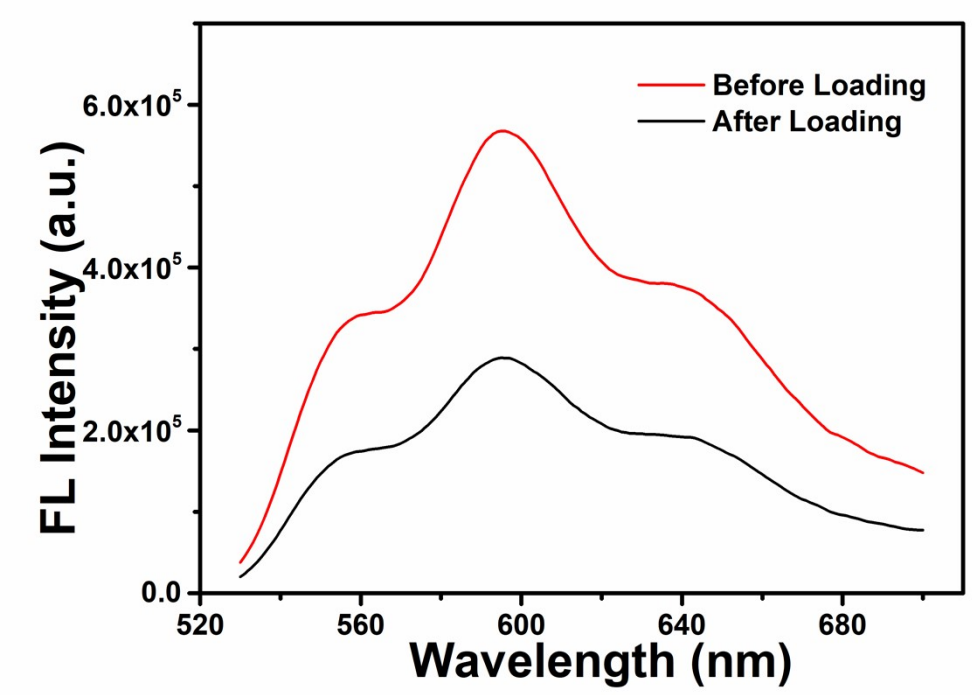

Figure S4. Fluorescence spectra of supernatant before and after Dox loading.

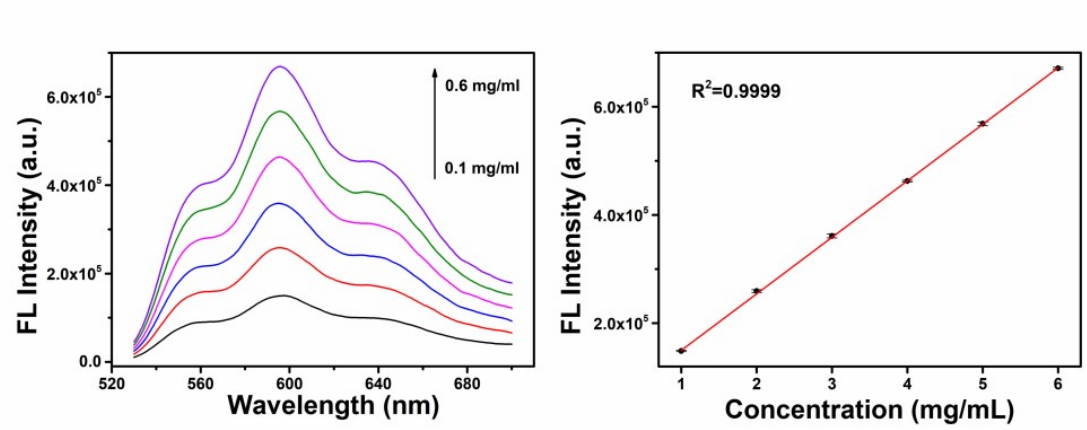

Figure S5. Fluorescence spectra of Dox at different concentrations (left) and standardization curve for fluorescence intensity of Dox (right).

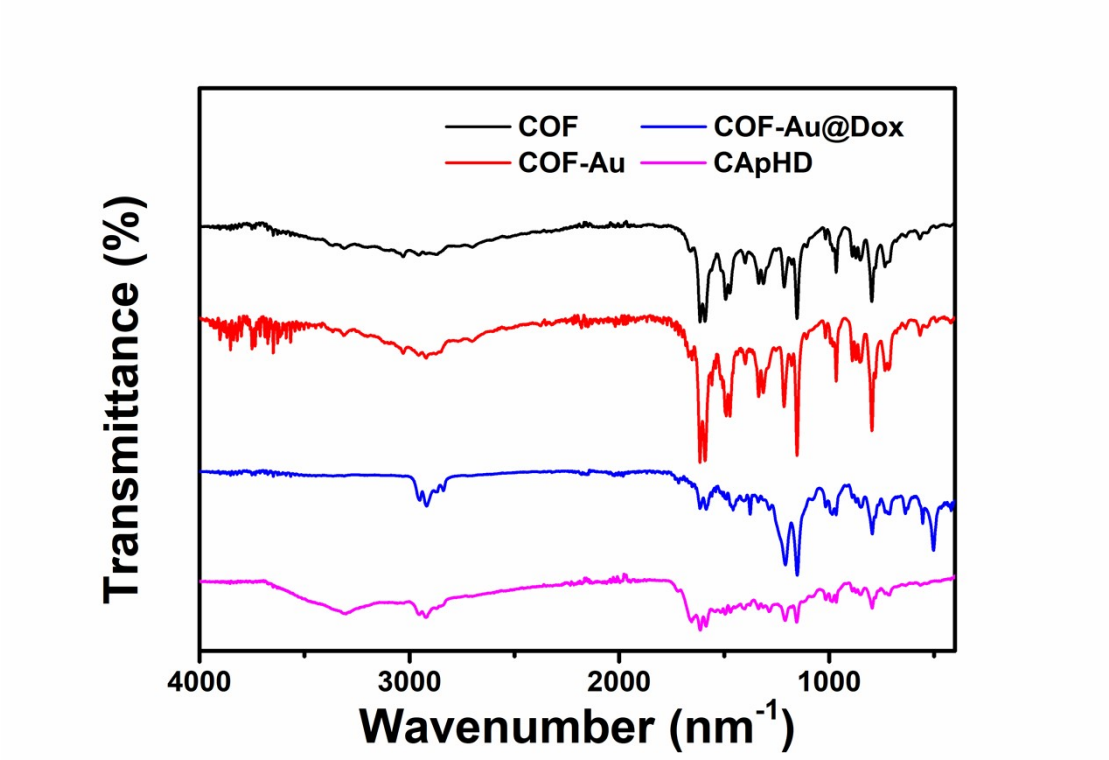

Figure S6. FTIR spectra of four-step synthesized NSs.

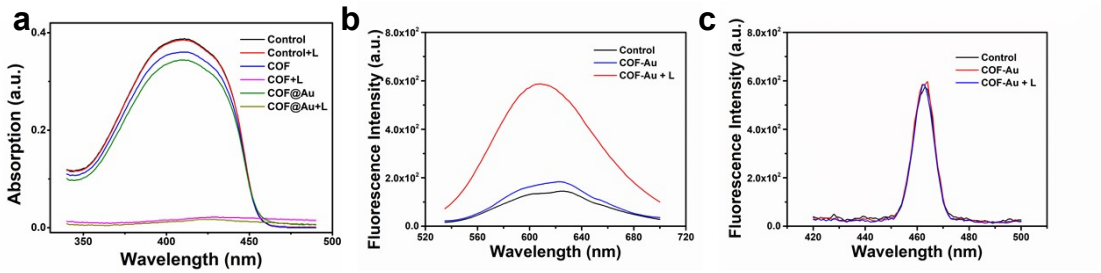

Figure S7. (a) UV-Vis spectra of DPBF for $^1O_2$ detecting; (b) Fluorescence spectra of DHE under different treatment to detect $O_2^{\bullet-}$ generation; (c) Fluorescence spectra of coumarin under different treatment to detect OH• generation.

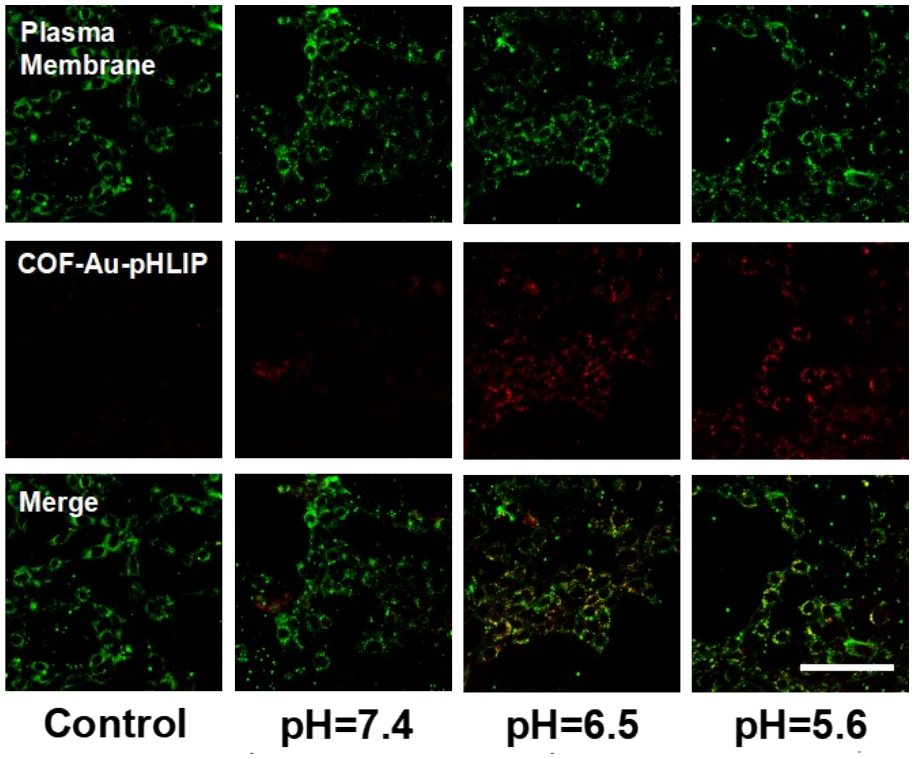

Figure S8. Plasma membrane anchoring capability of COF-Au-pHLIP at different pH conditions. Scale bar = 100 μm.

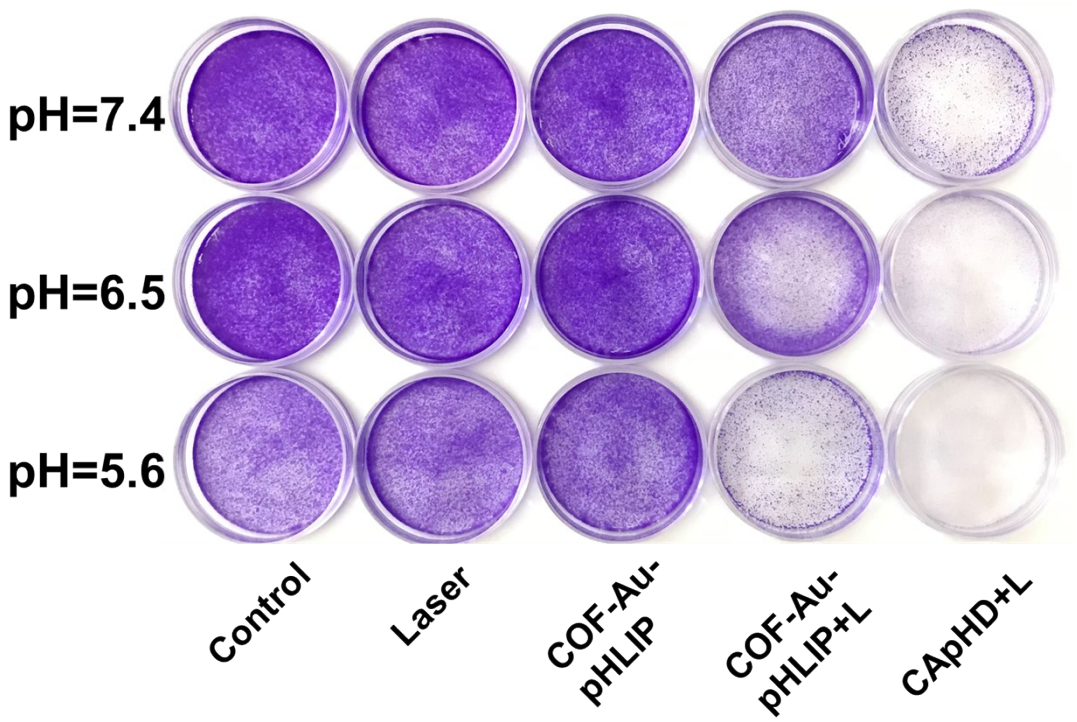

Figure S9. Photograph of 4T1 cells surviving fractions after the different treatments. "L" means that the cells were exposed to a 635 nm laser (0.625 W/cm^2) for 10 min.

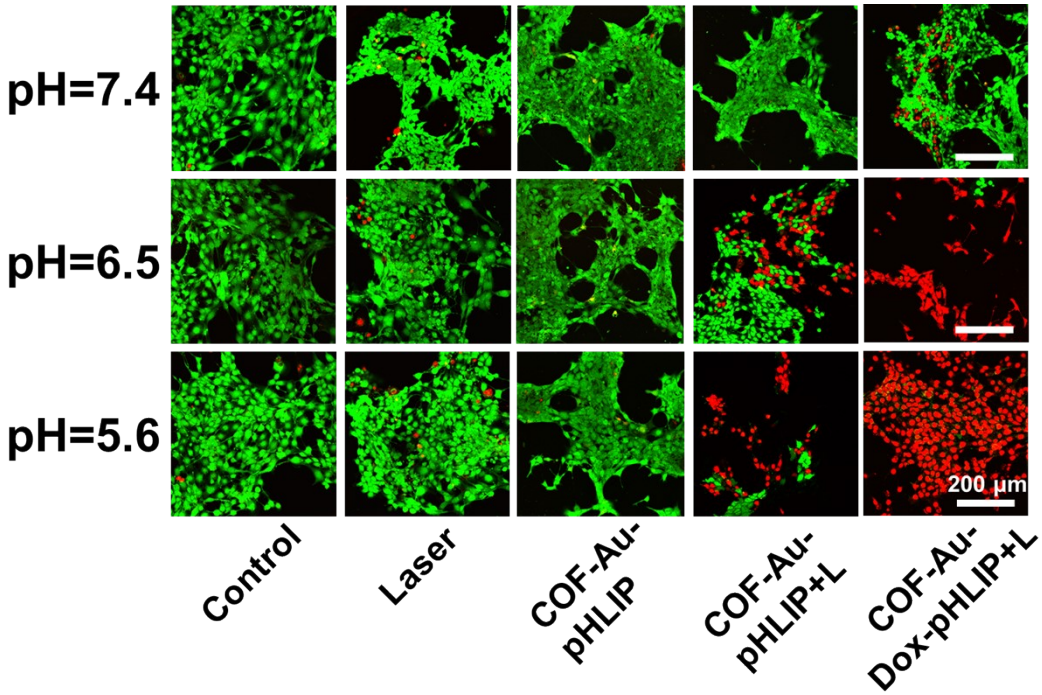

Figure S10. Live/dead cell staining assay of 4T1 cells with different treatments. "L" means that the cells were exposed to a 635 nm laser (0.625 W/cm^2) for 10 min.

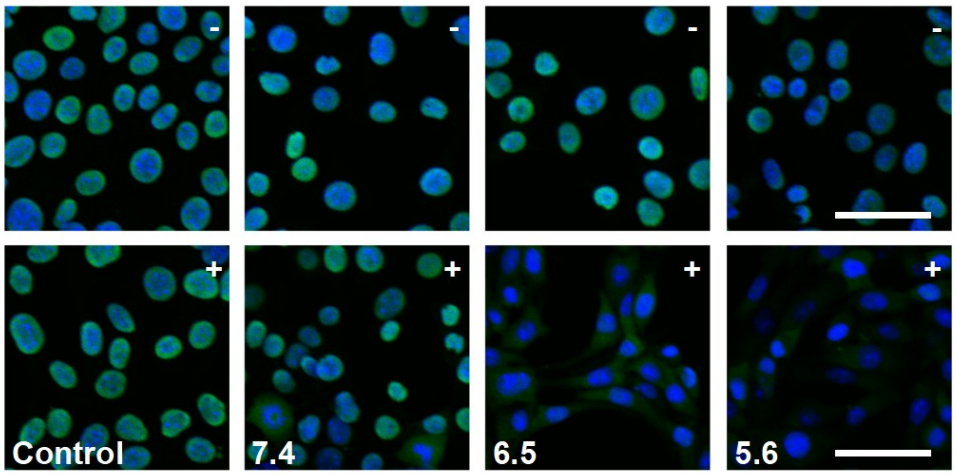

Figure S11. CLSM images of HMGB1 immunofluorescent staining (scale bar = 50 μm). "+" means that the cells were exposed to a 635 nm laser (0.625 W/cm^2) for 10 min.

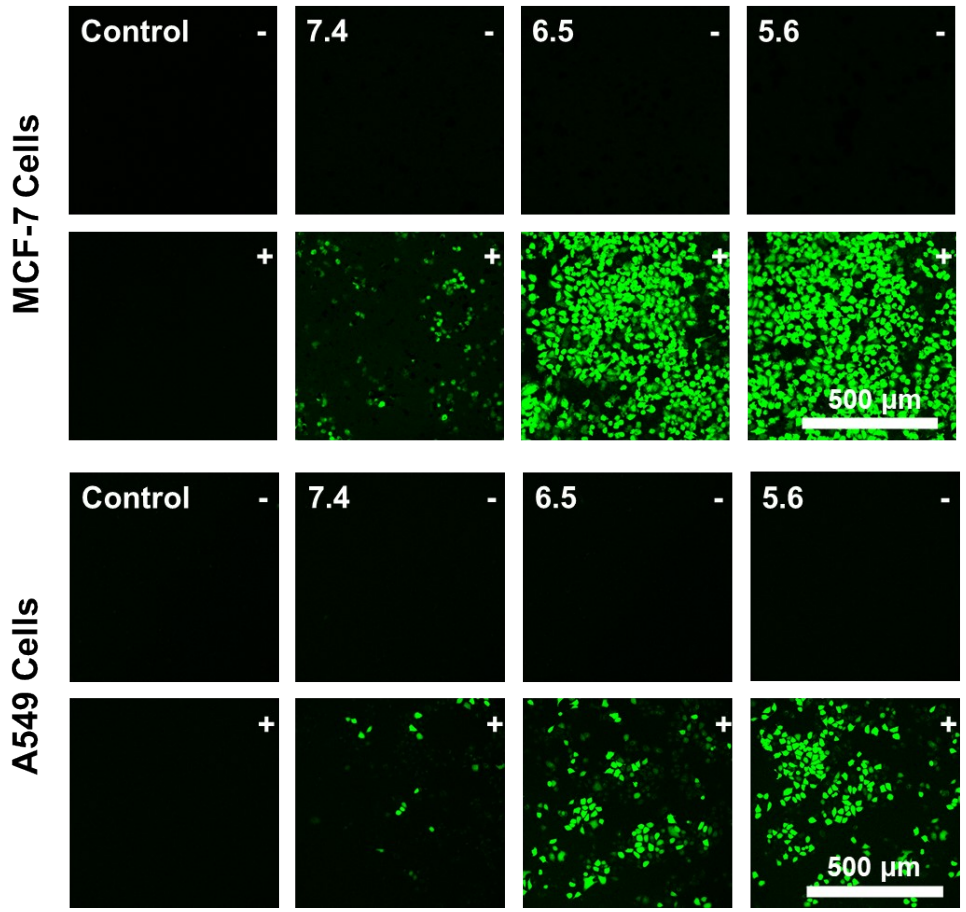

Figure S12. CLSM images of MCF-7 cells or A549 cells at different treatment with DCFH-DA staining. "+" means that the cells were exposed to a 635 nm laser (0.625 W/cm^2) for 10 min.

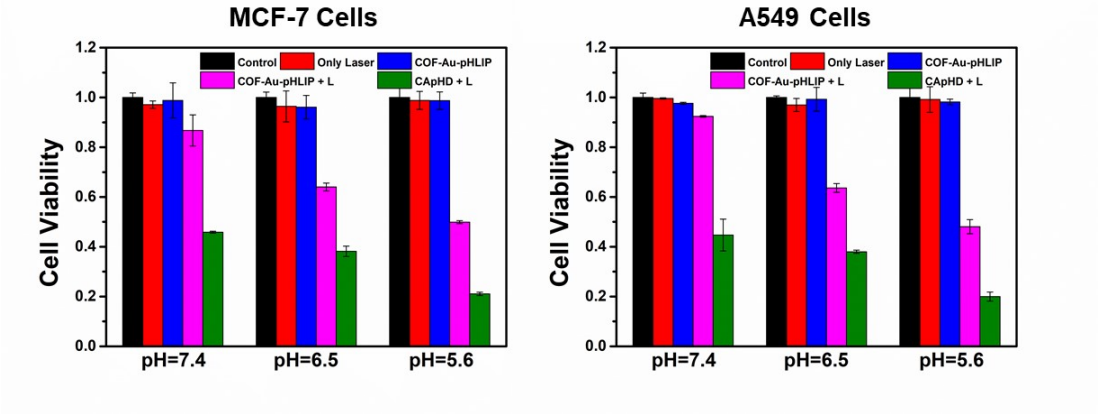

Figure S13. *In vitro* therapeutic effect of MCF-7 cells or A549 cells evaluated by MTT assay. Experimentally, the cells were incubated with NPs (0.1 mg/mL) for 1 h and exposed to a 635 nm laser (0.625 W/cm^2) for 10 min.

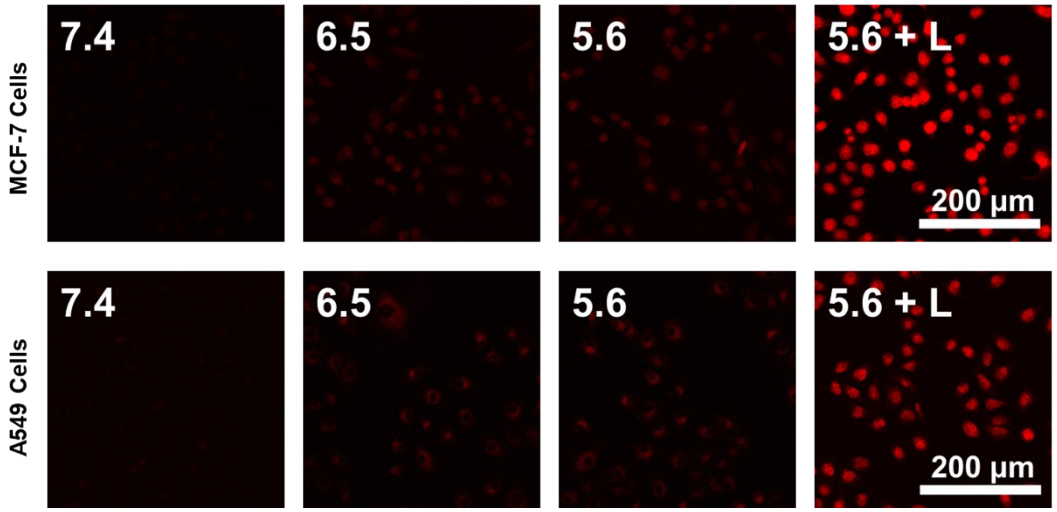

Figure S14. CLSM images of Dox release in MCF-7 cells or A549 cells under different conditions. "L" means that the cells were exposed to a 635 nm laser (0.625 W/cm^2) for 10 min.

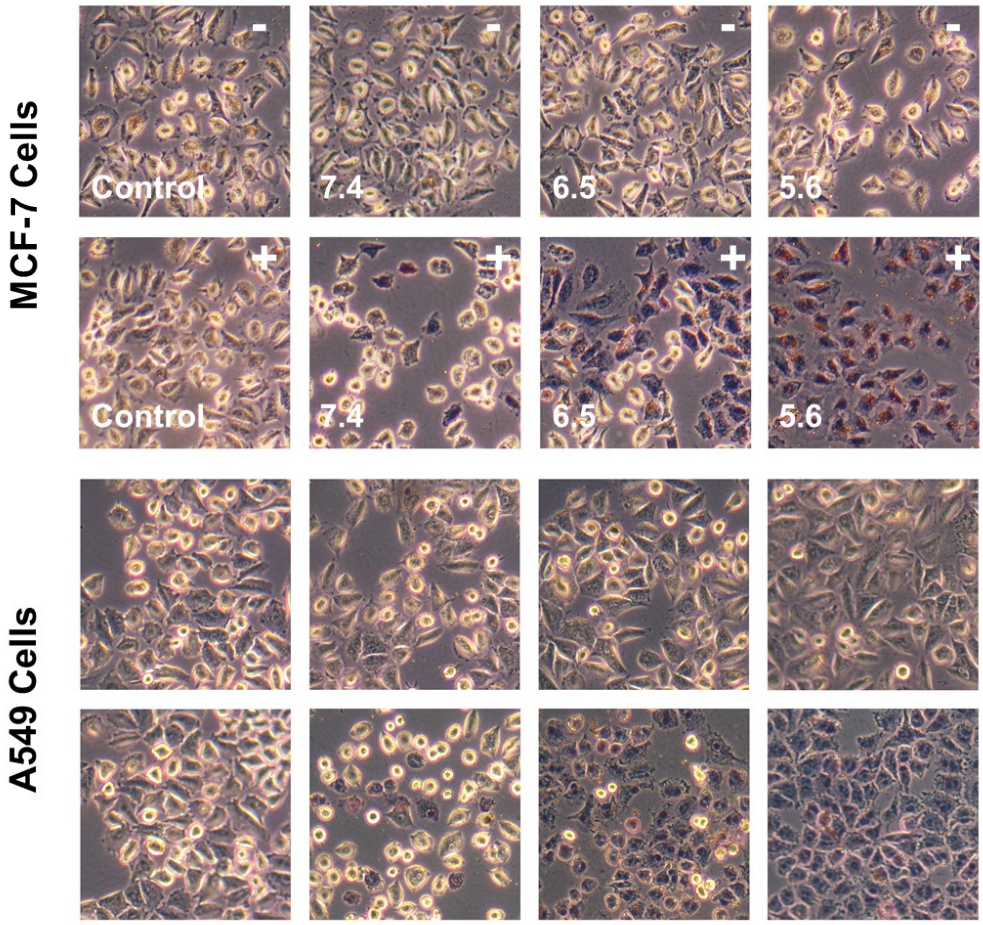

Figure S15. Trypan blue staining assay in MCF-7 cells or A549 cells to explore the integrity of cell membranes. "+" means that the cells were exposed to a 635 nm laser (0.625 W/cm^2) for 10 min.

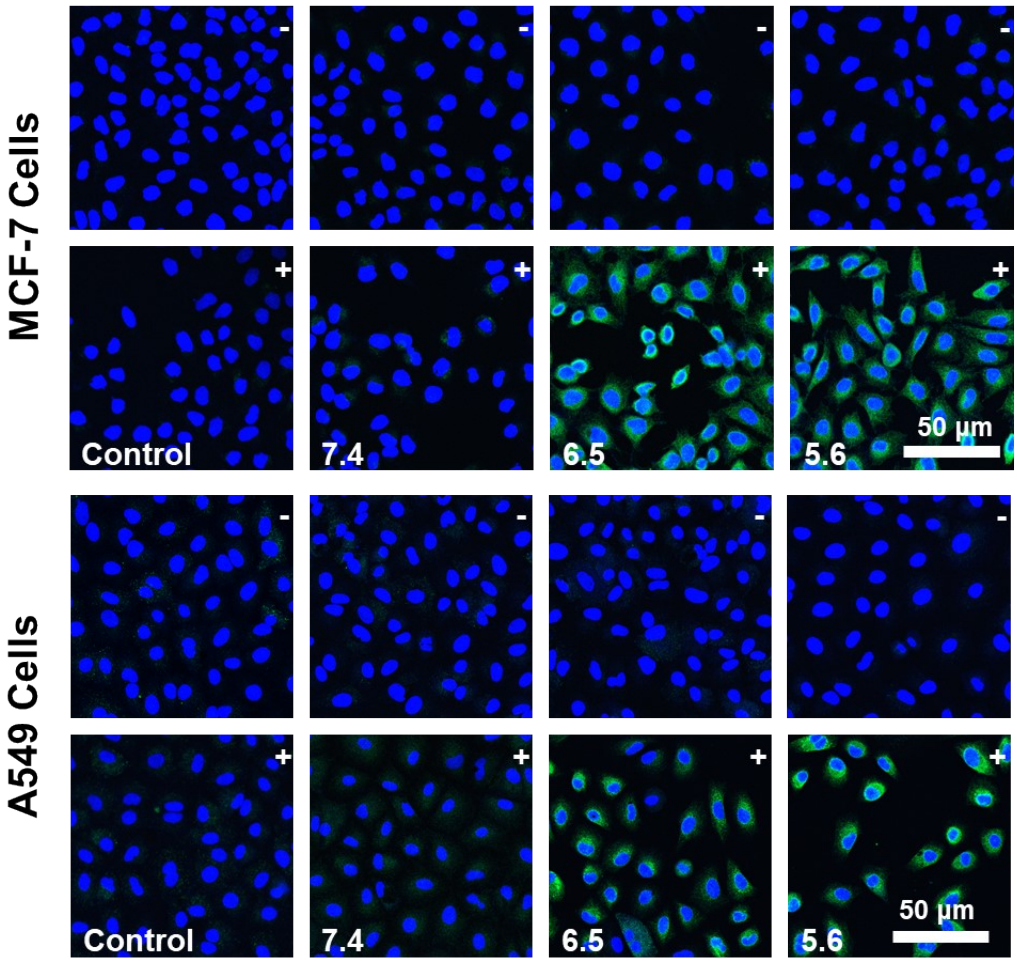

Figure S16. CLSM images of MCF-7 cells or A549 cells for CRT immunofluorescent staining. "+" means that the cells were exposed to a 635 nm laser (0.625 W/cm^2) for 10 min.

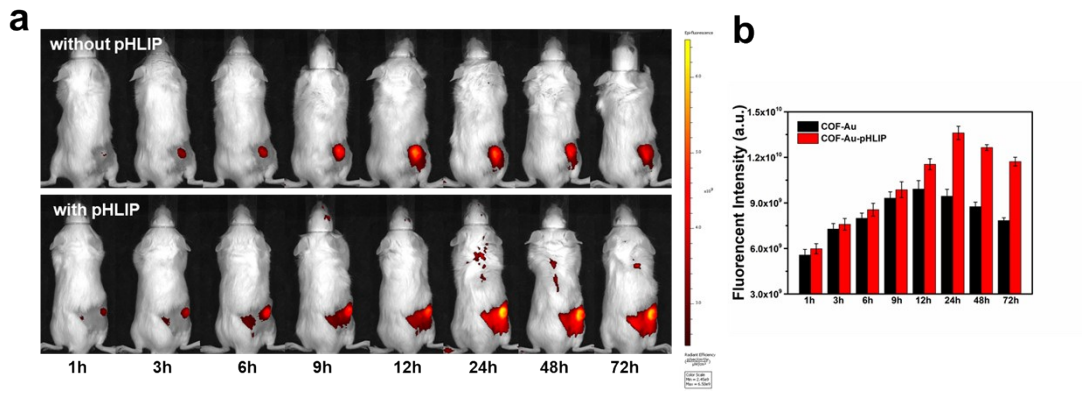

Figure S17. Fluorescence imaging of tumor-bearing balb/c mice after injection.

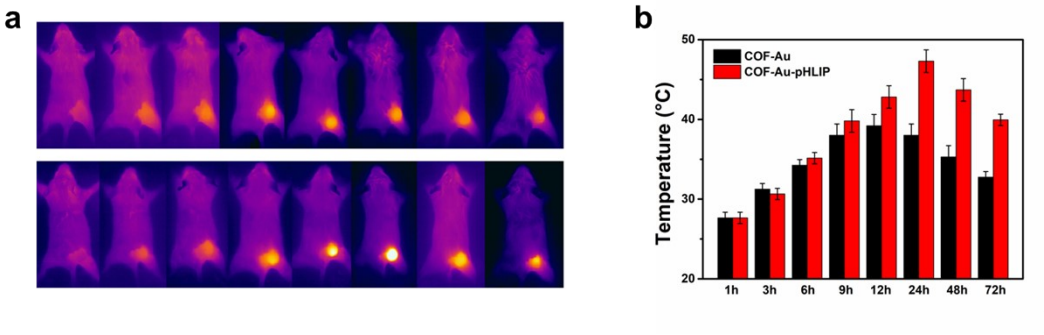

Figure S18. Thermal images (a) and temperature quantify (b) of tumor-bearing mice after intravenous injection of COF-Au or COF-Au-pHLIP at different times and irradiation with 635 nm laser for 10 min at tumor site.

ki67	**Names of group**	Control	Only Laser	COF-Au-pHLIP	COF-Au-pHLIP+L	CApHD+L
	Positive Cells %	**49.2%**	**34.8%**	**43.6%**	**11.8%**	**2.4%**
CD3	**Names of group**	Control	Only Laser	COF-Au-pHLIP	COF-Au-pHLIP+L	CApHD+L
	Positive Cells %	**3.3%**	**3.7%**	**8.3%**	**21.4%**	**22.8%**

Figure S19. The quantification analysis for Ki67 immumohistochemical staining images and CD3 immunofluorescent staining images.

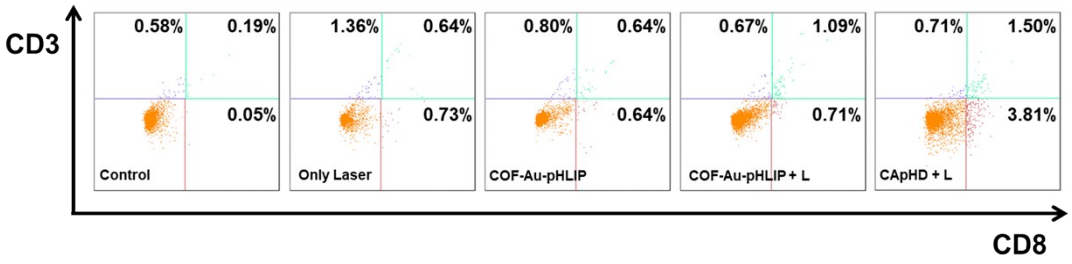

Figure S20. The cytotoxic T lymphocytes (CD3+CD8+) infiltrated into the tumor analyzed by the flow cytometry at Day 5 after different treatment. "L" means that the tumors were exposed to a 635 nm laser (0.625 W/cm^2) for 10 min.

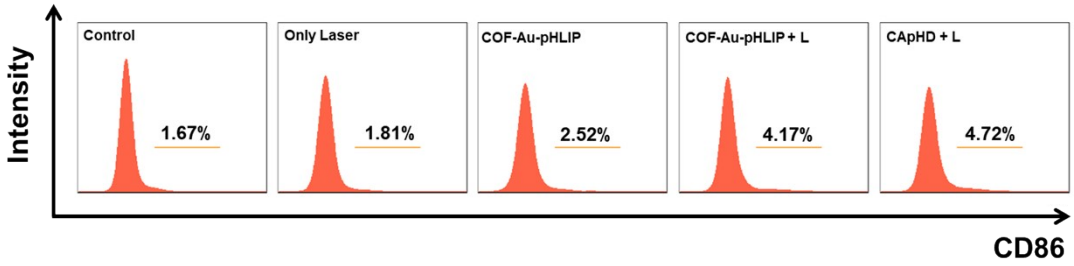

Figure S21. Antigen presenting cells (CD86+) infiltrated into the tumor analyzed by the flow cytometry at Day 5 after different treatment. "L" means that the tumors were exposed to a 635 nm laser (0.625 W/cm^2) for 10 min.

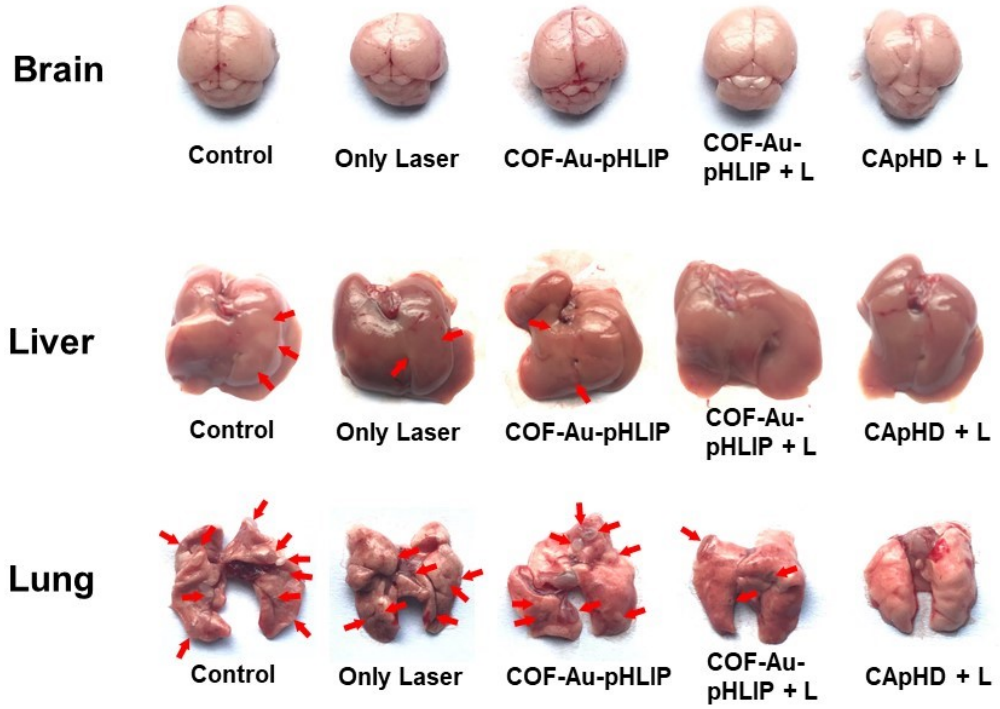

Figure S22. Photograph of brains, livers and lungs for metastasis observation in different groups. "L" means that the tumors were exposed to a 635 nm laser (0.625 W/cm^2) for 10 min.

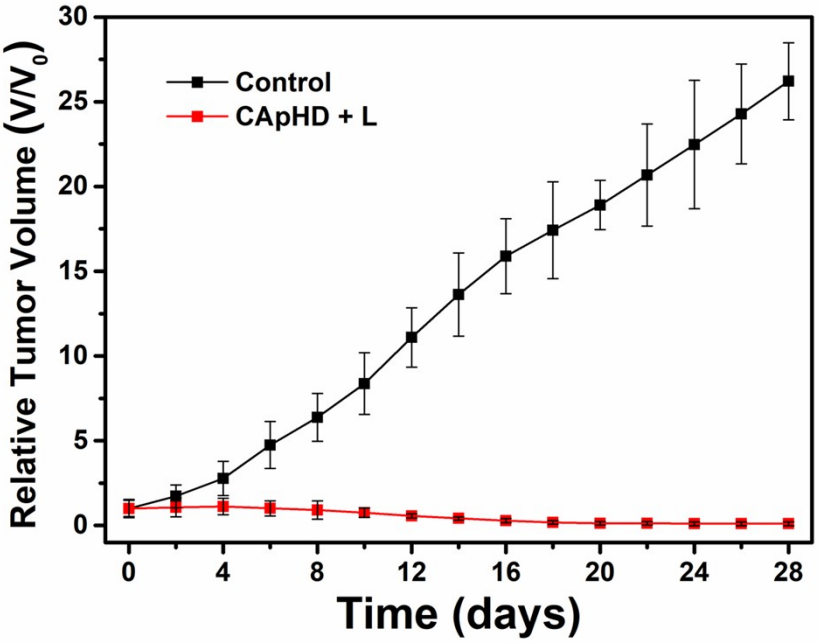

Figure S23. Relative tumor volume change curves during treatment of four weeks.

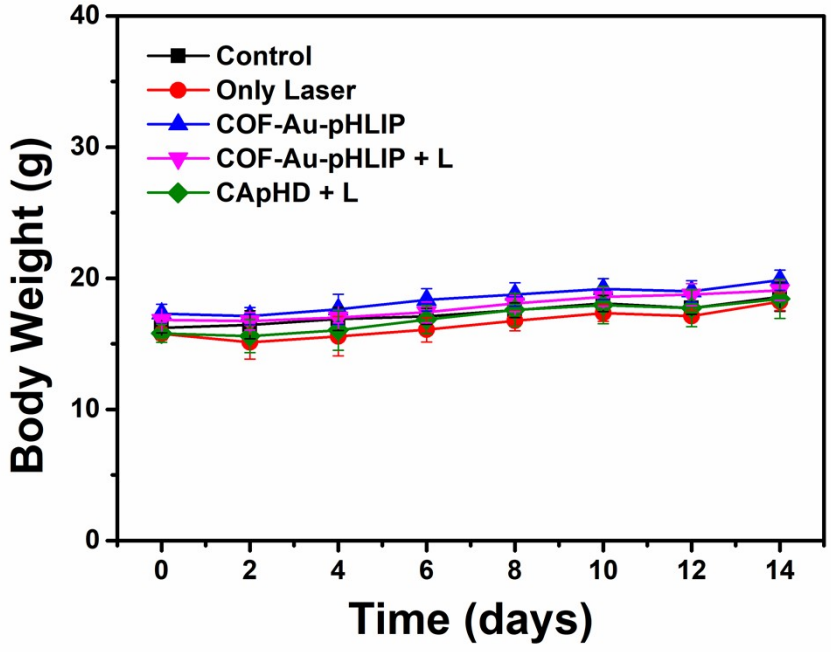

Figure S24. Body weight of the mice during treatment.

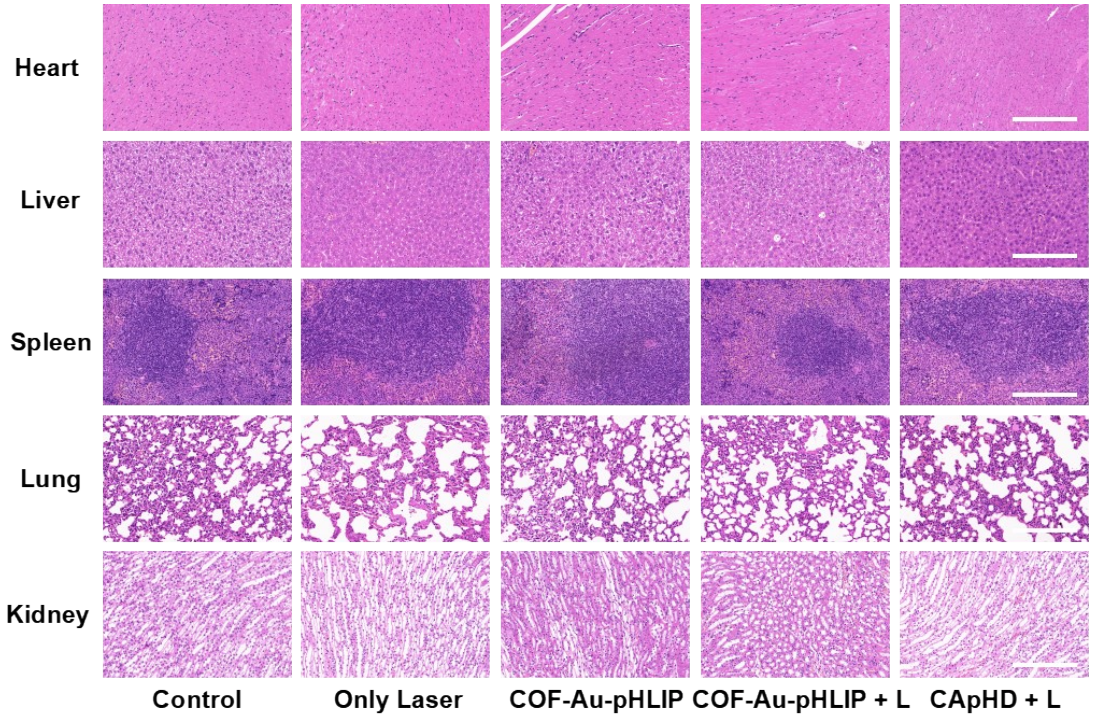

Figure S25. H&E staining of major organs after treatment (scale bar = 200 μm).