Supporting Information

NIR-Light Triggered Photodynamic Therapy Combined with Hypoxia Activated Chemotherapy for Anti-Tumor Effect

Chemical and Reagents

All commercially available compounds were used without further purification unless otherwise noted. All water used was Milliq water (18.2 MΩ). Olecin acid (OA), oleylamine (OM), tetraethoxysilane (TEOS) and cyclohexane (MCH) were purchased from Aladdin (China). 1-octadecene (ODE), Ethanol absolute (EtOH) and 1，3-diphenylisobenzofuran (DPBF) were obtained from Innochem (China). ERBium (III) chloride anhydrous ($ErCl_3$), Ytterbium chloride ($YbCl_3$), Sodium trifluoroacetate (CF_3COONa), Gadolinium trichloride ($GdCl_3$), Sodium hydroxide (NaOH), Ammonium chloride (NH_4Cl), 8-aminopyrene-1,3,6-trisodium trisulfonate (APTS), Trifluoroacetic acid (CF_3COOH) were purchased from Sigma-aldrich (USA). Hexadecy ltrimethyl ammonium bromide (CTAB) was obtained from Alfa (China). 3-aminopropyltriethoxysilane (APTES) was purchased from Beyotime (China). Chlorin e6 (Ce6) and Tirapazamine (TPZ) were purchased from MCE (USA). 2',7'- dichlorofluorescin diacetate (DCFH-DA) and Mouse Interleukin-6 ELISA kit were obtained from Solarbio (China). BisBenzimide H33342 (Hoechst 33342), Lyso-Tracker Green and Calcein/AM double staining kit were obtained from Beyotime (China). Annexin V/PI apoptosis kit and mouse Interleukin-12 ELISA kit were purchased from Elabscience (China). Cell counting kit-8 (CCK-8) was ordered from NCM (China). Goat anti-rabbit IgG H&L (Alexa Fluor® 488) and Recombinant anti-calreticulin antibody (EPR3924)-ER marker (Alexa Fluor 488) were obtained from Abcam (USA). HMG-1 polyclonal antibody was purchased from Immunoway (USA).

Instruments

UV/Vis spectra were collected on a DS5 spectrophotometer (Edinburgh, UK). Transmission electron microscopic (TEM) images were captured on a JEM-1200EX (JEOL, Japan) transmission electron microscope. In vitro cytotoxicity assay was carried out on a Multiskan SkyHigh (Thermo Fisher, USA). Confocal microscopic images were obtained using an FluoviewFV1000 confocal microscope (Olympus, Japan) at 60× and 40× magnification. The flow cytometry assays were carried out using a BECKMAN (CytoFLEX, USA). The in vivo fluorescence imaging was performed with IVIS®Lumina III Spectrum in vivo imaging system (PerkinElmer, Massachusetts).

Synthesis of NaGdF4:Yb,Er UCNPs

CF_3COONa (0.5 mmol) and $Ln(CF_3COO)_3$ (Ln = Gd, Yb, Er, with a total of 0.5 mmol and a 2% Er content) were added to a 100 mL three-neck flask containing 5 mmol oleic acid and 5 mmol 1-octadecene. After mixing, the mixture was heated to 120 °C, and the oxygen was removed by vacuum pumping and heated stably for 30 min. The mixture was continued to be heated to 310 °C under nitrogen protection and kept for 50 min. After cooling, the mixture was transferred to a 50 mL centrifuge tube and centrifuged (10000 rpm, 10 min) with the addition of ethanol. The precipitate was dispersed with 10 mL of cycloethane to produce α-$NaGdF_4$:Yb,Er.

Synthesis of core-shell $NaGdF_4$:Yb,Er@$NaGdF_4$ UCNPs

0.5 mmol CF_3COONa, 0.5 mmol $Gd(CF_3COO)_3$, 5 mmol oleic acid and 5 mmol 1-octadecene were added to a three-neck flask, followed by the introduction of previously synthesized α-$NaGdF_4$:Yb,Er. The mixture was then heated to 120 °C and vacuum-pumped for 30 min to eliminate oxygen. Afterward, the vacuum was turned off and heating was continued to 310 °C for 60 min

under the protection of nitrogen. Upon reaction completion, the heating was removed for natural cooling to room temperature, and the solution was subsequently transferred to a 50 mL centrifuge tube. An appropriate amount of ethanol was added, centrifuged at 10000 rpm for 10 min. 10 mL cyclohexane was added to the resulting precipitate and sonicated for 30 s to disperse homogeneously, ultimately yielding $NaGdF_4$:Yb,Er@$NaGdF_4$ UCNPs in the cyclohexane phase.

$NaGdF_4$:Yb,Er@$NaGdF_4$ UCNPs in the cyclohexane phase transfer to aqueous phase

1 mL of $NaGdF_4$:Yb,Er@$NaGdF_4$ UCNPs was mixed with 1 mL HCL solution (0.01 mol/L) using ultrasonic dispersion for 10 min. Then, the mixture was stirred for 3 h at room temperature, centrifuged at 10000 rpm for 2 min, the supernatant was removed, and an ethanolic solution of 1 mL HCL was added. After ultrasonic dispersion, the supernatant was removed by centrifugation at 10000 rpm for 20 min, and an ethanolic solution of 1 mL HCL was added. After centrifugation, 1 mL of deionized water was added and sonicated for dispersion before centrifugation at 10000 rpm for 20 min, then 1 mL of deionized water was added and sonicated for dispersion.

Synthesis of UCs@MSN (UM)

1 mg Cetyltrimethyl ammonium bromide (CTAB) was weighed in a 50 mL beaker, and dissolved in 20 mL of deionized water with stirring. Then, 400 μL of the prepared UCNPs was introduced dropwise and stirred overnight until transparency was achieved. The above clarified solution was poured into a 100 mL round-bottom flask, and deionized water was added to 60 mL, followed by 6 mL absolute ethanol and 100 μL sodium hydroxide solution. Next, 80 μL of Tetraethoxysilane (TEOS) dissolved in 1 mL ethanol was added dropwise to the flask and the reaction was stirred at 60 °C for 1.5 h. After cooling to room temperature, the mixture was collected into a 50 mL centrifuge tube and centrifuged at 10000 rpm for 5 min. The precipitate was washed twice with ethanol and dispersed in absolute ethanol. 0.06 g of ammonium chloride was weighed, dissolved in 30 mL absolute ethanol, and the sample prepared above was added and stirred at 60 ° C for 2 h. After the reaction, it was allowed to cool to room temperature and centrifuged at 10000 rpm for 5 min. The precipitate was collected and washed three times with ethanol, and finally dispersed in absolute ethanol to produce the desired UCNPs@MSN (UM).

Synthesis of UCs@MSN-Ce6/TPZ (UMCT)

600 μL of the synthesized UM was centrifuged (10000 rpm, 10 min) and the supernatant was removed, and 600 μL of Phosphate Buffered Saline (PBS) was added to disperse. 100 μL of TPZ solution (2.5 mg/mL) and 100 μL of Ce6 solution (10 mg/mL) were added, mixed and stirred for 24 h at room temperature in the dark. After 24 h, the cells were centrifuged again (10000 rpm, 10 min), and the supernatant and precipitate were separated into fresh centrifuge tubes. The precipitate was dispersed in 500 μL of PBS, centrifuged (10,000 rpm, 10 min), and washed three times to yield the final product: UCNPs@MSN-Ce6/TPZ (UMCT).

Drug loading

The supernatants of UMCT were collected, and the absorption spectra of Ce6 and TPZ in the supernatants were measured using a UV spectrophotometer. To establish a standard curve, a range of Ce6 and TPZ solutions with varying concentrations were prepared, and their absorbance values were recorded. This allowed for the calculation of unloaded Ce6 and TPZ drug concentrations in the supernatants based on the UV absorption standard curves. The encapsulation efficiency was determined using the formula: encapsulation efficiency = (original drug concentration - drug concentration in the supernatant) / original drug concentration × 100%.

Singlet oxygen ($^1O_2$) generation analysis

First, a solution of 1,3-diphenylisobenzofuran (DPBF, 1 mg/mL) was prepared with DMSO in the dark. Then 10 μL of DMSO solution of DPBF (1 mg/mL) was mixed with 2 mL of synthetic UMCT (100 μg/mL), and the initial absorbance of the sample was measured using a UV-vis spectrophotometer. After irradiation with or without 980 nm laser at 1.2 W/cm^2 for various durations (2, 4, 6, 8, 10 min), the absorbance was remeasured with a UV/VIS spectrophotometer.

Cell culture

Breast cancer (MCF-7) cells were cultured in DMEM supplemented with 10 % Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin solution. The cell cultures were stored in a humidified atmosphere with 5% CO_2 at 37 °C.

Cellular uptake assay

MCF-7 cells were seeded in 35 mm confocal dishes (~1 × 10^5 cells per well, for fluorescence imaging) or 6-well plates (~2 × 10^5 cells per well, for flow cytometry). Following a 4-hour incubation in Opti-MEM medium containing UMCT, the cells were washed with 1 mL of PBS and were then fixed using 4% paraformaldehyde for 10 min. After another series of three PBS washes, the cells were stained with 500 μL of the nuclear dye Hoechst 33342 (10 μM) and incubated for 10 min at room temperature in a dark environment. Finally, images were acquired by Confocal Laser Scanning Microscope (CLSM). MCF-7 cells, cultured in 6-well plates, were subjected to trypsin digestion and centrifugation (500 rpm, 5 min) following the aforementioned processing steps. The cells were then washed twice with 1 mL of PBS, resuspended in 500 μL of PBS, and finally, the intracellular fluorescence signal was captured by flow cytometry.

Co-localization assay

MCF-7 cells (~1 × 10^5 cells per well) were seeded and incubated overnight in 35-mm glass-bottom confocal culture dishes. Then cells were incubated with UMCT for distinct durations (1, 2, and 4 h). After washing three times with 1 mL PBS, an appropriate amount of Lyso Tracker was added and incubated for 15 min. The cells were again washed three times with 1 mL PBS before adding 1 mL of 4% paraformaldehyde to immobilize them for 10 min. After an additional PBS wash, 500 μL of the nuclear dye Hoechst 33342 was applied and incubated in darkness at room temperature for 10 min. Images were subsequently captured using CLSM.

ROS generation in living cells

MCF-7 cells were seeded in 35 mm confocal dishes (~1 × 10^5 cells per well, for fluorescence imaging) or 6-well plates (~2 × 10^5 cells per well, for flow cytometry). Following a medium change to Opti-MEM, the MCF-7 cells were exposed to PBS, UM, UMC, and UMCT for 4 h. Afterward, the cells were irradiated with a 980 nm laser at 1.2 W/cm² for 10 min, with irradiation pulses administered every minute. The cells were then washed twice with 1 mL of PBS, followed by two washes with 1 mL of DMEM. Next, 500 μL of DMEM containing 2',7'-dichlorofluorescein diacetate (DCFH-DA, 10 μM) was added to each well, and the cells were incubated for an additional 20 min. Excess dye was removed by washing the cells with 1 mL of DMEM, followed by two washes with 1 mL of PBS. After staining with 500 μL of the nuclear dye Hoechst 33342 for 10 min at room temperature in the dark, the cells were washed with 1 mL of PBS before imaging with CLSM. MCF-7 cells cultured in six-well plates underwent the same treatment and staining protocol. After washing with 1 mL of PBS, the cells were digested with trypsin. The resulting cell suspension was transferred to a 15 mL centrifuge tube and spun at 800 rpm for 5 min. The cells were then washed twice with 1 mL of PBS and resuspended in 500 μL of PBS. Ultimately, flow cytometry was employed to assess and compare variations in DCFH-DA fluorescence intensity across the different treatment groups.

In vitro cytotoxicity assay

MCF-7 cells were seeded in 96-well dishes at a density of 1×10^4 cells per well and incubated overnight in a normoxic environment with 20% oxygen. Cells designated for normoxia or hypoxia treatments were maintained in normoxia or moved to a hypoxia chamber (1% oxygen) for 12 h. Following this, Opti-MEM medium supplemented with PBS, UM, UMC, and UMCT was introduced to each well for a duration of 4 h. Subsequently, the cells underwent irradiation with a 980 nm laser at 1.2 W/cm² for 10 min, with irradiation pulses administered every minute. After PBS washing and addition of 100 μL of complete medium, the hypoxic treatment cells were returned to hypoxic conditions for an additional hour before being transferred back to normoxia and incubated alongside the normoxia treatment cells for 24 h. Likewise, 16HBE (human bronchial epithelioid) cells were plated and treated similarly with Opti-MEM containing UM, UMC, and UMCT for 4 h. After PBS washing, the cells were refreshed with complete medium and incubated for a further 24 h. To assess cell viability, 10 μL of CCK-8 reagent was added to each well and incubated for 1 h, followed by absorbance measurement at 450 nm using a microplate reader.

Live/dead cell staining experiment

MCF-7 cells, seeded in 35-mm glass-bottom confocal culture dishes, were first incubated in normoxia (20% oxygen) for 12 h. Subsequently, cells in the normoxia treatment group remained in normoxia, while those in the hypoxia treatment group were transferred to a hypoxia incubator (1% oxygen) for another 12 h. Following this, the medium was changed to Opti-MEM, and PBS, UM, UMC, and UMCT were introduced for a duration of 4 h. The cells were then exposed to a 980 nm laser for 10 min (1.2 W/cm^2, with 1-min intervals for 5 min) before being incubated for an additional hour. The cells underwent two washes with 1 mL of PBS, followed by two washes with detection buffer. Cells from the various treatment groups were subsequently co-cultured with calcein AM and PI dyes in an incubator for 30 min. After a final wash with 1 mL detection buffer, 1 mL of PBS was added, and fluorescence images were captured using CLSM.

Cell apoptosis assay

MCF-7 cells were plated in six-well dishes at a concentration of 2×10^5 cells per well and incubated overnight in a normoxic environment (20% oxygen). Cells designated for the normoxia group remained in these conditions, while those in the hypoxia group were moved to a hypoxia incubator (1% oxygen) for 12 h. Following a medium switch to Opti-MEM, the cells were exposed to PBS, UM, UMC, and UMCT for an additional 4 h, maintaining their respective oxygen conditions. Subsequently, the cells underwent irradiation with a 980 nm laser for 10 min (1.2 W/cm^2, with 1-min intervals every 5 min) and were then incubated for another hour under their respective oxygen conditions. After PBS washing, trypsin was applied for digestion, and the cell suspension was collected and centrifuged (800 rpm, 5 min), then washed twice with PBS. Cells were then resuspended in 100 μL Buffer, stained with 2.5 μL of Annexin V-FITC and PI dyes, and incubated in the dark at room temperature for 15 min Finally, the volume of each tube was adjusted to 500 μL, and cell apoptosis was and analyzed via flow cytometry.

Immunofluorescence staining assay

MCF-7 cells were seeded at a density of 1×10^5 per well in 35-mm glass-bottom confocal dishes and incubated overnight. Cells were then exposed to Opti-MEM medium supplemented with PBS, UM, UMC, and UMCT for 4 h, followed by irradiation with a 980 nm laser for 10 min (1.2 W/cm^2, with 1-min intervals). Afterward, the medium was washed with PBS and replaced with fresh complete medium for further incubation overnight. The cells were then washed three times with PBS, fixed

with 1 mL of 4% paraformaldehyde for 10 min, and permeabilized using 500 μL of 0.1% Triton X-100 per dish for another 10 min. This was followed by three washes with 500 μL PBST every 5 min. Next, the cells were blocked with 5% fetal bovine serum for 30 min and incubated with 200 μL of anti-CRT (1:500) and anti-HMGB1 (1:200) antibodies for 1 h at room temperature or overnight at 4 °C. After three washes with 500 μL PBST for 5 min, the corresponding fluorescent secondary antibody (AlexaFluor® 488-labeled) was added and incubated for 1 h at room temperature in the dark. Finally, the plates were washed twice with 500 μL PBST, and images were captured using CLSM.

Mice and tumor models

All in vivo experiments were carried out complying with NIH guidelines, with the protocol approved by the Institutional Animal Care and Use Committee of Beijing Tuberculosis and Thoracic Tumor Research Institute. BALB/c mice aged 6-8 weeks (female,18-22 g) were purchased from Vital River Animal Laboratories (Beijing, China) and maintained in a sterile environment with access to water and food. Xenograft tumor models were established by inoculating 4T1 cells (1×10^6 cells/100 μL in 1:1 (v/v) PBS and Matrigel, BD bioscience) into the left flank of the mice. When the tumor volumes reached the appropriate size, the mice were used for further in vivo experiment.

In vivo biodistribution

When the tumor sizes reached 100-200 mm^3, the mice were anesthetized and intravenously injected with UMCT into the tail vein of the 4T1 tumor-bearing mice. At the pre-determined time points (0, 1, 3, 6, 12, 24 h), mice were anesthetized with isoflurane and imaged with an IVIS system. The mice were euthanized at the specified time points postinjection and their major organs including heart, liver, spleen, lung, kidney, and tumor were carefully removed for visualization under the imaging system.

In vivo antitumor assay

When the tumor sizes reached 50 mm^3, the tumor bearing mice were randomly divided into 8 groups. PBS, UM, UMC, and UMCT were administered via tail vein injection every other day for a total of 3 times. Six hours post-injection, tumors was irradiated with a 980 nm laser (1.2 W/cm^2, 1-min intervals for 5 min). Body weight and tumor volume (calculated as (length × $width^2$) / 2) were monitored every other day. On the 16th day of treatment, mice were euthanized, and their tumors were collected, photographed, and weighed. Tumor tissues were then fixed in 4% paraformaldehyde and embedded in paraffin. Moreover, the tumor and major organs were sectioned for histopathological analysis with hematoxylin and eosin (H&E) staining and triphosphate nickend labeling (TUNEL) immunofluorescence assay.

Haematological indixes and blood biochemistry.

Healthy mice underwent injection via the tail vein with PBS, UM, UMC, and UMCT every other day for a total of three times. After two weeks of treatment, whole blood and serum were collected, and major organs (heart, liver, spleen, lung, and kidney) were harvested. Serum and whole blood were subjected to biochemical analysis and blood cell counting, respectively. Serum levels of cytokines (including IL-12 and IL-6) were measured by Enzyme-linked immunosorbent assay (ELISA). Furthermore, the major organs were fixed with 4% paraformaldehyde, subsequently embedded, and sectioned for H&E staining to evaluate their biosafety.

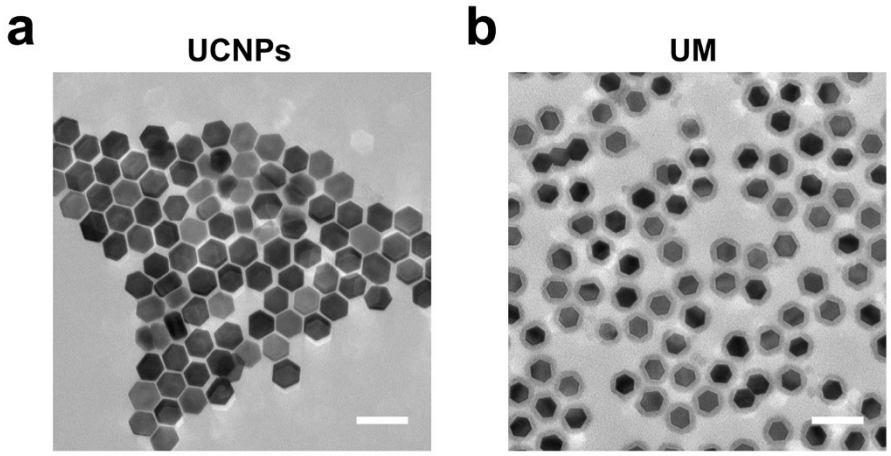

Figure S1 TEM images of $NaGdF_4$:Yb,Er@$NaGdF_4$ (UCNPs, a) and UCs@MSN (UM, b).

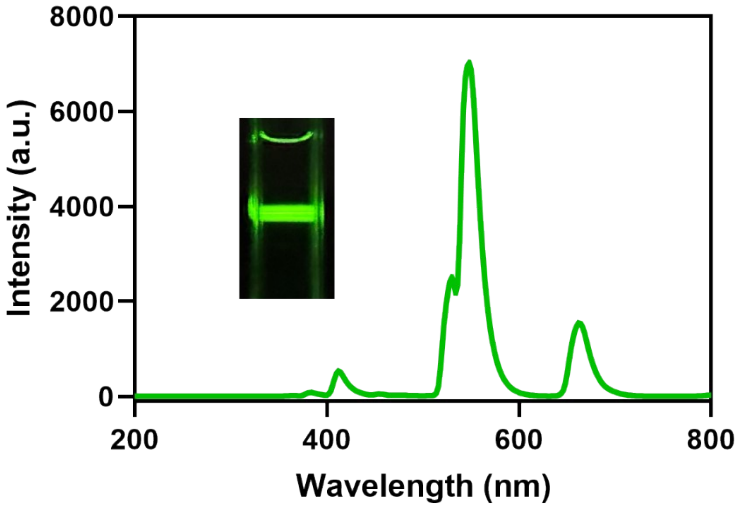

Figure S2. Upconversion luminescence spectra of UCNP under excitation at 980 nm. Inset shows the photographs of the solution of UCNP under 980 nm laser illumination.

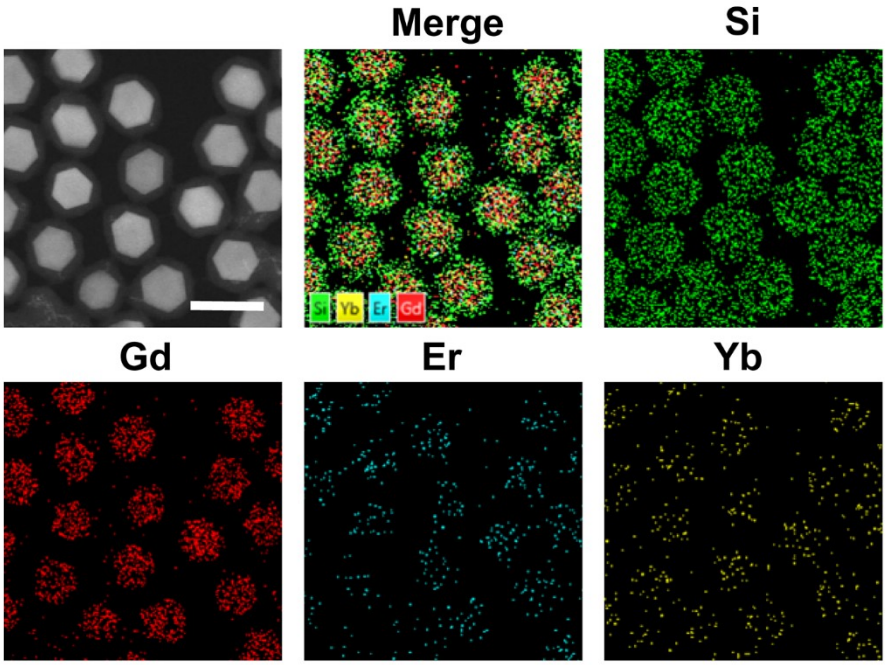

Figure S3 Elemental mapping images of UM. Scale bar, 100 nm.

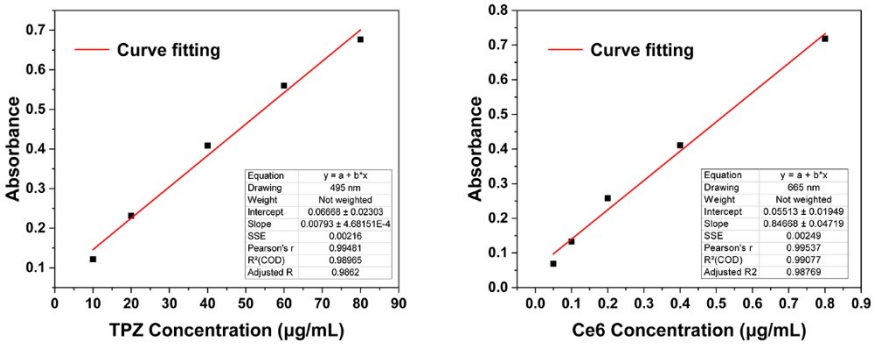

Figure S4 The standard curve of TPZ and Ce6.

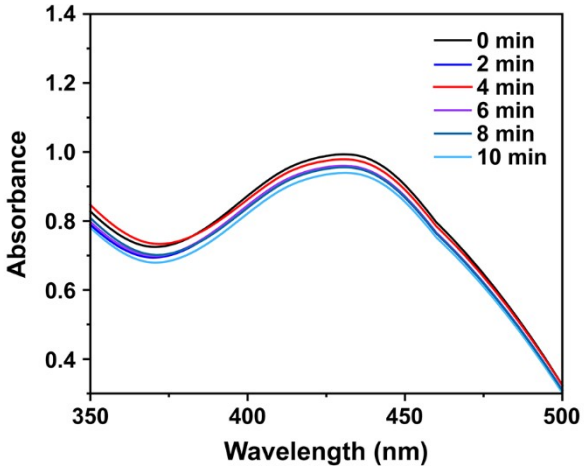

Figure S5 UV–vis absorption spectra of DPBF after incubation with UMCT in the absence of 980 nm laser irradiation.

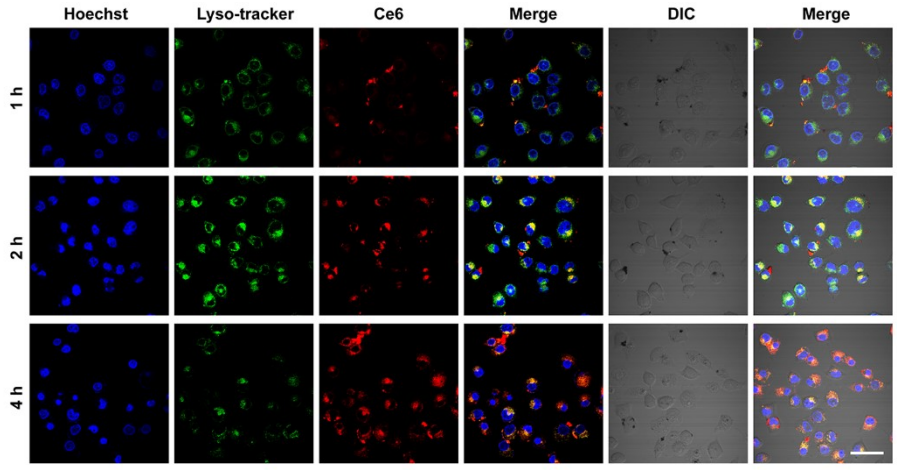

Figure S6 CLSM images of MCF-7 cells after incubation with UMCT for 1 h, 2 h and 4 h. The endo/lysosomes were stained by Lyso-Tracker Green. Scale bar, 50 μm.

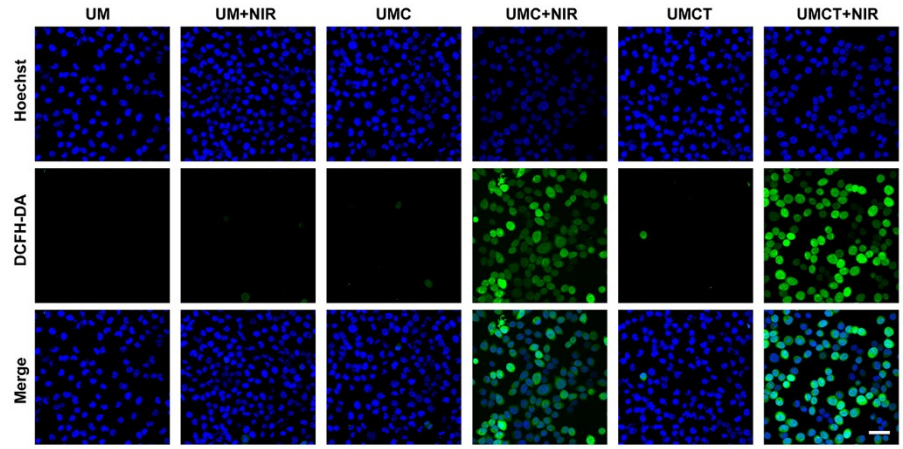

Figure S7 CLSM images of ROS detected by DCFH-DA probe in MCF-7 cells after treatment with different formulations. Scale bar, 50 μm.

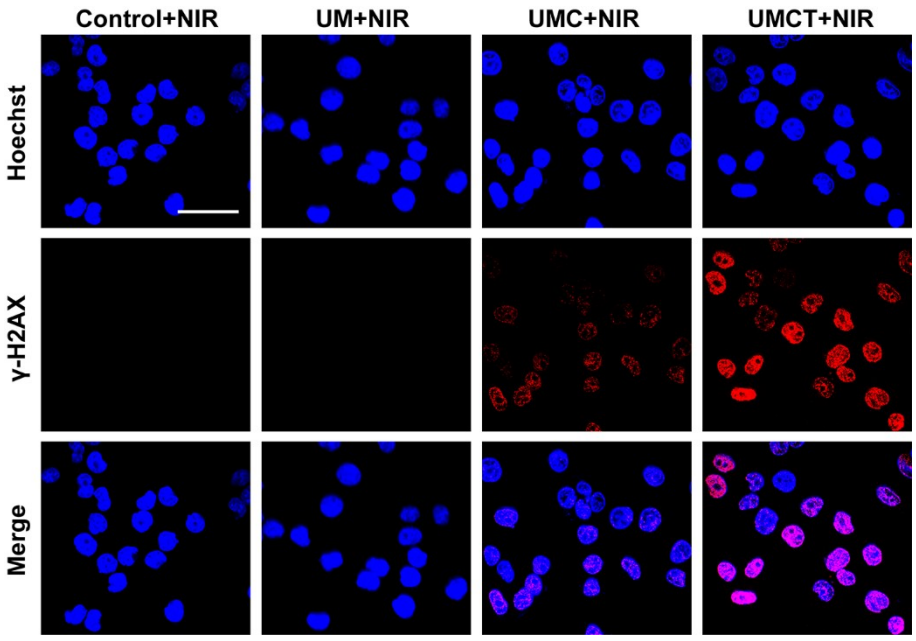

Figure S8 Representative fluorescence images of γ-H2AX-stained MCF-7 cells indicating DNA damage after different treatments. Scale bar: 50 μm.

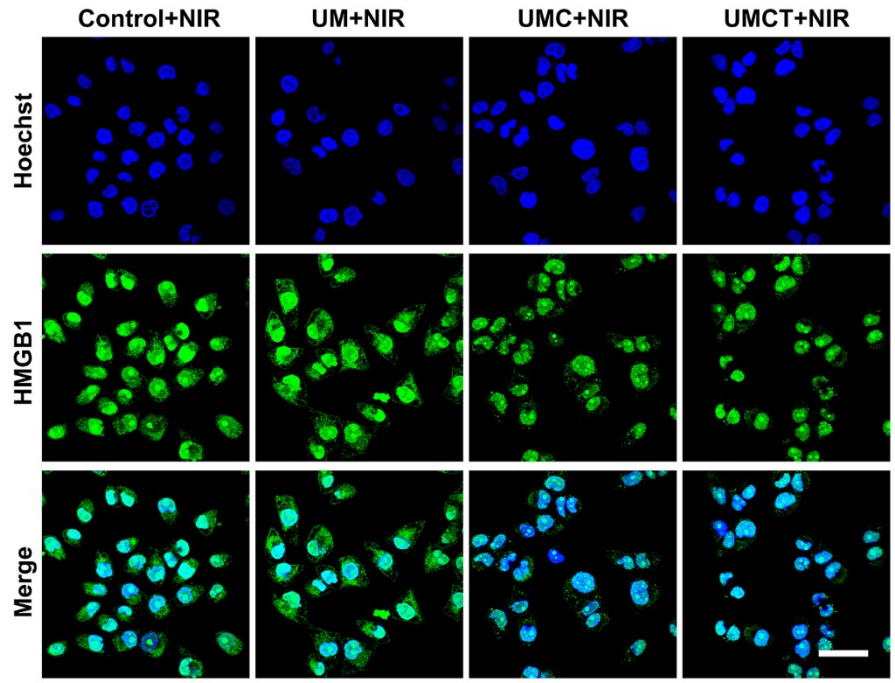

Figure S9 Immunofluorescence staining of HMGB1 in MCF-7 cells with different treatments with and without NIR light irradiation. Scale bar, 50 μm.

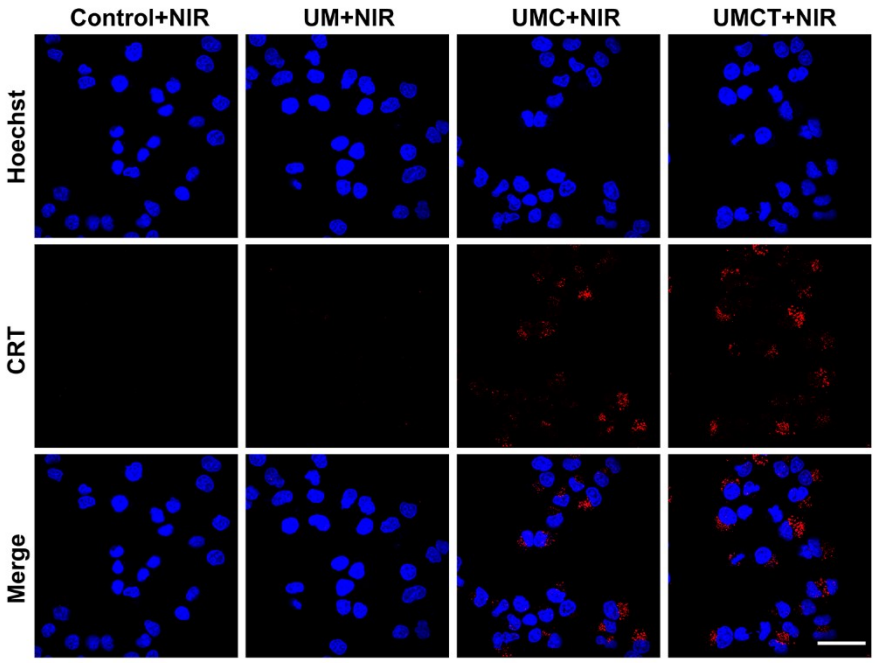

Figure S10 Immunofluorescence staining of CRT in MCF-7 cells with different treatments with and without NIR light irradiation. Scale bar, 50 μm.

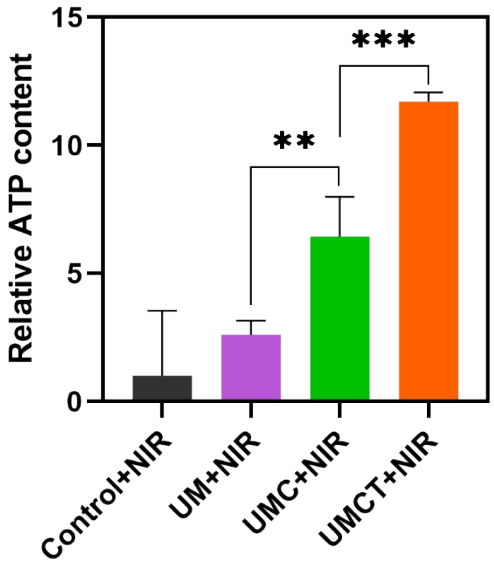

Figure S11 Relative ATP content of MCF-7 cells after treatment with different nanomaterials under 980 nm laser.

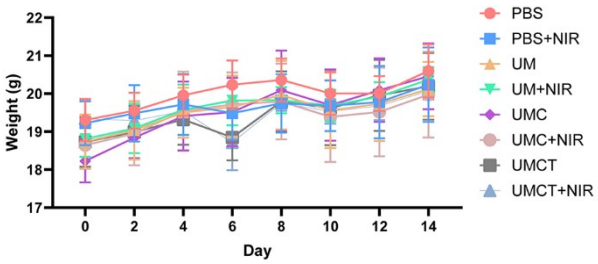

Figure S12Time-dependent changes in body weight of 4T1 tumor-bearing mice subjected to various treatments.

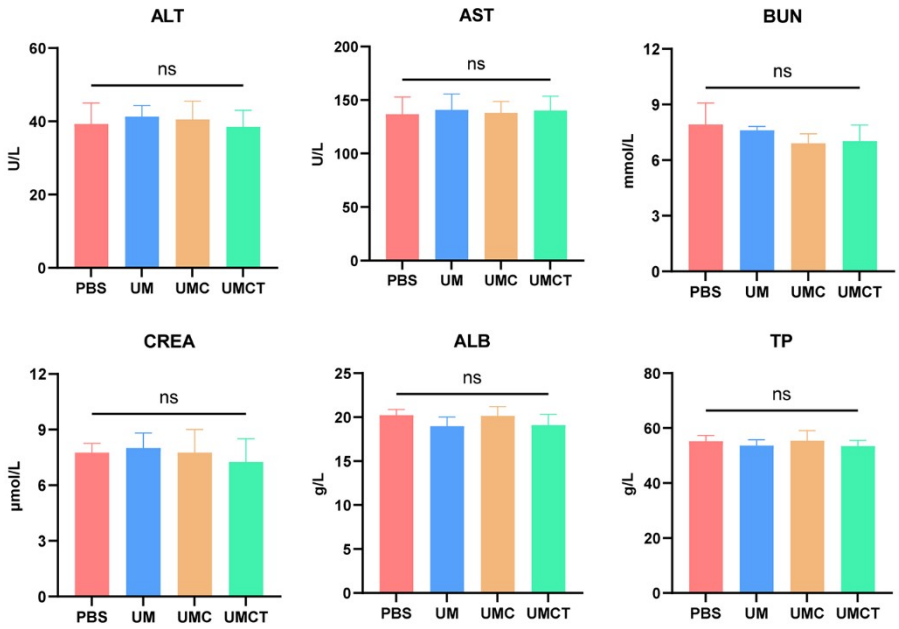

Figure S13 Serum biochemistry analysis of 4T1 tumor-bearing mice after various treatments to investigate the potential systemic toxicity.

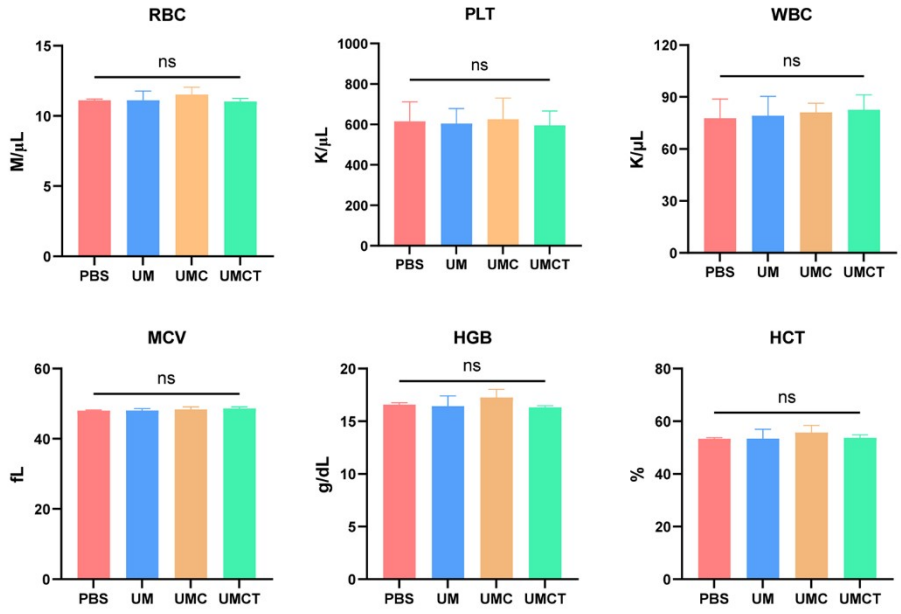

Figure S14 Whole blood analyses from 4T1 tumor-bearing mice after different treatments.

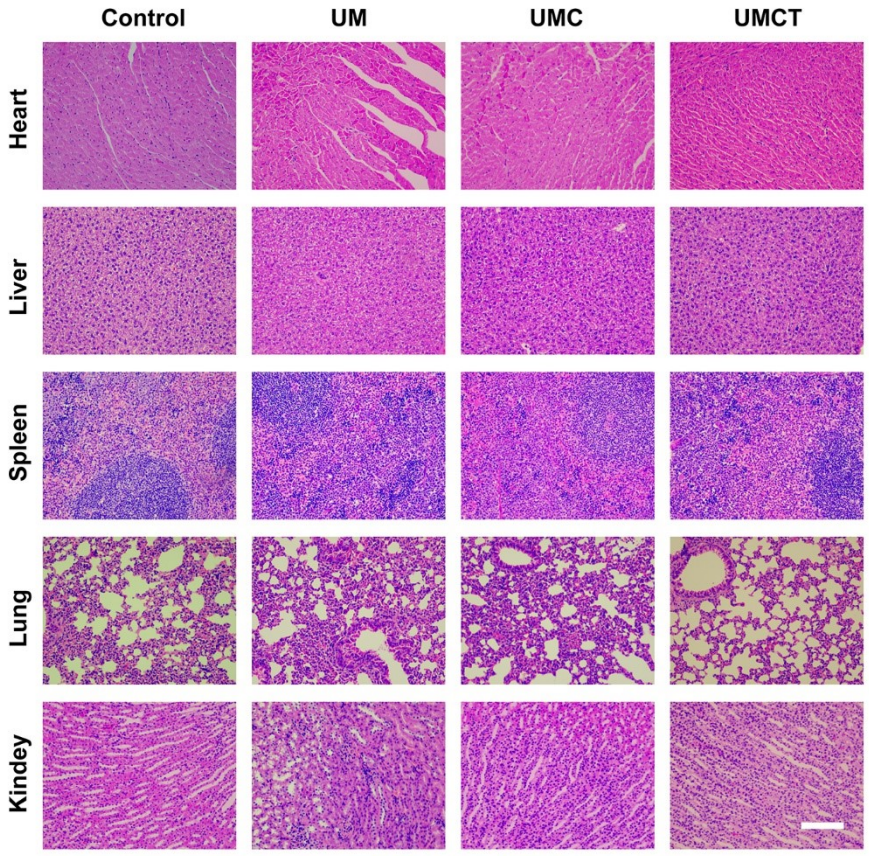

Figure S15 Histological analysis of hematoxylin and eosin (H&E) stained tissue slices of major organs (heart, liver, spleen, lung and kidney) after various treatments. Scale bar, 200 μm.

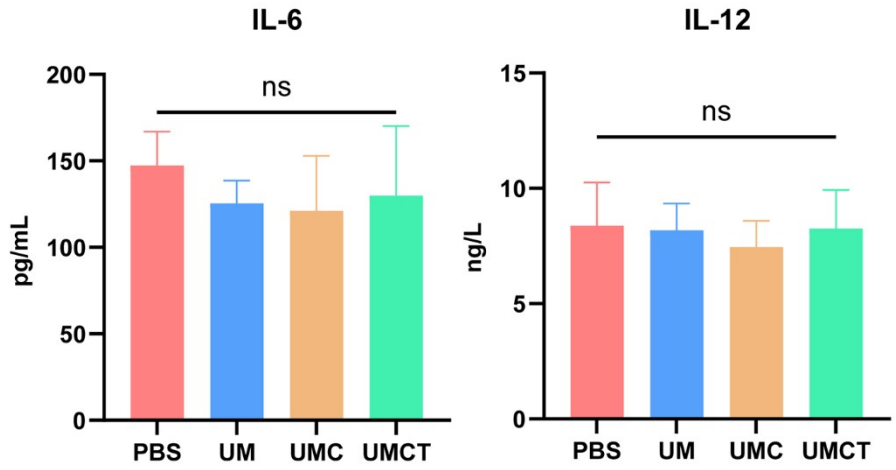

Figure S16 Interleukin-6 (IL-6) and Interleukin-12 (IL-12) levels of serum from 4T1 tumor-bearing mice after various treatments using enzyme-linked immunosorbent assay (ELISA).