Electronic Supplementary Information for:

Stable π-Radical Nanoparticles as Versatile Photosensitizer for Effective Hypoxia-Overcoming Photodynamic Therapy

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These authors contributed equally: X. Cui and G. Lu

1. Supporting Figures

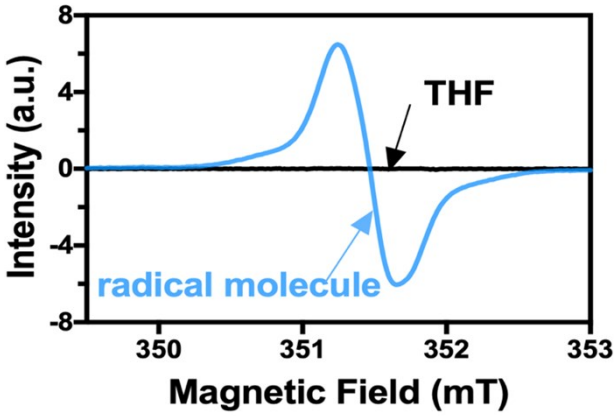

Figure S1. ESR spectra of the PTM-TPA radical molecule in THF and THF solvent as control group.

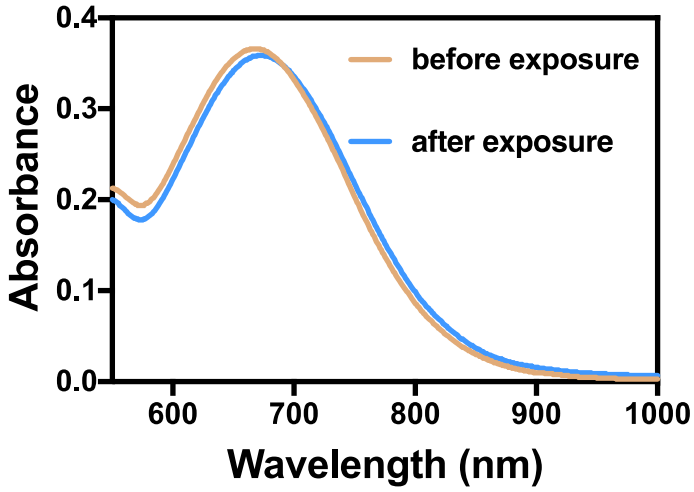

Figure S2. Absorption spectra of the PTM-TPA radical molecule before and after irradiation with a 635 nm laser (0.4 W/cm^2) under ambient conditions for one hour.

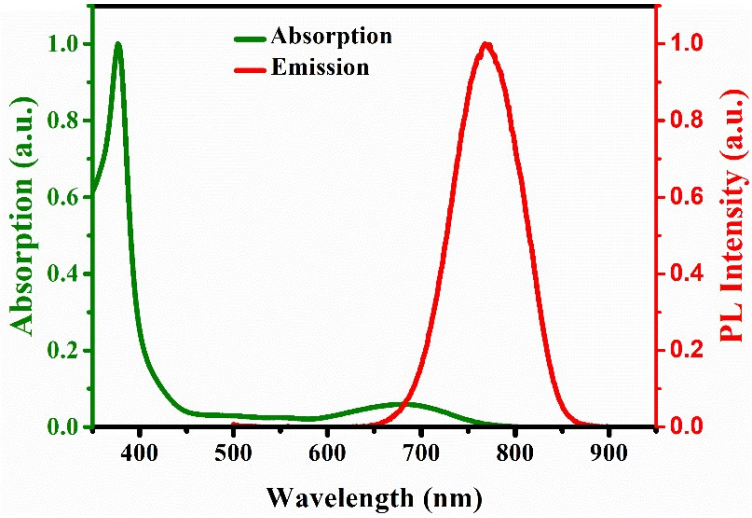

Figure S3. Absorption and fluorescence spectra of the PTM-TPA radical molecule in cyclohexane.

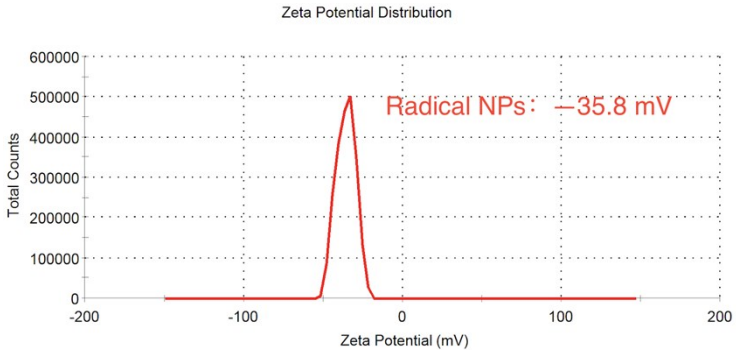

Figure S4. Zeta potential of the Radical NPs in water.

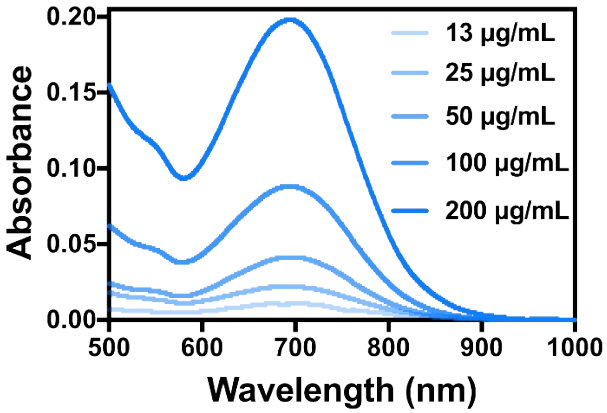

Figure S5. Absorption spectra of the Radical NPs at different concentrations.

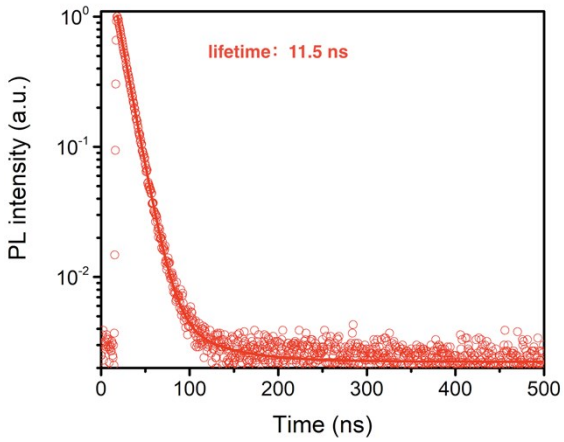

Figure S6. PL decay curve at room temperature of the Radical NPs.

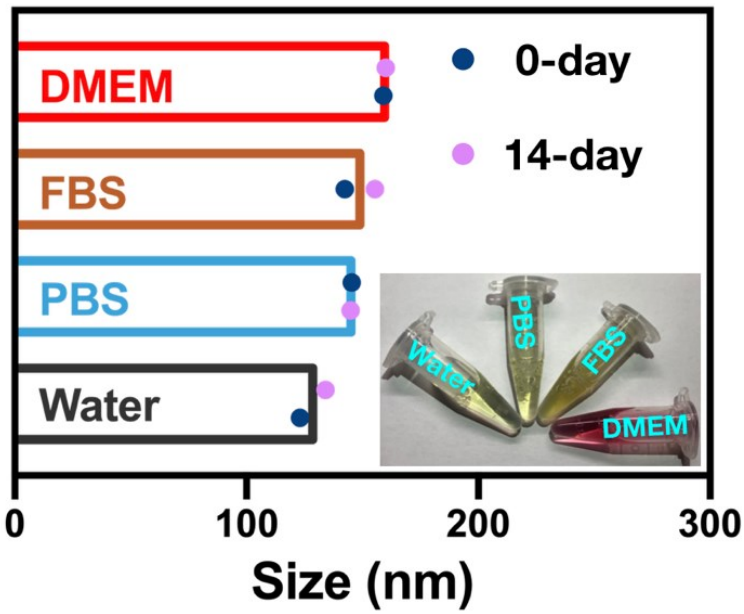

Figure S7. Size stability of nanoparticles in different physiological mediums (The inset is a digital photograph of Radical NPs dispersed in water, PBS, FBS and DMEM).

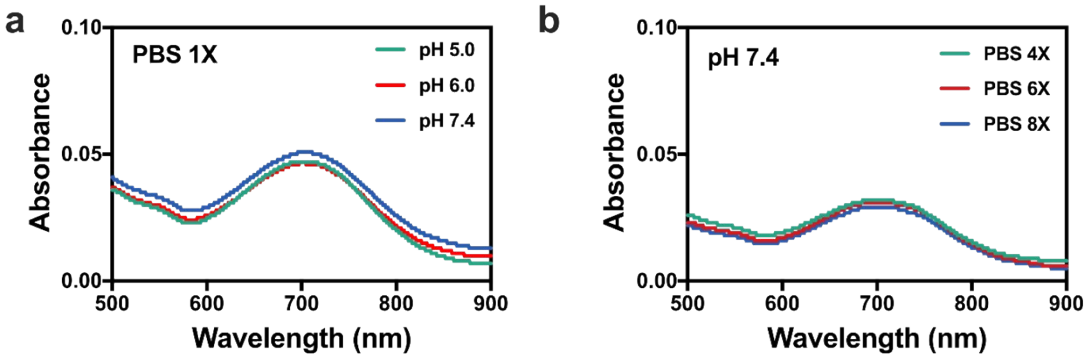

Figure S8. Absorption spectra of the Radical NPs in PBS with (a) different pH values and (b) ions concentrations.

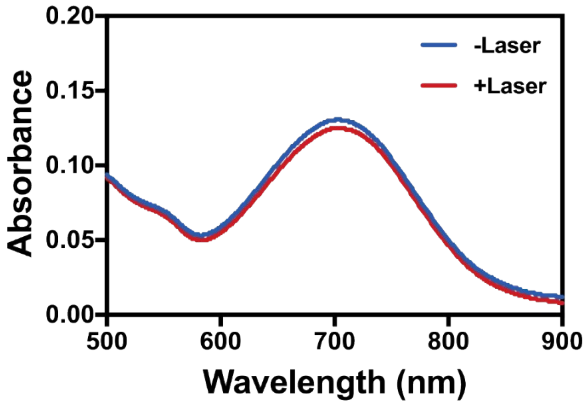

Figure S9. Absorption spectra of the Radical NPs before and after exposure to 635 nm laser irradiation (0.4 W/cm^2) for 1 hour.

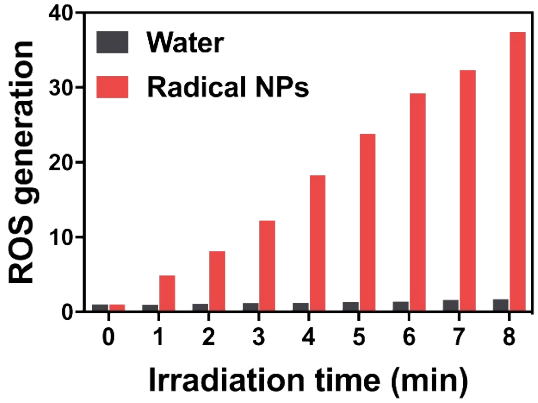

Figure S10. ROS generation evaluated by DCFH with or without NPs addition.

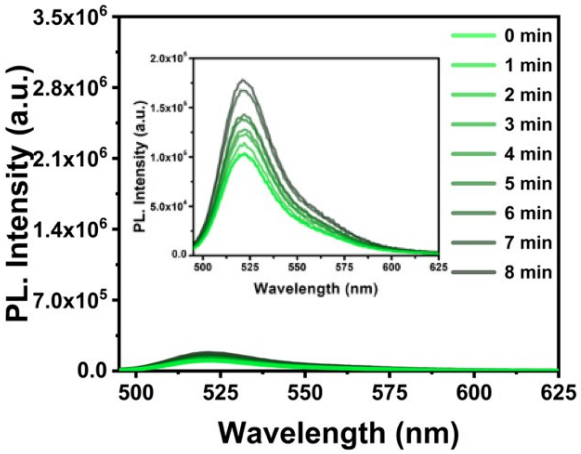

Figure S11. DCFH fluorescent spectra in the absence of the Radical NPs (488 nm excitation), inset is the enlarged spectra.

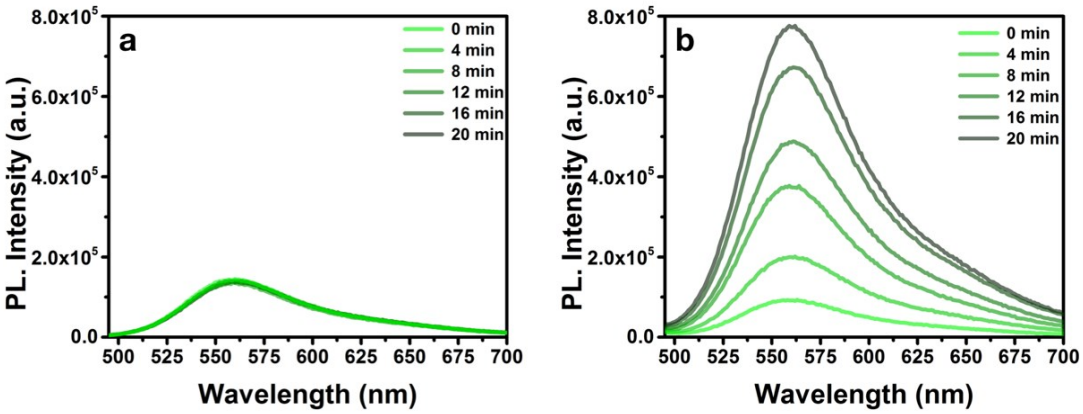

Figure S12. SOSG fluorescent spectra incubated with (a) water and (b) the Radical NPs (488 nm Excitation).

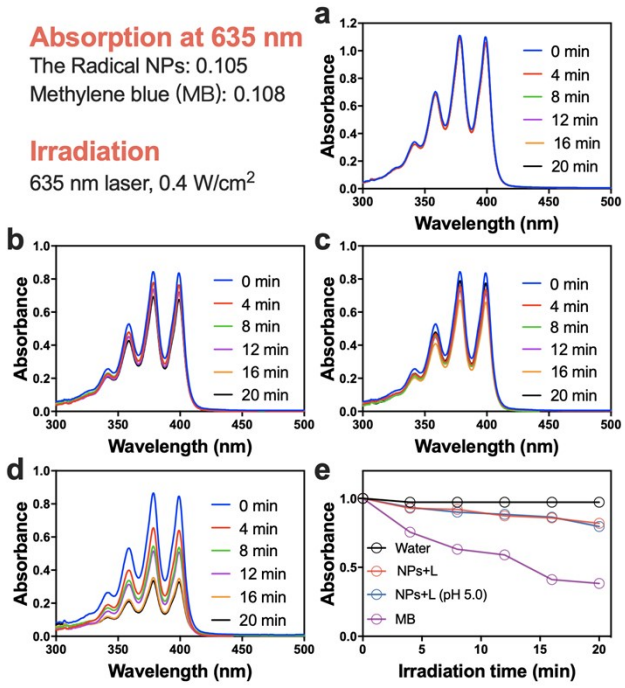

Figure S13. Time-dependent decay of ADPA induced by $^1O_2$ generation: (a) only water, (b) the Radical NPs, (c) the Radical NPs in pH 5.0, and (d) methylene blue. (e) The change in ADPA absorption at 380 nm as a function of the irradiation duration.

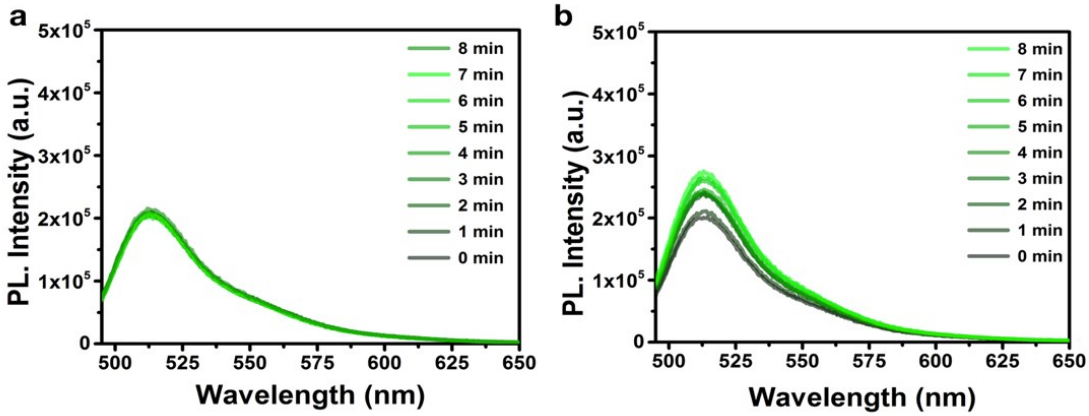

Figure S14. HPF fluorescent spectra (a) without or (b) with the Radical NPs (488 nm excitation).

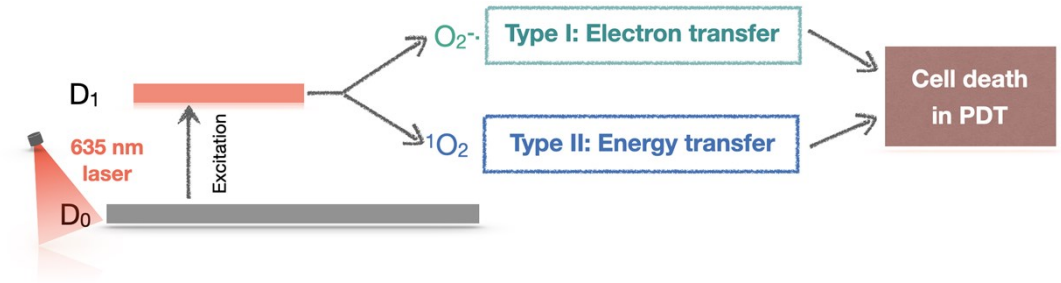

Figure S15. Proposed mechanism of photodynamic therapy.

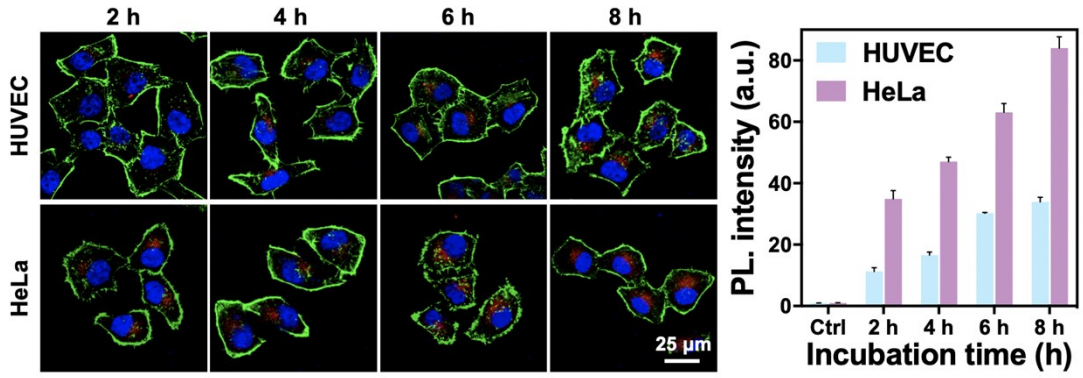

Figure S16. Confocal fluorescence images of HUVEC cells and HeLa cells treated with the Radical NPs in different time durations. The fluorescence of the Radical NPs is shown in red, the cell membrane stained with Rhodamine phalloidin is shown in

green, and the cell nuclei stained with Hoechst 33324 is shown in blue, scale bar is 25 µm.

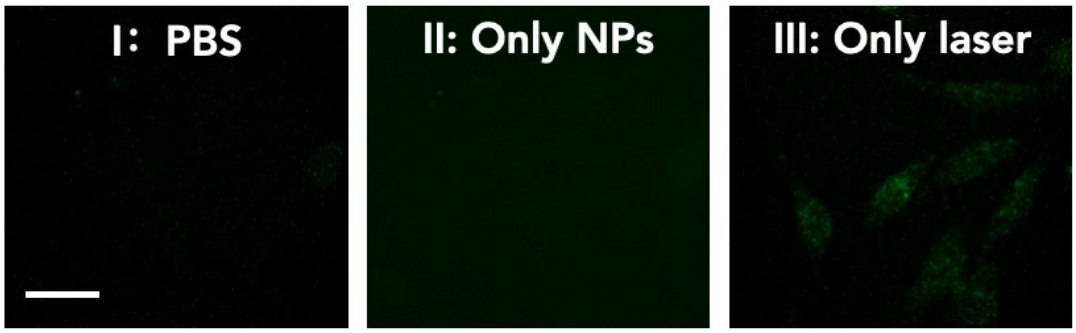

Figure S17. Intracellular ROS generation using DCFH-DA as a probe (I) PBS, (II) only NPs and (III) only laser irradiation, scale bar is 30 µm.

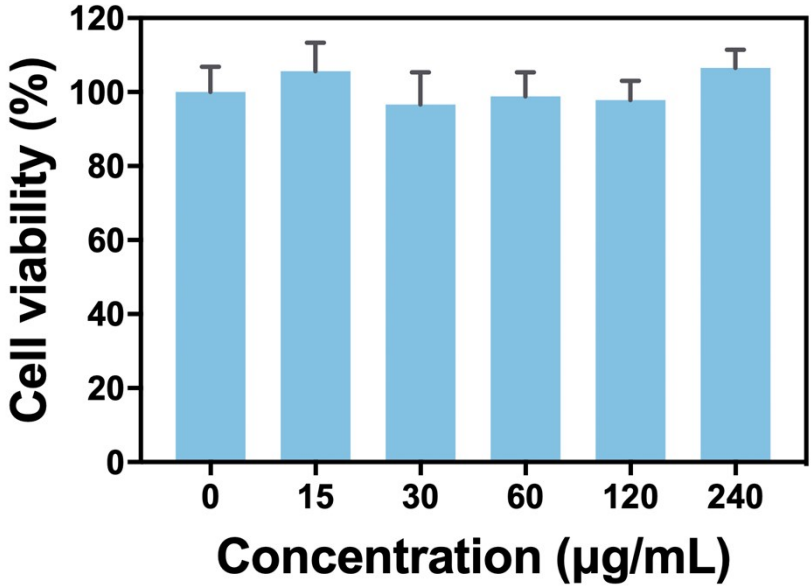

Figure S18. NIH-3T3 cell viability incubated with various concentrations of the Radical NPs (24 h).

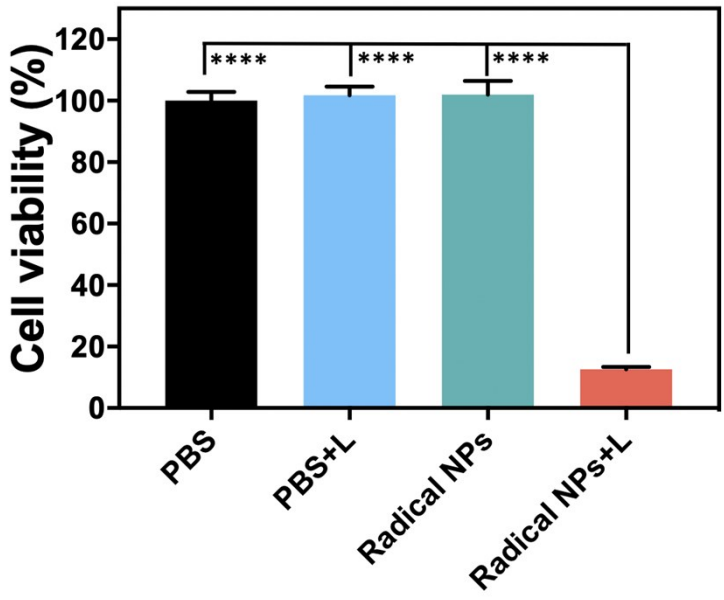

Figure S19. HeLa cell viability with various formulations: only PBS (PBS), PBS with laser irradiation (PBS+L), only Radical NPs (Radical NPs) and Radical NPs plus laser irradiation (Radical NPs+L). (****P < 0.0001)

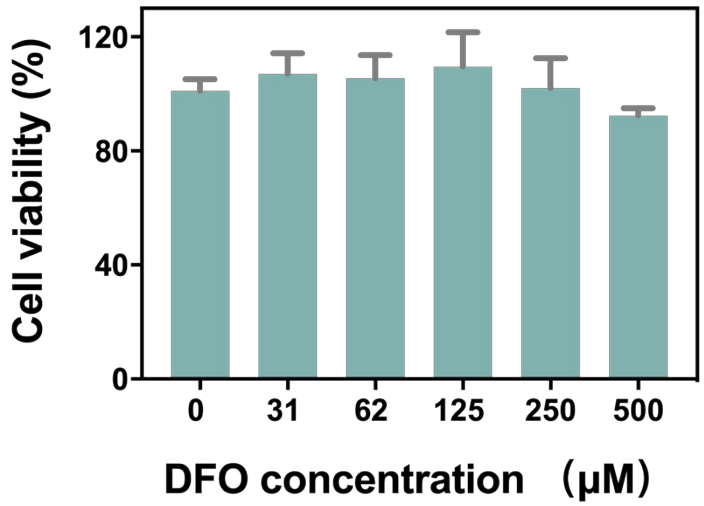

Figure S20. HeLa cell viability incubated with various concentrations of DFO.

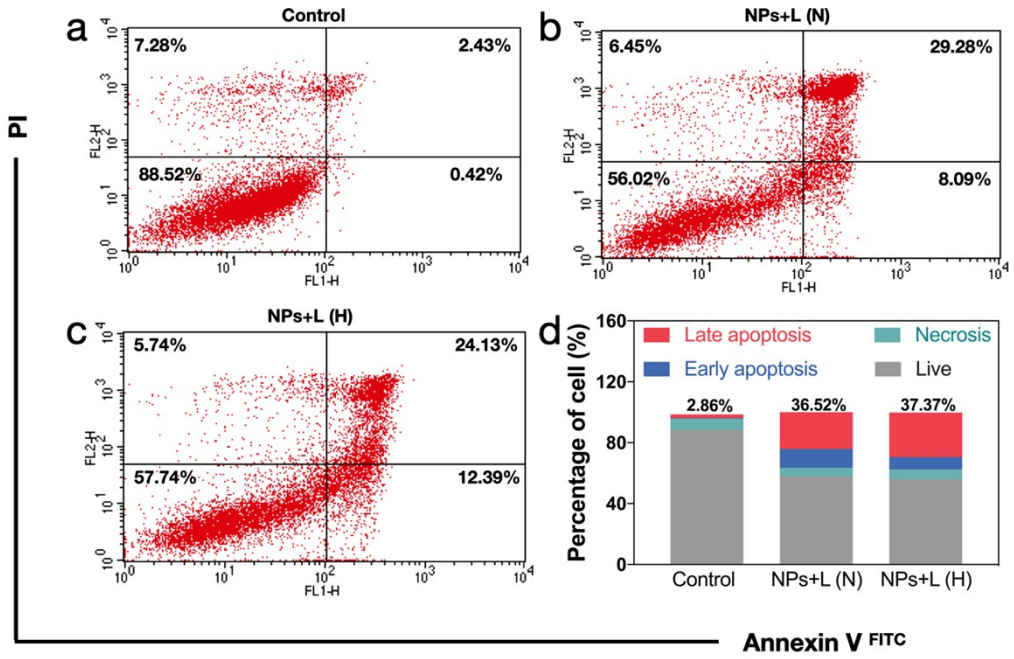

Figure S21. Apoptosis and necrosis ratio of HeLa cells with (a) control (only cells), NPs plus laser irradiation in (b) normoxia and (c) hypoxia using flow cytometry with Annexin V ^{FITC} and PI staining. (d) Apoptosis (early and late apoptosis) analysis of HeLa cells after various treatment.

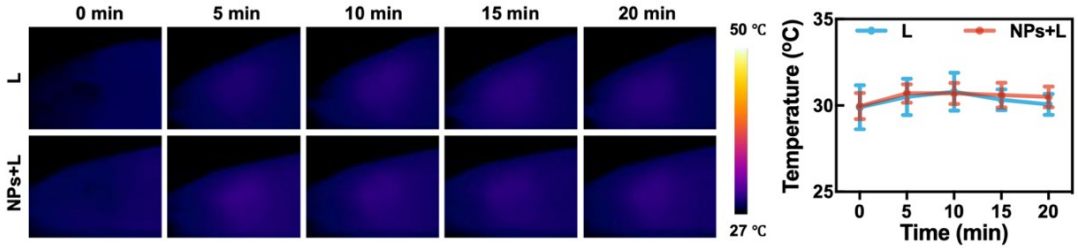

Figure S22. Infrared thermal images of HeLa-tumor-bearing nude mice after various treatments and temperature change profiles of tumors under laser irradiation with or without NPs addition.

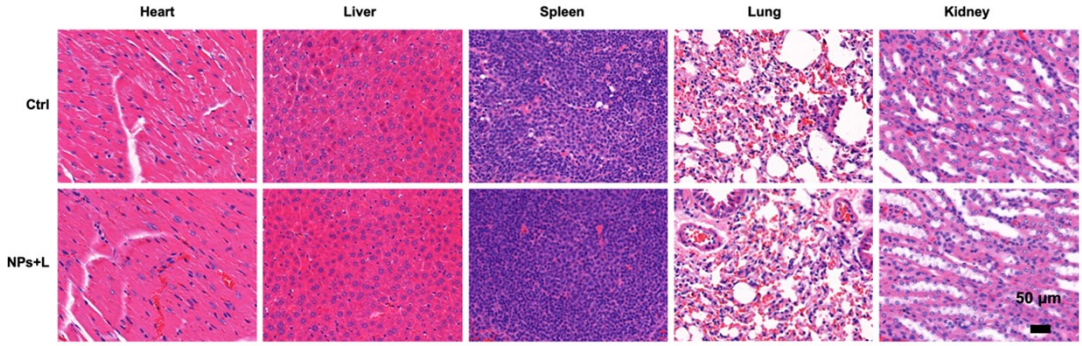

Figure S23. H&E staining immunohistochemical staining of main organs (heart, liver, spleen, lung and kidney) collected from mice after various treatments (Ctrl: healthy mice without tumor as control group, NPs+L: tumor bearing mice treated with NPs plus irradiation), scale bar is 50 μm.

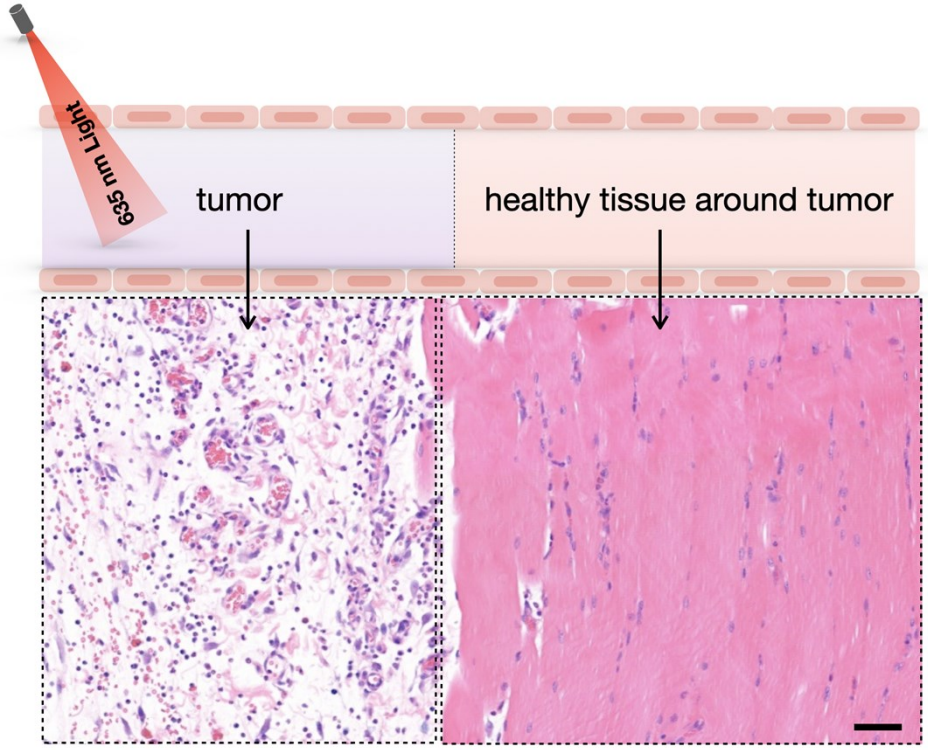

Figure S24. H&E staining immunohistochemical staining of healthy tissues around tumor, scale bar is 50 μm.

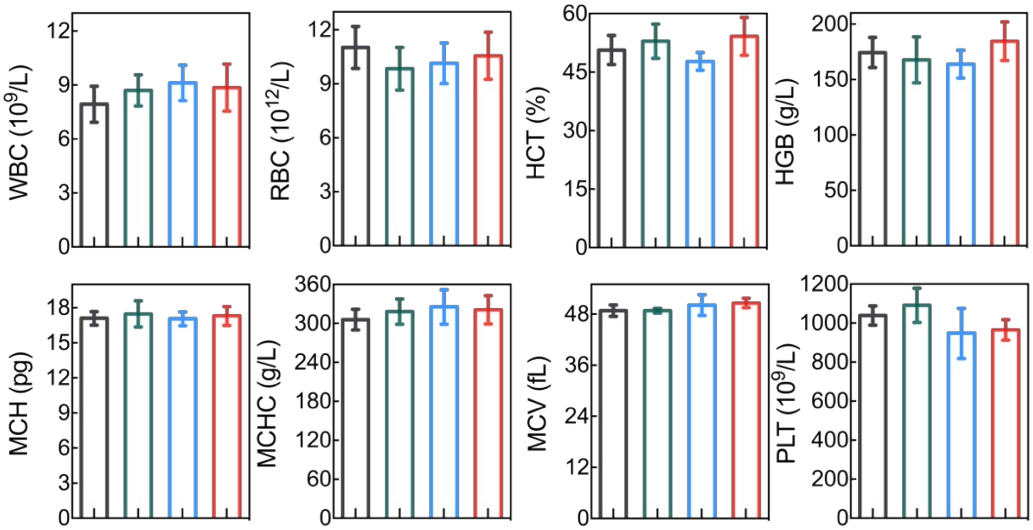

Figure S25. Complete blood count assay of different treatment groups (Black column represents only PBS; green column represents only laser; blue column represents only NPs; red column represents NPs+Laser).

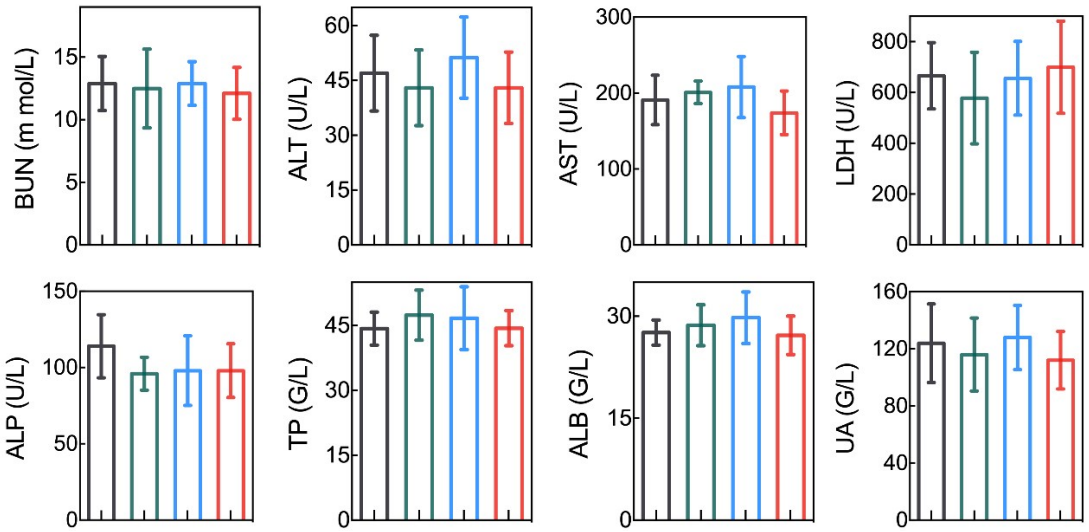

Figure S26. Blood biochemistry data of mice: BUN, ALT, AST, LDH, ALP, TP, ALB, UA levels in the blood after various treatments (Black column represents only PBS; green column represents only laser; blue column represents only NPs; red column represents NPs+Laser).

2. Experimental Procedures

2.1. Materials: DSPE-PEG2000, 2',7'-Dichlorofluorescin (DCFH), 2',7'-Dichlorofluorescin diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl tetrazolium bromide (MTT), deferoxamine (DFO), 9,10-anthracenedipropionicacid (ADPA), and Protoporphyrin IX (PPIX) were bought from Sigma-Aldrich. Trypsin-EDTA (no phenol red), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Phosphate buffered saline (PBS, pH 7.4, 10X), penicillin-streptomycin (PS) were from Gibco Thermo Fisher. Singlet oxygen sensor green (SOSG), Annexin V-FITC conjugate, propidium iodide (PI), Rhodamine phalloidin (for staining cell membrane), Hoechst 33342 (for staining cell nuclei) and Image-iT™ Green Hypoxia Reagent were purchased

from Invitrogen Thermo Fisher. Hydroxyphenyl fluorescein (HPF) was purchased from ENZO Life Science. Chlorin e6 (Ce6) was purchased from Frontier Scientific. DHR 123 was bought from Aladdin Company. Tetrahydrofuran (THF) and dimethyl sulfoxide (DMSO) were ordered from J&K Scientific Ltd. All chemicals were used without further purification.

2.2. Characterizations: Size and morphology of the Radical NPs were investigated on transmission electron microscope (TEM, Philips CM-200 FEG). Size of NPs were measured using a Malvern Zetasizer Nano ZS. Absorption properties of molecule and nanoparticles were obtained using a Shimadzu-1700 spectrometer. Photoluminescence (PL) spectra of materials and probes were explored by Edinburgh FLS 980 and Horiba Fluoromax-4 Spectrofluorometer. Fluorescence decay of the Radical NPs was measured by Edinburgh FLS 980 using 375 nm laser source. ESR signal of radical molecule in THF and Radical NPs in PBS were detected using the ESR technique without addition of any trapping agent (E500, Bruker). Leica TCS SPE/SP5 laser confocal scanning microscope were used for confocal fluorescence imaging.

2.3. Radicals NPs Preparation.

The Radical NPs were prepared using a nanoprecipitation method. In a typical synthesis, 500 µL PTM-TPA radical molecule (0.5 mg/mL in THF) and 500 µL DSPE-PEG2000 (5 mg/mL in THF) were respectively prepared and then fully mixed under ultrasonication. 1 mL of mixed solution was added dropwise into 9 mL of DI water

under vigorous stirring at room temperature. After 48 h of continuous stirring, the THF in solution is almost completely removed and the solution was filtered by 450 nm filter. Finally, the sample was collected with a 100,000 kDa ultrafiltration tube and the concentrated solution was stored at 4 °C for future use.

2.4. Total ROS Detection by DCFH.

DCFH fluorescence intensity was applied to detect reactive oxygen species. Typically, 0.6 mL DCFH (concentration: 10 nM) was mixed with 0.1 mL the Radical NPs solution (working concentration: 100 μg/mL). Control group were prepared by adding 0.1 mL water in 0.6 mL DCFH. After that, the mixtures were exposed to 635 nm irradiation (0.4 W/cm^2), and the photoluminescence (PL) intensities of DCFH at ~525 nm were recorded every minute in a photoluminescence spectrofluorometer (488 nm excitation).

2.5. Detection of $^1O_2$ Generation in Normoxia by SOSG.

$^1O_2$ generation in solution were monitored with SOSG via its fluorescence intensity. Briefly, SOSG in methanol (working concentration: 5.5 μM) was mixed with the Radical NPs solution (working concentration: 50 μg/mL) and irradiated by 635 nm laser (0.4 W/cm^2). Then the fluorescence intensities of SOSG at 525 nm of different irradiation intervals (0, 5, 10, 15 and 20 min) were recorded in a photoluminescence spectrofluorometer (488 nm excitation).

2.6. Quantum Yields of $^1O_2$ Using ADPA.

The generation of $^1O_2$ was detected chemically using ADPA which would be bleached to its corresponding endoperoxide upon react with $^1O_2$. The reaction was monitored spectrophotometrically by recording the decrease in optical densities at 380 nm. Methylene blue was used as a calibration standard (Φ =52%), and the absorption maxima of methylene blue and the Radical NPs were adjusted to below 0.2 OD. 20 μL ADPA (1 mg/mL in DMSO) was mixed with 1 mL of NPs dispersion. The samples were irradiated with a 635 nm laser (0.4 W/cm^2), and their absorption at 380 nm were recorded every 4 min in a UV-vis spectrophotometer. $^1O_2$ quantum yield of the Radical NPs were calculated according to previous reports.[1-3]

2.7. Detection of $O_2^{-\bullet}$ in Stimulated Hypoxia.

DHR 123 probe fluorescence intensity was applied to detect $O_2^{-\bullet}$ generation. Typically, DHR 123 (working concentration: 10 μM) was mixed with Ce6 (working concentration: 50 μg/mL) or Radical NPs (working concentration: 50 μg/mL). In order to extract oxygen for the stimulated hypoxia, the prepared solution in a tightly sealed Schlenk tube was quickly frozen using liquid nitrogen for 5 min, then pull vacuum for 5 min while it is frozen. By pulling vacuum, most of oxygen can be removed to stimulate hypoxia. This procedure was repeated three time, and then the mixtures in a tightly sealed Schlenk tube were directly exposed to 635 nm laser irradiation (0.4 W/cm^2). After irradiation for 5 min, the PL intensity of DHR 123 at 525 nm were recorded in a photoluminescence spectrofluorometer (488 nm excitation).

2.8. Detection of Extracellular •OH.

HPF probe fluorescence intensity was applied to detect •OH generation. Typically, HPF (working concentration: 10 μM) was mixed with Radical NPs (working concentration: 50 μg/mL). After that, the mixtures were exposed to irradiation from a 635 nm laser (0.4 W/cm^2). Finally, the PL intensity of HPF at 525 nm were measured in a photoluminescence spectrofluorometer (488 nm excitation).

2.9. *In Vitro* Cell Imaging.

To monitor the uptake of the Radical NPs, cancer cell (HeLa) or healthy cells (HUVEC) were seeded in glass bottom dishes (5 × 10^3 cells per well) and incubated for 24 h. Then the Radical NPs (final concentration: 50 μg/mL) was added to the dish and incubated for different time periods (2, 4, 6, 8 hours). After fixed with 4% (w/v) paraformaldehyde, the nuclei and membrane of the cells were stained with hoechst 33342 and rhodamine phalloidin respectively, and then imaged by confocal laser scanning microscopy (Leica TCS SPE). Hoechst 33342 was excited with UV, emitting 450-500 nm fluorescence. Rhodamine phalloidin was excited using a 561 nm laser, and emission was collected in the range of 590-630 nm. The Radical NPs were excited with UV, and emission was collected in the range of 700-800 nm. For flow cytometry analysis, the cells were seeded into 24-well plates and treated the Radical NPs as above. The collected cells were analyzed by flow cytometry (BECKMAN COULTER, Cytoflex LX).

2.10. Intracellular Hypoxia Evaluation.

Intracellular hypoxia was decided by the fluorescence intensity of Image-iT™ Green Hypoxia Reagent. It is non-fluorescent when live cells are in an environment with normal oxygen concentrations (20%) and becomes fluorescent when oxygen levels are decreased. HeLa cells were seeded in 27 mm nunc glass base dish cultured with DMEM (Invitrogen) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) in 5% CO_2, 20% O_2 at 37 °C in a humidified incubator. Then Image-iT™ Green Hypoxia Reagent (working concentration: 10 μM) was added to the dish and carefully mixed. After staining for 30 min, the original culture medium was replaced by 2 mL fresh medium containing 40 μL DFO (stock concentration: 5 mM, working concentration: 200 μM) and the treated cells were returned to incubator (5% CO_2, 20% O_2 at 37 °C). DFO induces hypoxia by chelating iron for excretion and DFO stabilizes HIF-1alpha from proteolysis by inhibiting the activity of iron-dependent prolyl hydroxylases subsequently reducing the potential for oxygen transport. After incubation for another 4 h, the cells were washed thoroughly with PBS three times. Finally, the intensity of green fluorescence was recorded on a Leica TCS SPE Spectral Confocal Microscope under 488 nm excitation.

2.11. Intracellular ROS Detection in Normoxia and Hypoxia.

HeLa cells were seeded in 27 mm nunc glass base dish cultured with DMEM (Invitrogen) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin

(Gibco) in 5% CO_2, 20% O_2 at 37 °C in a humidified incubator. For hypoxia, the original culture medium was replaced by 2 mL fresh medium containing 40 μL DFO (stock concentration: 5 mM, working concentration: 200 μM) and 70 μl of Radical NPs (final concentration: 100 μg/mL). For normoxia, the original culture medium was replaced by 2 mL fresh medium containing 70 μl of Radical NPs (final concentration: 100 μg/mL). Then the hypoxic and normoxic cells were returned to incubator (5% CO_2, 20% O_2 at 37 °C) and incubated for 6 h. Next, 2 μl 10 mM of DCFH-DA (working concentration: 10 μM) was added for another 30 min staining. Then HeLa cells were washed with PBS three times and irradiated by 635 nm laser (0.4 W/cm^2, 5 min). At last, green fluorescence emission from DCF excited by 488 nm was recorded on a Leica TCS SPE Spectral Confocal Microscope.

2.12. HeLa Cell Cytotoxicity by MTT Assay.

The HeLa cells were seeded on 96-well plates in DMEM (with 10% FBS, 1% penicillin/ streptomycin) in 5% CO_2, 20% O_2 at 37 °C in a humidified incubator. After growing overnight, the cells were used for experiments. For hypoxia, the original culture medium in each well was replaced by 200 μl fresh medium containing 4 μL DFO (stock concentration: 5 mM, working concentration: 200 μM) and different concentrations of the Radical NPs (0 to 240 μg/mL). For normoxia, the original culture medium was replaced by 200 μl fresh medium containing different concentrations of (0 to 240 μg/mL). Then the hypoxic and normoxic cells were returned to incubator (5% CO_2, 20% O_2 at 37 °C). After 6 h incubation, for PDT evaluation, the Radical NPs were treated

with irradiation by 635 nm laser (0.4 W/cm^2, 30 min). For PPIX NPs (2.5 μg/mL), the cells were irradiated with 635 nm laser (20 mW/cm^2, 2 min). The plates were quickly return back to incubator for another 12 h incubation. Then, 20 μL of MTT (5 mg/mL in PBS) was diluted ten times with DMEM and was added to each plate. After 4 h, MTT was replaced by the addition of 200 μL of DMSO. Cell cytotoxicity was measured by the absorbance value at 570 nm using a BioTek Powerwave XS microplate reader.

2.13. Apoptosis Assay

HeLa cells were seeded in six-well plates and incubated at 37 °C overnight (5% CO_2, 20% O_2). Five groups were prepared, only PBS (PBS), only laser irradiation (L), only Radical NPs (NPs), the Radical NPs (240 μg/mL) with laser irradiation (0.4 W/cm^2, 30 min) (NPs+L). After 12 h, the medium was collected, and the cells were washed by PBS three times (collected). The cells were collected by trypsinization, centrifugation (700 g, 3 min). Then cells were washed with cold PBS once and annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) three times gently and carefully. Next, cells were resuspended in 100 μL of binding buffer, and a total of 5 μL of Annexin V-FITC conjugate and propidium iodide (PI, 1 mg/mL in DMSO) solutions were added to the cells and stained for 15 min at room temperature. Afterward, extra 400 μL of binding buffer was added. Then the samples were analyzed by a flow cytometer (BD Bioscience FACS Calibur) excited by 488 nm.

2.14. *In Vivo* Antitumor Activity and Biosafety.

Balb/c nude (female) mice, 5 weeks of age, were purchased from Vital River Laboratories (Beijing, China) and were housed in a specific pathogen-free room. For investigating the therapeutic efficacy of the Radical NPs *in vivo*, the antitumor effects were conducted on HeLa tumor-bearing nude mice. Before commencing PDT treatment, HeLa cells were subcutaneously injected around 3 weeks. When the tumors grew to about 60 mm^3 in volume, these mice were divided into four groups randomly with five mice in each group. The mice then received tail intravenous injections at 0, 2, 4, 6 day and the Radical NPs was administered at an equivalent dose (200 μL, 2 mg/mL). Different formulations were administered, including only PBS (PBS), only NPs (NPs), only laser (L), NPs plus laser (NPs+L), respectively. After injection for 12 h, the laser irradiation (635 nm, 0.6 W/cm^2 for 30 min) at 1, 3, 5, 7 day was administrated to mice. No obvious thermal effect was observed in the process of irradiation. Both mouse body weights and tumor volumes were recorded every two days during the whole treatments. After 14 days of treatment, the mice were sacrificed, and tumor tissue, major organs (heart, liver, spleen, lung, kidney) and healthy tissues around tumor were taken for hematoxylin and eosin (H&E) staining at 12 h after treatment. To further evaluate the safety of different formulations *in vivo*, blood samples were collected from mice with various treatments for blood biochemical parameters evaluation, mainly including blood urea nitrogen (BUN), alanine aminotransferase (ALT), transaminase (AST), aspartate lactate dehydrogenase (LDH), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), uric acid (UA). The hematology markers were also evaluated, including white blood cells (WBC), red

blood cells (RBC), haematocrit (HCT), hemoglobin (HGB), mean corpuscular haemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), platelets (PLT).

2.15. Photothermal Properties of the Radical NPs.

Mices treated with PBS (control) or Radical NPs were subjected to laser irradiation (635 nm, 0.6 W/cm^2) for 30 min. The temperature was collected with a thermal imaging camera (Fluke, Ti400, IR Fusion Technology).

2.16. Statistical Analysis

Statistical Analysis: All data were expressed in this article as mean result ± standard deviation (s.d.). All figures shown in this article were obtained from three or more independent experiments with similar results unless specific mention. The statistical analysis was performed by using excel software. Statistical evaluation was performed using unpaired Student's two-sided t test analysis. Asterisks indicate significant differences (*P < 0.01, **P < 0.005, ***P < 0.001, ****P < 0.0001).

References

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