Supporting Information

Inducing Tumor Ferroptosis via pH-Responsive NIR-II Photothermal Agent Initiating Lysosomal Dysfunction

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Materials

All regents and chemicals were purchased from commercial source and used without further purification. $POCl_3$, CH_2Cl_2, CH_3CH_2I, anhydrous dimethyl formamide (DMF), 1-ethylpiperazine, Ac_2O, AcONa, toluene, Et_3N and $NaHCO_3$ were obtained from Energy Chemical. Indocyanine green(ICG), 1,3-diphenylisobenzofuran(DPBF), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and coumarin-3-carboxylic acid (3-CCA) were obtained from Shanghai Macklin Biochemical Technology Co., Ltd. Liperfluo (LPO) was acquired from Dojindo Laboratorise (Kumamoto, Japan). The standard MDA assay kit was acquired from Beijing Solarbio Science & Technology Co., Ltd. GPX4 rabbit polyclonal antibody and Cell Counting Kit-8 (CCK-8) were acquired from Beyotime Biotechnology. Phosphate buffer saline was used to prepare all aqueous solutions. Bafilomycin A1 (Baf A1) and LysoTracker Green DND-26 were purchased from Sigma Aldrich (St. Louis, MO, USA). Female BALB/c mice (4-6 weeks old and weighted 25-30 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China) and all animals received care incompliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences Animal Care and Use Committee (SIAT-IRB-150304-YYS-CLT-A0131-1).

Instruments

^1H were obtained by a Bruker ARX 600 MHz spectrometer, ^{13}C NMR were obtained by a Bruker ARX 400 MHz spectrometer. High Resolution Mass Spectra (HRMS) was obtained by a GCT Premier CAB 048 mass spectrometer operating in MALDI-TOF mode. Absorption spectra were measured on a RarDMFian 50 Conc UV-Visible spectrophotometer at 25 °C. Fluorescence spectra were recorded on an Edinburgh FS5 fluorescence spectrophotometer at 25 °C. The 808 nm laser was obtained by FC-E-808. The cytotoxicity was measured on Microplate reader (MultiskanGO). Thermal images were recorded using a FTIR T420 thermal camera. Confocal laser scanning microscopy (CLSM) imaging on Leica STELLARIS5. Fluorescence images of mice were performed on a Caliper Life Science IVIS LuminaIIXGI-8 small animal NIRF imaging and anesthesia system (America).

Synthesis and characterization of IR-PE

Compounds 1, 2 and Cy7-Cl were prepared according to the reported procedure.[1] Cy7-Cl (63.9 mg, 0.1 mmol) was dissolved in anhydrous DMF (3 mL), and 1-ethylpiperazine (45.6 mg, 0.4 mmol) and a few drops of triethylamine was added. The mixture was stirred at 80 °C for 30 minutes under an argon atmosphere. The solvent was removed under reduced pressure, then the crude product was purified to afford a blue solid (30%).

^1H NMR (600 MHz, MeOD) δ7.78 (d, J = 13.5 Hz, 2H), 7.43 (d, J = 7.2 Hz, 2H), 7.35 (t, J = 7.6 Hz, 2H), 7.16 (t, J = 7.0 Hz, 4H), 5.98 (d, J = 13.5 Hz, 2H), 4.07 (q, J = 7.2 Hz, 4H), 3.79 (t, J = 4.7 Hz, 4H), 3.60 (q, J = 7.0 Hz, 4H), 2.82 (s, 4H), 2.66 (q, J = 7.2 Hz, 2H), 2.54 (t, J = 6.5 Hz, 4H), 1.86-1.82 (m, 2H), 1.35 (t, J = 7.2 Hz, 7H), 1.28 (s, 1H), 1.22 (t, J = 7.2 Hz, 3H), 1.17 (t, J = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, MeOD) δ 169.00 (s), 142.22 (d, J = 44.0 Hz), 141.78 (m), 140.50 (s), 128.24 (s), 124.17 (d, J = 73.4 Hz), 123.44 (m), 121.86 (s), 109.20 (s), 95.72 (s), 56.92 (s), 54.09 (s), 52.21 (s), 37.89 (s), 27.76 (s), 24.49 (s), 21.73 (s), 16.97 (s), 10.57 (s).

Calculation of the photothermal conversion efficiency (PCE)

Firstly, prepare a stock solution of IR-PE and ICG with a concentration of 1 mg/mL in DMSO, then dilute it with PBS (pH 3.5) to a working concentration of 10 μg/mL. In the photothermal performance test, the solutions containing IR-PE and ICG (10 μg/mL, pH3.5) in the PE tube (200 μL) were exposed to 808 nm irradiation (0.1 W/cm^2) for 10 min, and then cooled down to room temperature. The temperature variation at intervals of 30 seconds (testing room temperature: 25 °C) during this process was recorded by a camera. The photothermal conversion efficiencies (η) were measured according to the formula.[2]

$$\eta = \frac{hs_1(T_{max1} - T_{sur}) - hs_2(T_{max2} - T_{sur})}{I \times (1 - 10^{-A})}$$

$$hs = \frac{C \times m}{\tau s}, \quad \tau s = \frac{t}{-ln\theta}, \quad \theta = \frac{T - T_{sur}}{T_{max} - T_{sur}}$$

Among them, T_{max} and T_{sur} are the maximum steady state temperature and the environmental temperature, respectively. Subscripts 1 and 2 respectively represent the test substances and solvents. A is the absorbance of the material at the laser wavelength, and I is the power density. Tmax refers to the highest temperature, C refers to the specific heat capacity of the solvent, m refers to the quality of the solvent. τs is the associated time constant and t refers to the cooling time, θ is a dimensionless parameter, known as the driving force temperature.

ROS generation detection in vitro

1,3-diphenylisobenzofuran (DPBF) was utilized as indicators of reactive oxygen species (ROS). In the experiment, we first dissolved DPBF in acetonitrile under dark conditions to prepare a 1 mM DPBF stock solution. Then, we prepared IR-PE and indocyanine green (ICG) with a concentration of 1 mg/mL using dimethyl sulfoxide (DMSO) as the solvent and diluted them with a pH 3.5 PBS solution to a working solution concentration of 10 μg/mL. After that, 50 μM of DPBF was added to the above solutions (10 μg/mL, pH 3.5) and the mixture was illuminated with an 808 nm laser at 0.1 W/cm² for 120 seconds, and we recorded the absorption spectrum at 410 nm every 15 seconds. The efficiency of ROS generation (A/A_0) was calculated by comparing the absorbance at 410 nm after illumination to the absorbance intensity (A_0) without illumination.

Cell cytotoxicity study by CCK-8 assay

When A549 cells reach approximately 80%-90% confluence on the entire surface area of the culture flask, remove the culture medium from the flask, and then wash the cells with 5 mL of PBS three times. Next, add 1 mL of trypsin for digestion to detach the cells from the flask. Once digestion is complete, add culture medium to stop the digestion and gently pipette to ensure that the cells are in a suspended state. Subsequently, adding 200 μL to each well to seed the cells onto a 96-well plate. Incubate the plate in a 37°C, 5% CO_2, and 95% O_2 carbon dioxide incubator for 24 hours until the cells adhere to the surface. After that, add different concentrations of IR-PE solution (0, 2 , 5, 10, 15, 20, 25, 30, 35 μg/mL) with six wells for each concentration, and incubate the plate in the carbon dioxide incubator for 3 hours. Discard the supernatant from the plate wells, wash the cells with PBS three times. Add 100 μL of CCK-8 reagent to each well and continue

incubating for 4 hours. Finally, measure the optical density (OD) of each well at 450 nm using a microplate reader.

Cell imaging

To determine the intracellular ROS level, A549 cells were pre-seeded in 8-well Chambered Coverglass w/non-removable wells for 24 h, and then incubated with PBS (control), PBS + Laser (808 nm, 0.1 W/cm^2, 5 min), IR-PE (5 μg/ml), IR-PE (5 μg/ml) + Laser (808 nm, 0.1 W/cm^2, 5 min) for 24 h. Next, those cells were stained with DCFH-DA (5 μM) for 30 min. After washing three times with PBS, the intracellular fluorescent emission of DCFH-DA was observed by Leica STELLARIS5 confocal laser scanning microscope (Ex = 488 nm, Em = 500-550 nm). The relative fluorescent intensity was quantified via ImageJ software.

To assess the intracellular •OH levels, A549 cells were pre-seeded in 8-well Chambered Coverglass w/non-removable wells for 24 h, and then incubated with PBS (control), PBS + Laser (808 nm, 0.1 W/cm^2, 5 min), IR-PE (5 μg/ml), IR-PE (5 μg/ml) + Laser (808 nm, 0.1 W/cm^2, 5 min) for 24 h. Next, those cells were stained with 3-CCA (10 μM) for 35 min. After washing three times with PBS, the intracellular fluorescent emission of 3-CCA was observed by Leica STELLARIS5 confocal laser scanning microscope (λ_{Ex} = 405 nm, λ_{Em} = 425-475 nm). The relative fluorescent intensity was quantified via ImageJ software.

To assess the intracellular LPO production, A549 cells were pre-seeded in 8-well Chambered Coverglass w/ non-removable wells for 24 h, and then incubated with PBS (control), PBS + Laser (808 nm, 0.1 W/cm^2, 5 min), IR-PE (5 μg/ml), IR-PE (5 μg/ml) + Laser (808 nm, 0.1 W/cm^2, 5 min) for 24 h. Next, those cells were stained with LPO (5 μM) for 35 min. After washing three times with PBS, the intracellular fluorescent emission of LPO was observed by Leica STELLARIS5 confocal laser scanning microscope (λ_{Ex} = 488 nm, λ_{Em} = 500-550 nm). The relative fluorescent intensity was quantified via ImageJ software.

MDA assay

A549 cells were seeded into 6-well plates with fresh DMEM containing 10% FBS (2.0 mL per well) for 24 h, then incubated with PBS (control), PBS + Laser (808 nm, 0.1 W/cm^2, 5 min), IR-PE (5 μg/ml), IR-PE (5 μg/ml) + Laser (808 nm, 0.1 W/cm^2, 5 min) for 24 h. After that, the culture solution was removed, those cells were disrupted and the cell lysate was collected. Subsequently, the standard MDA assay kit (Beijing Solarbio Science & Technology Co., Ltd) was adopted to measure the concentration of MDA content. Absorbance of supernatants was tested with a microplate reader (Molecular Devices, SpectraMax® iD3, USA) at 532, 600 nm (OD532, OD600) according to the equation formula.

$$\Delta OD = OD(\text{Test}) - OD(\text{Blank})$$

$$MDA\text{（nmol/mg prot）} = 0.1075\times (\Delta OD532 - \Delta OD600)$$

Western blotting experiments

Briefly, A549 cells were seeded into 6-well plates for 24 h, then cells were treated with following treatments: PBS (control), PBS + Laser (808 nm, 0.1 W/cm^2, 5 min), IR-PE (5 μg/ml), IR-PE (5 μg/ml) + Laser (808 nm, 0.1W/cm^2, 5 min) for 24 h. Next, those A549 cells were washed with cold PBS twice and added with 60 μL of ice-cold lysis buffer. When those cells were disrupted, the cell lysate was collected and boiled for 5 min at 95 °C. Subsequently, the cell

lysates underwent SDS-polyacrylamide gel electrophoresis (SDS-PAGE) operation and were further transferred to polyvinylidene fluoride (PVDF) membrane. Then, PVDF membrane were blocked with tris-buffered saline (containing Tween-20 + 5% skim milk), and immunoblotted with Glutathione Peroxidase 4 (GPX4) rabbit polyclonal antibody (1:1000, Beyotime), anti-GAPDH antibody (1:2000, Servicebio) for 15 h, followed by the further incubation of horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000, Beyotime) for 1 h. Finally, the specific protein bands were captured with an enhanced chemiluminescence detection system.

Supplementary Figures

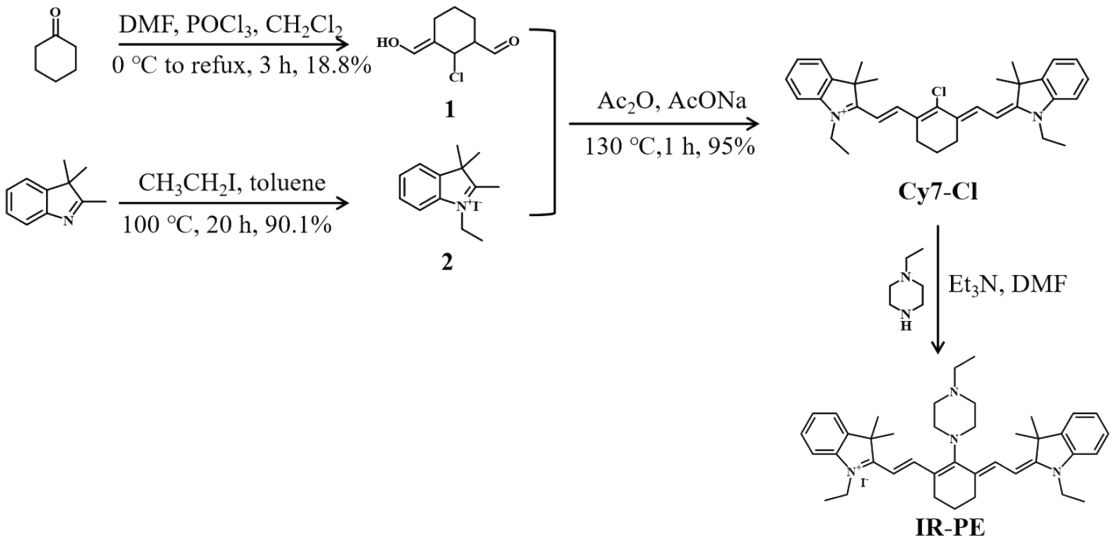

Figure S1. Synthetic route to IR-PE.

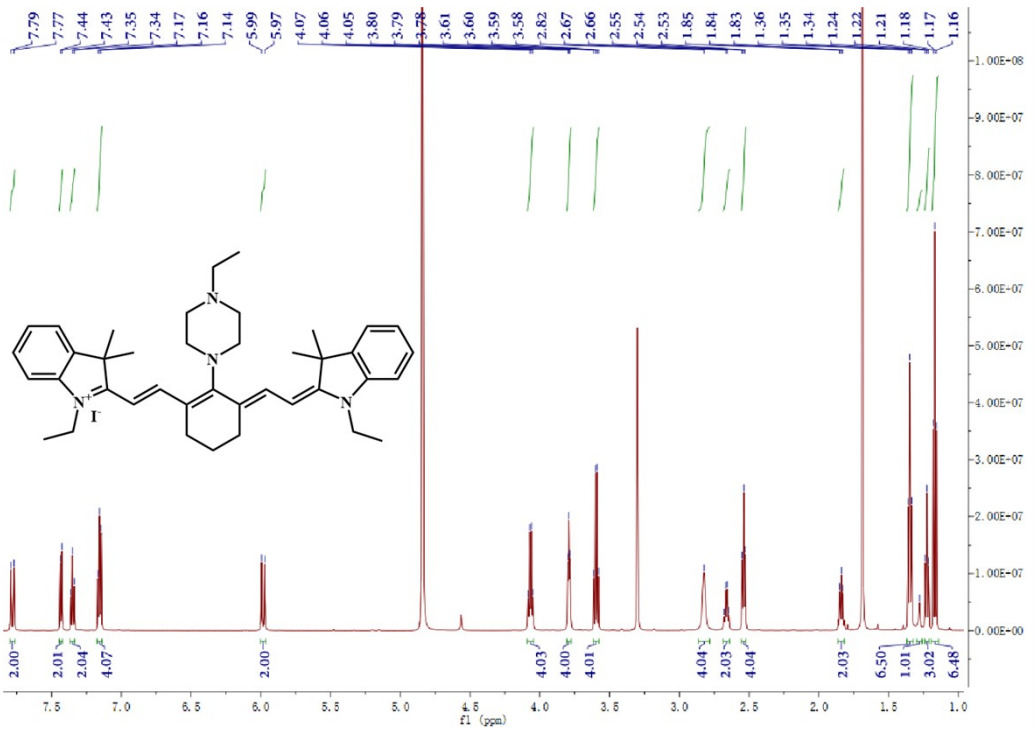

Figure S2. ^{1}H NMR spectrum of IR-PE in CD_3OD.

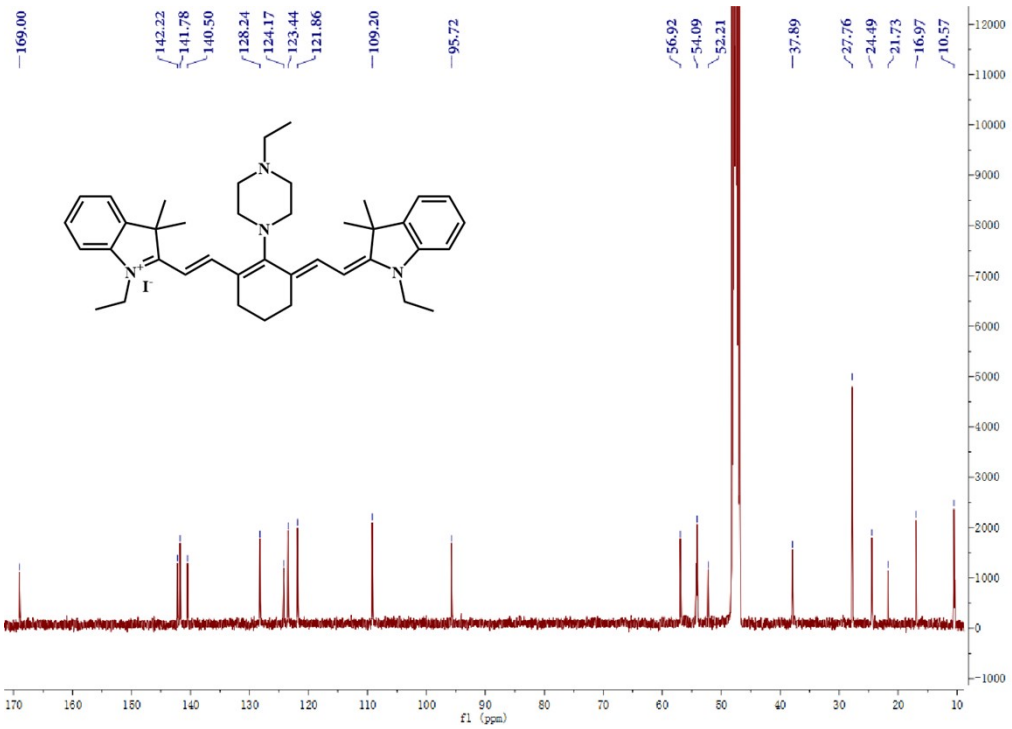

Figure S3. ^{13}C NMR spectrum of IR-PE in CD_3OD.

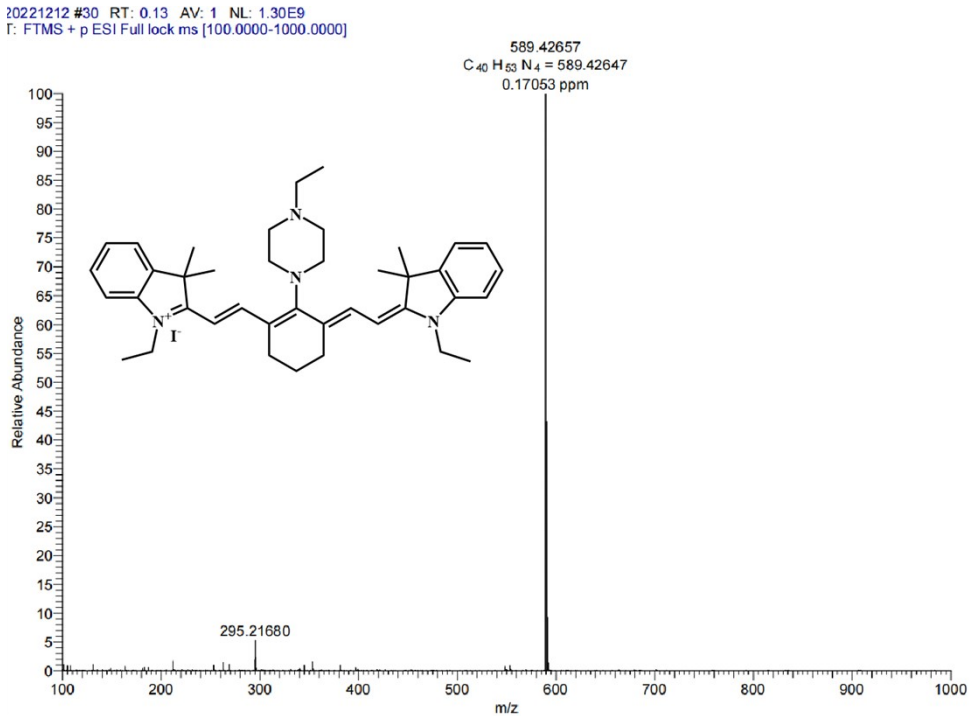

Figure S4. High resolution mass spectrum (MALDI-TOF) of IR-PE.

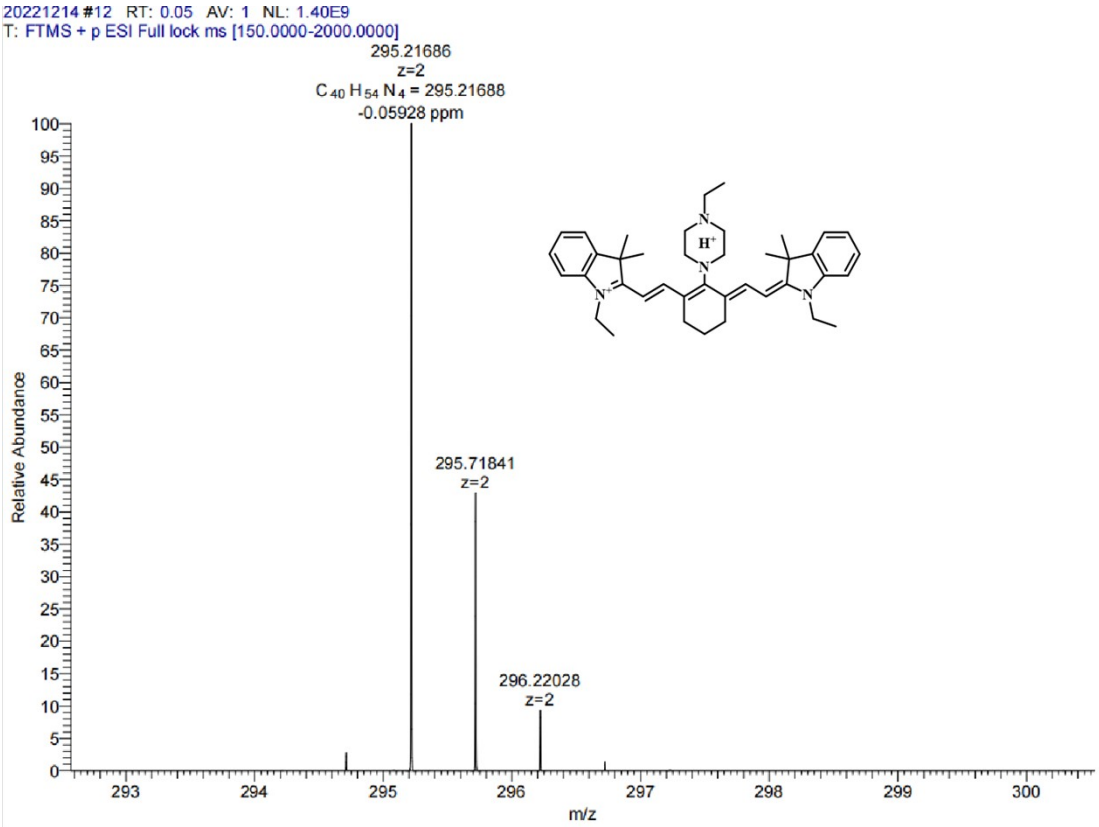

Figure S5. High resolution mass spectrum (MALDI-TOF) of IR-PEH.

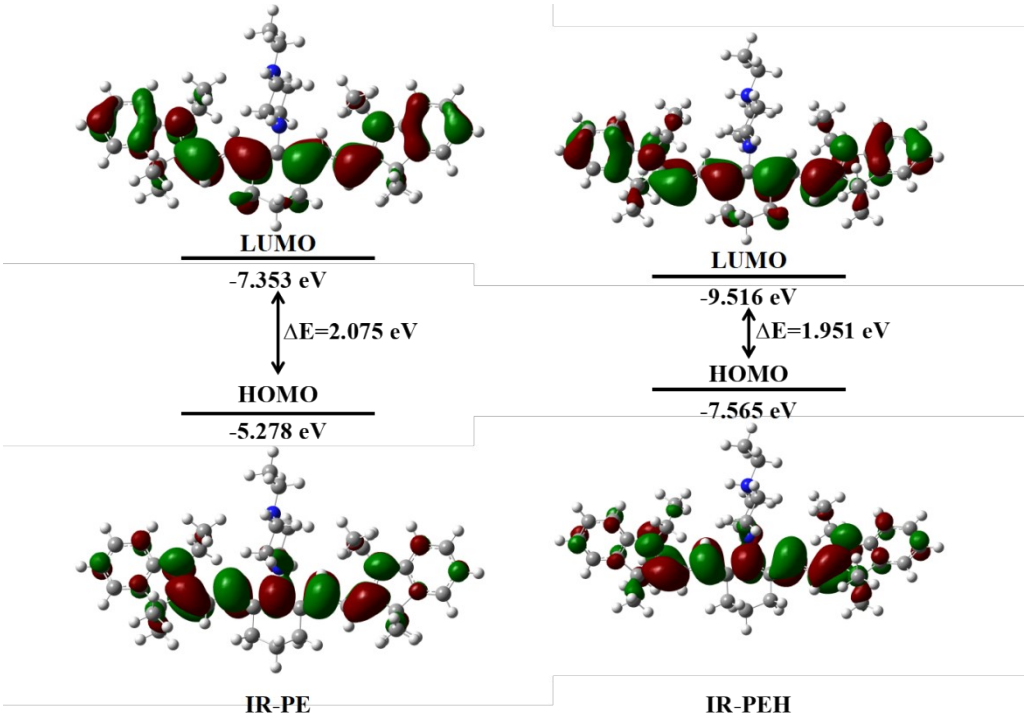

Figure S6. Optimized molecular structures and calculated HOMO-LUMO energy gaps of IR-PE and IR-PEH.

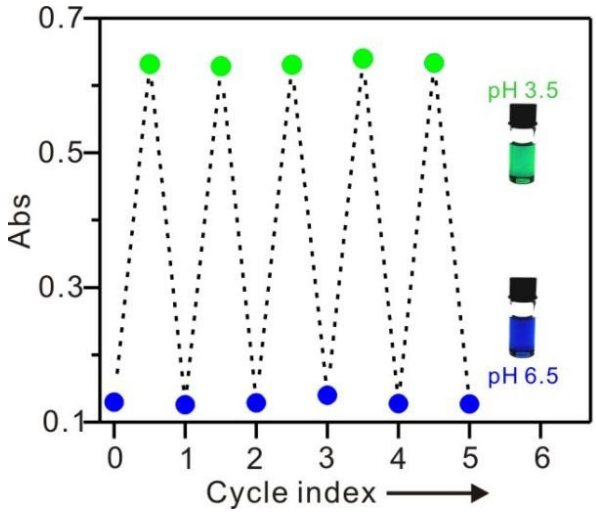

Figure S7. Normalized absorption of IR-PE upon exposure to the redox cycle between pH6.5 and pH3.5 for 5 times.

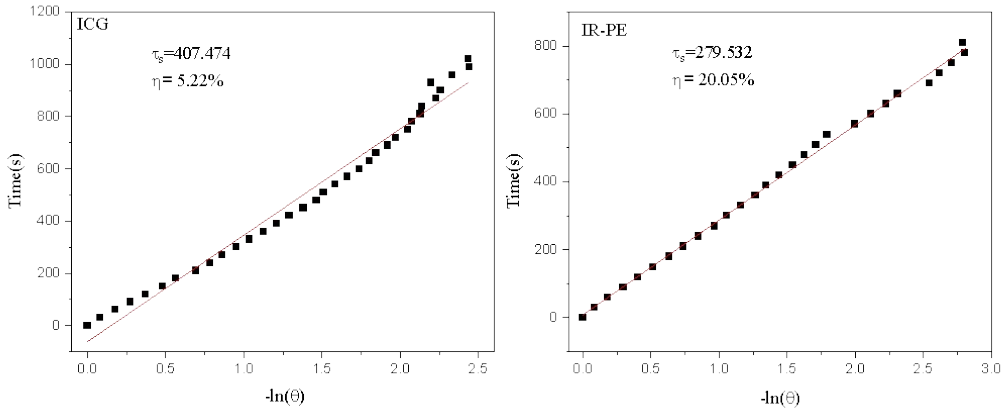

Figure S8. Photothermic heating curves of ICG and IR-PE solutions under 808 nm irradiation (0.1 W/cm^2) for 10 min followed by cooling to room temperature. Linear correlation of the cooling times versus negative natural logarithm of driving force temperatures.

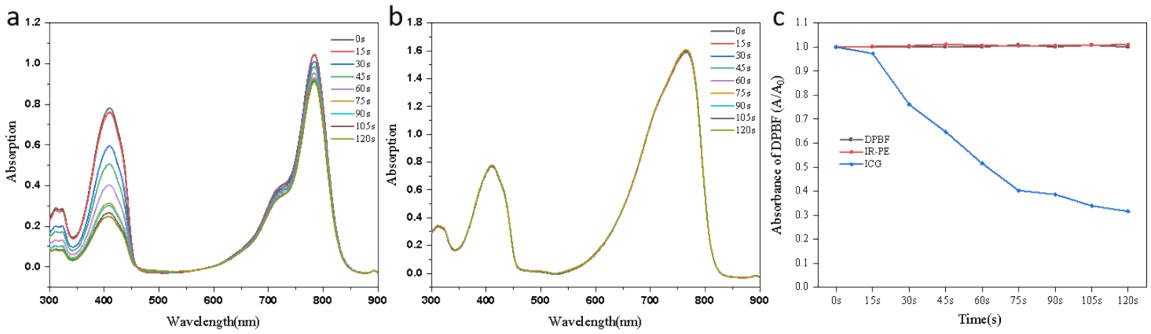

Figure S9. Absorption spectra of ICG (10 μg/mL) (a) and IR-PE (10 μg/mL) (b) in PBS (pH 3.5) containing DPBF (50 μM) upon 808 nm irradiation (0.1 W/cm^2). (c) Comparison of ROS generation of ICG and IR-PE in PBS under upon 808 nm irradiation (0.1 W/cm^2) for 120 s.

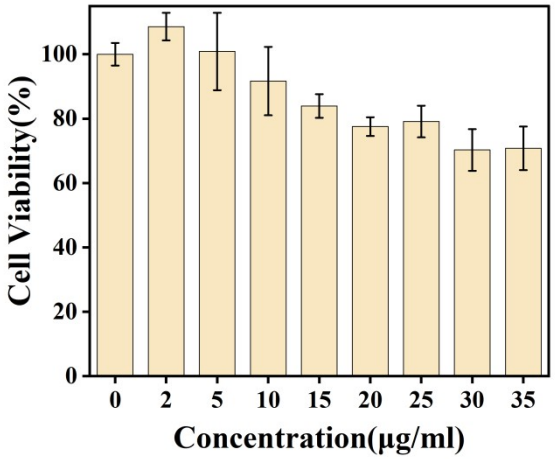

Figure S10. The cytotoxicity of IR-PE against A549 cells for 24 h.

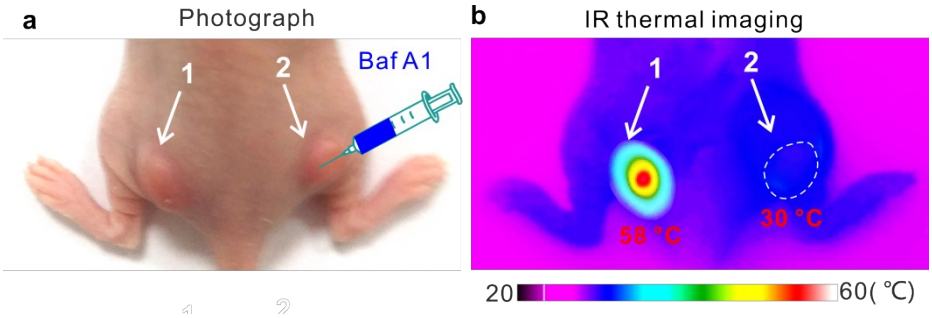

Figure S11. Photograph (a) and IR thermal image (b) of a Balb/c nude mouse with two A549 tumors on the left and right hind legs 10 h after intratumoral injection of IR-PE irradiated with 808 nm laser (0.1 W/cm^2, 20 min). 1: Tumor without treatment, 2: Tumor injected with Baf A1 solution to up regulate the pH of tumor.

References:

[1] K. Hanaoka, Y. Kagami, W. Piao, T. Myochin, K. Numasawa, Y. Kuriki, T. Ikeno, T. Ueno, T. Komatsu, T. Terai, T. Nagano, Y. Urano, *Chem. Commun.* **2018**, *54*, 6939-6942.

[2] S. Liu, X. Zhou, H. Zhang, H. Ou, J.W.Y. Lam, Y. Liu, L. Shi, D. Ding, B.Z. Tang, Molecular motion in aggregates: manipulating TICT for boosting photothermal theranostics. *J. Am. Chem. Soc.* **2019**, *141*, 5359-5368.