Supplementary Information

A cascaded amplification carrier-free nanoplatform for synergistic photothermal/ferroptosis therapy via dual antioxidant pathway disruption in cervical cancer

Dihai Gu [1], Xiao Liang [2,*], Zitong Shao [5], Haonan He [5], Lin Zhu [2], Shali Qiu [3,*], Zhen Liu [2], Senlin Wang [1], Haijiao Chen [2], Shusheng Ci [4], Hongshuai Wu [2,*],Yihong Wang [1,*]

[1] School of Chemistry and Chemical Engineering, Southeast University, Nanjing 210089, PR China

[2] Wuxi Key Laboratory of Biomaterials for Clinical Application, Department of Oncology, Jiangyin Clinical College of Xuzhou Medical University, Wuxi 214400, PR China

[3] Department of Pathology, Jiangyin Clinical College of Xuzhou Medical University, Wuxi 214400, PR China

[4] School of Basic Medical Sciences, Nanjing Medical University, Nanjing 211166, P. R. China

[5] The First Clinical Medical College, Nanjing Medical University, Nanjing 210029, P. R. China

Experimental Section

Materials. 1,3,5,7-tetramethyl-*meso*-CF_3-BODIPY (TCF_3), 4-hydroxybenzaldehyde, methylene blue (MB), 1,10-Phenanthroline-5,6-dione, Glutathione (GSH), 9,10-anthracenediyl-bis(methylene) dipropanoic acid (ABDA), and 6-aminofluorescein were purchased from Shanghai Macklin Biochemical Co., Ltd. Propargyl bromide and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (98%) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. N_3-PEG_{2000}-Folate, 4′,6-diamidino-2-phenylindole (DAPI), nicotinamide adenine dinucleotide phosphate (NADPH), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), GSH/GSSG Assay Kit, $NADP^+$/NADPH assay kit, lipid peroxidation MDA assay kit, Cell Counting Kit-8 (CCK-8), 4% paraformaldehyde, Crystal violet staining solution, Calcein-AM/PI assay

kit, Mitochondrial membrane potential (MMP) assay kit, Calcein-AM/PI Assay Kit, and 5′, 6, 6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanineiodide (JC-1) were purchased from Beyotime Biotechnology Co., Ltd (China). LPO probe (C11-BODIPY$^{581/591}$) was purchased from Thermo Fisher Scientific Co., Ltd. Fetal bovine serum (FBS) were supplied by ExCell Biotech Co., Ltd. GPX4, FSP1, and ACSL4 were supplied by Cell Signaling Technology. Tissue slice staining was supported by Wuhan Servicebio Technology Co., Ltd.

Synthesis of 4-(2-Propyne-1-yloxy)benzaldehyde. According to previously reported synthetic procedure[1], potassium carbonate (5.0 g, 36.12 mmol) and 4-hydroxybenzaldehyde (3.66 g, 29.97 mmol) were dissolved in 50 mL of acetone and stirred at 50°C for 30 min. Propargyl bromide (5.5 mL of 80 wt% solution in toluene, 30 mmol) was then added, and the mixture was carried out under reflux for 3 h. After the reaction mixture was cooled to room temperature, it was subjected to vacuum filtration, and the filtrate was washed three times with acetone. The resulting filtrate was evaporated under reduced pressure to obtain a light-yellow solid, which was used directly used in the next step without further purification, achieving a yield of 99%.

Synthesis of L-MNF. In a nitrogen atmosphere, 4-(2-Propyn-1-yloxy)benzaldehyde (2.62 g, 15.75 mmol), 1,10-phenanthroline-5,6-dione (3.15 g, 15.00 mmol), 6-aminofluorescein (5.21 g, 15.00 mmol) and ammonium acetate (23.10 g, 300 mmol) were dispersed in 120 mL of glacial acetic acid. The mixture was then heated to 120°C and stirred for 24 h. After the reaction was complete, the solution was cooled to room temperature, and the pH was adjusted to 7.0 with concentrated aqueous ammonia. The resulting precipitate was filtered, washed with water, and dried under vacuum to obtain the desired compound, which was used directly in the next step without additional purification, achieving a yield of 67.03%. The product was characterized by ^{1}H NMR (600 MHz, DMSO-d_6) with the following chemical shifts: δ 10.23 (s, 2H), 9.11 (dd, J = 4.3, 1.8 Hz, 1H), 9.04 (dd, J = 8.0, 1.8 Hz, 1H), 9.01 (dd, J = 4.2, 1.7 Hz, 1H), 8.55 (d, J = 1.9 Hz, 1H), 8.04 (dd, J = 8.0, 2.0 Hz, 1H), 7.89 (dd, J = 8.0, 4.3 Hz, 1H), 7.65 (dd, J = 8.4, 1.7 Hz, 1H), 7.63 – 7.55 (m, 2H), 7.56 – 7.47 (m, 2H), 7.09 – 7.05 (m, 1H), 7.06 – 6.99 (m, 2H), 6.74 (dd, J = 8.8, 6.5, 2.4 Hz, 3H), 6.68 (dd, J = 8.6, 2.4 Hz, 1H),

6.60 (d, J = 8.6 Hz, 1H), 4.86 (d, J = 2.4 Hz, 2H), 3.61 (t, J = 2.3 Hz, 1H).

Synthesis of IrFlu. A solution of Ir(III)-dimer (0.15 mmol) and L-MNF (0.30 mmol) were prepared in CH_2Cl_2/CH_3OH solvent mixture (21 mL, v/v = 2:1). The resulting mixture was heated under reflux in a nitrogen atmosphere for 24 h in the dark. After the reaction, the mixture was cooled to room temperature, and a six-fold excess of NH_4PF_6 (based on molar ratio) was added while stirring vigorously for 1 h, resulting in the .

precipitation of a solid. The precipitate was collected by filtration, washed with a small amount of ether, and dried under vacuum. The crude product was purified by column chromatography on neutral Al_2O_3, using CH_2Cl_2/CH_3OH (10:1, v/v) as the eluent, yielding a solid product with a 55% yield. The product was characterized by ^{1}H NMR (600 MHz, DMSO-d_6), displaying the following chemical shifts: δ 9.35 (dt, J = 8.2, 1.7 Hz, 1H), 8.31 – 8.25 (m, 3H), 8.17 (dd, J = 8.3, 5.1, 1.7 Hz, 2H), 8.00 – 7.86 (m, 7H), 7.62 – 7.53 (m, 4H), 7.51 – 7.47 (m, 1H), 7.15 – 6.93 (m, 10H), 6.70 – 6.59 (m, 5H), 6.34 – 6.26 (m, 2H), 4.87 (t, J = 2.1 Hz, 2H), 3.62 (s, 1H).

Synthesis of IrFlu-PEG$_{2000}$-FA. A mixture containing IrFlu (26.52 mg, 20.0 μmol), N_3-PEG-FA (Folate polyethylene glycol-N_3, 2000 MW) (40 mg, 20.0 μmol), $CuSO_4 \cdot 5H_2O$ (5.05 mg, 20.2 μmol) and NaVc (9.61 mg, 40.0 μmol) was dissolved in anhydrous, degassed DMSO (10 mL) and stirred at 50°C under a nitrogen atmosphere for 24 h. Upon completion of the reaction, the solution was dialyzed using a dialysis bag with a molecular weight cutoff of 2500 Da to remove excess Na^+, Cu^{2+}, and unreacted reagents. The product was then isolated by lyophilization, resulting in a yield of 51.41%. The ^{1}H NMR spectrum of IrFlu-PEG$_{2000}$-FA showed a broad peak at 3.49 ppm (attributed to the CH_2 groups in PEG$_{2000}$), confirming the successful conjugation of PEG. The product was characterized by ^{1}H NMR (600 MHz, DMSO-d_6), displaying the following chemical shifts: δ 9.33 (d, J = 8.1 Hz, 1H), 8.17 (d, J = 11.7 Hz, 1H), 4.54 (s, 1H), 3.49 (s, 97H).

Preparation of TCF$_3$P. In a two-neck round-bottom flask, 1,3,5,7-tetramethyl-*meso*-CF_3-BODIPY (TCF_3) (100 mg, 0.315 mmol) and 4-(diphenylamino)benzaldehyde (344 mg, 1.26 mmol) were dissolved in 30 mL of toluene. Piperidine (26.04 mg, 0.30 mmol)

and acetic acid (31.23 mg, 0.52 mmol) were then added under a nitrogen atmosphere, and the reaction mixture was heated to reflux at 110 ℃ for 12 h. Upon completion, the solvent was evaporated under reduced pressure, yielding a residue that was subsequently purified via silica gel column chromatography (using PE/EA =2/1 mixture as the eluent) to afford the BODIPY product as a dark solid in 87% yield. The product was characterized by ^{1}H NMR (400 MHz, CD_2Cl_2) with the following chemical shifts: δ 7.59 (s, 1H), 7.55 (s, 1H), 7.49 (d, J = 2.0 Hz, 2H), 7.47 (d, J = 2.1 Hz, 2H), 7.35 – 7.26 (m, 11H), 7.15 – 7.09 (m, 11H), 7.01 (s, 2H), 6.99 (s, 2H), 6.83 (s, 2H), 2.36 (q, J = 3.2 Hz, 6H).

Cellular uptake of Ir-TCF$_3$P-FA NPs. The MCF-7 and HeLa cell were seeded in confocal dishes and incubated for 24 h. Following this incubation, the cells were treated with 5 μM Ir-TCF$_3$P-FA NPs for varying exposure durations of 0, 2, 4, 6, and 8 h. Subsequently, the cells were washed three times with PBS and immediately examined by CLSM (Olympus FV-1000, Japan), employing an excitation wavelength of 450 nm and an emission wavelength of 480 ± 20 nm.

Western blotting assays. Western blotting was employed to evaluate the expression of ferroptosis-related proteins FSP1, GPX4 and ACSL4. HeLa cells were assigned to four experimental groups: two groups treated with Ir-TCF$_3$P-FA NPs, one exposed to 450 nm laser irradiation and the other subjected to dual laser irradiation (450 nm + 808 nm) (450 nm + 808 nm, the sign “450 nm + 808 nm” here indicates both irradiation with 450 nm wavelength light at the density of 10 mW cm^{-2} for 10 min and 808 nm wavelength light at the density of 0.6 W cm^{-2} for 5 min). The remaining two control groups received either normal medium or free Ir-TCF$_3$P-FA NPs. After 12 h of incubation period, cells were rinsed with PBS and lysed using radioimmunoprecipitation assay (RIPA) buffer to extract proteins, which were quantified via the bicinchoninic acid (BCA) assay. The protein extracts were then separated by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Blocking was performed using 3% bovine serum albumin (BSA) or 5% skim milk, followed by overnight incubation with primary antibodies at 4°C. After washing with tris-buffered

saline with 0.1% Tween 20 (TBST), the membranes were incubated with secondary antibodies conjugated with fluorescent tags. Protein bands were visualized using a chemiluminescence detection system.

Monitoring of cellular mitochondria morphology by TEM. HeLa cells were seeded in a 6-well plate and treated with different conditions. Following treatment, the cells were detached using trypsin, washed with cold PBS, and fixed in glutaraldehyde (5%) at 4 °C for 1 h. Subsequently, the fixed cells were further processed by fixation in osmium tetroxide and dehydration with ethanol. Ultrathin sections of the cells were prepared and subsequently counterstained with uranium acetate (3%) and lead citrate (0.3%). Mitochondria morphology was then observed by TEM.

Calcein-AM/PI assay. Calcein acetoxymethyl ester (Calcein-AM) and propidium iodide (PI) were employed to differentiate viable from non-viable cells. HeLa cells were allocated into four experimental groups: a control group treated with PBS, and three groups treated with Ir-TCF_3P-FA NPs (5 μM) for 8 h. After incubation, one of the Ir-TCF_3P-FA NPs-treated groups was exposed to 450 nm laser irradiation (10 mW cm^{-2}, 10 min), while another group received dual laser irradiation (450 nm + 808 nm). After an additional 6 h of incubation, the cells were harvested and co-stained with Calcein-AM and PI for 15 min. The stained cells were then transferred to glass slides, and fluorescence images were obtained using CLSM.

Fluorescence (FL), photoacoustic (PA), and photothermal (PT) imaging *in vivo*. Female nude mice (5 weeks) were purchased from Shanghai Slack Laboratory Animal Co., Ltd., and the experiments were implemented in accordance with protocols approved by the Animal Experimental Ethics Committee of Southeast University. HeLa tumor models were obtained by subcutaneous injecting 4×10^6 HeLa cells. When the tumor size reached around 60 mm^3, the HeLa-bearing mice were randomly divided into four groups. Then the mice were intravenously injected with Ir-TCF_3P-FA NPs. The mice were anesthetized and imaged by IVIS small animal imaging system at scheduled time intervals (0.5, 3, 6, 12, 20, and 24 h). Drawing inspiration from the notable photothermal attributes of Ir-TCF_3P-FA NPs, tumor imaging was conducted at various post-injection time points (0, 3, 6, 12, 20, and 24 h) using a hybrid PA/Ultrasonic image

system (Vevo® LAB, Administrator MX250) operating at 808 nm wavelength. Finally, at the optimal uptake time point, the tumor area was irradiated by NIR (808 nm, 0.6 W cm^{-2}), and the temperature change of the tumor site was monitored by thermal imaging camera (Fluke, Ti480U). Finally, after injection for 20 h, mice were euthanized and tumors and major organs (heart, liver, spleen, lung, and kidney) were collected and imaged.

Statistical analysis. All the data are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using GraphPad Prism 9.0 software (San Diego, CA, USA). Group comparisons were performed using analysis of variance (ANOVA) followed by Student's t-test to determine significant differences. For significance levels, the following annotations were adopted: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001

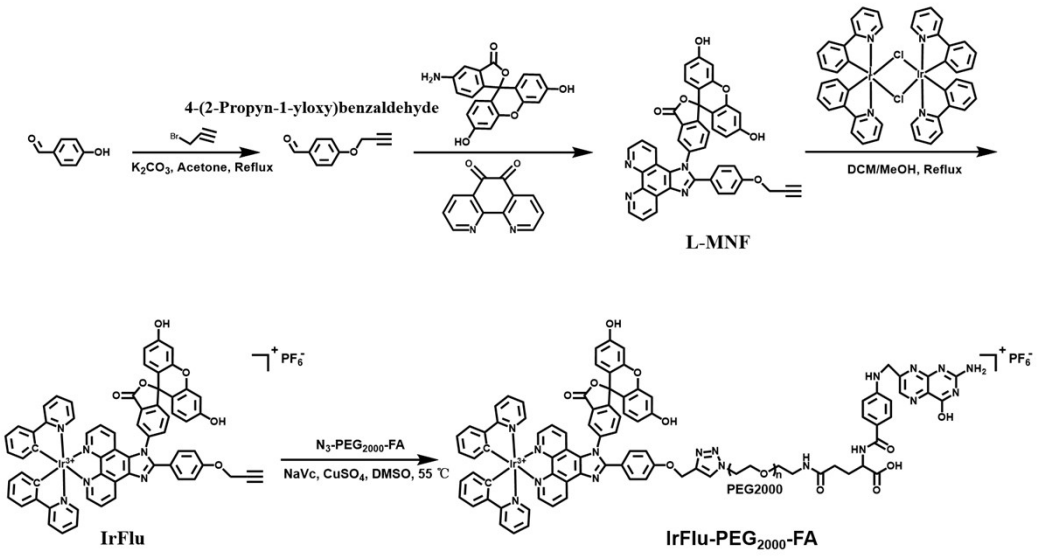

Fig. S1 The synthetic route of ligands and complexes.

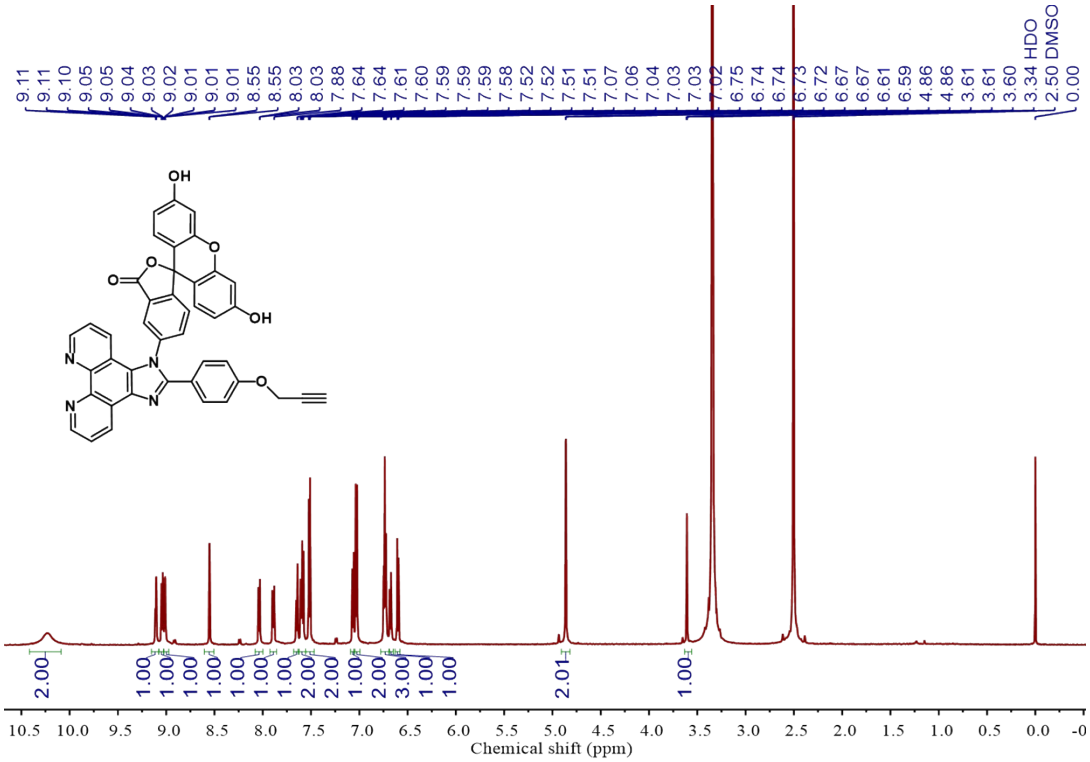

Fig. S2 ^{1}H NMR spectrum of L-MNF in DMSO-d_6 at 298K.

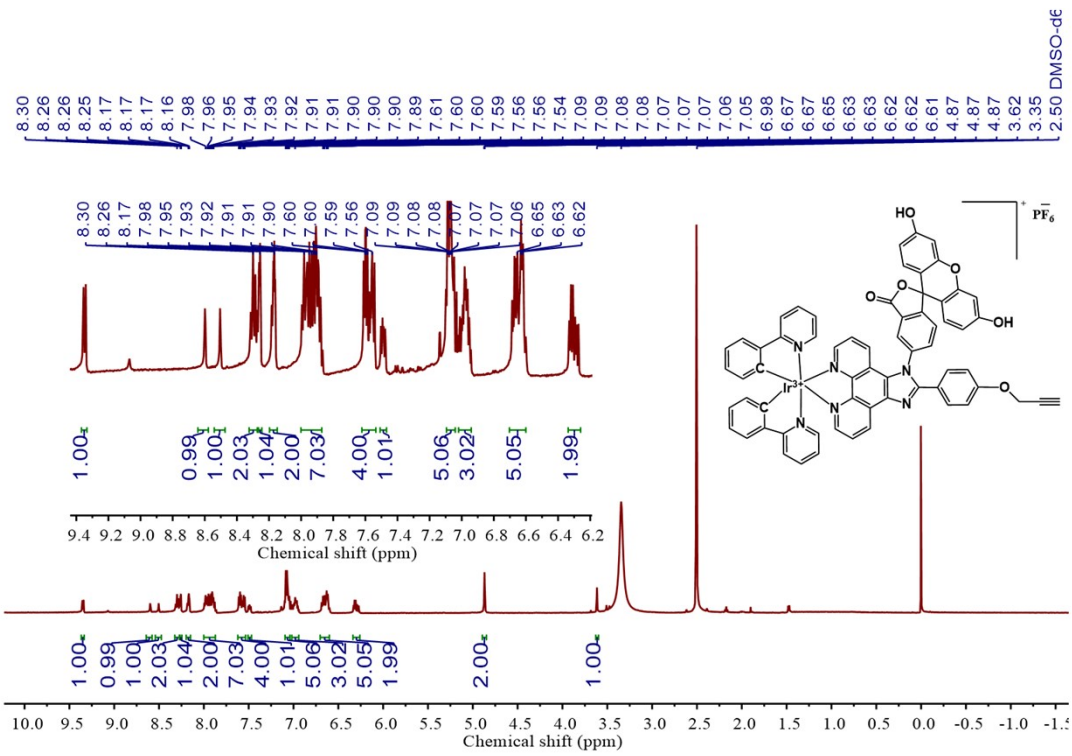

Fig. S3 ^{1}H NMR spectrum of IrFlu in DMSO-d_6 at 298K.

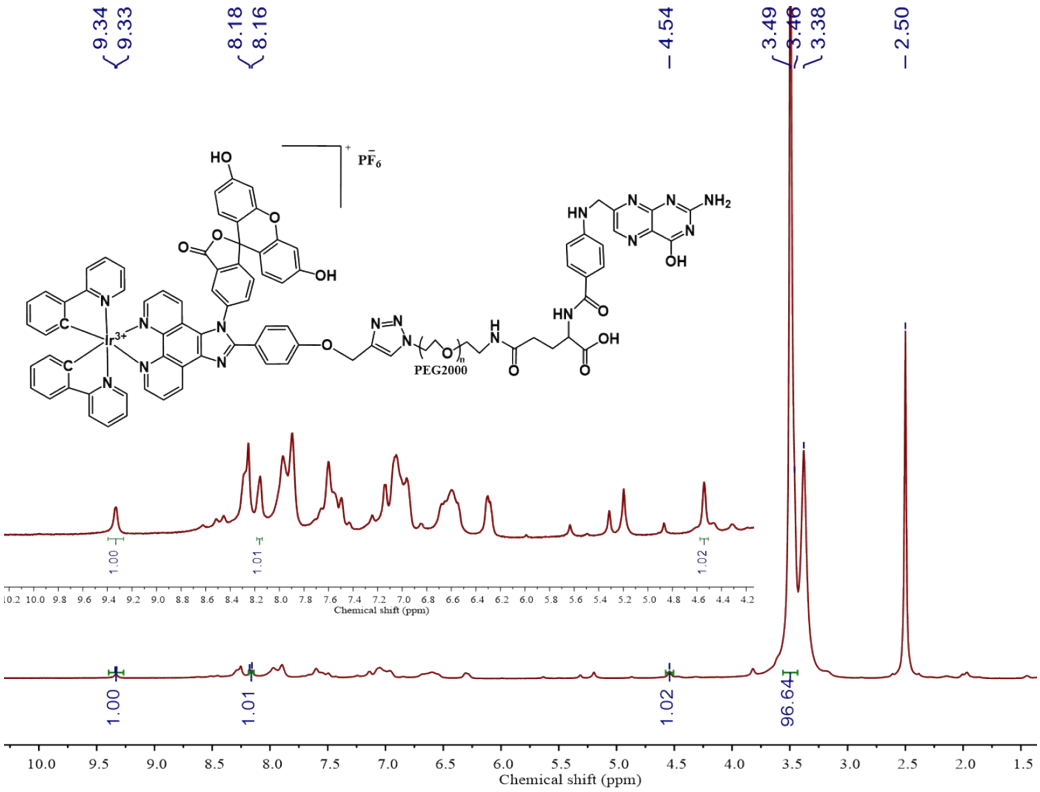

Fig. S4 ^{1}H NMR spectrum of IrFlu-PEG$_{2000}$-FA in DMSO-d_6 at 298K.

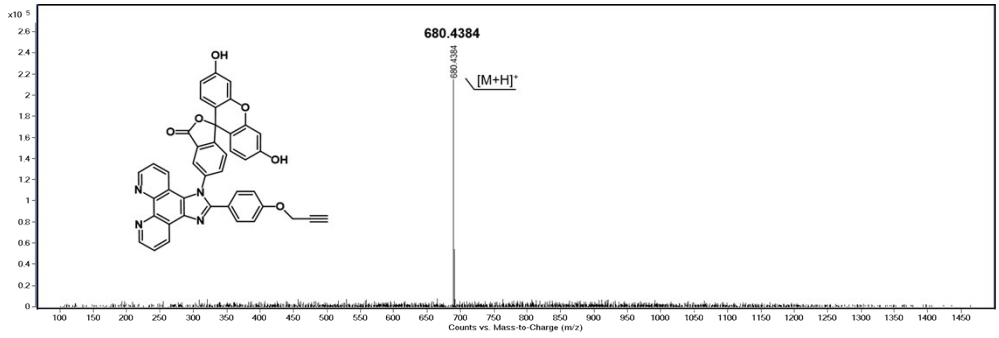

Fig. S5 ESI-MS spectrum of L-MNF in CH_3OH. $[M+H]^+$, 680.4384.

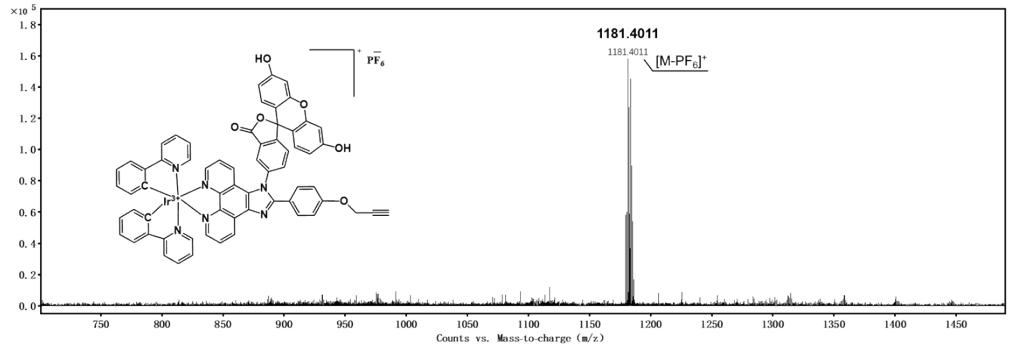

Fig. S6 ESI-MS spectrum of IrFlu in CH_3OH. $[M\text{-}PF_6]^+$, 1181.4011.

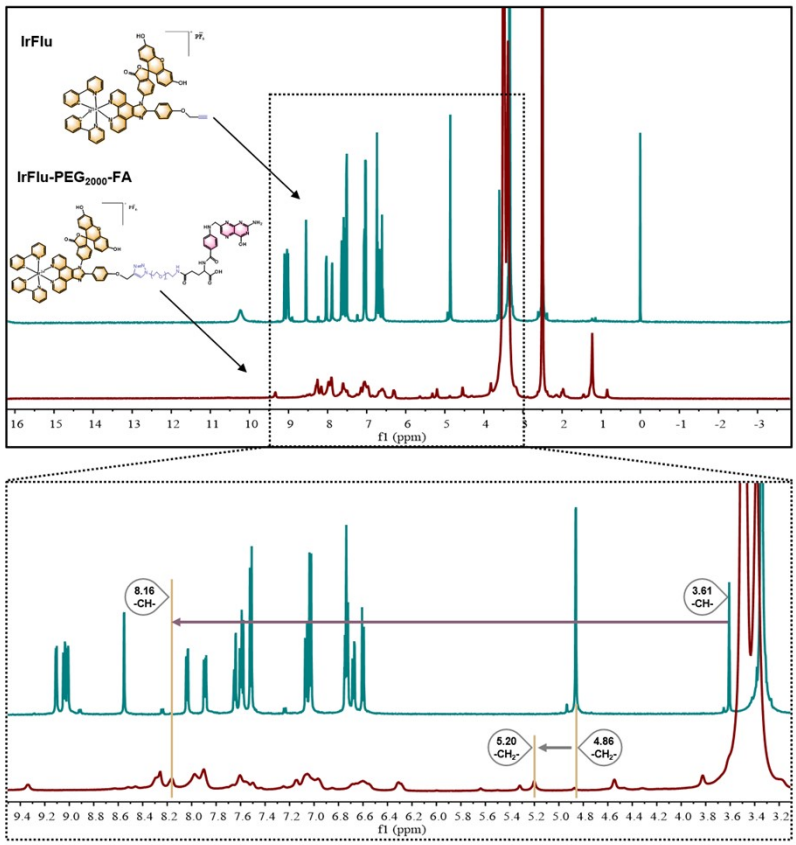

Fig. S7 ^1H NMR spectra of IrFlu and IrFlu-PEG_{2000}-FA. DMSO-d_6 as the solvent.

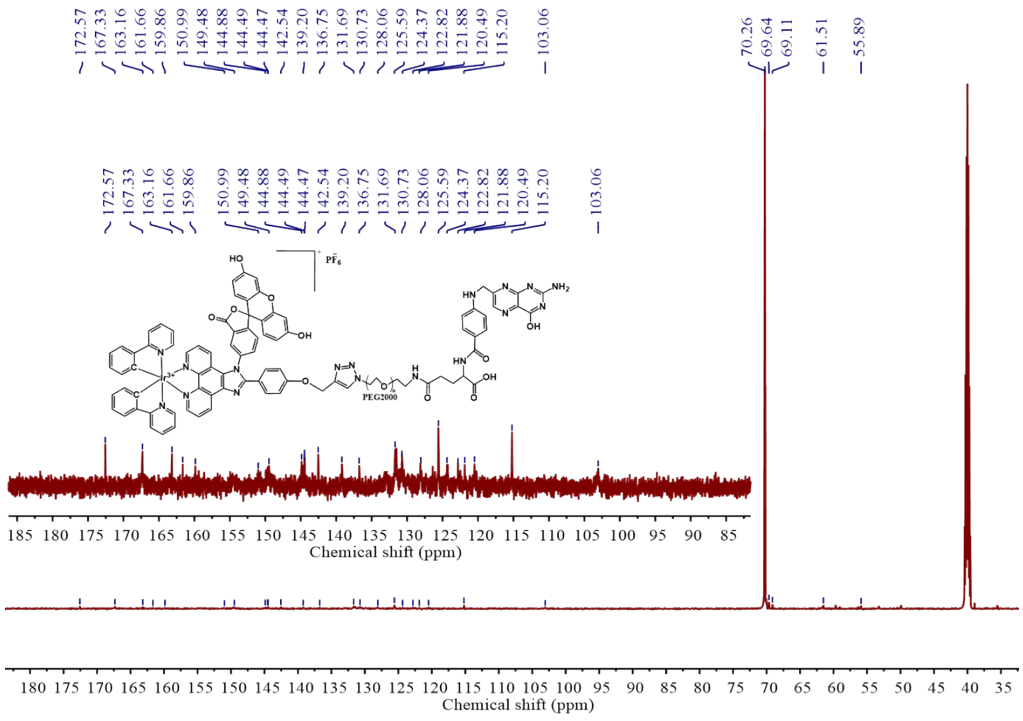

Fig. S8 ^{13}C NMR spectrum of IrFlu-PEG_{2000}-FA in DMSO-d_6 at 298K.

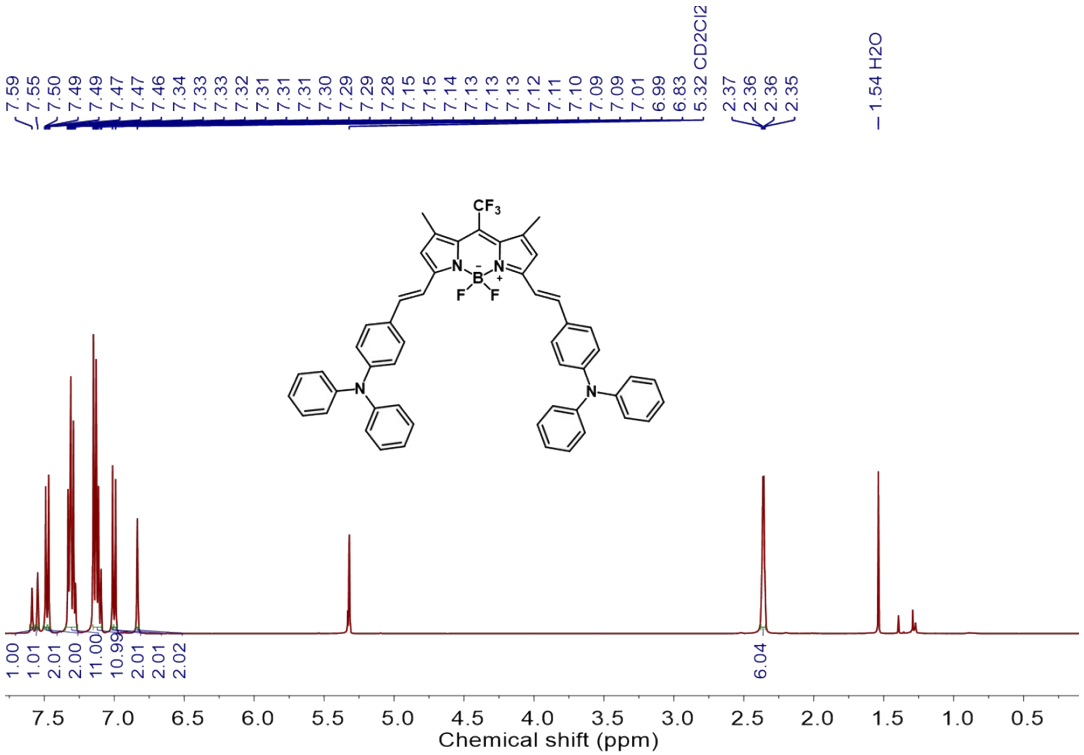

Fig. S9 ^{1}H NMR spectrum of TCF_3P in CD_2Cl_2 at 298K.

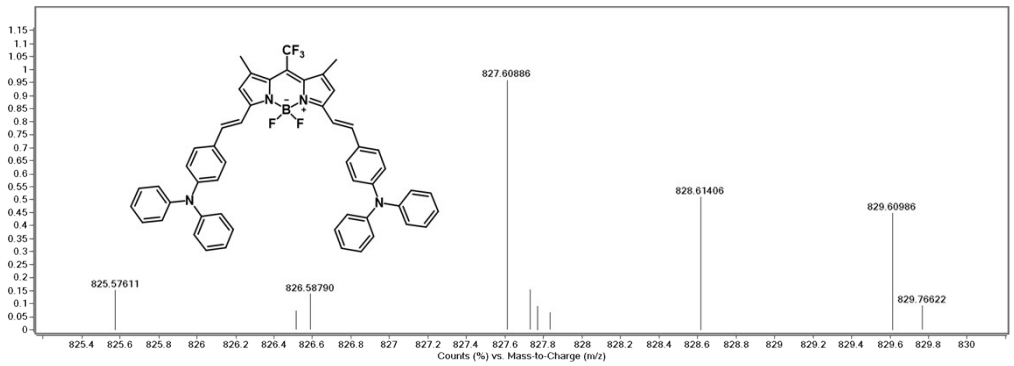

Fig. S10 ESI-MS spectrum of TCF_3P.

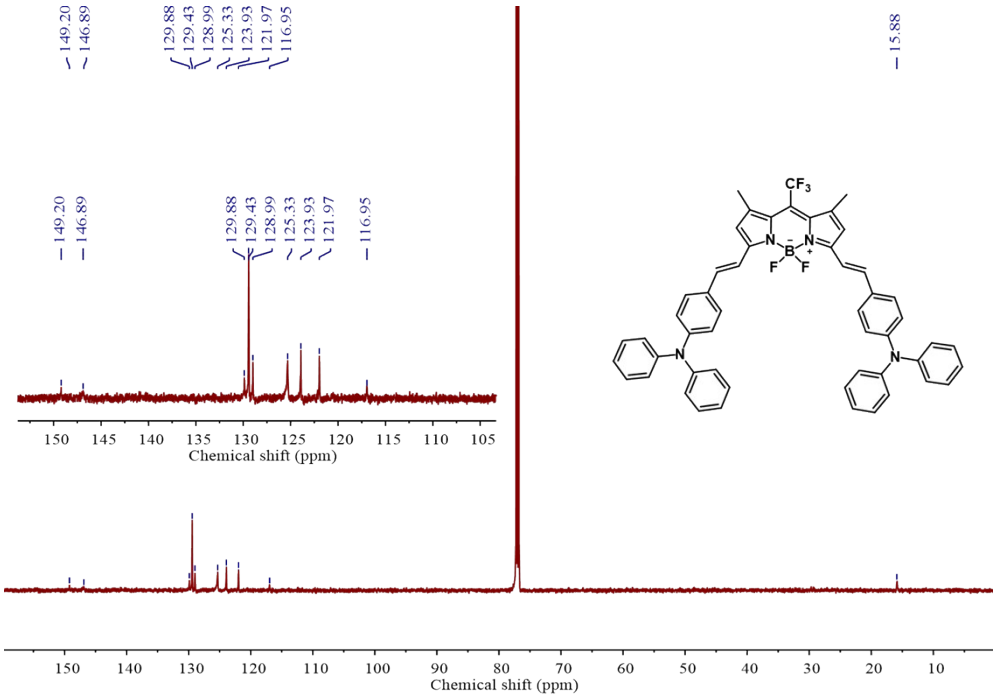

Fig. S11 ^{13}C NMR spectrum of TCF_3P in $CDCl_3$ at 298K.

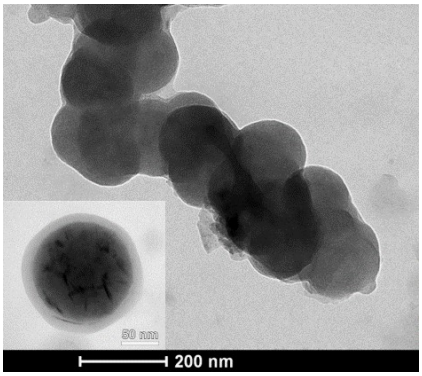

Fig. S12 TEM image of Ir-TCF_3P-FA NPs.

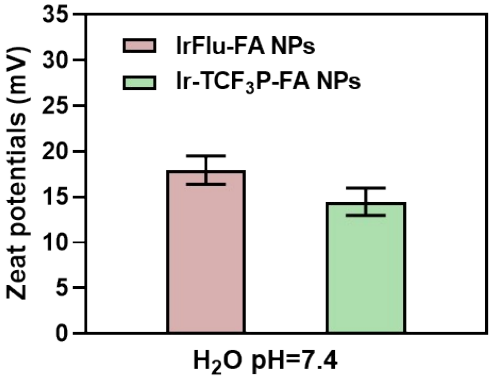

Fig. S13 Zeta potentials of IrFlu-FA NPs and Ir-TCF_3P-FA NPs in H_2O (pH = 7).

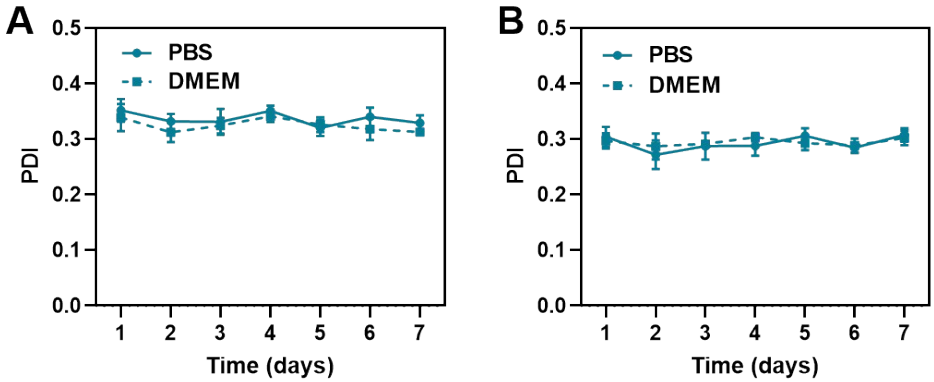

Fig. S14 Time-dependent PDI of (A) IrFlu-FA NPs and (B) Ir-TCF_3P-FA NPs in physiological buffer solutions (PBS) and DMEM medium (containing 10% fetal bovine serum) solutions.

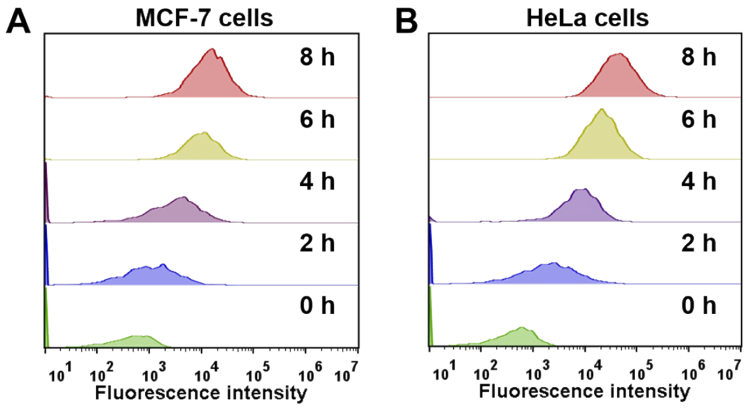

Fig. S15 Flow cytometry analysis of (A) MCF-7 cells and (B) HeLa cells after incubation with Ir-TCF_3P-FA NPs (5 μM) at different times.

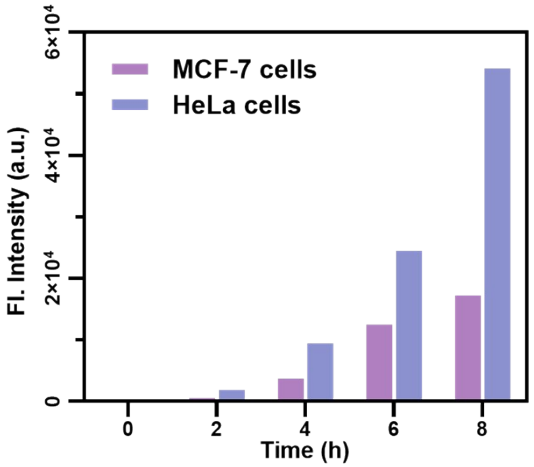

Fig. S16 Fluorescence intensity of MCF-7 cells and HeLa cells after incubation with Ir-TCF_3P-FA NPs (5 μM) for different times.

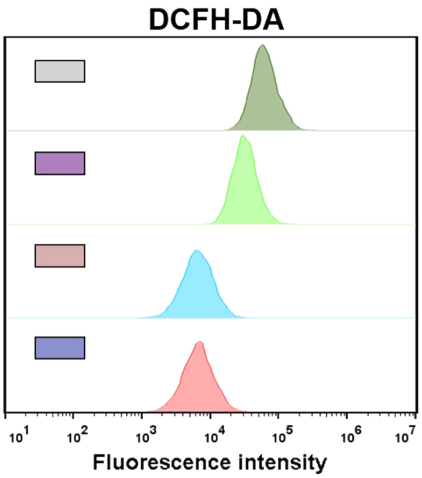

Fig. S17 Flow cytometry of ROS generation in different treatments.

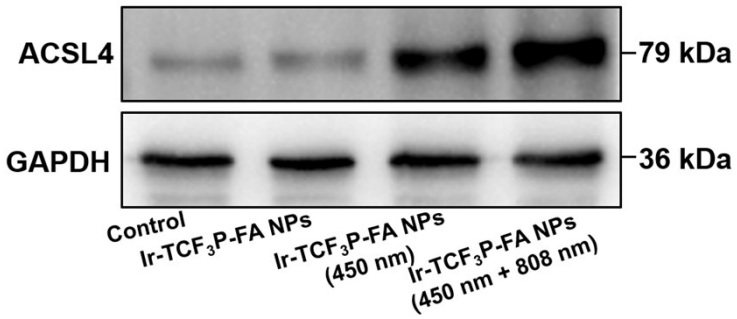

Fig. S18 After different treatments, the expression of ACSL4 in HeLa cells were tested by western blot.

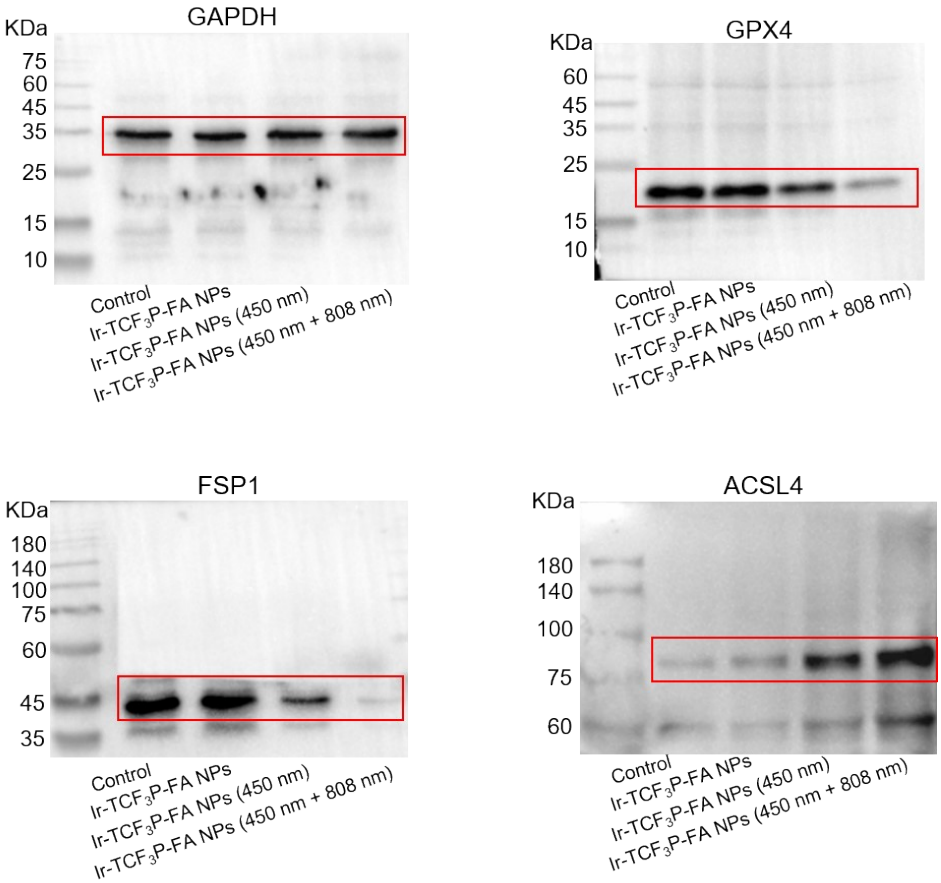

Fig. S19 Western-blot assay for the expressions of GPX4, FSP1, and ACSL4 in HeLa cells after different treatments.

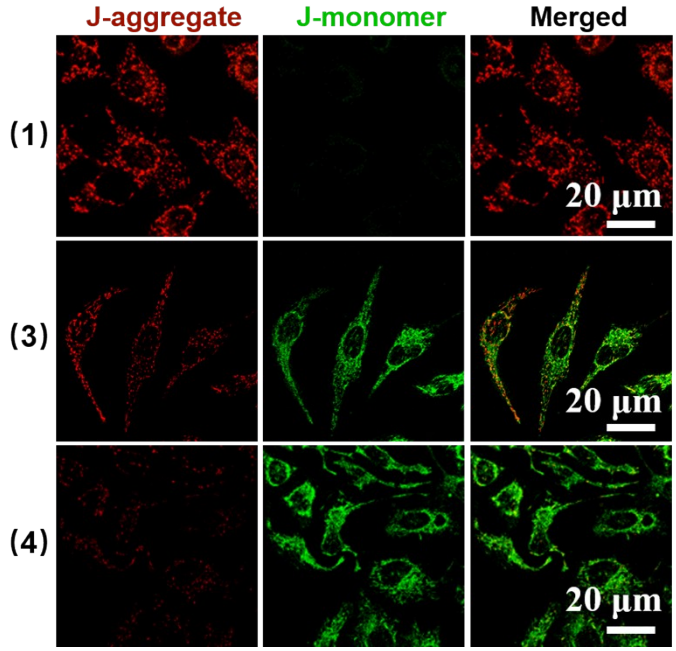

Fig. S20 CLSM images of mitochondrial membrane potential (MMP) in HeLa cells treated with various formulations. (1): Control, (3): Ir-TCF_3P-FA NPs (450 nm), (4): Ir-TCF_3P-FA NPs (450 nm + 808 nm).

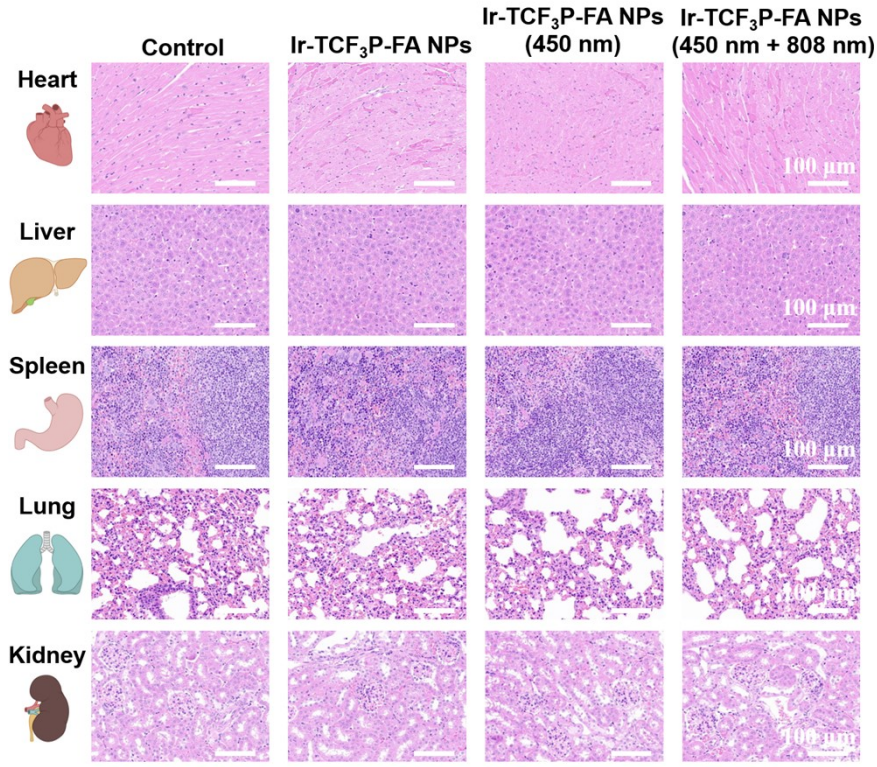

Fig. S21 H&E staining of the major organ slices (heart, liver, spleen, lung, and kidney) after 16 days of various treatments.

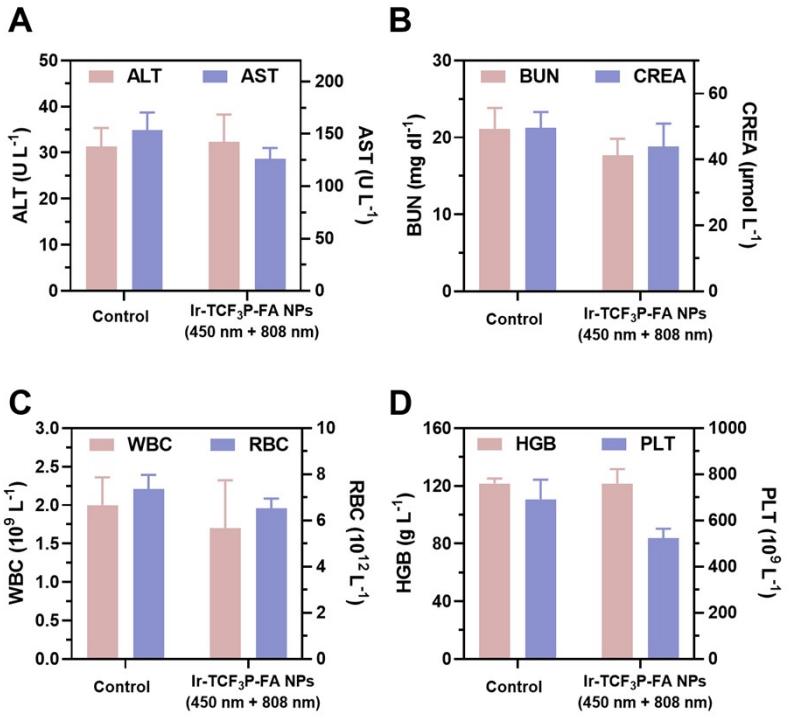

Fig. S22 (A) Hepatic enzymes (ALT and AST), (B) renal function markers (BUN and CREA), and (C-D) hematological parameters (WBC, RBC, HGB, and PLT) of mice after 16 days of treatment with Ir-TCF$_3$P-FA NPs.

References

[1] L. Wei, X. He, C. Liu, M. Kandawa-Shultz, G. Shao, Y. Wang, Biotin-Functionalized Iridium-Based Nanoparticles as Tumor Targeted Photosensitizers for Enhanced Oxidative Damage in Tumor Photodynamic Therapy, ACS Appl. Nano Mater., 7 (2023) 1170-1180.