Supporting information for

Oxygen Self-Produced Nanoplatform for Relieving Hypoxia and Breaking Resistance to Sonodynamic Treatment of Pancreatic Cancer

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Part A: additional experimental details

Materials

Tetraethoxysilane (TEOS, A.R), ammonia solution ($NH_3 \cdot H_2O$) (25~28%, A.R), acetonitrile, methanol, sodium carbonate anhydrous (Na_2CO_3, A.R) and anhydrous ethanol (EtOH, A.R) was purchased from Sinopharm Chemical Reagent Co., Ltd. IR 780 iodide, 3-aminopropyltriethoxysilane (APTES) and 1H,1H,2H,2H-Perfluorodecyltriethoxysilane (PDES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), fluorescein isothiocyanate (FITC) and ascorbic acid were purchased from Sigma-Aldrich CO., LTD. O_2 level indicator ($[Ru(dpp)_3]Cl_2$), oxygen free radiacals assay kit (DCFH-DA), 4',6-diamidino-2-phenylindole (DAPI), annexin V-FITC apoptosis detection kit and calcein & PI apoptisis assay kit and cell counting kit (CCK-8) were obtained from Beyotime Institute of Biotechnology, Shanghai, China. 1,3-diphenylisobenzofuran (DPBF), 2,2,6,6–tetramethylpiperidine (TEMP) and hypoxyprobe-1 plus kit (Hypoxyprobe, Inc., Burlington, MA, USA) were purchased from Shanghai Yare Biotechnology Co., Ltd, Shanghai. Deionized (DI) water was used in all experiments.

Characterizations

FETEM (field emission transmission electron microscopy) analysis was conducted with a JEM 2100 F electron microscope operated at 200 kV to characterize the mesopores and hollow structure. Nitrogen adsorption–desorption isotherms were measured at 77 K on a Micromeritics Tristar 3000 analyzer. The pore-size distributions were calculated using adsorption isotherm branches by the BJH method. Pore volume and specific surface area were calculated by using BJH and BET methods, respectively. Fourier-transform infrared (FTIR) spectra were recorded on a Nicolet Avatar 370 FT-IR spectrophotometer using KBr pellets. UV–vis absorption spectra were obtained with a Shimadzu UV-3101PC UV–vis absorption spectrophotometer. The Zeta potential and size distribution of samples was measured by dynamic light scattering (DLS) on Malvern Nano-ZS90. Ultrasound irradiation with low power density was conducted on a portable ultrasound apparatus (Chattanooga, USA). B mode ultrasound imaging was conducted on GE LOGIQ E9. Different cell stages were tested on Flow cytometry of Beckman Coulter, Inc., and confocal observation was conducted on Olympus confocal microscopy. O_2 characteristic peak in GC was recorded on GC-2060 gas

chromatograph equipped with a flame ionization detector and 5A zeolite as chromatographic column.

Release measurement of IR780 molecules from IR780@O_2-FHMON

Three groups were set, and they were No US (pH=7.4), No US (pH=6.0) and US (pH=6.0). In detail, 3 mg of IR780@O_2-FHMON was added into the dialysis bag (cutoff molecule: 3000) and sealed by plastic clip, and the whole dialysis bag was placed in a non-transparent centrifugal tube holding 25 mL of PBS (pH=7.4 or 6.0). Finally, the centrifugal tube was fastened in the sample slot of the electronic shaker at an oscillation rate of 200 rpm/min at 37 °C. In the group of No US, the incubation remained normal. In contrast, US irradiation was accompanied at four given time points (4 h, 8 h, 12 h, 18 h) within 24 h. During experiment, 3 mL of solution in centrifugal tube was sucked out for UV-vis measurement at some certain intervals (0 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 18 h, 24 h), and the intensity of IR780 characteristic peak at 780 nm was monitored, and the release profile of IR780 can be obtained *via* the absorbance standard curve of IR780 as a function of mass concentration of IR780 molecules.

Cell culture

PANC-1 cells, mouse fibroblast cell line (L929) and alpha mouse liver 12 (AML-12) cells were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. PANC-1 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), streptomycin (100 mg/mL) and penicillin (100 units/mL) in a humidified 5% CO_2 atmosphere at 37 °C for 24 h. Afterwards, they were transferred to a 37°C hypoxic incubator (MCO-5M CO_2 incubator, Sanyo, Moriguchi, Japan) with constant mixed atmosphere consisting of O_2, CO_2 and N_2 (0.1%: 5% : 94.9% in volume) for further cellular-level use.

L929 cells were still cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), streptomycin (100 mg/mL) and penicillin (100 units/mL) in a humidified 5% CO_2 atmosphere at 37 °C before use. AML-12 cells were cultured in a 90% 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and DMEM/F-12 (supplemented with 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL selenium, and 40 ng/mL dexamethasone) and 10% FBS and streptomycin (100 mg/mL) and penicillin (100 units/mL) in a humidified atmosphere consisting of 5% CO_2 and 95% air before use.

Cytotoxicity evaluation

Normoxic mouse fibroblast cells (L929) and alpha mouse liver (AML-12) cells were chosen to evaluate biosafety of such as oxygen self-produced SDT nanoplatform. AML-12 cells and L929 cells seeded in 6-well culturing plates at a density of 5×10^5 cells per well were cultured in the normal conditions (humidified 5% CO_2 atmosphere at 37 °C) for 24 h, and allowed to adhere overnight. Five groups were set, *i.e.*, control, US, US+FHMON, US+IR780@FHMON, IR780@O_2-FHMON and US+IR780@O_2-FHMON, respectively. After adhering, the corresponding treatments in the five groups were carried out, wherein US irradiation was applied on the bottom of dish and agar gel with 1 cm in thickness was inserted between US transducer and culturing dish in case of hyperpyrexia. The US parameters were indicated as follows: power density=1 W/cm^2, transducer frequency=1 MHz, duration time= 20 s per cycle, total cycle=9, interval between two cycles=30 s. The mass concentration of above samples dispersed in DMEM with 10% FBS was 500 μg/mL. At two given time points (12 h, 24 h) after hermetical incubation, the culture media in each group were discarded and replaced by 100 μL of FBS-free culturing medium that contained 10 v% CCK-8, and the viable cells were detected *via* CCK8 assay kit (Dojindo Molecular Technologies, Kumamoto, Japan). Absorbance intensity at 450 nm was measured and the viable percentage was calculated *via* comparing to the absorbance of control at λ = 450 nm, using a microplate reader (Bio-TekELx800, USA). Statistical analysis was performed using the Student's two-tailed t-test, and three independent experiments were repeated.

As for the mass concentration-dependent cytoxicity of FHMON, L929 cells and AML-12 cells that were seeded in a 96-well cell-culture plate at a density of 1×10^4 cells per well were cultured in the normal conditions at 5% CO_2 moist atmosphere for 24 h, and allowed to adhere overnight. After that, the culture medium was replaced by fresh medium containing various concentrations of FHMON (0, 10, 25, 50, 100, 200, 500 and 800 μg/mL). With a further 24 h-incubation, the culture media were discarded, and other measuring procedures were identical to those in above experiment.

***In vitro* proliferation inhibition test *via* the O_2-supplyed SDT strategy**

PANC-1 cells were seeded (1×10^5 cells in 2 mL of DMEM per well) in 6-well plates, and they were adherent to the plate for overnight under hypoxia condition. Five groups were set, and they were control, US, US+FHMON, US+IR780@FHMON, IR780@O_2-FHMON and US+IR780@O_2-FHMON, respectively. After adhering, the corresponding treatments in the five groups were carried out in hypoxic incubator, and US irradiation was applied on the bottom of dish,

between which agar gel with 1 cm in thickness was inserted in case of high fever. The employed parameters of US were indicated as follows: power density=1 W/cm^2, transducer frequency=1 MHz, duration time= 20 s per cycle, total cycle=9, interval between two cycles=30 s during which the hypoxic incubator gate was shut. The employed mass concentration of any sample was 500 μg/mL (that is, 46.5 μg/mL based on I780) and dispersed in DMEM with 10% FBS. At several given time points (0, 6 h, 12 h, 24 h) after hermetical incubation in hypoxic incubator, the culture medium in each group was discarded. The cell viability (V_t) at some given time point was determined by CCK8 method, and V_0 corresponded to the cell viability obtained immediately without further incubation after various treatments in above five groups. The cell proliferation rate (P_t) was obtained according to the following equation:

$$P_t = (v_t - v_0)/v_0 \times 100\% \qquad (1)$$

Power-dependent and mass concentration-dependent proliferation inhibition experiments were carried out under the same conditions simultaneously, wherein power density varied within a range of 0- 1.5 W/cm^2 and the mass concentration was tunable between 0 and 800 μg/mL. Three independent dishes per group at each time point were included. Noticeably, PBS used for cell washing as well as DMEM cell culture media was deoxygenated *via* bubbling with nitrogen gas before use.

Animals

Female BALB/c mice with an average age of 7 weeks (~20 g) were used for the PANC-1 tumour xenograft and corresponding assessments of *in vivo* O_2-supplied SDT and its accumulation. Female Kunming mice with an average age of 6 weeks (~20 g) were used to evaluate the biosafety of IR780@O_2-FHMON and blood terminal half-life. All the mice were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. All the animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee and the care regulations approved by the administrative committee of laboratory animals of Guangxi Medical University.

PANC-1 xenografted tumor model

Human pancreatic carcinoma cells, *i.e.*, Panc-1 cells, were supplied by China Infrastructure of Cell Line Resources, and cultured in DMEM containing 10% heat-inactivated fetal bovine serum and 1% antibiotics (streptomycin and penicillin) (Invitrogen). The cultures were maintained in a

humidified mixed atmosphere with a fixed volume ratio of $O_2/N_2/CO_2$ (0.1:94.9:5) at 37 °C.[1] Nude mice (Balb/C nu/nu) with an average body weight of about 20 g were supplied by Laboratory Animals Center of Guangxi Medical University, and were kept in sterilized cages with supply of filtered air, sterile food, and water. For developing solid tumor in nude mice, the procedures were carried out as described previously. In detail, 0.1 mL of cell suspension (1×10^6 cells) in PBS was injected subcutaneously into the flank of nude mice using a 1 mL injector. After 1 week, the solid tumor emerged, and tumors was not used until they reached 100-120 mm^3 (experimental day 0). Tumor burden associated with general well-being, weight, tumor volume, survival rate and tumor metastasis was monitored *via* direct observation. All *in vivo* animal experiments were performed according to the care regulations approved by the administrative committee of laboratory animals of Guangxi Medical University and were in accordance with the protocols approved by Institutional Animal Care and Use Committee (IACUC).

***In vivo* accumulation of IR780@O_2-FHMON in tumor**

Panc-1 xenografted tumor-bearing nude mice were randomly averaged into two groups (n=6), IR780@O_2-FHMON and US+IR780@O_2-FHMON, and the injection dose of Si is 300 mg Si/Kg; the ultrasound parameter is 1.0 W/cm^2-100%-20 s per cycle and 9 cycles in total with a 10 min interval between two cycles; the injection method is tail intravenous. After treatments in above two groups, at some given time points, the treated mice were anatomical, and PANC-1 tumours were collected weighed and then digested with the mixed solution of nitric acid and perchloric acid (volume ratio: 3:1). After that, quantitative analysis for Si atom was carried out *via* inductively coupled plasma-atomic emission spectrometry (ICP-AES) method. The accumulation percentage (A) of Si was calculated according to the following equation:

$$A = m_t/m_{ID}\times100\% \qquad (2)$$

wherein m_t was the measured weight of Si in tumour, and the constant m_{ID} was the initial dose of Si.

***In vivo* hypoxic regions staining**

Aiming at hypoxia immunofluorescence staining, intratumoral injection of Pimonidazole hydrochloride (0.6 mg/mouse) was carried out after *in vivo* SDT treatments. Subsequently, the tumor was harvested *via* surgical resection and slices (8 μm) were obtained. To visualize it, antipimonidazole mouse monoclonal antibody C (dilution 1:200, Hypoxyprobe Inc.) and Alex 488-conjugated goat antimouse (dilution 1:200, Jackson Inc.) were used as primary antibody and

secondary antibody, respectively, for further staining and fluorescence imaging. To monitor blood vessels, rat-antimouse CD31 antibody performed as the primary antibody, and Cy3-conjugated donkey−antirat antibody acted as the secondary antibody, to indicate the tumor blood vessels. DAPI was used to detect cell nuclei. When the survival rate was measured, the nude mice were not euthanized at the end of experimental period (day 28) until they reached 1500 mm^3 in consideration of the care regulations. The significant differences were analyzed using the Student's two-tailed t test (*P < 0.05, **P < 0.01 and ***P < 0.001).

Determination of blood terminal half-life

200 μl of IR780@O_2-FHMON solution in PBS was intravenously injected into Kunming mice (n = 8). Then, 20 μL of blood was collected from the tail vein at some given time points (2 min, 5 min, 10 min, 30 min, 1h, 2 h, 4 h, 6 h, 12 h and 24 h) and then diluted to 5 mL using ultrapure water with ethylenediaminetetraacetic acid disodium salt (EDTA-2Na, 10 mM) as anticoagulant. The Si concentration in blood was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Agilent 700 Series, USA). Origin was used for data analysis for the best-fit line and blood terminal half-life based on the one-component pharmacokinetic model.

***In vivo* biosafety evaluation**

Healthy Kunming mice were randomly divided into three groups (n=6): control, 30 days and 60 days, respectively. At the given time interval (60 day) after the *i.v.* injection of IR780@O_2-FHMON NPs (in 200 μL PBS) at a high dosage of 100 mg Si/ kg, these mice were sacrificed by injecting overdose anesthetics, and dissected, among which their major organs (heart, liver, spleen, lung and kidney) and blood were then harvested for pathological studies and biochemistry studies, respectively. The hematoxylin and eosin (H&E) stained tissue slices were observed on an optical microscope (LEICA DFC420 C, Leica) to inspect the potential histological changes. Various biochemical indexes associated with liver function and blood parameters after 30 days and 60 days post-injection of IR780@O_2-FHMON NPs were detected using the standard biochemistry test.

References:

(1) McEwan, C.; Kamila, S.; Owen, J.; Nesbitt, H.; Callan, B.; Borden, M.; Nomikou, N.; Hamoudi, R. A.; Taylor, M. A.; Stride, E.; *et al*. Combined Sonodynamic and Antimetabolite Therapy for the Improved Treatment of Pancreatic Cancer Using Oxygen Loaded Microbubbles as a Delivery Vehicle. *Biomaterials* **2016,** *80*, 20-32.

Part B: Supplementary figures

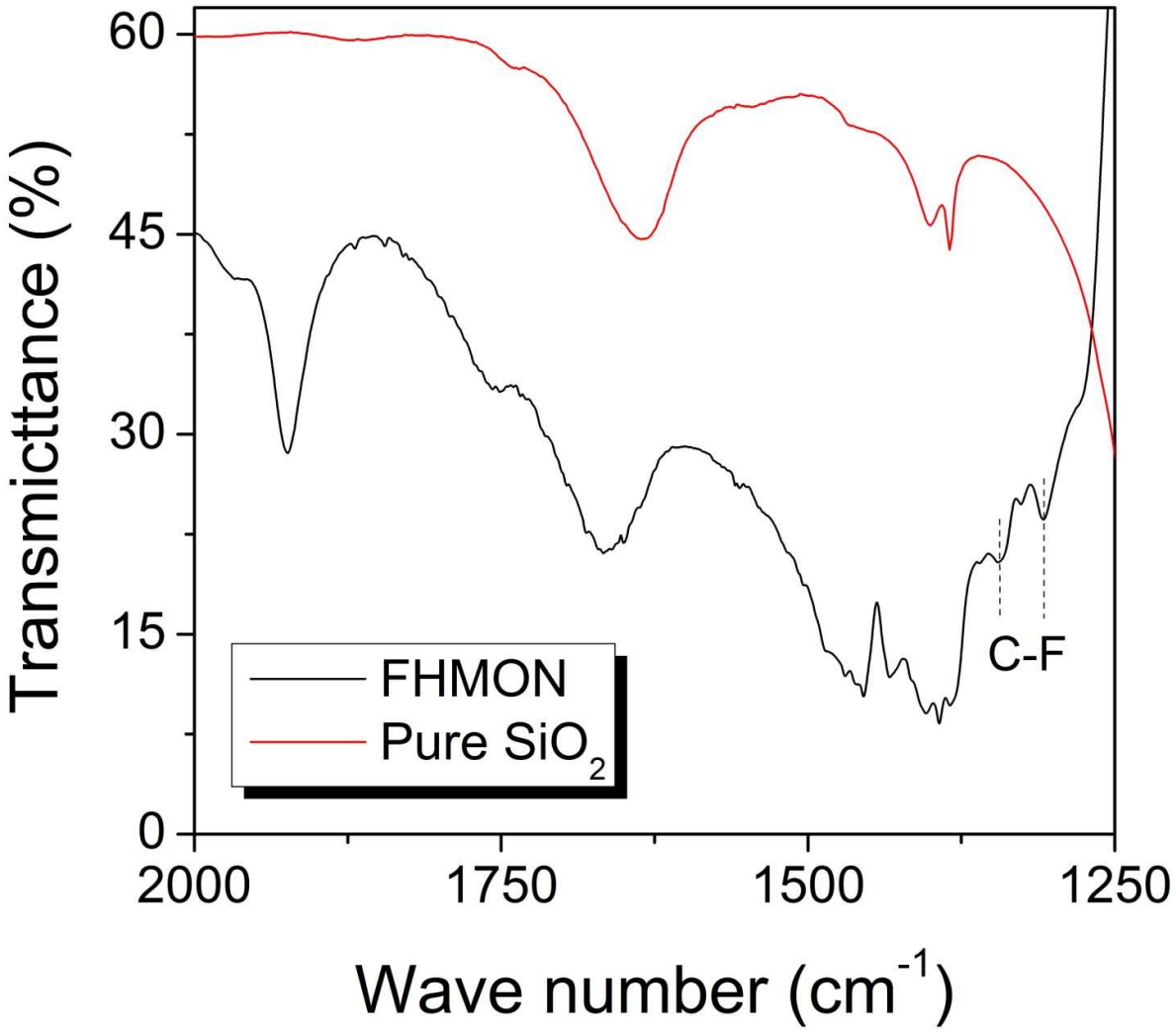

Figure S1. Fourier Transform Infrared Spectroscopy (FTIR) spectra of FHMON and pure SiO_2 completely deriving from the hydrolysis and condensation of TEOS.

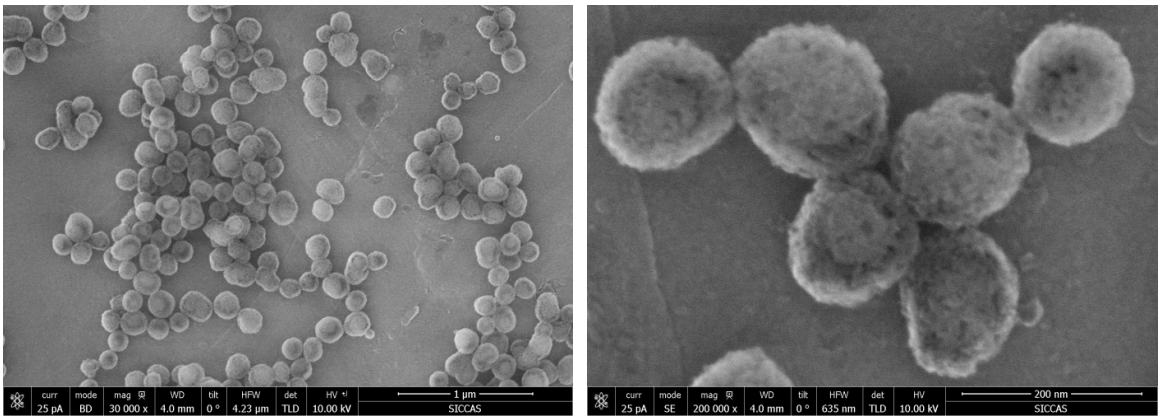

Figure S2. Low-fold (a) and high-fold (b) scanning electronic microscopic (SEM) images of FHMON carriers.

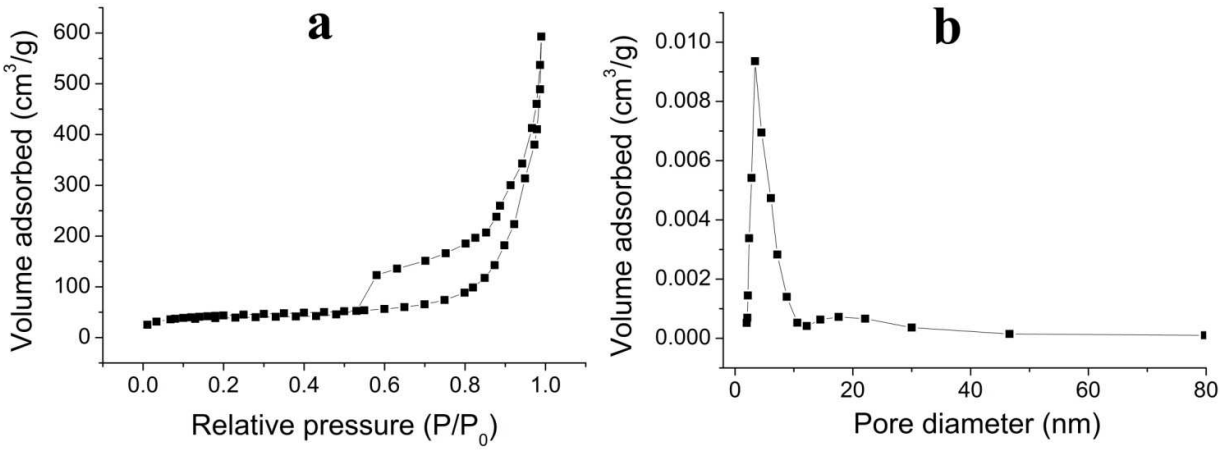

Figure S3. N_2-adsorption/desorption isotherm and pore diameter distribution of FHMON carrier.

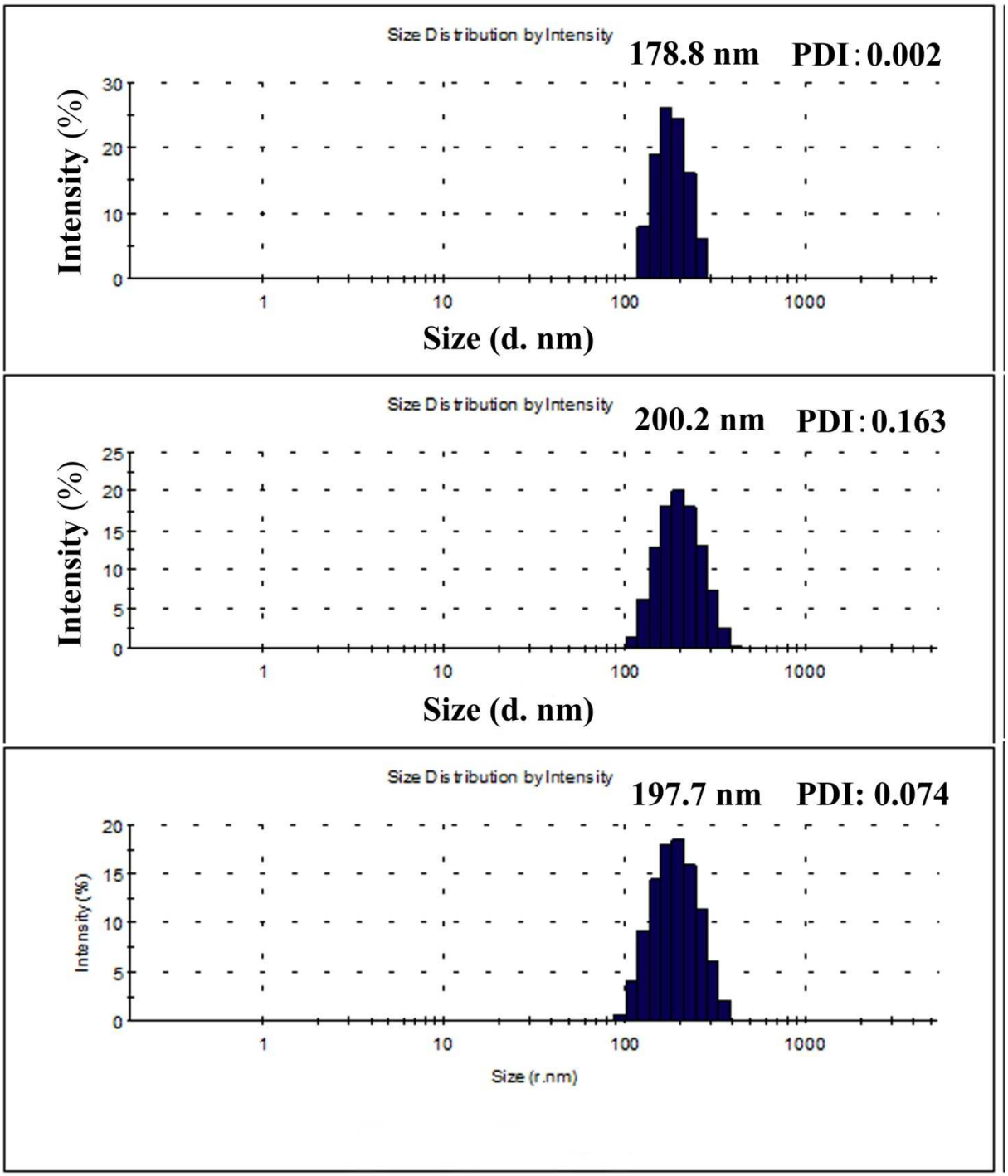

Figure S4. Size distributions of FHMON (upper), IR780@FHMON (middle) and IR780@O_2-FHMON using dynamic light scattering (DLS) method.

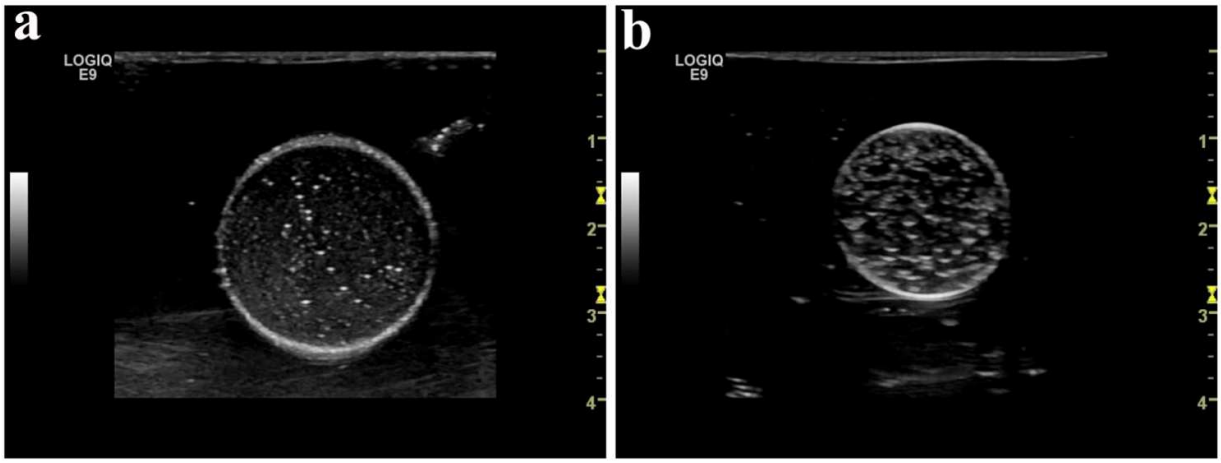

Figure S5. Ultrasonic images of IR780@O_2-FHMON before (a) and after heating (b).

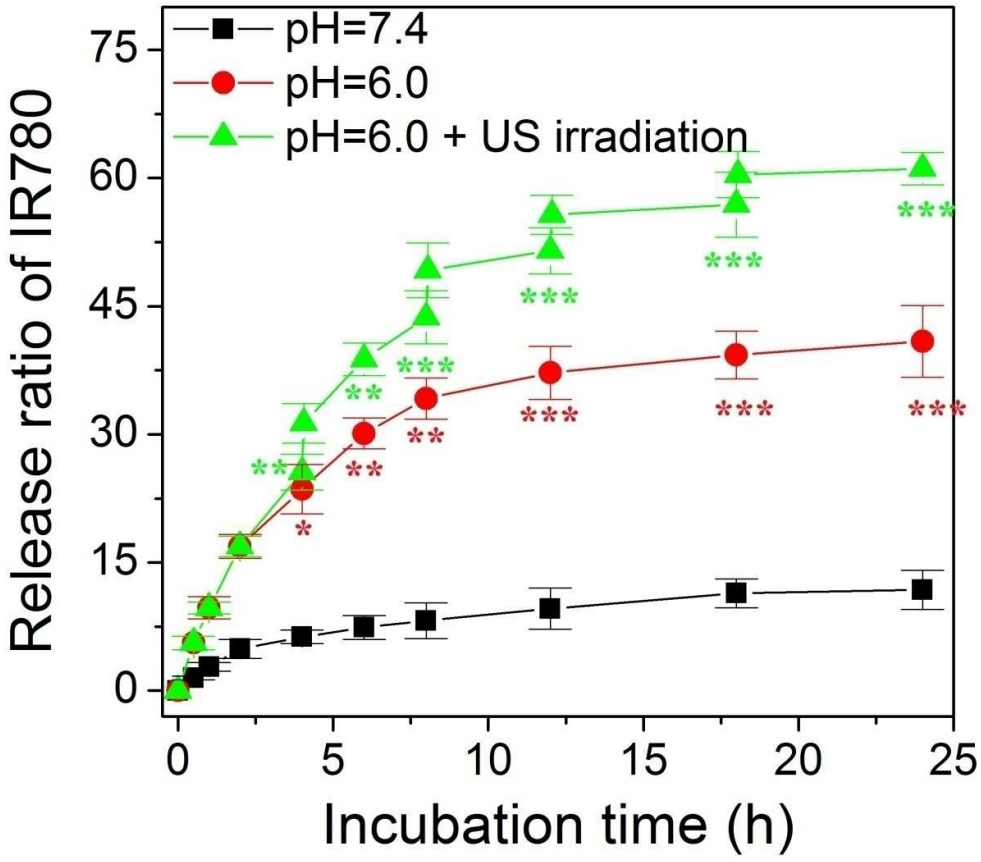

Figure S6. Release curves of IR780 from IR780@O_2-FHMON in PBS buffer solution (pH=7.4 and 6.0) with and without US irradiation (n=3), '*', '**' and '***' represent $p<0.01$, 0.005 and 0.001, respectively. Data is presented as the mean value ± SD (n=3).

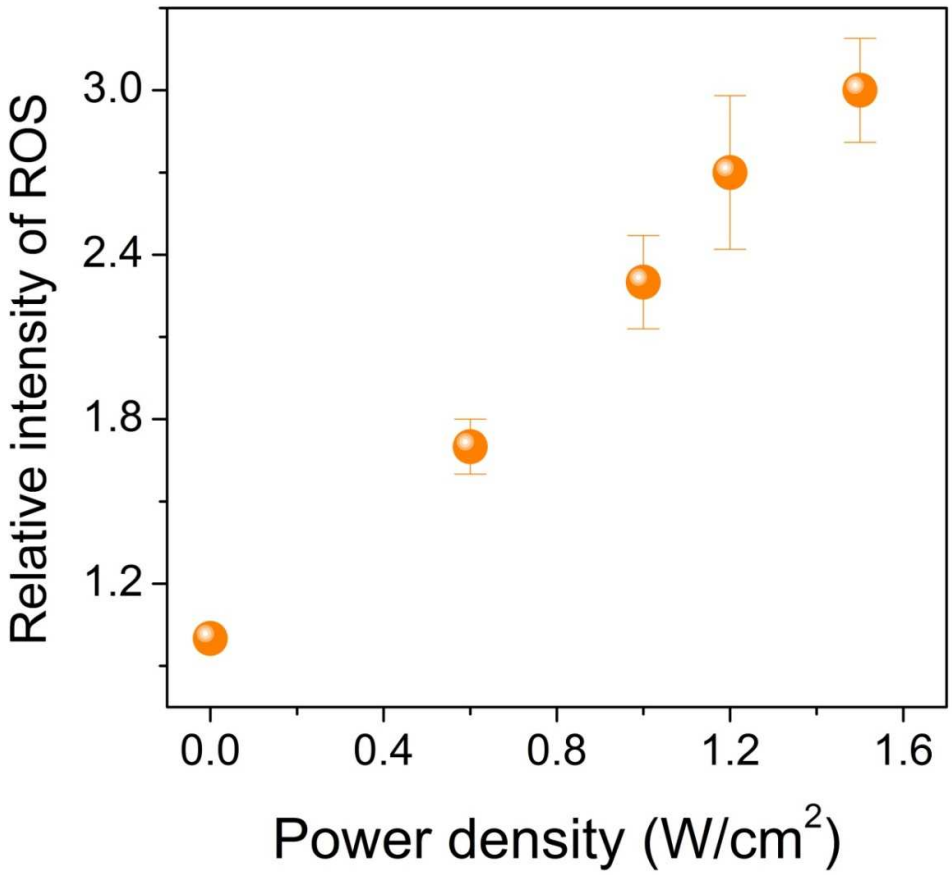

Figure S7. Power-density-dependent ROS production of IR780@O_2-FHMON upon exposure to US with variable power density by using flow cytometry to detect BCF fluorescence, and data is presented as the mean value ± SD (n=3).

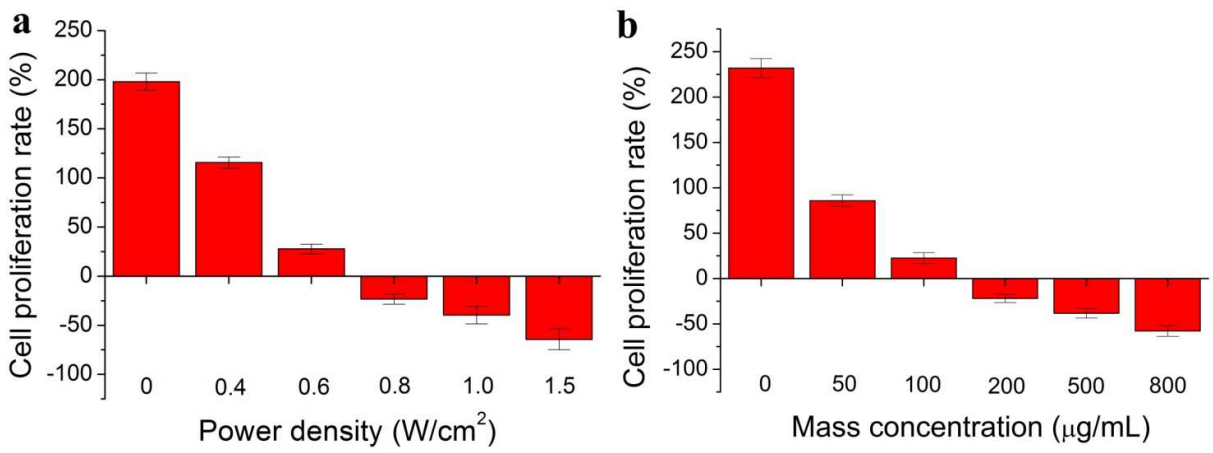

Figure S8. Cell proliferation rates of IR780@O_2-FHMON with variable power densities (a) and mass concentrations (b); and data is presented as the mean value ± SD (n=3).

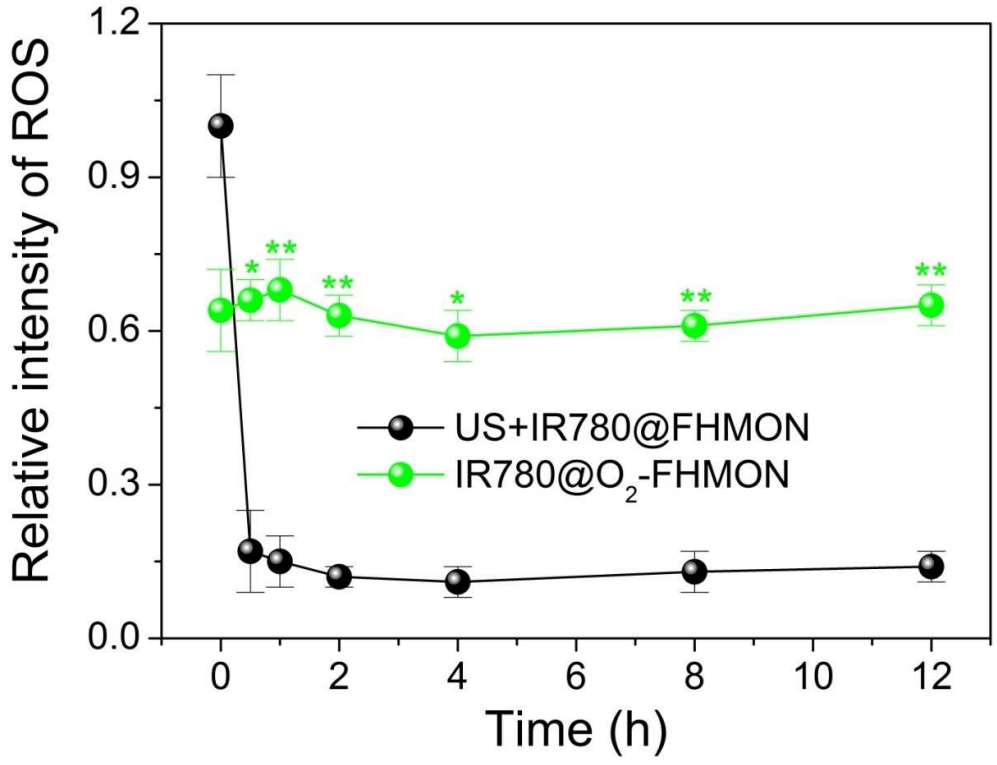

Figure S9 Time-dependent relative ROS production in hypoxic PANC-1 tumor treated with two different treatments, *i.e.*, US+IR780@FHMON and IR780@O_2-FHMON. Significance is determined *via* comparing to US+IR780@FHMON, and '*' and '**' represent $p<0.01$ and 0.005, respectively; Data is presented as the mean value ± SD (n=3).

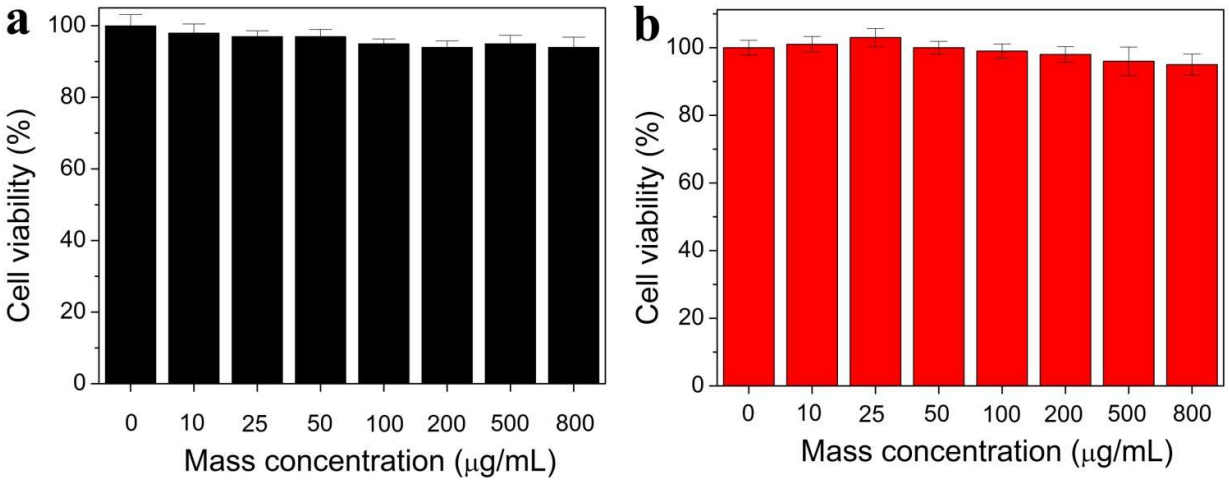

Figure S10. The cell viabilities of L929 (a) and ALM-12 (b) cells after incubations with FHMON carriers of variable mass concentrations for 24 h.

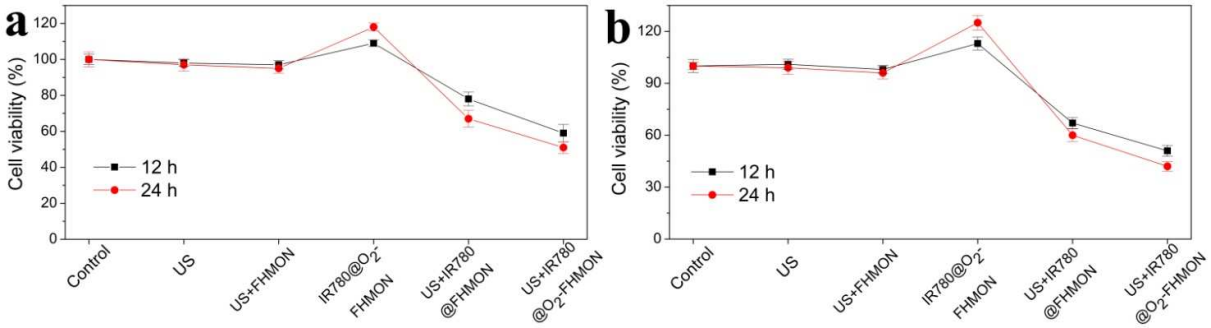

Figure S11. Cell viability of L929 cells (a) and AML-12 cells (b) after treatments with different groups (*i.e.*, control, US, US+FHMON, IR780@O_2-FHMON, US+IR780@FHMON and US+IR780@O_2-FHMON) for two certain time points (12 h and 24 h). Data is presented as the mean value ± SD (n=3).

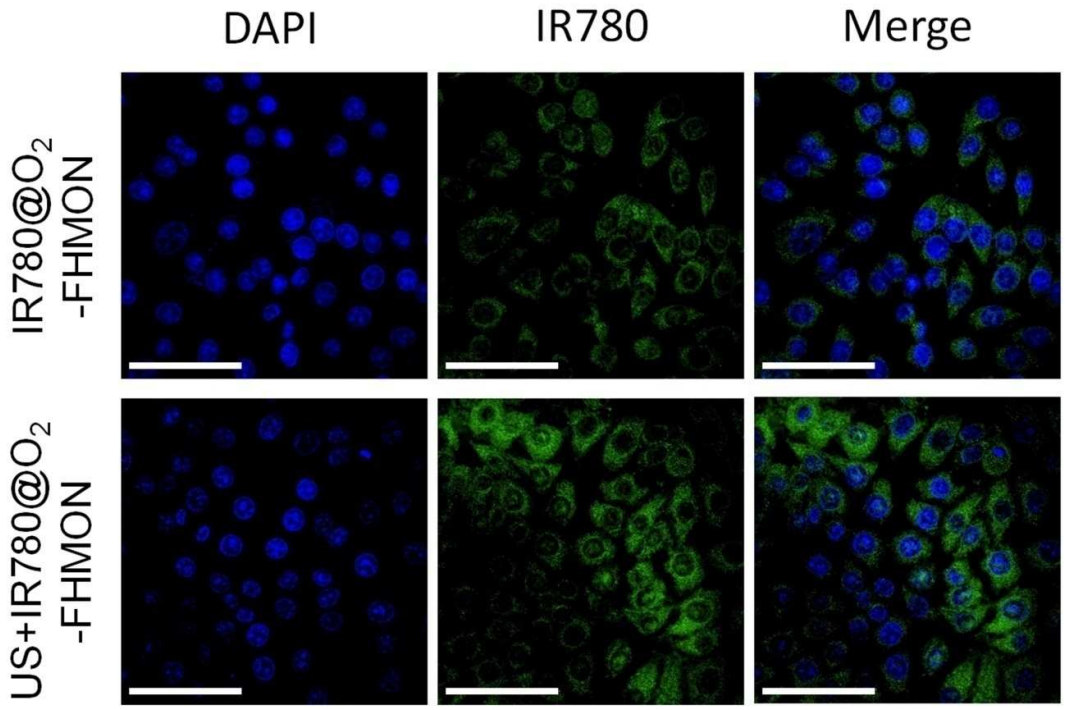

Figure S12. CLSM of hypoxic PANC-1 cells after treatment with two groups (*i.e.*, IR780@O_2-FHMON and US+IR780@O_2-FHMON), scale bar: 40 μm.

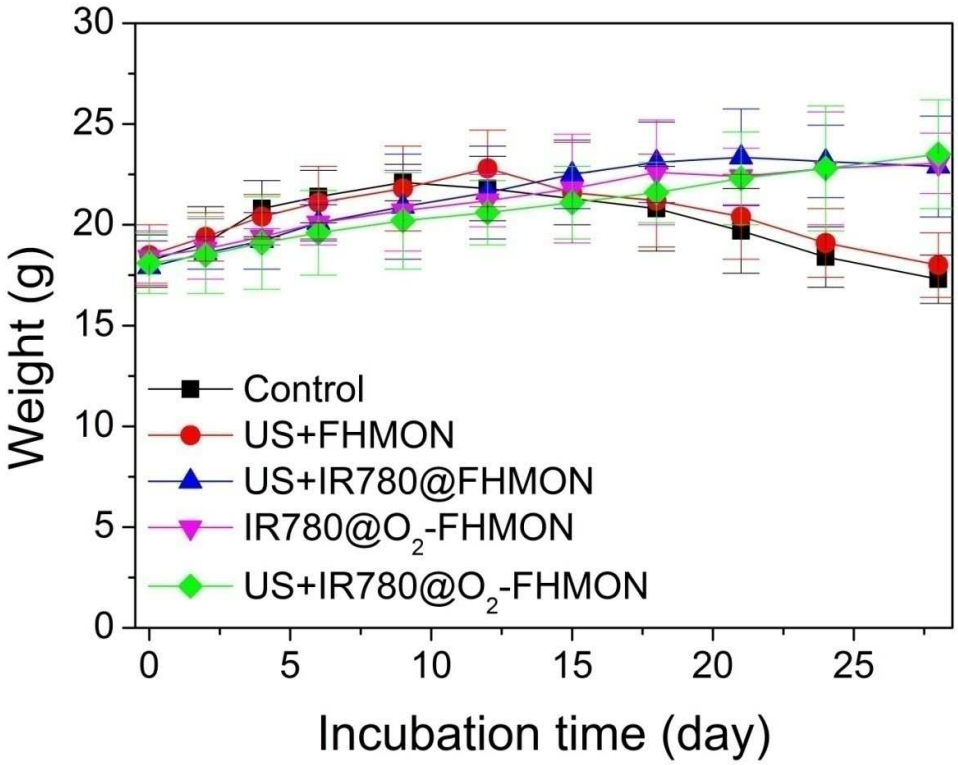

Figure S13. Weight variation of nude mice-bearing PANC-1 solid tumor in all groups. Data is presented as the mean value ± SD (n=6)

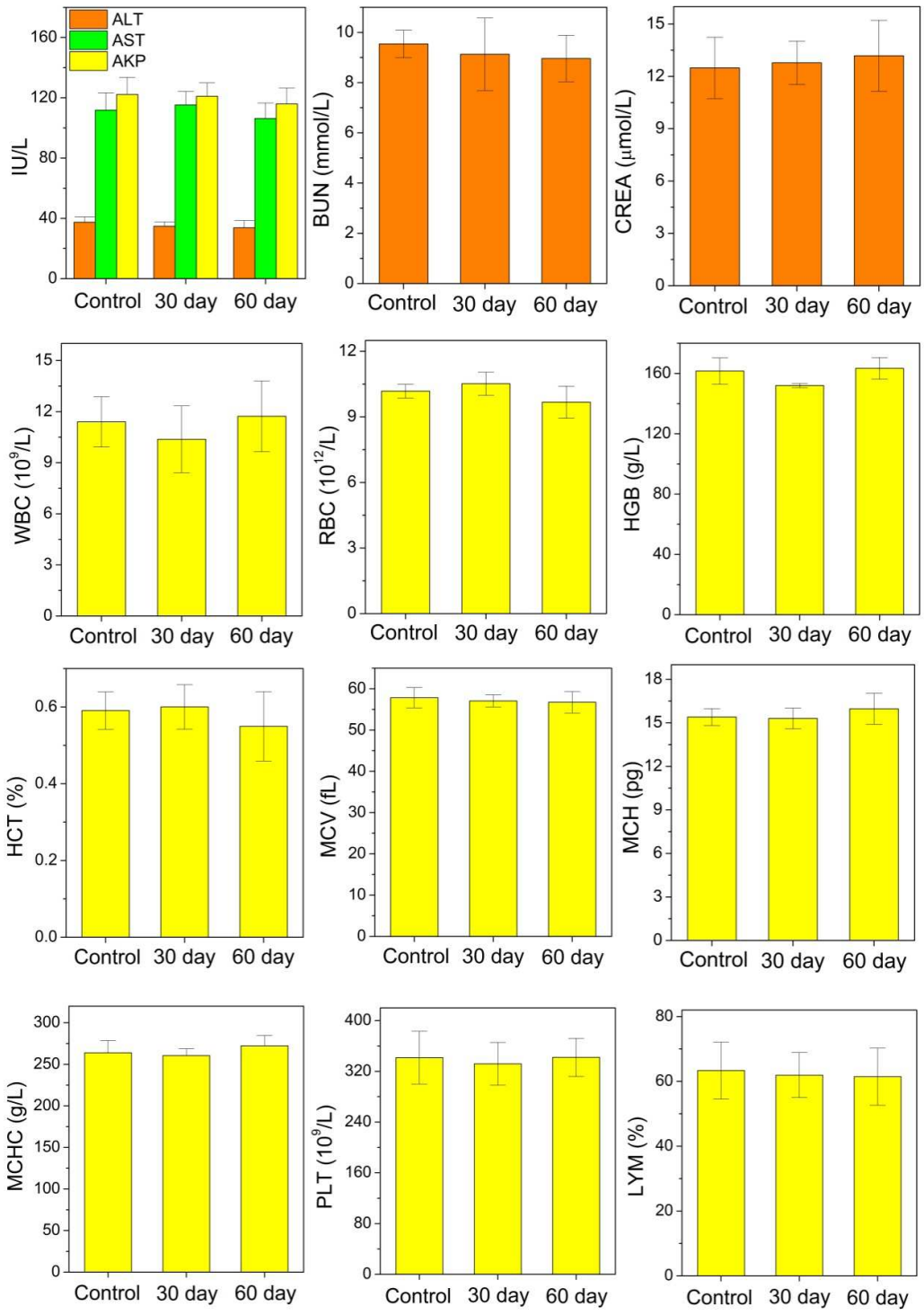

Figure S14. Blood and biochemical indexes of Kunming mice after 30 day and 60 day i.v. post-injection of IR780@O_2-FHMON, Data is presented as the mean value ± SD (n=6).

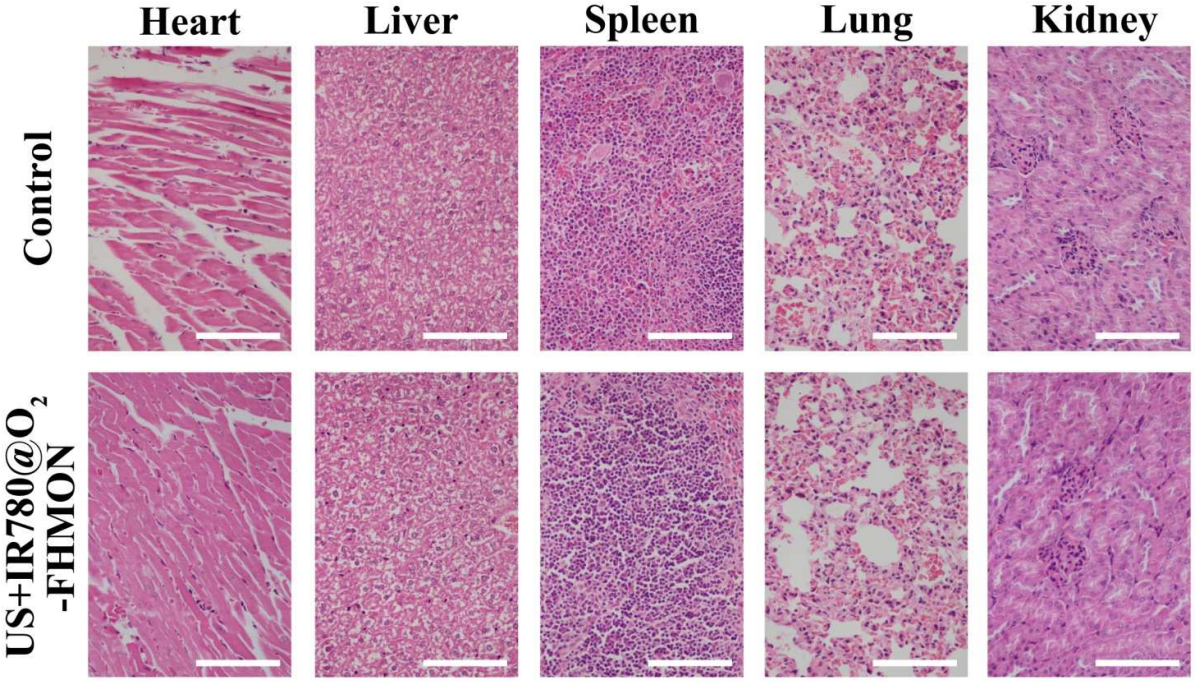

Figure S15. Optical microscopic images of primary organ slices of nude mice after 60 days post-treatment by two groups (*i.e.*, control and US+IR780@O_2-FHMON), scale bar: 200 μm.