Electronic Supplementary Material (ESI) for Nanoscale Horizons.

Electronic supplementary information **for:**

Ultrasound Activation of Liposome for Enhanced Ultrasound Imaging and Synergistic Gas and Sonodynamic Cancer Therapy

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EXPERIMENTAL SECTIONS

Chemicals. Cholesterol, 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[(carboxyl(polyethylene glycol) 2000] (DSPE-PEG_{2000}) were obtained from Avanti Polar Lipids Inc (USA). 2,2'-azobis[2-(2-imidazolin-2-yl) propane] dihydrochloride (AIPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), cobalt(II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$) and Phalloidin-Alexa Fluor 555, 2',7'-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS), DMEM medium, penicillin/streptomycin (100×) and fetal bovine serum (FBS) were supplied by Life Technologies (Carlsbad, CA). 4',6-diamidino-2-phenylindole (DAPI), Calcein-AM, PI and Lysotracker Deep Red were obtained from Thermo Fisher Scientific Inc (USA). Deionized (DI) water (18.2 MΩ.cm) used in all experiments was prepared by Milli-Q system (Millipore, USA). All other chemicals were of analytical grade and were used as received from Sigma-Aldrich.

Instruments. Transmission electron microscopy (TEM) images were captured on a HT7700 transmission electron microscope with an accelerating voltage of 100 kV. Dynamic light scattering (DLS) was measured on a Malvern Zetasizer nano ZS instrument. The fluorescence spectra were measured with a fluorimeter Hitachi F-4600 (Hitachi Co. Ltd., Japan). The hematoxylin and eosin (H&E)-stained slices were observed with a fluorescent inverted microscope (Nikon Ti2, Japan). A Visualsonics

Vevo 2100 (Visual Sonics) with an MS-550D 40 MHz transduce. Ultrasound irradiation for sonodynamic therapy was conducted by an WED-300. The fluorescence photographs were obtained on a confocal laser scanning fluorescence microscope (CLSM) (Nikon C2, Japan). Ultraviolet-visible light (UV-vis) absorption spectra were collected using a UH4150 Spectrophotometer (Hitachi Co. Ltd., Japan). Intracellular uptake and cell apoptosis was obtained by CLSM.

Statistical Analysis. The data were shown as mean ± standard deviation (SD), and the significance between two groups of the data in this work was analyzed on the basis of Student's two-tailed *t* test. Values of *P < 0.01 and **P < 0.001 and ***P < 0.0001 were considered statistically significant.

Preparation and Characterization of Lip-AIPH Nanoparticles. The Lip-AIPH nanoparticles were prepared using a thin-film hydration method. In brief, the lipid formulation (DPPC (Avanti Polar Lipids Inc.), DSPE-PEG_{2000} (Avanti Polar Lipids Inc.) and cholesterol at a molar ratio of 7:1:3 were dissolved in 2 mL mixed solution of chloroform and methanol with ratio of 9:1. The solvent was evaporated under reduced pressure for at least 2 h, and the product was dissolved in PBS. The film was hydrated with 4 mL PBS containing AIPH (5 mg ml^{-1} in PBS). The suspension was then extruded 20 times through cellulose acetate membranes (100 nm pore-size) by using a mini-extruder (Avanti Polar Lipids Co. Ltd., USA). The solution was dialyzed against PBS

for 48 h in a dialysis tube with a molecular mass cut-of 1000 kDa to remove the excess organic solvents and free AIPH molecules. The dialysis media were changed with fresh PBS at least twice a day. A UV-vis spectrophotometer was employed to quantify the AIPH concentration in the Lip-AIPH solution. The size of Lip-AIPH was determined using a Malvern Zetasizer nano ZS instrument. The morphology of the Lip-AIPH was characterized by transmission electron microscope (TEM, HT7700).

In Vitro **Release of Fluorescent Dye and Generation of Nitrogen Gas.** US-activated release profile of the encapsulated molecule from the Lip-AIPH was further tested. The fluorescein isothiocyanate (FITC) as a model drug was encapsulated in the liposome (Lip-AIPH). The solution of Lip-AIPH (1 mL 1 mg mL^{-1}) was firstly stored in a transparent plastic tube (diameter 5 mm, length 2.5 cm, sealed with a latex membrane). The whole tube was covered by the US probe, and then ultrasonic gel was then placed between the ultrasonic source and the plastic tube. US (1.0 MHz) with different parameters and durations were applied to trigger the release of AIPH. Finally, the solution was transferred to a 1.5 mL plasmonic tube and centrifuged. Then 200 μL of supernatant was taken out and the absorption intensity of FITC at 520 nm was tested to calculate the release behavior. In the following, US with different power density and irradiation times was applied to trigger the generation of gas microbubbles. Moreover, one piece of pork with 2 cm thick was placed between the US probe and the tube to simulate the tissue and block the US, and the generation of gas bubble was recorded after US irradiation (1.0 MHz, 2.5 W cm^{-2}).

***In vitro* Generation of Alkyl Free Radical.** The generation of alkyl radicals from Lip-AIPH was further examined. Upon US triggering, the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was used as an indicator to test the generation of free radicals. UV-vis spectra of the generated $ABTS^*$ from the mixed solution of ABTS and Lip-AIPH aqueous solution was measured. US (1.0 MHz) with different parameters (power intensity and irradiation duration) were further applied to trigger the generation of alkyl free radical. The absorbance intensities at 734 nm (between 500-800 nm) before and after US irradiation were recorded.

DCFH was also used as another ROS indicator to investigate the ROS generation. 5 mL methanol with DCFH-DA (1 mM) was mixed with 2 mL NaOH (0.01 M), followed by mixing them for 0.5 h. Next, 10 mL PBS (10×10^{-3} M, pH = 7.4) was added in the mixed solution. Then, 150 μL Lip-AIPH solutions (500 μg mL^{-1}) were mixed with 150 μL DCFH (40 μM). The mixture was irradiated with US (1.0 MHz) at different power intensity (0, 0.5, 1.0, 1.5, 2.0 and 2.5 W cm^{-2}). The fluorescence signal of the solution was recorded.

***In virto* US imaging.** A Visualsonics Vevo 2100 (Visual Sonics) with an MS-550D 40 MHz transducer at B-mode was used for the sonography. For *in vitro* imaging, 1 mL of PBS, blank liposome (1 mL, 1 mg mL^{-1}) and Lip-AIPH (1 mL, 1 mg mL^{-1}) was stored in the transparent plastic pipe, respectively (diameter: 5 mm, length: 2.5 cm, sealed with a latex membrane). Then, the pipe was fixed in degassed water for examining the

performance of PBS, blank liposome and Lip-AIPH as US contrast agents. The pipe was radiated by US (1.0 MHz, 2.5 W cm^{-2}) for 30 min, and the US images was recorded at different points of 1, 5, 15 and 30 min.

Cell Therapy Studies. MCF-7 cells were cultured in a humidified incubator in the setting of a partial pressure of 5% CO_2 at 37 °C in DMEM, which was supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). Then, the cells were seeded in a 96-well microplate and allowed to adhere overnight. The cell viability was measured using a standard CCK-8 viability assay according to the instructions by the manufacturer. Next, the cells were treated with varied concentrations of Lip-AIPH (0, 30, 60, 250, 500 μg mL^{-1}). The experimental group was irradiated by US (1.0 MHz, 2.5 W cm^{-2}, 1min) in the dark. After washing with PBS for three times, CCK-8 was added into each well.

For florescence imaging, MCF-7 cells were seeded in CLSM-specific dishes and cultured in DMEM medium containing 10% FBS for 24 h. After different treatments, the cells were stained with both calcein AM (calcein acetoxymethyl ester) and PI (propidium iodide) in CLSM-specific dishes for 15 min, and then living cells and dead cells were observed by a Confocal Laser Scanning Microscope. The cells were incubated in the culture medium containing $CoCl_2$ at the concentration of 100 μM as the hypoxic group.

Intracellular Generation of Free Radical. MCF-7 cells were cultured in a humidified incubator in the setting of a partial pressure of 5% CO_2 at 37 °C in DMEM, which was

supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). After MCF-7 cells were seeded in a CLSM-specific dish for 24 h. They were treated with Lip-AIPH, and then washed with PBS. Next, the cells were cultured with DCFH-DA (10 μM) at 37 °C for 60 min in the dark and irradiated with US (2.5 W cm^{-2}, 1.0 MHz). For the hypoxic group, cells were incubated in the culture medium containing $CoCl_2$ at the concentration of 100 μM. The Confocal Laser Scanning Microscope (Nikon C2, Japan) was used to acquire the fluorescence images.

The Cellular Uptake Studies of the Lip-AIPH. For observing the cellular uptake, MCF-7 cells were seeded into confocal dishes at a density of 5×10^4 cells per dish. After 24 h incubation, the medium was replaced by fresh medium containing FITC-labeled Lip-AIPH (with concentration at 500 μg mL^{-1}) and the cells were incubated for 2 h at 37 °C. Then, the cell was treated with or without US irradiation (1.0 MHz, 2.5 W cm^{-2}) for 20 s. The cells were washed with PBS and stained with Lysotracker Deep Red and DAPI for 30 min. The fluorescence imaging of the cells were observed using a fluorescence microscope.

The Cell Morphology Analysis. The MCF-7 cells were harvested at 37 °C in a 5% CO_2 humidified atmosphere for cellular morphology experiments. MCF-7 cells were seeded into confocal dishes and incubated for 24 h. Then the medium was replaced by fresh medium containing Lip-AIPH (with concentration at 500 μg mL^{-1}) and incubated

for 2 h at 37 °C. After the cells treated with US (1.0 MHz, 2.5 W cm^{-2}, 60s), the cell morphology was observed by confocal laser scanning microscopic.

Animal Models. All animal experiments were performed strictly under the guidelines of the "National animal management regulations of China" and approved by the Animal Ethics Committee of Fujian Medical University. BALB/c nude mice (weight ≈20 g) were obtained from Shanghai SLAC laboratory Animal Co., Ltd. The tumor-bearing mice were prepared by subcutaneously injecting of MCF-7 cells (1×10^7 cell, 100 μL) into the back of right hind leg. When tumor size reach ~90 mm^3, the mice were randomly divided into four groups (n = 5) as follows: (I) control group (200 μL, PBS only), (II) US only (200 μL, PBS), (III) Lip-AIPH only (200 μL, 500 μg mL^{-1}), (IV) Lip-AIPH (200 μL, 500 μg mL^{-1}) plus US irradiation (2.5 W cm^{-2}, 1.0 MHz, 10 min). At 24 h post-intravenous injection, the whole tumor region was irradiated with US (1.0 MHz, 2.5W cm^{-2}, 10 min). The body weight of the mice and the size of the tumor were recorded every other day The tumor volume was calculated based on the equation of $V = d^2 \times D/2$ (where d and D mean the minimum and maximum diameter of the tumor in millimeters, respectively). The total therapeutic observation lasted for 14 days, and the mice were euthanized when the tumor size reach ~1200 mm^3.

In vivo **Enhanced US Imaging.** The tumor bearing mice were anesthetized by inhalation of 2% isoflurane with 1% oxygen before US imaging. A Lip-AIPH solution (200 μL, 500 μg mL^{-1}) was administered by tail vein injection. After 2 h of intravenous

injection, the tumor was irradiated with US (1.0 MHz, 2.5W cm^{-2}) for 10 min. The tumors of mice were imaged at different time points with an US instrument Visualsonics Vevo 2100.

Histology Studies. Mice were sacrificed by cervical vertebra dislocation after imaging and therapy. Subsequently, the tumor and major organs in each treatment group were collected and stored in 10% formalin. The ultrathin sections were successively obtained for staining by hematoxylin and eosin (H&E). To detect DNA fragmentation during apoptosis in tumor tissue, the commercially available colorimetric terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) apoptosis assay kits were used.

Supporting Figures:

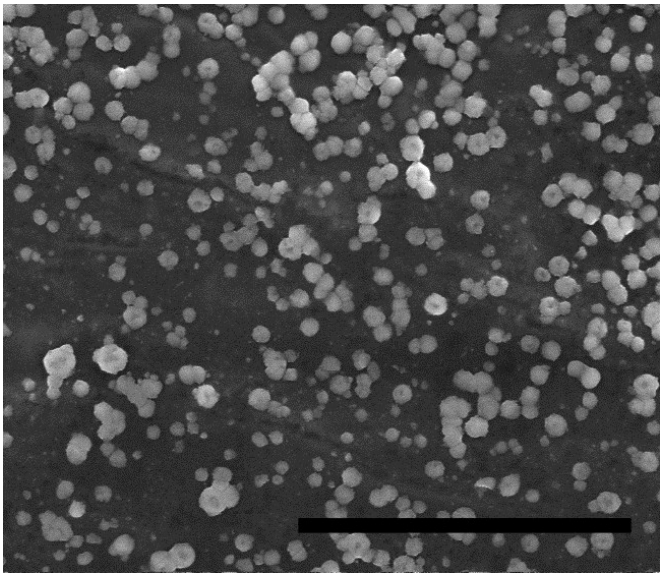

Figure S1. SEM image of the Lip-AIPH (scale bar: 3 μm).

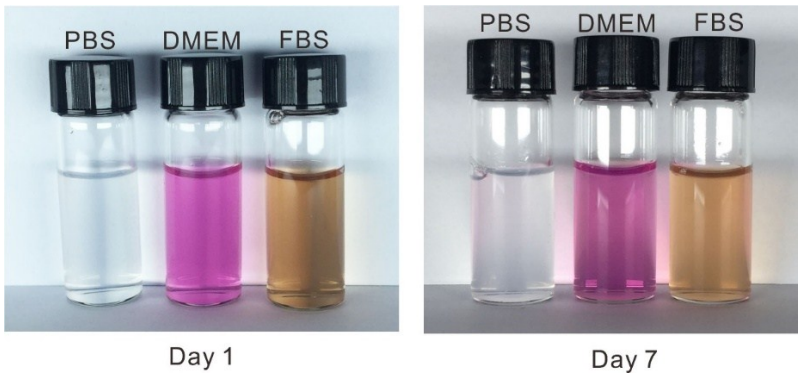

Figure S2. The stability of the Lip-AIPH liposomes in physiological mediums before and after 7 dyas incubation.

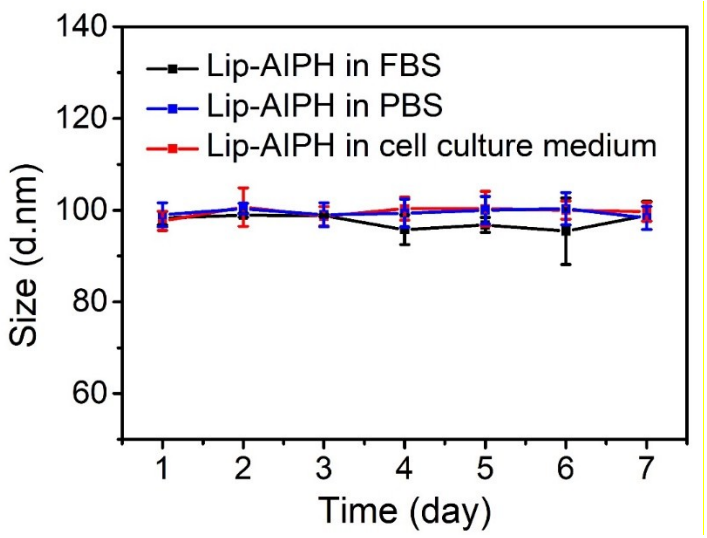

Figure S3. The size variation of Lip-AIPH as function of post-incubation time in PBS, FBS and cell culture medium. All data are means ± s.d.; n = 3.

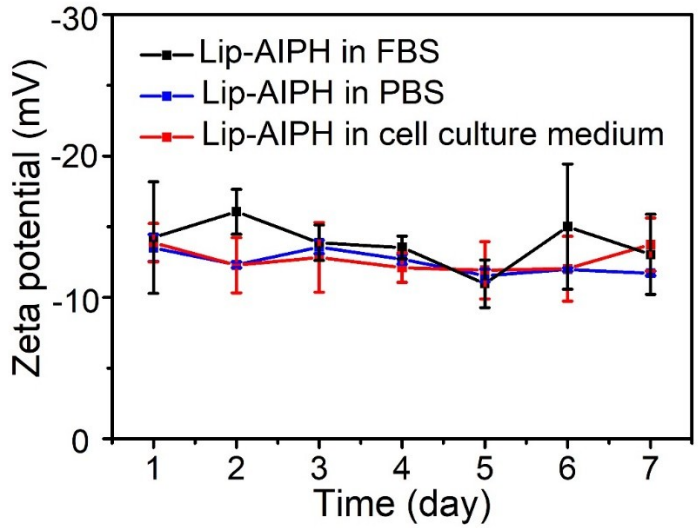

Figure S4. The zeta potential of Lip-AIPH in PBS, FBS and cell culture medium at different time points. All data are means ± s.d.; n = 3.

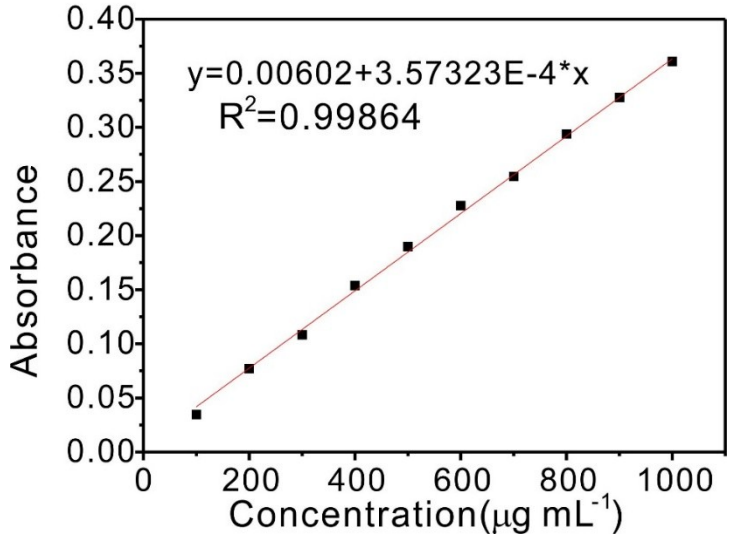

Figure S5. Standard curve of AIPH concentration (X axis) and UV-vis absorbance intensity (Y).

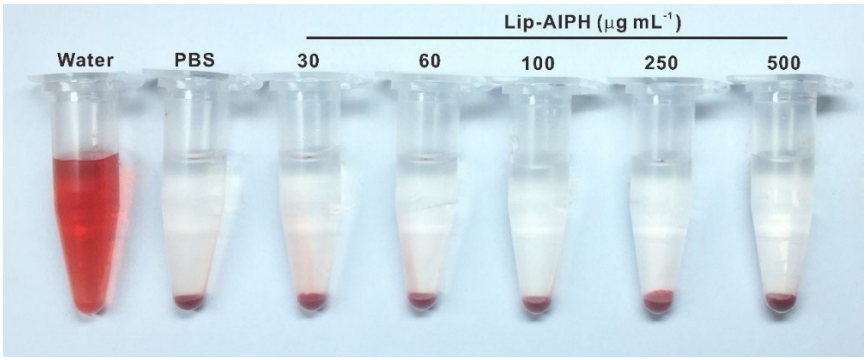

Figure S6. Photographs of red blood cells treated with water, PBS and Lip-AIPH at 37 ^{o}C for 3 h.

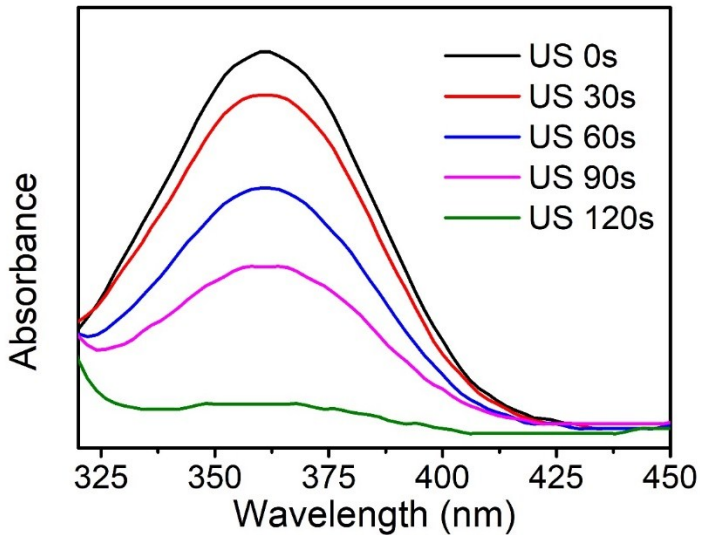

Figure S7. The absorption of AIPH (500 μg mL^{-1}, 200 μL) under different US irradiation time (0, 30 ,60, 90, 120 s).

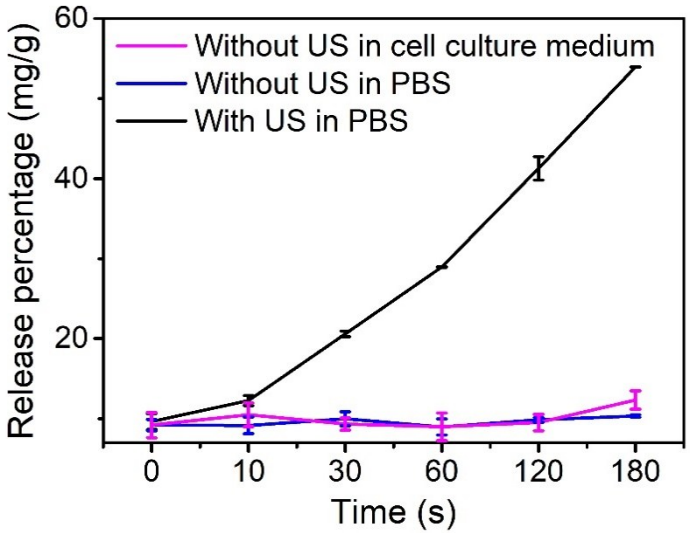

Figure S8. The leakage percentage of the cargos in the presence or absence of ultrasound radiation. All data are means ± s.d.; n = 3.

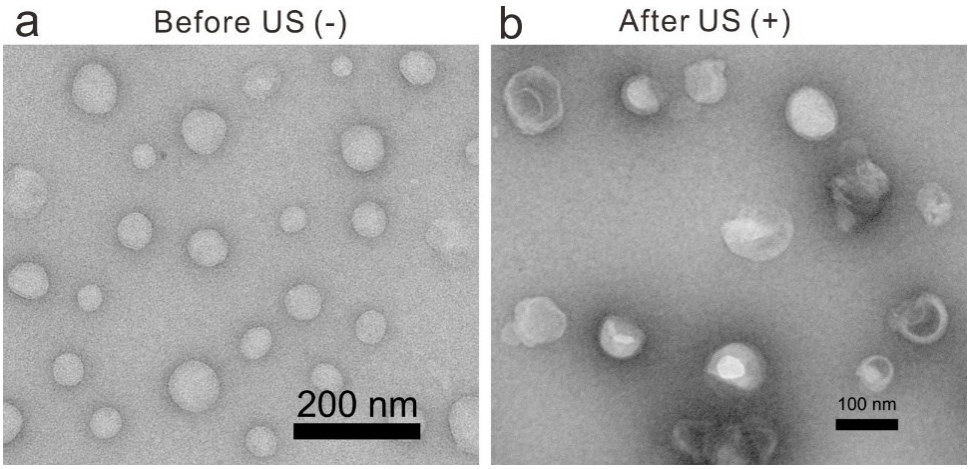

Figure S9. TEM images of Lip-AIPH before (a) and after (b) US irradiation (1.0 MH).

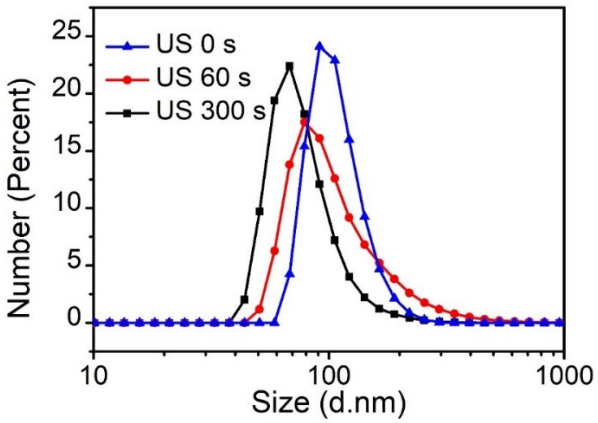

Figure S10. Size distribution of Lip-AIPH before and after US irradiation (1.0 MHz, 2.5 W cm^{-2}).

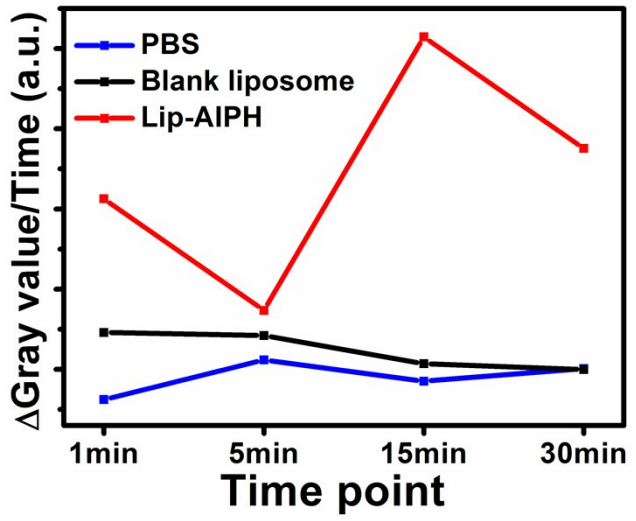

Figure S11. The growth rate of gray value in groups of PBS, blank liposome and Lip-AIPH at different time points (1, 5, 15, 30 min).

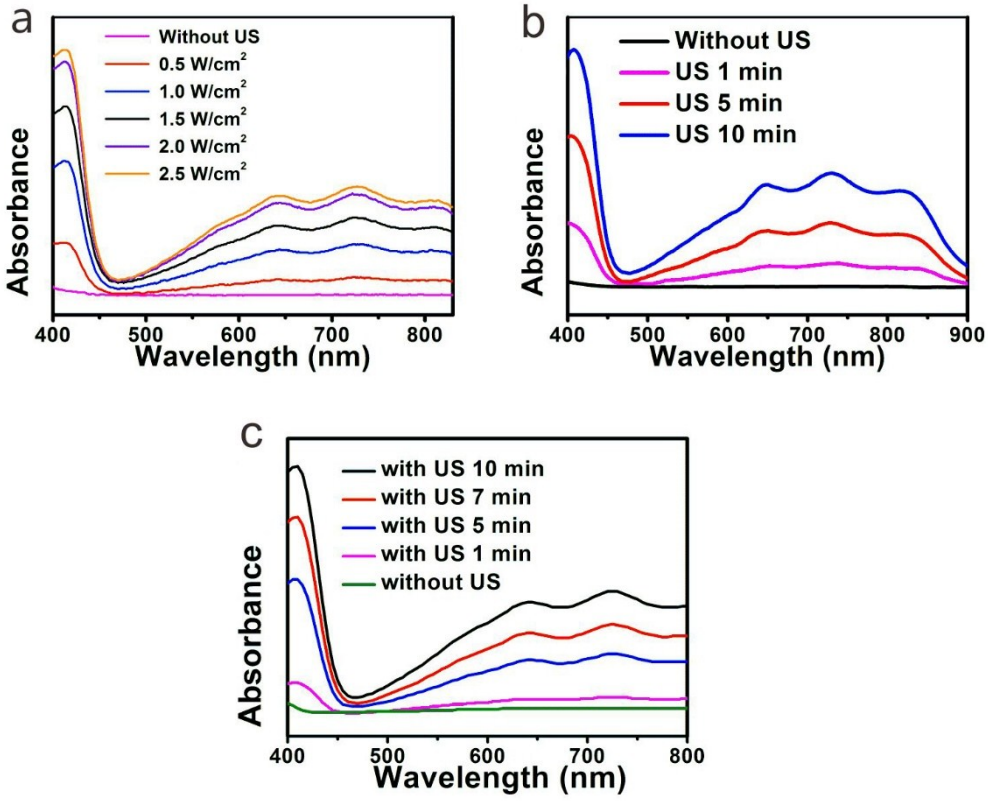

Figure S12. (a) Generation of ABTS* as induced by the free radicals released from AIPH under US irradiation with various power intensities (0, 0.5, 1.0, 1.5, 2.0, 2.5

W/cm^2, 1.0 MHz, 1 min) and (b) different irradiation time (0, 1, 5, 10 min, 2.5 W/cm^2, 1.0 MHz). (c) The generation of free radical from the tube containing Lip-AIPH under a piece of pork with 2 cm thick upon US irradiation (0, 1, 5, 7, 10 min, 2.5 W/cm^2, 1.0 MHz).

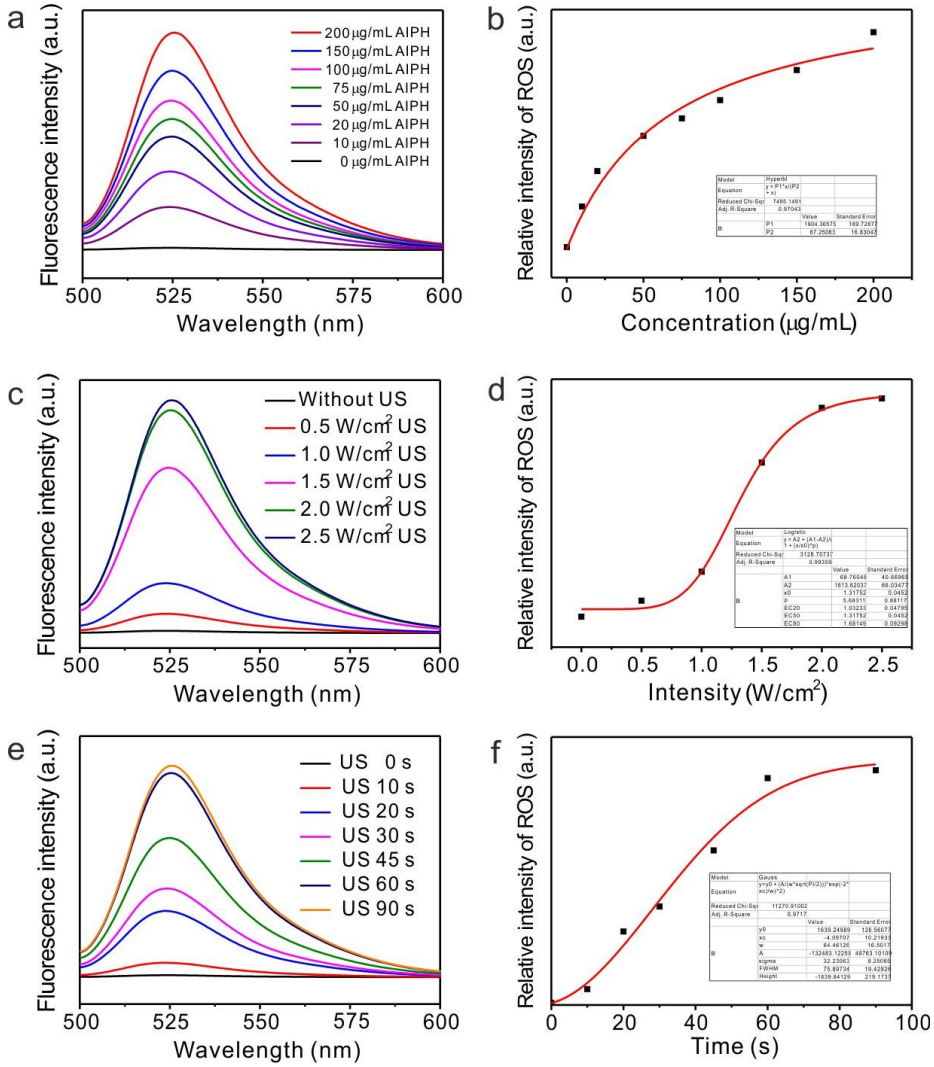

Figure S13. (a) The fluorescence spectra of DCFH in the solution of AIPH with different concentration under US irradiation (1.0 MHz, 2.5 W cm^{-2}, 1 min). (b) Concentration-dependent ROS production of AIPH at different concentration of 0, 10, 20, 50, 75, 100, 150, 200 μg/mL upon US irradiation. (c) The fluorescence spectra of

DCFH of the solution of AIPH under US irradiation with different power densities (0, 0.5, 1.0, 1.5, 2.0, 2.5 W cm^{-2}). (d) US intensity-dependent ROS production of AIPH upon exposure to US with variable power densities. (e) ROS generation of AIPH indicated by the fluorescence of DCFH after US irradiation for different times (0, 10, 20, 30, 45, 60, 90 s). (f) Time-dependent ROS production of AIPH upon exposure to US with variable duration by detecting DCFH fluorescence.

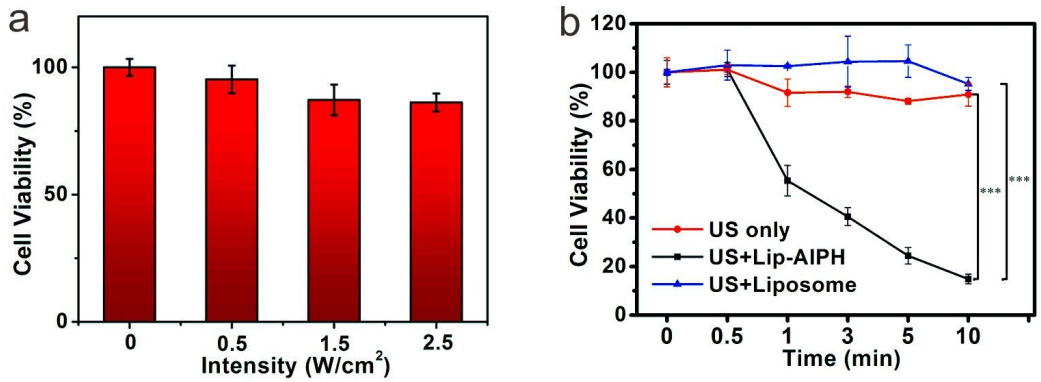

Figure S14. (a) The cell viability after treatment with US at different power intensity (0, 0.5, 1.5, 2.5 W/cm^2, 1.0 MHz, 1 min). (b) The cell viability after treatment with PBS, Lip-AIPH and blank liposome and US irradiation for 0.5, 1, 3, 5, 10 min. All data are means ± s.d.; n = 3.

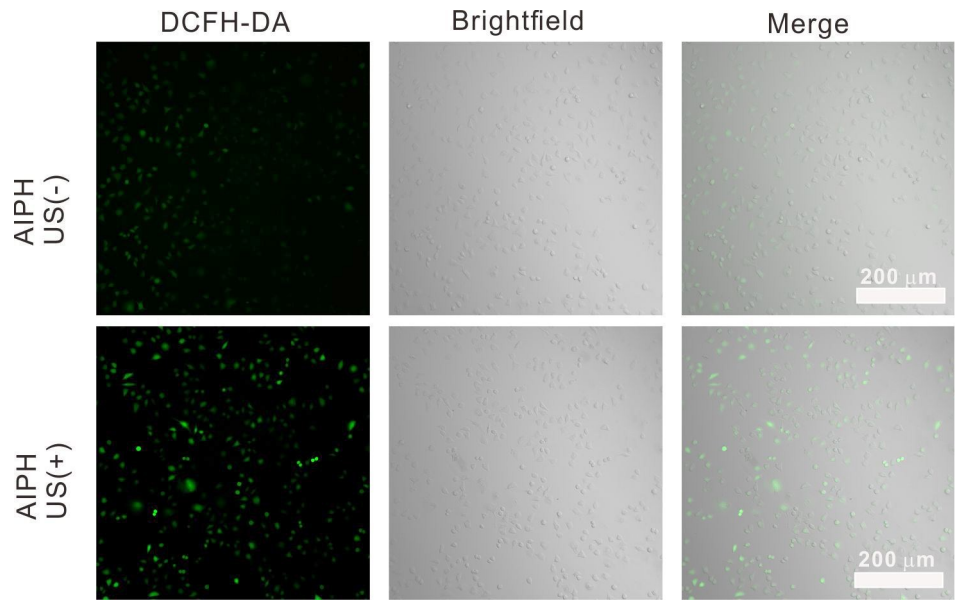

Figure S15. Detection of ROS in MCF-7 cells treated with AIPH with and without US irradiation. The MCF-7 cells were treated with AIPH (200 μg/ml) in culture media for 2 h and then irradiated by US (1.0 MHz, 2.5 W cm^{-2}, 10 min) or not (Scale bar: 200 μm).

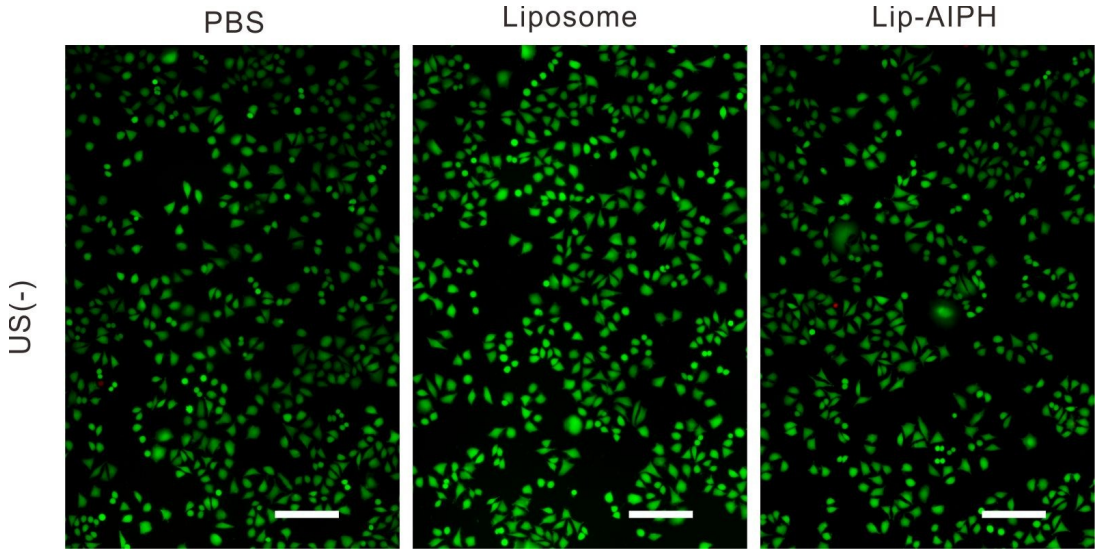

Figure S16. Confocal laser scanning microscopic images of MCF-7 cells costained with calcein AM (live cells, green fluorescence) and PI (dead cells, red fluorescence) after incubated with PBS, blank liposome and Lip-AIPH without US irradiation (Scale bar: 200 μm).

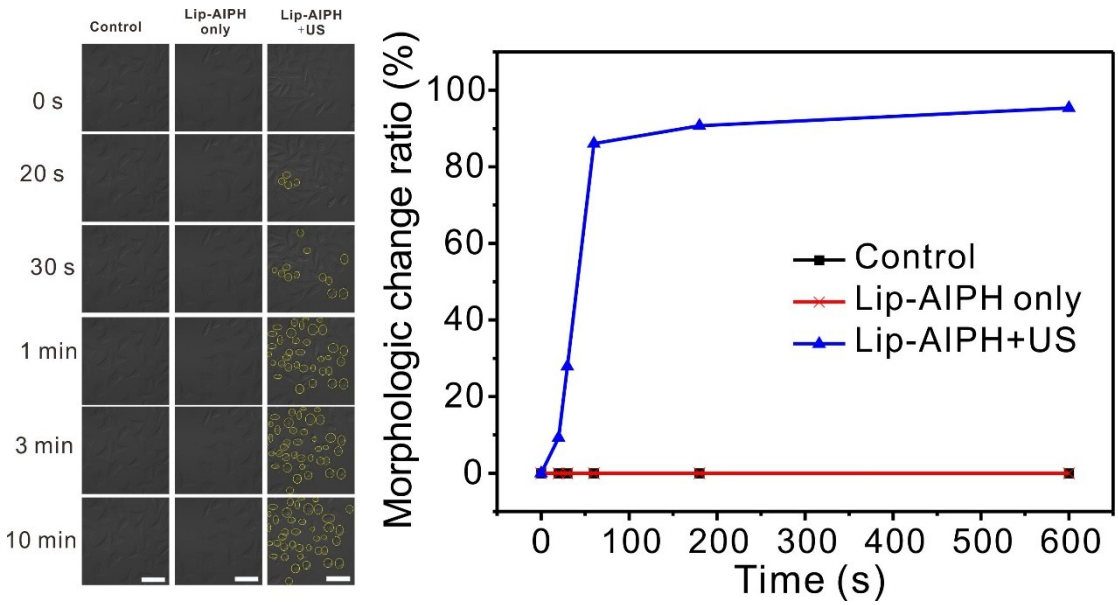

Figure S17. The cell morphologic changes after treatment with different samples and US irradiation (1.0 MHz, 2.5 W cm^{-2}) for different times (0, 20, 30, 60, 180, 600 s) (scale bar is 60 μm).

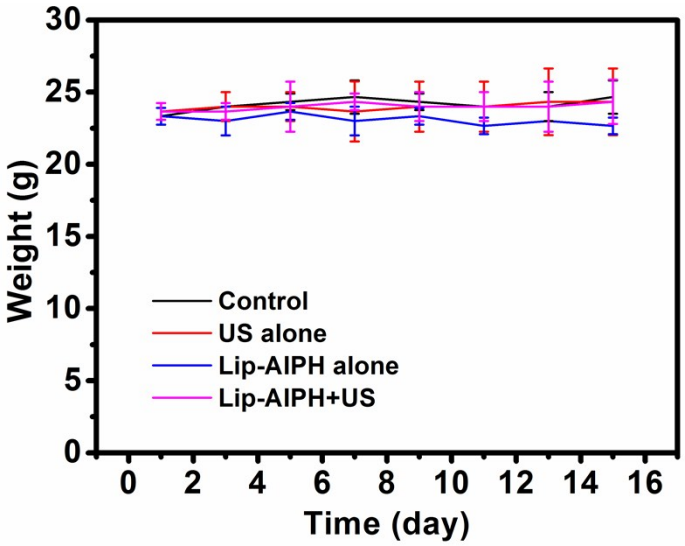

Figure S18. Time-dependent body-weight curves of MCF-7 tumor-bearing nude mice in different treatment groups. All data are means ± s.d.; n = 5.

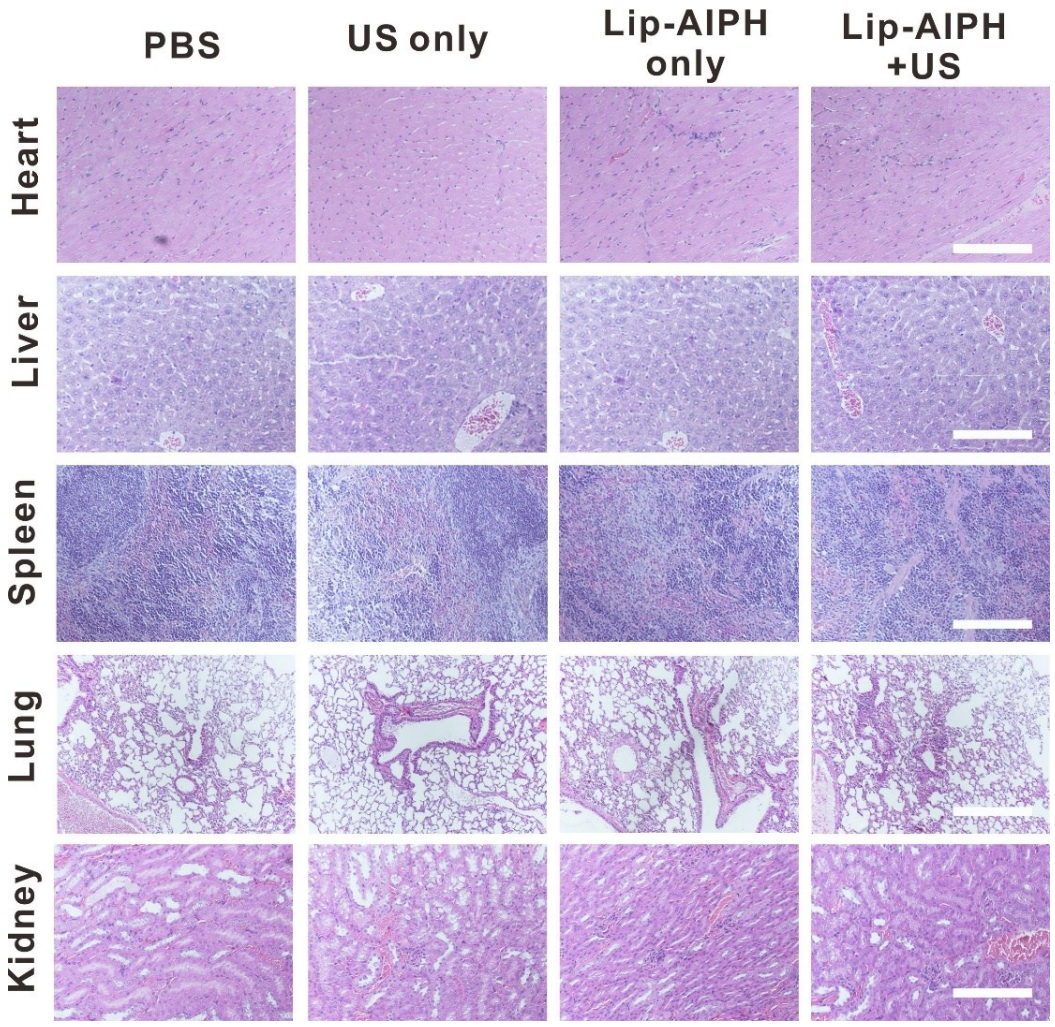

Figure S19. H&E-stained tissue sections of major organs (heart, liver, spleen, kidney and lung) of mice in different treatment groups (scale bar is 100 μm).