Supplementary Information for:

A Single-step Multi-level Supramolecular System for Cancer Sonotheranostics

Experimental Section

Materials: Indocyanine green (ICG) were purchased from J&K Scientific Ltd. (Beijing, China). DSPE-PEG (2000), lecithin, cholesterol and reactive oxygen species assay kit (DCFH-DA) were purchased from Sangon Biotech (Shanghai) Co. Ltd. 4-nitrophenyl chloroformate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), thiophene-2-thiol,4,6-Dia-midino-2-phenylindole (DAPI) were obtained from Sigma Aldrich Co. Ltd. (MO, USA). Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from HyClone Inc. All other chemicals were purchased from Sigma Aldrich in analytical grade and used without further purification.

Isothermal titration calorimetry (ITC): We used a MicroCal ITC 200 microcalorimeter (GE Healthcare) to detect the Fe^{3+} ions binding with ICG and Fe^{3+}/ICG respectively binding with lecithin and cholesterol at 20 °C. Buffer solution was prepared as adding 70.5 mL CH_3OH, 11.5 mL $CHCl_3$ and 1 mL n-hexane into 167 mL Hepes buffer (10 mM, pH = 6.8) and was standing about 24 h. Fe^{3+} analyte and the ligand solutions (ICG, lecithin and cholesterol) were prepared by adding them into above buffer solution. The final concentrations were respectively 0.05 mM and 1 mM. The ligands were injected 19 times and 2 μL per time for injection. Measurements were repeated twice, and the data was handled by OriginPro 8 software.

Synthesize of Fe^{3+}/ICG@MB and Characterization: Firstly, to prepare Fe^{3+}/ICG, 2.5 mg ICG was dissolved in 12.5 mL mixed solvents (CH_3OH / H_2O = 2/1), then 12.5 mL Fe^{3+} (64.5 μM, $FeCl_3$) was

added and stirring for 3 h. To form Liposome, 5 mg lecithin, 2.7 mg Cholesterol and 0.8 mg DSPE-PEG (2000) were respectively dissolved in chloroform. They were then combined in a flask with sonication for 5 min. Fe^{3+}/ICG mixture was added into the liposome and sonicated for 5 min, then let sit for 5 min. Solvent was removed by rotary evaporation in 60 °C water bath. The self-assembly lipid film was set to 65 °C water bath for 1 min before 250 μL perfluoropropane lipid (C_3F_8, PFP, Aladdin). Sonication may be necessary to the resuspending of the lipid film. Sonication tank with 33% of 20 kHz was used to disperse the lipid film. The flask was filled with the gas and stored at 4 °C.

Fe^{3+}/ICG@MB was then characterized by Zetasizer Nano-ZS instrument (Malvern Instruments, Malvern, UK), scanning electron microscopy- energy dispersive spectrum (SEM-EDS) (DXS-10ACRT), UV-Vis (THERMO), Spectral Domain Optical Coherence Tomography (SD-OCT), ultrasound imaging system (Vevo 2100 Imaging System), fluorecence imaging system (Carestream FX Pro) and samples which were storage at 4 °C were counted around 500,000 MBs mL^{-1}. Flow cytometry was also used to measure the size changes of MBs.

DCFH detection in tubes and in vitro: To activate DCFH in tubes, 10 μL of DCFH-DA (10 mM) was added into 400 μL NaOH solution (10 mM) and the mixture was standing for 30 min without light, then it was neutralized with 2mL PBS (10 mM, pH = 7.4). A mixed solution of activated DCFH with different concentration (i.e., 0, 2, 5, 10, 20, 50 μg mL^{-1}) of free ICG or Fe^{3+}/ICG or Fe^{3+}/ICG@MB at the volume ratio of 1:1 was sonicated with 1MHz , 1.61 W cm^{-2} ultrasound by a multi-function ultrasound device (Selfridge, Beijing). UV-Vis system was applied to detect the fluorescence production at an excitation wavelength of 488 nm, emission wavelength of 525 nm.

The intracellular ROS levels were also determined by using LM3 cells (a human hepatocellular carcinoma cell line) and Hepa1-6 (a mice hepatic cancer cell line) respectively. Typically, Hep1-6

cells in Dulbecco's modified Eagle's medium (DMEM) were seeded into 6-well culture plates at a density of 1×10^6 cells per well. After incubation for 12 h at 37 °C in a CO_2 incubator, the medium was replaced with fresh medium containing blank MB or free ICG or Fe^{3+}/ICG@MB (ICG, 20 μg mL^{-1}) respectively, and then incubated at 37 °C in a CO_2 incubator for 6 h. The culture medium was removed and the cells were rinsed three times with PBS buffer. The DCFH-DA (10 μM L^{-1}, dissolved in culture medium without FBS) was added and incubated at 37 °C in a CO_2 incubator for 20 min. The culture medium was removed and the cells were rinsed three times with culture medium without FBS. After treated with sono-activation at a frequency of 1 MHz, 30% pulse width and an average intensity of 1.61 W cm^{-2} for 5 min separately. Similarly, LM3 was treated in the same way. Inversed Fluorescent Microscope images was using to reveal ROS generation.

Cytotoxicity test, cell uptake and SDT efficacy in vitro: The cytotoxicity, cell uptake and SDT efficacy of Fe^{3+}/ICG@MB and other materials were evaluated in *vitro* using LM3 cells and Hepa1-6 respectively. Cytotoxicity test was studied using the methyl thiazolyl tetrazolium (MTT) assay. Typically, Hepa1-6 cells were seeded into 96-well culture plates at a density of 1×10^4 cells per well. After incubation for 12 h at 37 °C in a CO_2 incubator, the medium was replaced with fresh medium containing 20 μL dispersions (Fe^{3+}/ICG@MB) at final ICG concentrations (0, 2, 5, 10, 20, and 50 μg mL^{-1}), and then incubated for another 24h. Next, the medium was replaced with culture medium and 10 μL of MTT solution (5 mg mL^{-1}, dissolved in PBS) was added to each well. Moreover, the mixture was incubated for 4 h. The MTT solution was carefully removed and 150 μL dimethyl sulfoxide (DMSO) was added for 10 min in order to solubilize the violet formazan crystals. Thereafter, the absorbance of each well was measured using a Thermo Scientific Multiskan MK3

ELISA reader (Thermo Scientific) at 570 nm. Similarly, the cytotoxicity of those materials for LM3 was also evaluated in the same way.

The optimal concentration of MTT assay was selected in the cellular uptake test. Hepa1-6 cells and LM3 cells were seeded into confocal dish at a density of 1×10^5 cells per well respectively. After incubated for 12 h, the medium was replaced with fresh medium containing 20 μL Fe^{3+}/ICG@MB at final ICG concentration of 10 μg mL^{-1}. After 6h incubation, the culture medium was removed, and the cells were rinsed three times with PBS buffer and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Then removed the paraformaldehyde solution and rinsed three times with PBS buffer. Next, the Diphenyl phenylindole (DAPI) was diluted to 300 nM with PBS and was incubated with cells for 15 min at room temperature. Finally, rinsed the confocal dish several times to remove all free DAPI. The cellular uptake was observed by confocal microscope (Olympus, Japan).

Next, various concentrations of blank MB, free ICG, and Fe^{3+}/ICG@MB as sonosensitizer agents were added into the Hepa1-6 cells and LM3 cells for in *vitro* cancer cell killing. Hepa1-6 cells and LM3 cells were seeded into 96-well culture plates at a density of 1×10^4 cells per well. After incubated for 12 h, the medium was replaced with fresh medium containing 20 μL dispersions (blank MB, free ICG, and Fe^{3+}/ICG@MB) at final ICG concentrations (0, 5, 10 μg mL^{-1}). After incubation for 6h, all of the materials entered the cytoplasm well, cells of every well were treated with the focused ultrasound with a frequency of 1MHz, 30% pulse width and an average intensity of 1.61 W cm^{-2} for 5 min separately. Moreover, the cells were incubated for another 18 h. The MTT assay was used to assess cell killing efficiency as the same method as above.

Establishment of orthotopically implanted HCC model: Animal experiments were ap-proved by Animal Care and Use Committee (CC/ACUCC) of Xiamen University. Male BALB/C nude mice obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd, weigh approximate 25 g. The orthotopic transplant HCC model were established as described previously literature.[1] Briefly, a laparotomy performed in anesthetized nude mouse. 25 μL LM-3/Luc cells (1×10^7 cells mL^{-1}) injected into the right liver lobe of the nude mice. 7 days after the operation, bioluminescence imaging by IVIS Lumina II was used for screening out tumor-bearing mice to image after intraperitoneal injection of fluorescein substrate. At last, the tumor formation rate was 70%.

To simulate the human immune system, male C57BL/6 mice obtained from Xiamen University Laboratory Animal Center, mice weighing approximately 25 g, were used for assessing treatment effect. Tumor-bearing C57BL/6 mice obtained by the same method as above. Briefly, mice anesthetized, then 25 μL Hepa1-6/Luc cells (1×10^7/mouse) injected into their right liver lobe by a laparotomy. 15 days after the operation, bioluminescence imaging performed with IVIS Lumina II after intraperitoneal injection of fluorescein substrate to screen out tumor-bearing mice for treatment. The tumor formation rate was 70%.

In vivo ultrasound imaging and fluorecence imaging: Tumor-bearing nude mice were randomly divided into two groups (n = 5/group) and 100 μL Fe^{3+}/ICG@MB (ICG = 2.5 mg kg^{-1}) was intravenously injected. One group of mice used for imaging directly after administration. While the other group mice were administered ultrasound immediately to burst the microbubbles in order to achieve accumulation in tumors and this process was also recorded by ultrasound imaging system (Vevo 2100 Imaging System). In this group, cold degassed water (4 °C) used as the ultrasound couple medium, thus reducing hyperthermia during bursting. The frequency of the ultrasound was 1

MHz. Mice exposed to ultrasound for 2.86 W cm^{-2}, 5 min. The fluorescent signals were recorded by Carestream FX Pro at 0, 1, 2, 4, 8, 12 and 24 h and maximum excitation wavelength of 730 nm. Then all the mice euthanized. We collected tumors and major organs including heart, liver, spleen, lung, kidney and brain to visualize with Carestream FX Pro. Meanwhile, fluorecence imaging and tissue distribution of tumor-bearing mice was implemented as the same way.

In vivo sonodynamic: For reducing hyperthermia during treatment, cold degassed water (4 °C) used as the ultrasound couple medium. The temperature of ultrasonic probe showed no significant variation (< 3 °C) after treatment. In detail, the tumor-bearing C57BL/6 mice were randomly divided into four groups (n = 6/group): PBS group, blank MB group, ICG@MB group, Fe^{3+}/ICG@MB group. The mice of control group intravenously injected with 100 μL PBS. The mice of blank MB group were injected with blank MB (lecithin, 5 mg kg^{-1}) via the caudal vein, and the remaining two groups of mice were injected with ICG@MB (ICG, 2.5 mg kg^{-1}) and Fe^{3+}/ICG@MB (ICG, 2.5 mg kg^{-1}; lecithin, 5 mg kg^{-1}) via the caudal vein. The four groups suffered ultrasound 5 min immediately after administration to blast microbubble as the same conditions as fluorescence imaging. And all of the four groups were executed further ultrasound treatment 5 min at 6 h post-injection as the same conditions as blasting. Throughout the trial period, bioluminescence imaging was performed using IVIS Lumina II after intraperitoneal injection of fluorescein substrate, both the tumor total ROI and the mice weights were recorded every 5 days. Moreover, liver function in mice were compared before and after treatment.

Statistical analysis: All data are reported as the means ± the standard deviation (SD). Comparison of means was performed with Student's t test.

Supporting Figs

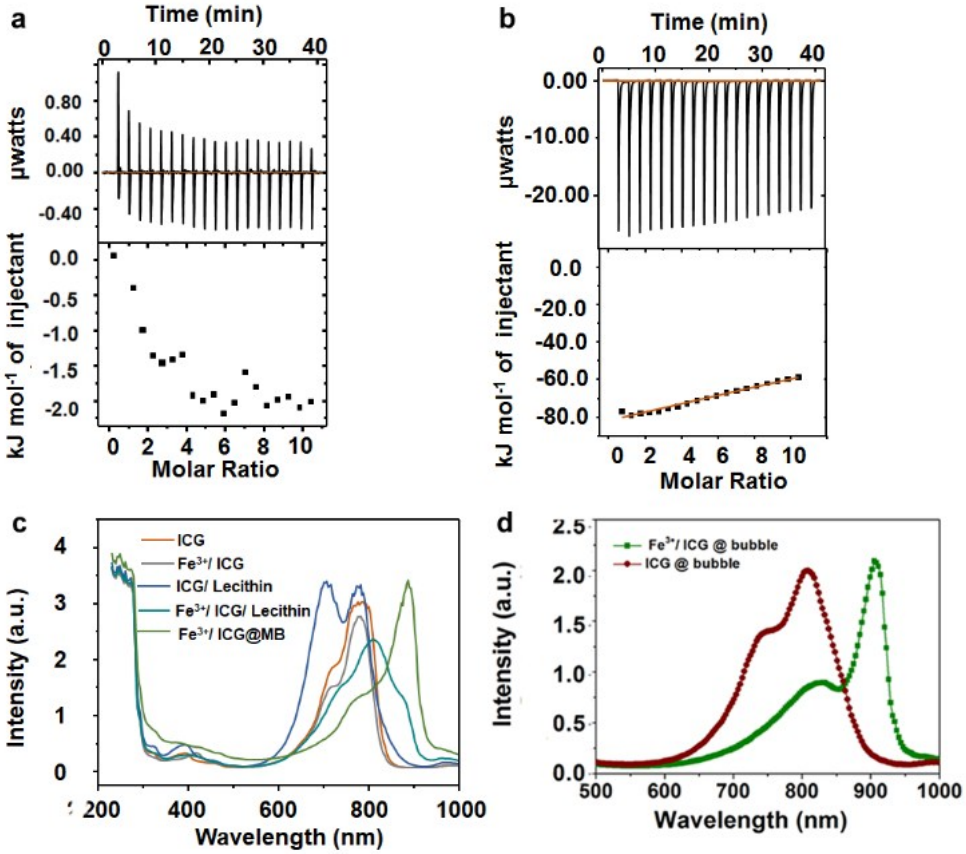


Fig S1. Isothermal titration calorimetry (ITC) data of ICG (1 mM) and Fe^{3+} (0.05 mM) following lecithin (1 mM) adding **a)** and cholesterol **b)** adding. **c)** UV-Vis specturm of ICG, Fe^{3+}/ICG, Fe^{3+}/ICG@lecithin, and Fe^{3+}/ICG@MB. **d)** The UV-Vis specturm at the same concentration of ICG between ICG@MB and Fe^{3+}/ICG@MB.

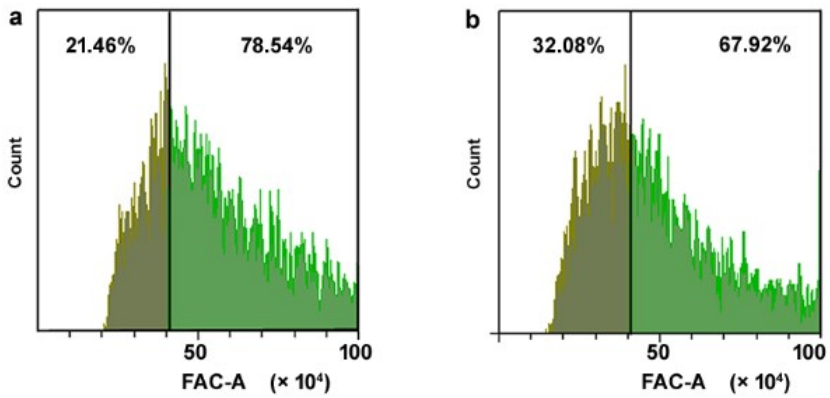
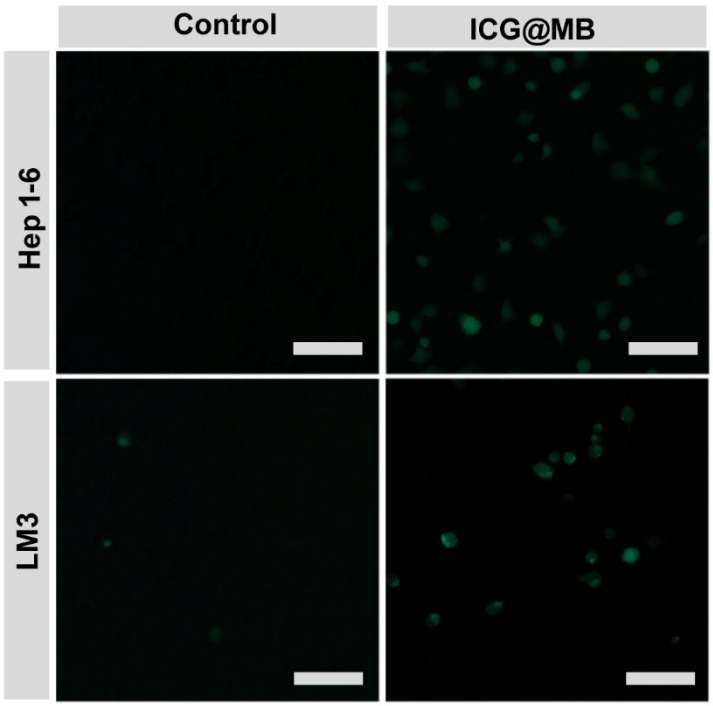


Fig S2. Flow cytometry data revealed the size distribution of Fe^{3+}/ICG@MB before **a)** and after **b)** sonication (1 MHz, 2.86 W cm^{-2}).



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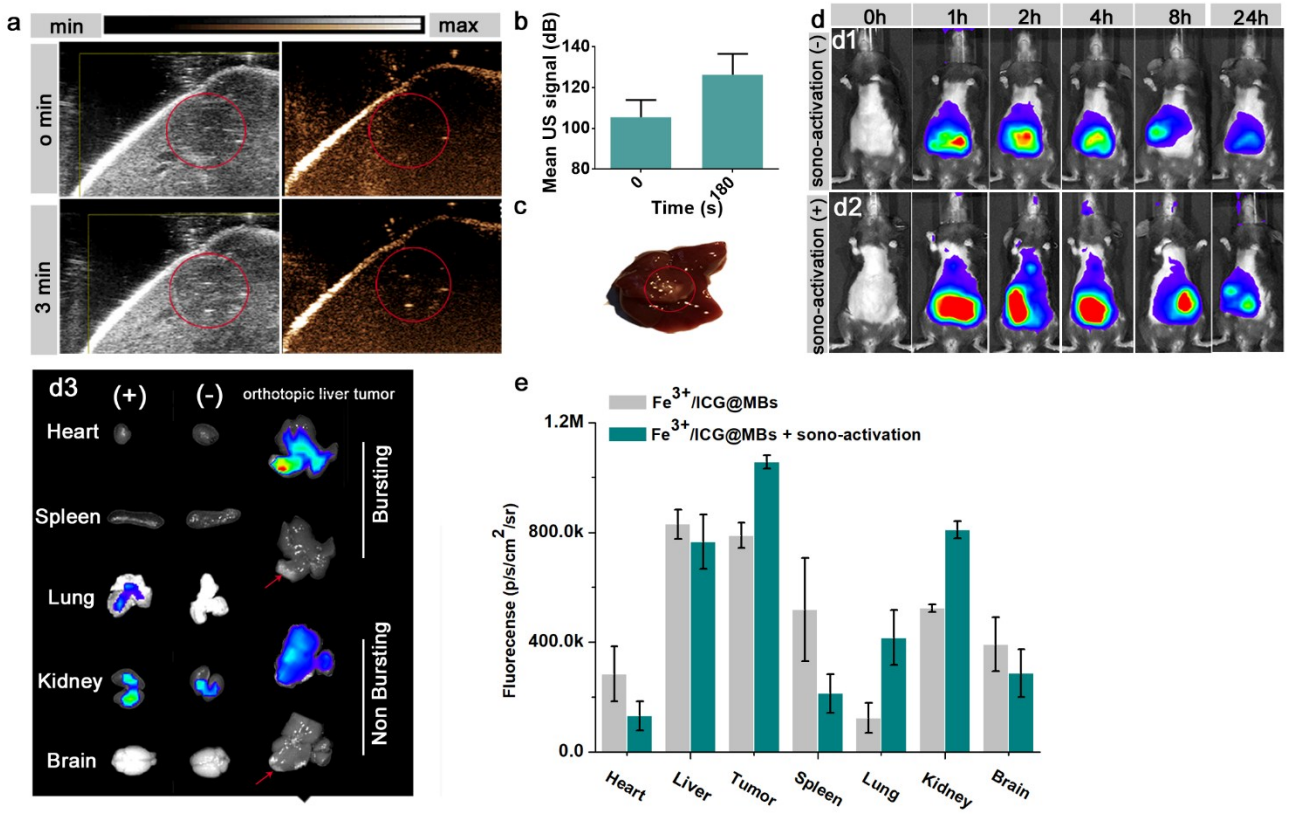
145 **Fig S3.** Inversed fluorescent microscope images of Hep1-6 and LM3 cells stained with DCFH-DA
146 after treated with PBS (Control) and ICG@MB (ICG 50 μg mL^{-1}) upon sono-activation. (Scale bar =
147 100 μm)



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149 **Fig S4. a)** Detection of HCC *in situ* by sonography using 13–24 MHz transducer in B-mode and

contrast mode. Sonication subjected at 1 min for 10s and tumor contrast was observably enhanced at 3rd min . **b**) The quantitative US signal measurements of Fe^{3+}/ICG@MB at different times (180 s). **c**) A photograph showing the liver with tumor. **d**) Time-dependent whole body NIR fluorescence images of orthotopic Hep1-6 tumor-bearing mice treated with Fe^{3+}/ICG@MB (ICG = 2.5 mg kg^{-1}) with **d1**) and without **d2**) sono-activation. Images were acquired at 0, 1, 2, 4, 8, 24 h post-i.v. injection. **d3**) The corresponding *ex-vivo* fluorescence images of resected organs and Hep1-6 tumor from the mice administrated with Fe^{3+}/ICG@MB at 24 h post-injection. **e**) A fluorescence intensity analysis of major organs and tumors at 24 h post-injection of Fe^{3+}/ICG@MB in orthotopic HCC-bearing mice.

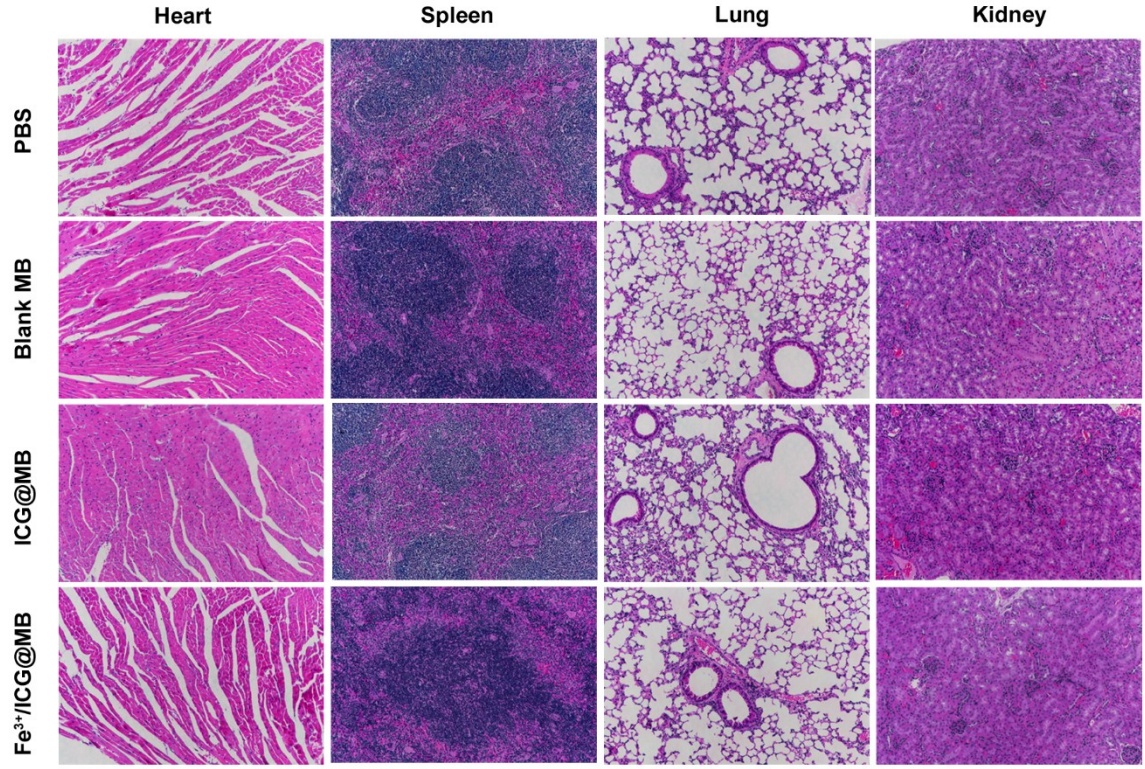

Fig S5. Hematoxylin and eosin (H&E) staining of organs from mice bearing HCC after Fe^{3+}/ICG@MB-based SDT.

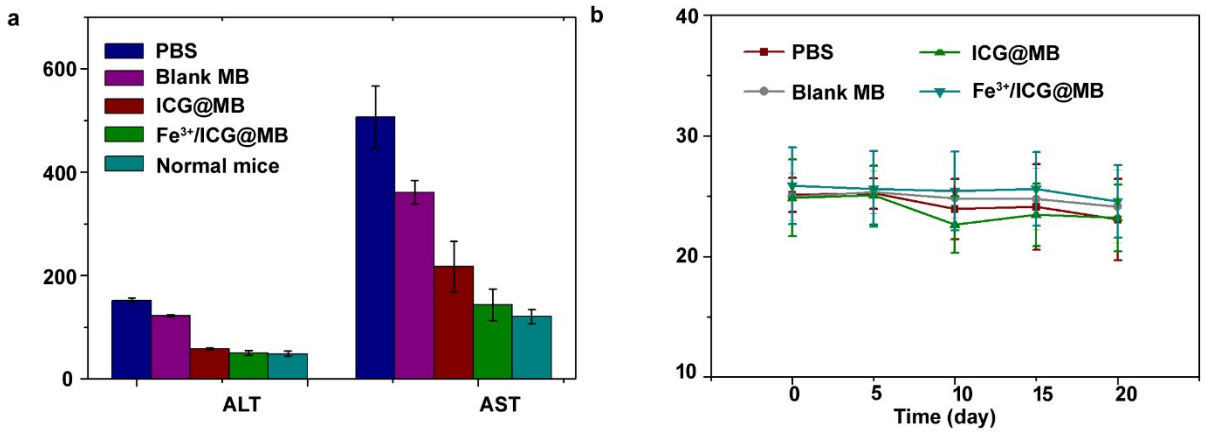

Fig S6. a) Serum chemistry of mice after 20 therapy days. b). The body weight curve for different

treatment group.

1 K. C. Kwon, E. Jo, Y. W. Kwon, B. Lee, J. H. Ryu, E. J. Lee, K. Kim, J. Lee, *Adv. Mater.* **2017**, 29, 1701146.