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Electronic Supplementary Information

Bioadhesive Metal-Phenolic Nanoparticles for Enhanced NIR Imaging-Guided Locoregional Photothermal /Antiangiogenic Therapy

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1. Experimental section

1.1 Materials

Sorafenib (SRF) and 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl tetrazolium bromide (MTT) were provided by Energy Chemical (China). Dulbecco's modified eagle's medium (DMEM), penicillin−streptomycin, and fetal bovine serum (FBS) were obtained from M&C gene technology Co., Ltd. (Beijing, China). Tannic acid, fluoresceinc diacetate (FDA), propidium iodide (PI), and Coumarin 6 (C6) were purchased from Aladdin reagent (Shanghai, China). IR820 was obtained from Sigma (USA). Iron-(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and Tween 80 were provided by Sinopharm Group Chemical Reagent Co., Ltd. (Wuhan, China). Lysotracker Red DND-99 was purchased from YEASEN Biotechnology Co., Ltd. (Shanghai, China).

1.2 Experimental methods

Preparation of TA-Fe nanoparticles. To prepare TA-Fe complex nanoparticles (NPs) loaded with SRF (SRF@TA-Fe), SRF solution (100 mg mL^{-1}, 100 μL) was mixed with tannic acid solution (40 mg mL^{-1}, 0.8 mL) and PEG5000 ethanol solution (4 mg mL^{-1}, 3.2 mL) under constant vorticity, then the mixture was added into water (50 mL) with stirring and $FeCl_3 \cdot 6H_2O$ aqueous solution (10 mg mL^{-1}, 390 μL) was immediately added. Notably, as soon as the $FeCl_3 \cdot 6H_2O$ was added, the solution color changed from colorless to blue, showing the formation of the polyphenol-iron network. Finally, the mixture was centrifugated twice at 8000 rpm for 15 min to remove excess drugs and organic phases.

To fabricate TA-Fe complex NPs loaded with SRF and IR820 (SIF@TA-Fe NPs), IR820 solution was added into SRF@TA-Fe solution in the dark, setting the mass ratio of SRF/IR820 as 5:1. After absorption overnight, the mixture was centrifugated at 8000 rpm for 15 min and the

precipitate was washed by deionized water for twice as well. The preparation of Blank@TA-Fe NPs and IR820@TA-Fe NPs were similar, except for relevant drugs. As for C6@TA-Fe, SRF was replaced by C6, which acts as the fluorescent tag.

Characterization of SIF@TA-Fe. The hydrodynamic size (diameter, nm), and size distribution (polydispersion index, PDI) of various NPs were measured by the dynamic light scattering (DLS, Malvern Zetasizer Nano ZS90, USA). The morphology of NPs was captured by transmission electron microscope (TEM, Hitachi HT7700, 100 kV, Japan). The absorbance of NPs loaded with IR820 was recorded using a UV-Vis spectrophotometer (TU-1810DSPC, Puxi General Instrumental Company, China). The loading efficiency of NPs was determined by high speed centrifugation. The concentration of IR820 was calculated based on the characteristic absorption peak at 835 nm. The concentration of SRF was measured by ultra performance liquid chromatography (UPLC) system. The loading efficiency (LE) and encapsulation efficiency (EE) were calculated according to the following equation:

$$LE\ (\%) = (W_1/W_0) \times 100\%$$

$$EE\ (\%) = (W_1/W_2) \times 100\%$$

Where W_1 was the amount of drugs (SRF or IR820) encapsulated in NPs, and W_0 was the total amount of NPs, respectively. W_2 was the initial amount of drugs added in the preparation.

***In vitro* release of SRF from SIF@TA-Fe.** The released performance of SRF from SIF@TA-Fe was determined by the dynamic dialysis method. Briefly, SIF@TA-Fe solution (1 mL) was transferred into a dialysis bag (MWCO 7000 Da). The release experiments were started by immersing the dialysis bag into 4 mL of PBS buffer (0.01 M, pH 7.4, 6.5 or 4.5, containing 0.1% Tween-80) with continuous shaking at 100 rpm and 37 °C. At each predetermined time interval, 4 mL of dialysate solution was withdrawn and replaced with equal volume of fresh release medium.

The amount of released SRF was detected by UPLC system (Agilent 1290 infinity, USA). All sorafenib samples were analyzed following this UPLC method: chromatographic separation was achieved on a reversed phase C18 column (250 mm × 4.6 mm, 5 μm) maintained at 40 °C with a mobile phase consisted of acetonitrile and water (85:15, v/v) at a flow rate of 0.5 mL min^{-1}. The absorbance of the eluent was monitored at 265 nm. Injection volume was 20 μL. Liner rang of SRF was from 0.5 μg mL^{-1} to 20 μg mL^{-1}. Retention time of SRF was 6.769 min.

Photothermal imaging. For exploiting the photothermal conversion performance of SIF@TA-Fe, different concentrations of SIF@TA-Fe (IR820, 0, 1.25, 2.5, 5, 10, 20, 40 μg mL^{-1}) were irradiated with an 808 nm-wavelength NIR laser at a power density of 0.5 W cm^{-2} for 5 min. SIF@TA-Fe (SRF 200 μg mL^{-1}, IR820, 40 μg mL^{-1}) solution was irradiated with an 808 nm-wavelength NIR laser at various power density (0.3, 0.5, 1.0, 1.5 W cm^{-2}) for 5 min. The temperature change of SIF@TA-Fe solution was recorded by an infrared thermal camera. To investigate the photothermal stability, SIF@TA-Fe (SRF 200 μg mL^{-1}, IR820 40 μg mL^{-1}) and SIF micelles (SRF 200 μg mL^{-1}, IR820 40 μg mL^{-1}) solution were irradiated under 808 nm laser (0.5 W cm^{-2}) for 5 min. Then the laser was shut down for cooling naturally to ambient temperature. The process of heat up (laser ON) and cool down (laser OFF) was repeated for five times cycle.

Surface adhesion of SIF@TA-Fe. To evaluate the excellent adhesion properties of SIF@TA-Fe, we determined the content of C6@TA-Fe in the cell surface through green flurescence of C6. For comparation, C6 micelles were acted as a control. B16F10 cells were seeded in 6-well plate (4 × 10^5 cells per well) and cultured for 24 h. C6@TA-Fe (C6, 1 μg mL^{-1}) or C6 micelles (C6, 1 μg mL^{-1}) were added into each well and incubated with cells for 1 min. Then the cells were washed by PBS for three times and captured by a luminescence microscope (MF52, Mshot, China). The

flurorecence intensity of C6 in cell was measured by a flow cytometry (CytoFLEX S, Becman, USA).

Scratch test. Scratch healing assay was carried out to elucidate the inhibiting angiogenesis effects of SIF@TA-Fe. Human umbilical vein endothelial cells (HUVECs) were precultured in 24-well plate at 8×10^4 per well. Then a scratch should be made in the cells slowly along the axis with the tip of a new 10-liter spear head. And the scratch should be as straight as possible. After the scratch was completed, the cells were gently washed by DMEM free of FBS for twice. SIF@TA-Fe (SRF 5 μg mL^{-1}, IR820 1 μg mL^{-1}), Blank@TA-Fe, and SIF micelles (SRF 5 μg mL^{-1}, IR820 1 μg mL^{-1}) were added into each well, respectively. At each predetermined incubation time interval (0, 24, or 48 h), the scratch healing of each group was imaged by a microscope.

Celluar uptake and location. We used C6@TA-Fe to evluate the cellular uptake of SIF@TA-Fe, because the NIR fluorescence of IR820 is hard to capture using a confocal laser scanning microscope (CLSM). B16F10 cells (5×10^4 per well) were seeded into confocal dish and cultured overnight. The old medium was replaced by C6@TA-Fe solution (C6, 1 μg mL^{-1}). The cells were washed by PBS for three times after incubation with C6@TA-Fe for different time. Subsequently, cells were incubated with Lyso-Tracker Red DND-99 (50 nM) for 20 or 30 min and observed by CLSM (FLUOVIEW FV3000, Olympus, Japan). Excitation at 488 nm and signal collection at 500-580 nm for C6; excitation at 594 nm and signal collection at 610-710 nm for Lyso-Tracker Red DND-99.

***In vitro* combined treatment.** The cytotoxicity of TA-Fe complex NPs on B16F10 cells were assessed through cell viability using MTT assays. Cells were seeded in 96-well plate (5000 cells per well) and cultured for 24 h. After that, the cells were incubated with different formulations in the dark for 2 h. The concentrations of SRF and IR820 were 20 μg mL^{-1} and 4 μg mL^{-1},

respectively. For comparsion, cells cultured in standard medium was acted as control. The cells were exposed to irradiation (808 nm, 0.3 W cm^{-2}) for 3 min or 5 min. After incubation for another 6 h, cells were washed by PBS for twice. After that, 100 μL of freshly prepared MTT (0.5 mg mL^{-1}) solution was added into each well. The MTT solution was carefully removed after 3 h of incubation, DMSO (120 μL) was added into per well and the plate was gently shaken for 10-20 min at 37 °C to dissolve all the formazan formed. The absorbance of MTT at 570 nm was determined by the microplate reader (Varioskan LUX, Thermo Scientific, USA). The cell viability was expressed by the ratio of the absorbance of the cells incubated with fromulations to that of the cells incubated with standard medium. Each experiment was repeated at least three times.

LIVE/DEAD cell staining. To further demonstrate the combined therapy efficacy of SIF@TA-Fe, B16F10 cells were stained with the FDA/PI mixed solution to identify dead (red fluorescence) and live (green fluorescence) cells. B16F10 cells were incubated with SIF@TA-Fe (SRF 40 μg mL^{-1}, IR820 8 μg mL^{-1}), or IR820@TA-Fe (IR820, 8 μg mL^{-1}) for 2 h, and then exposed to laser irradiation (808 nm, 0.3 W cm^{-2}, 3 min). Cells treated with SRF@TA-Fe (SRF 40 μg mL^{-1}) without irradiation, DMEM and DMEM with irradiation were set as control. The old medium was replaced by FDA/PI mixed solution (FDA 1.0 μg mL^{-1}, PI 10 μg mL^{-1}) and the mixed solution incubated with cells for 30 min. Subsequently, the cells were washed gentlely by PBS, and fixed by 4% paraformaldehyde for 15 min. Finally, B16F10 cells were imaged by luminescence microscope post being washed by cold PBS. The dead rate of B16F10 cells treated with different formulations was counted, which is based on the proportion of cells with red fluorescent signals, using the Image J software. Moreover, more than five fluorescent images of different field of vision in each group were counted.

Hemolysis assays. Blood samples were collected from healthy female C57 mice in heparinized test tubes. The blood was centrifuged at 500 g for 10 min to collect erythrocytes. The red blood cells (RBCs) were suspended in saline after being washed by saline for three times. 100 μL of RBCs solution was added into 900 μL of SIF@TA-Fe (the final concentration of NPs was from 1 to 10000 μg mL^{-1}). The final concentration of RBCs suspensions was 4%. Triton X-100 (0.1 % v/v) and saline were used as a positive and negative control, respectively. All mixtures were incubated in a shaking incubator (37 °C) for 2 h at the speed of 100 rpm. The mixtures were centrifuged at 500 g for 10 min to remove unbroken red blood cells and 100 μL of supernatant was transferred to a 96-well plate. The absorbance value of the supernatant was measured at 570 nm. The hemolysis rate was calculated using the following formula: Hemolysis (%) = (sample absorbance - negative control absorbance)/(positive control absorbance - negative control absorbance) × 100%.

Animals and tumor xenograft models. All animal experiments were performed in compliance with and approved by the Institutional Animal Care and Ethic Committee of Huazhong University of Science and Technology. Female C57 mice (6-weeks-old) were provided by Laboratory Animal Centre of Hubei, China. To establish tumor-bearing mouse model, 100 μL of B16F10 tumor cells (5.0×10^5) suspension was subcutaneously injected into the right rear flank of each mouse. When the tumor size reached approximately 100 mm^3, the mice were used for fluorescence imaging and antitumor therapy.

***In vivo* fluorescence imaging.** B16F10 melanoma-bearing mice were anesthetized and placed on an animal plate heated to 37 °C after intratumoral injection with different formulation solutions. The mice were imaged by a Maestro EX in vivo fluorescence imaging system (Caliper IVIS Lumina II). The excitation light was 650 nm and the light wavelength ranged from 680 to 810 nm

was collected with an exposure time of 2 ms for each image frame. The imaging results were quantified by the IVIS Lumina Living Image software.

***In vivo* antitumor efficacy.** C57 mice bearing B16F10 melanoma were randomly assigned into five groups (n = 14), which were treated with saline ("Saline" group, 50 μL), SIF micelles ("SIF micelles" group, 50 μL, SRF 0.5 mg mL^{-1}, IR820 0.1 mg mL^{-1}), SIF@TA-Fe NPs ("SIF@TA-Fe" group, 50 μL, SRF 0.5 mg mL^{-1}, IR820 0.1 mg mL^{-1}) , IR820@TA-Fe NPs and light irradiation ("IR820@TA-Fe + L" group, 50 μL, 0.1 mg mL^{-1}) and SIF@TA-Fe NPs and light irradiation ("SIF@TA-Fe + L" group, 50 μL, SRF 0.5 mg mL^{-1}, IR820 0.1 mg mL^{-1}), respectively. The mice were all treated by intratumoral injection on Day 0, and Day 3. For irradiation groups, the mice were exposed to NIR light (808 nm, 10 min) on Day 0 (0.3 W cm^{-2}) , Day 1 (0.3 W cm^{-2}), Day 3 (0.5 W cm^{-2}), and Day 4 (0.5 W cm^{-2}). For the groups without irradiation, mice were kept in the dark after administration. After different treatments, the tumor sizes and body weights were monitored every day. The tumor size was measured by a Vernier caliper, and calculated as volume = (long diameter of tumor) × (short diameter of the tumor)^2 /2. For the survival rate study, mice were euthanized when the tumor size exceeded 2000 mm^3.

Histological examination. Some of B16F10 melanoma-bearing mice were sacrificed at Day 3 to examine the tumor tissue change via histological examinations. Main organs (hearts, livers, spleens, lungs, kidneys) were collected at Day10 for evaluating the biosafety of different formulations. Then the organs and tumors were fixed in 4% paraformaldehyde, processed routinely into paraffin, and sectioned into slices. Hematoxylin and eosin (H&E) staining was carried out to stain the slices from main organs and tumor following standard protocols. For proliferation cell nuclear antigen (PCNA) staining and the apoptosis staining, the tumor slices were stained according to the manual instruction of PCNA Detection Kit (Servicebio, Beijing, China) and

terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL) Cell Death Detection Kit (Roche Applied Science), respectively. A optical microscopy (Zeiss, Germany) and a luminescence microscope were applied to capture images of slices.

Statistical analysis. Statistical significance comparisons for survival curve made by the log-rank test was performed using Prism 5.0 software (GraphPad Software). Statistical significance was analyzed using two-tailed unpaired Student's t-test (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001).

2. Figures

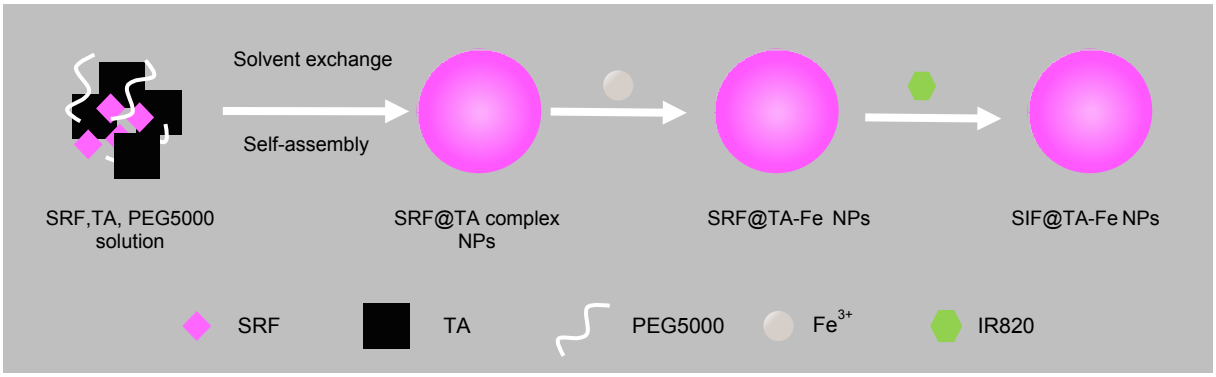

Fig. S1 General procedure for the fabrication of SIF@TA−Fe.

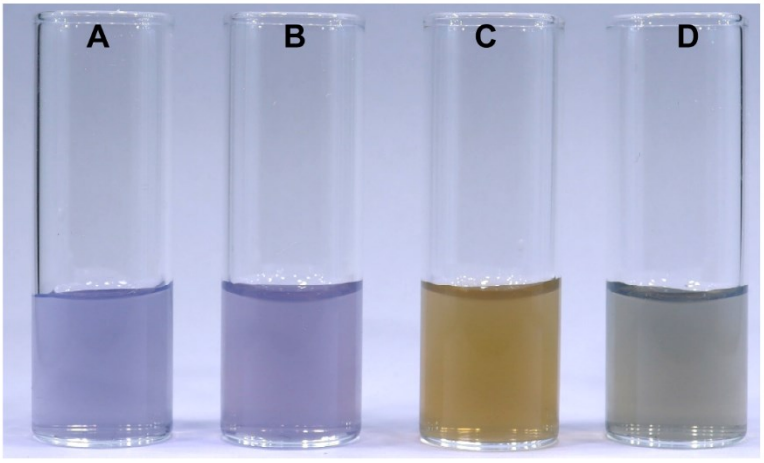

Fig. S2 TA-Fe complex NPs dispersed in water. Blank@ TA-Fe (A), SRF@TA-Fe (B), (C) IR820@TA-Fe, and SIF@TA-Fe (D).

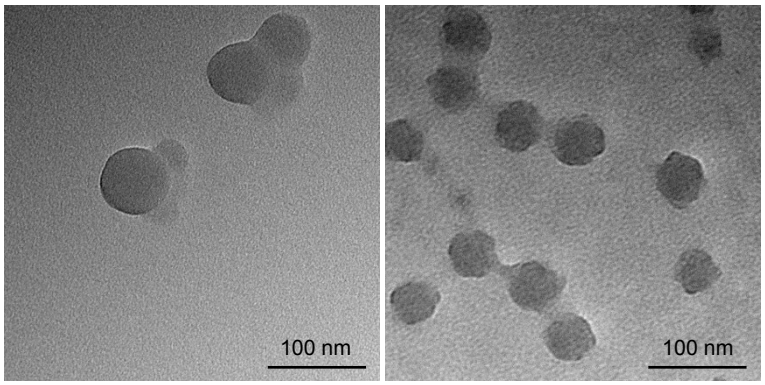

Fig. S3 The TEM images of SRF@TA-Fe (left), and IR820@TA-Fe (right).

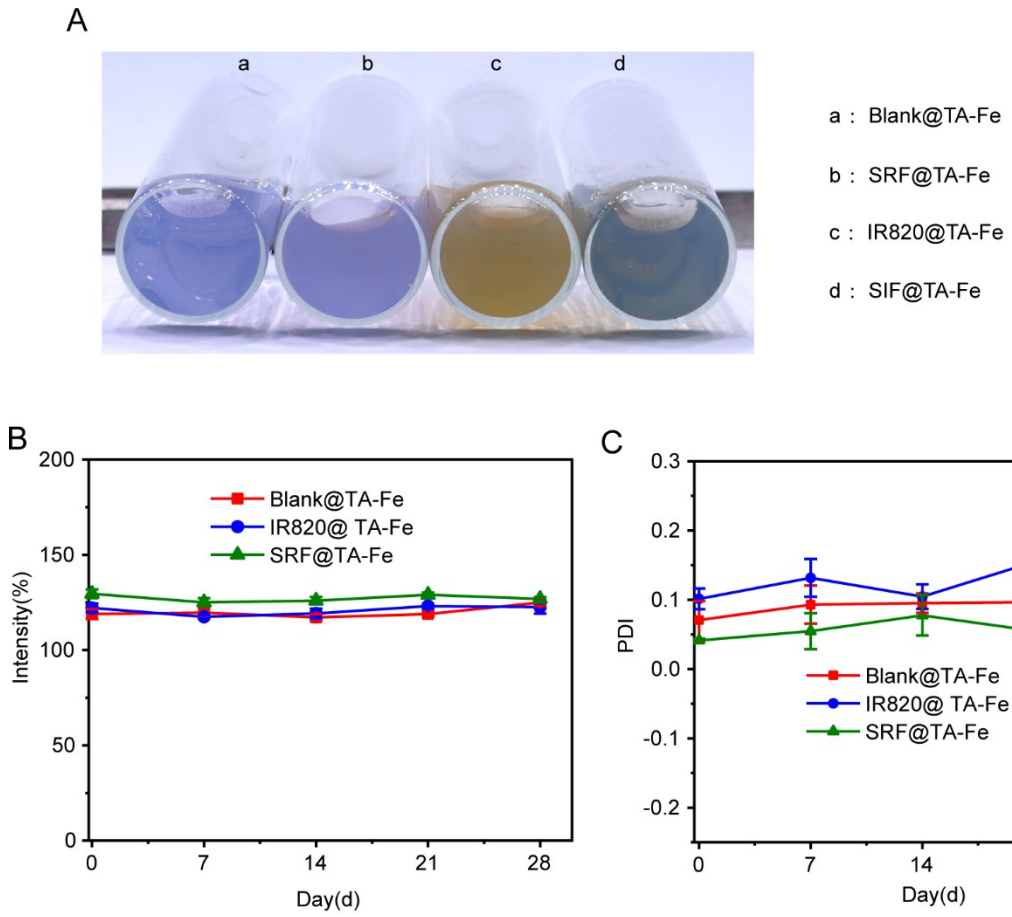

Fig. S4 (A) The optical image of various TA-Fe complex NPs in water (SRF 200 μg mL^{-1}, IR820 40 μg mL^{-1}) after four weeks storage in the dark at 4 °C. The size (B) and PDI (C) of Blank@TA-Fe, SRF@TA-Fe, and IR820@TA-Fe post storage for various time points in the dark at 4 °C.

Table S1. Characterizations of different formulations

(data are presented as mean ± SD, n = 3).

Formulations	Diameter [a] (nm)	PDI [b]	Zeta potential (mV)	LE of SRF [%]	EE of IR820 [%]
SRF@TA-Fe	129.5 ± 2.26	0.04 ± 0.003	-25.1 ± 0.56	29.6 ± 1.57	NA
IR820@TA-Fe	122.1 ± 2.38	0.10 ± 0.015	-22.3 ± 0.45	NA	94.5 ± 4.50
SIF@TA-Fe	128.8 ± 0.64	0.11 ± 0.007	-23.4 ± 0.35	34.8 ± 4.02	95.2 ± 4.80
Blank@TA-Fe	118.9 ± 2.61	0.07 ± 0.028	-21.9 ± 0.80	NA	NA
SIF micelles	113.2 ± 2.16	0.15 ± 0.019	-5.5 ± 0.35	4.0 ± 0.51	NA

[a] Intensity distribution determined by DLS, [b] PDI- polydispersity index.

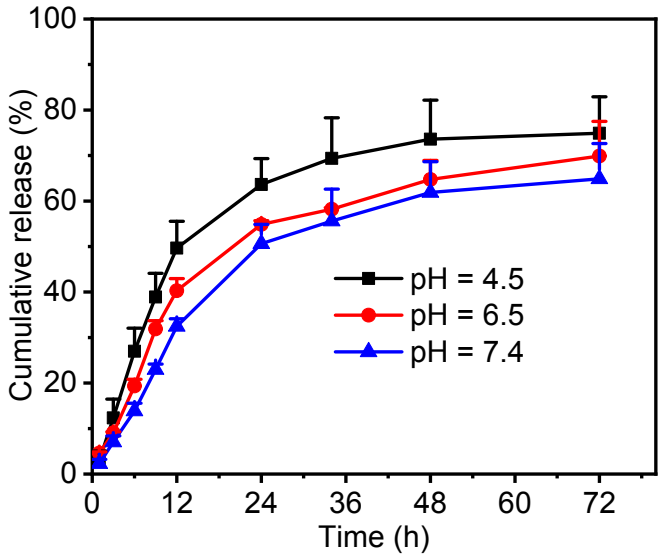

Fig. S5. The release of SRF from SIF@TA-Fe in PBS (0.1 M, pH = 4.5, 6.5, or 7.4) with 0.1% Tween 80.

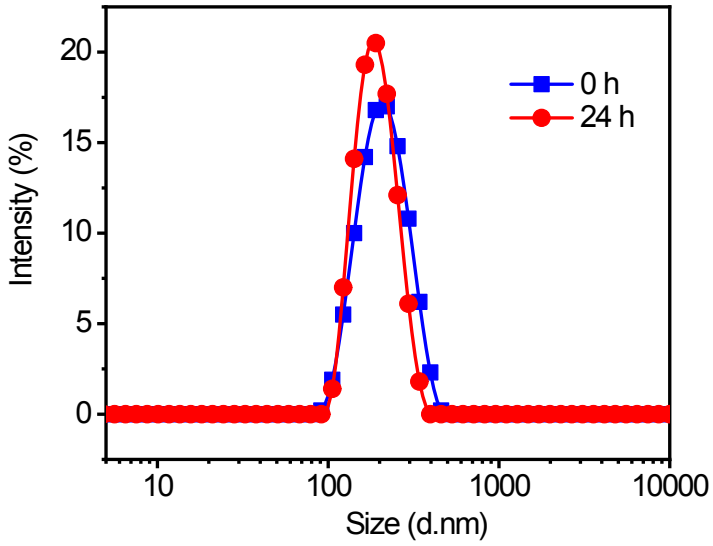

Fig. S6 The stability of SIF@TA-Fe in DMEM containing 10% FBS post 24 h of incubation at room temperture.

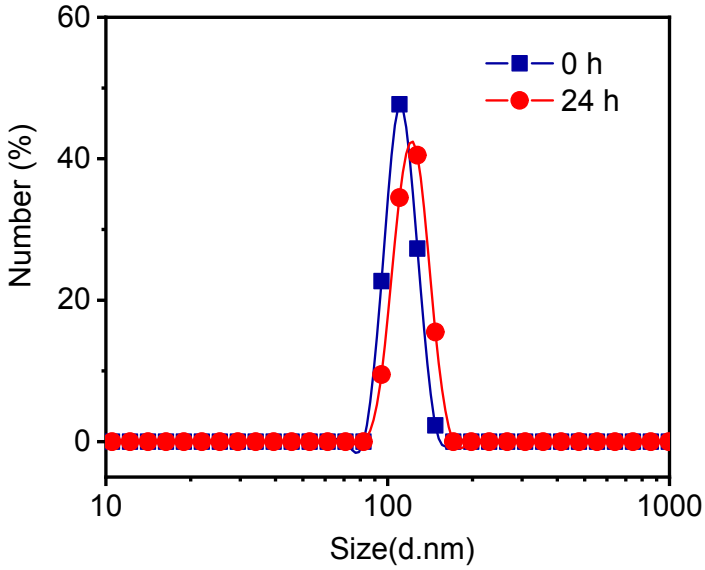

Fig. S7 The stability of C6 micelles in DMEM containing 10% FBS post 24 h of incubation at room temperture.

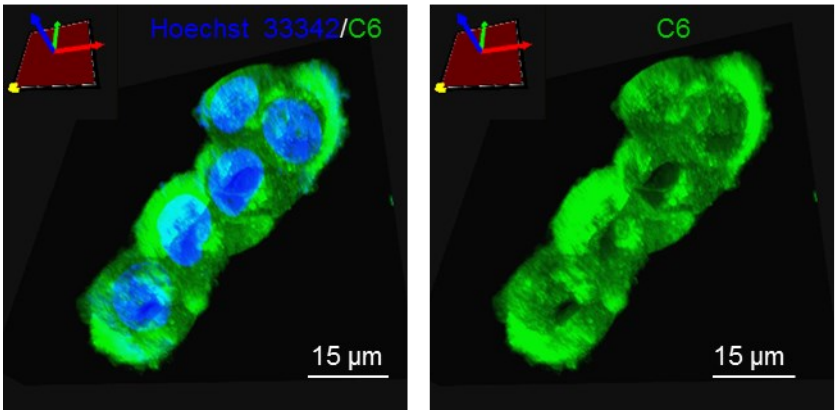

Fig. S8 Z-stack confocal images of B16F10 cells treated with C6@TA-Fe (C6, 1.0 μg mL^{-1}) for 1 min. Nuclei were stained with Hoechst 33342 (blue). Green channel was C6 (λex = 488 nm, λem = 500-580 nm).

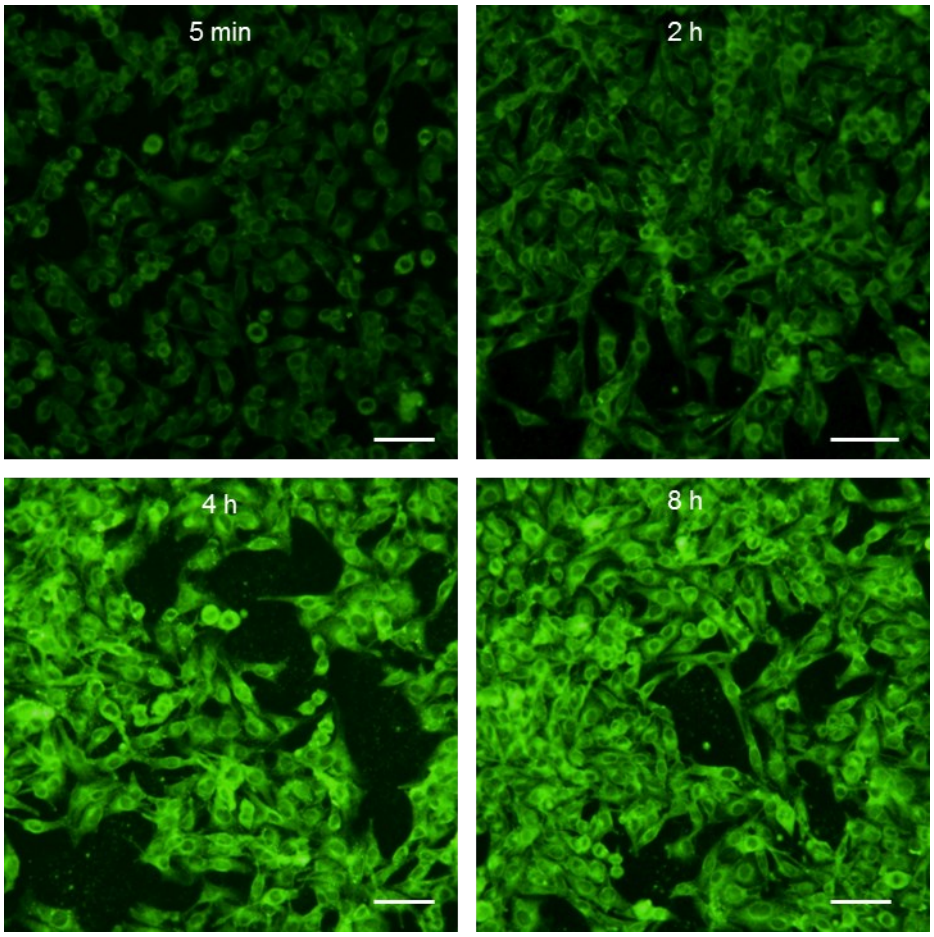

Fig. S9 The fluorescent images of B16F10 cells treated with C6@TA-Fe for different time. Scale bar = 100 μm.

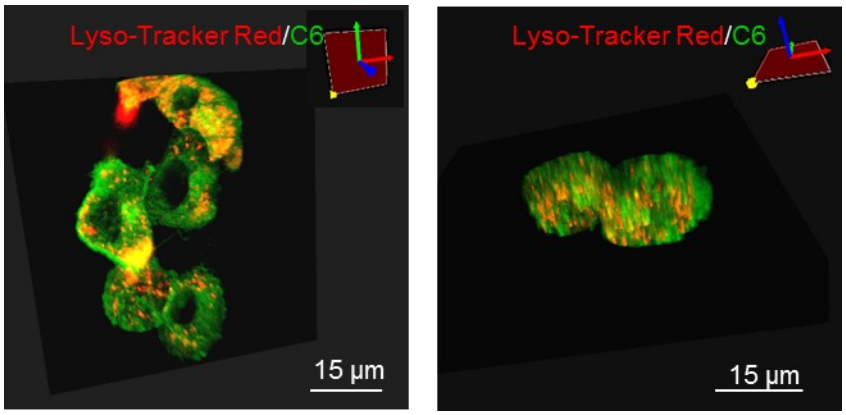

Fig. S10 Z-stack confocal images of B16F10 cells treated with C6@TA-Fe (C6, 1.0 μg mL^{-1}) for 24 h and stained by Lyso-Tracker Red DND-99 (50 nM). Green channel was C6 (λex = 488 nm, λem = 500-580 nm); Red channel was Lyso-Tracker Red DND-99 (λex = 594 nm, λem = 610-710 nm).

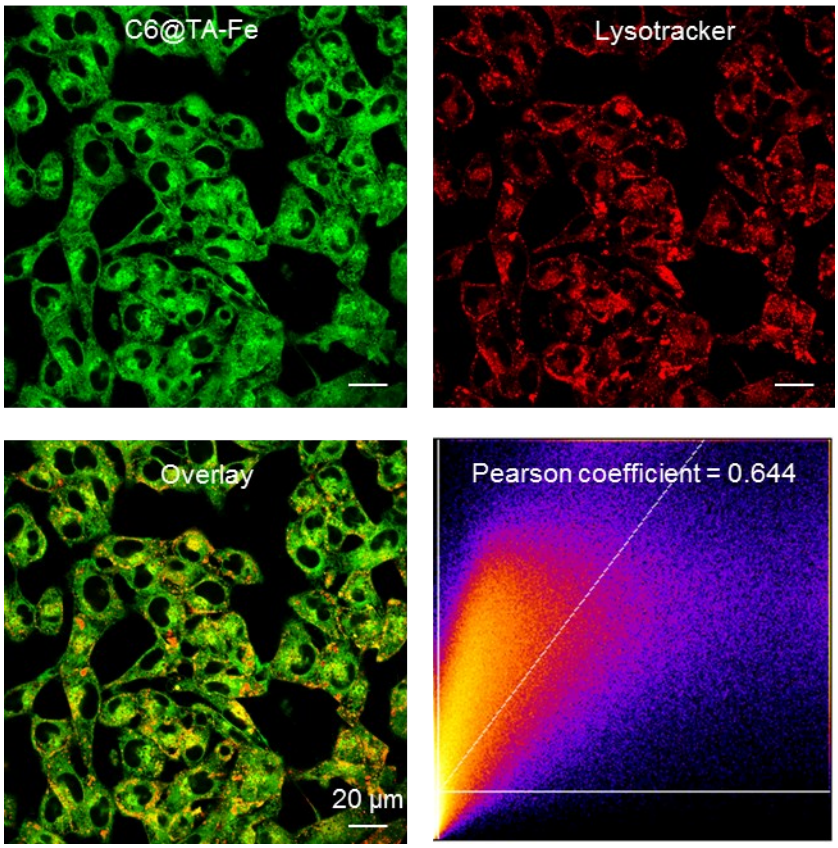

Fig. S11 CLSM images of B16F10 cells stained with Lyso-Tracker Red DND-99 (50 nM) after 24 h of incubation with C6@TA-Fe (C6, 1 μg mL^{-1}) in the dark. Red channel for Lyso-Tracker Red DND-99 (λ_{ex} = 594 nm, λ_{em} = 610-710 nm). Green channel for C6 (λ_{ex} = 488 nm, λ_{em} = 500-580 nm).

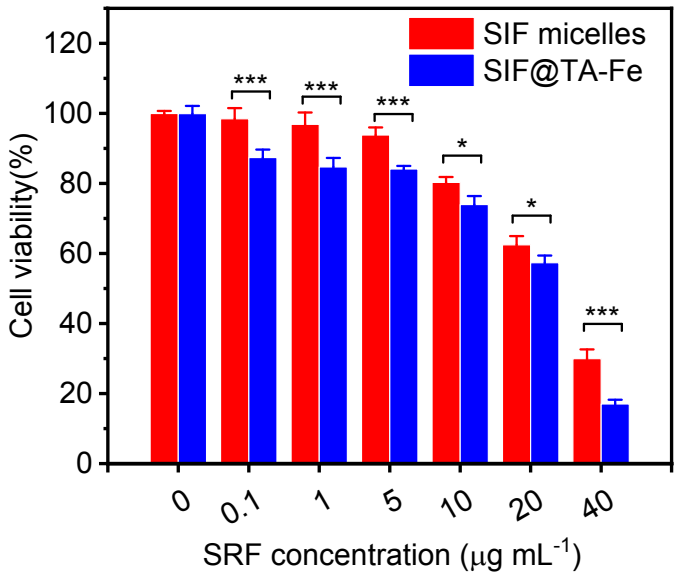

Fig. S12 Relative viability of HUVECs treated with different concentrations of SIF@TA-Fe or SIF micelles for 24 h without 808 laser irradiation. Data are presented as mean ± SD from a representative experiment (n = 5) from 3 independent experiments. Statistical significance was analyzed using two-tailed unpaired Student's t-test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

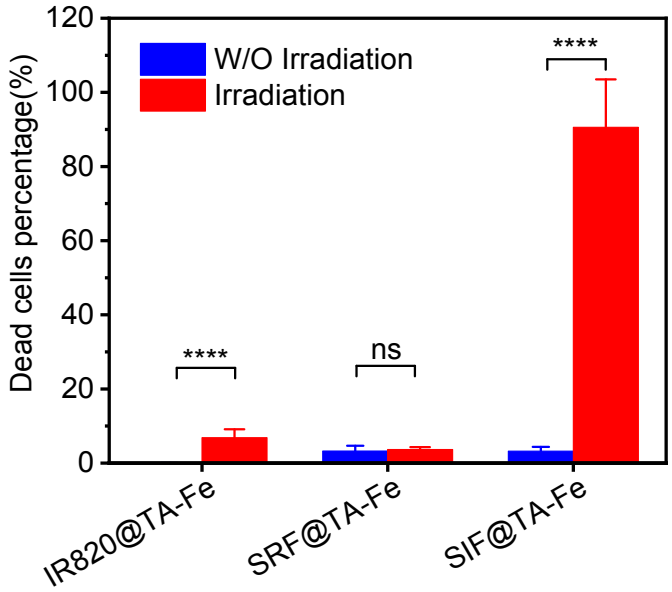

Fig. S13 Dead rate of B16F10 cells incubated with SIF@TA-Fe (SRF 40 μg mL^{-1}, IR820 8 μg mL^{-1}), SRF@TA-Fe (SRF 40 μg mL^{-1}), or IR820@TA-Fe NPs (IR820, 8 μg mL^{-1}) for 2 h, and exposed to 808 nm laser (0.3 W cm^{-2}, 3 min). ns, no significant difference; Statistical significance was analyzed by using two-tailed unpaired Student's t-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$).

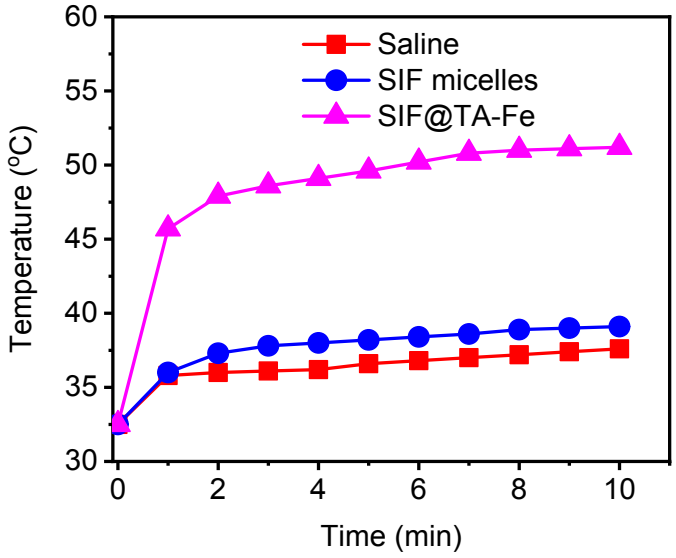

Fig. S14 The temperature changes in tumor area from mice post 24 h of intratumoral injection with SIF micelles, SIF@TA-Fe, or saline. Laser irradiation: 808 nm, 0.5 W cm^{-2}, 10 min.

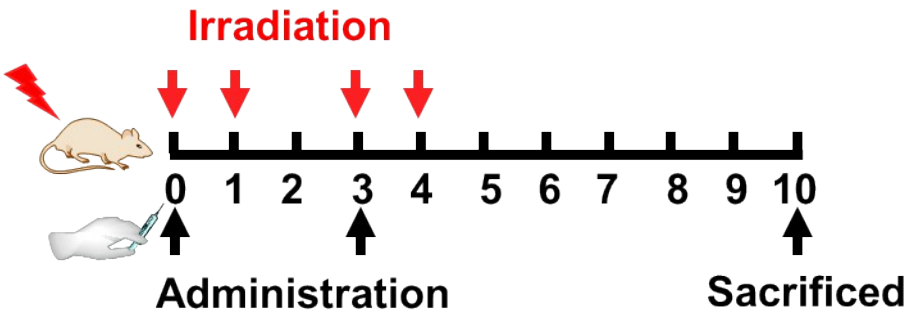

Fig. S15 The time axis of the *in vivo* antitumor study of B16F10 melanoma-bearing mice when the tumor volume reached approximately 100 mm^3.

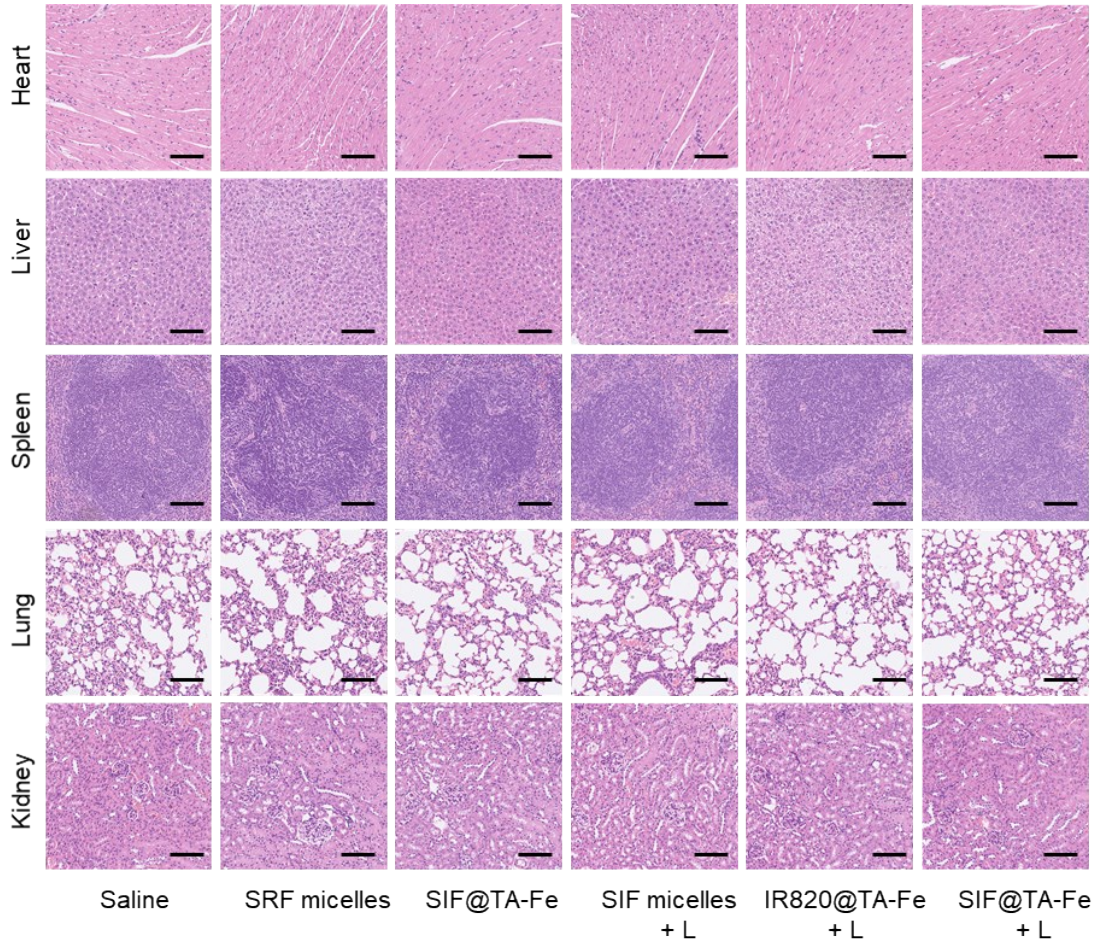

Fig. S16 Images of various H&E-stained organ slices from B16F10 melanoma-bearing mice after different treatments on Day 10 post receiving the first treatment. Scale bar: 100 μm.

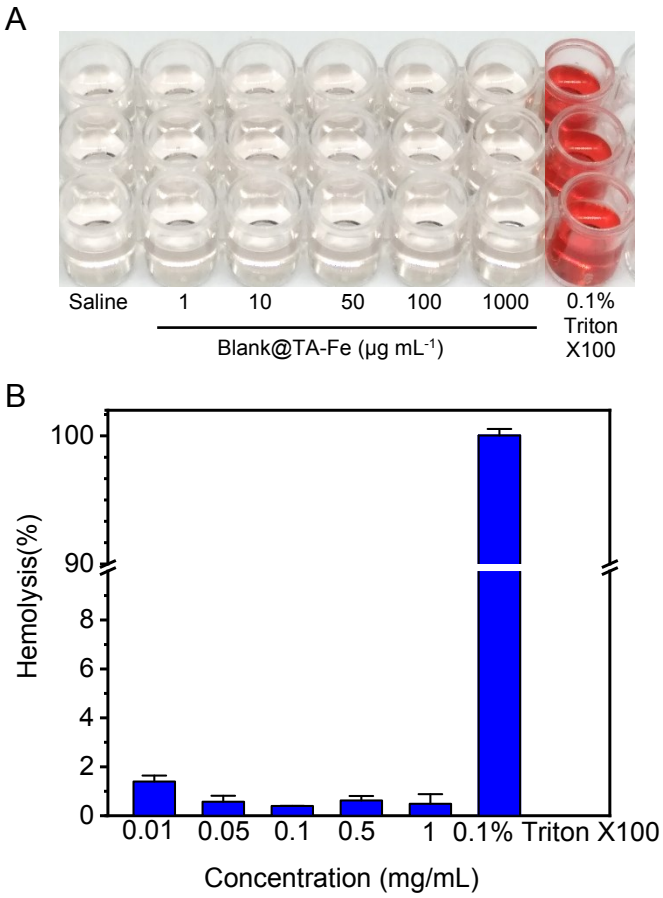

Fig. S17 (A) The optical images of erythrocytes suspension after incubation with Blank@TA-Fe for 2 h. (B) The hemolysis percentage induced by Blank@TA-Fe.

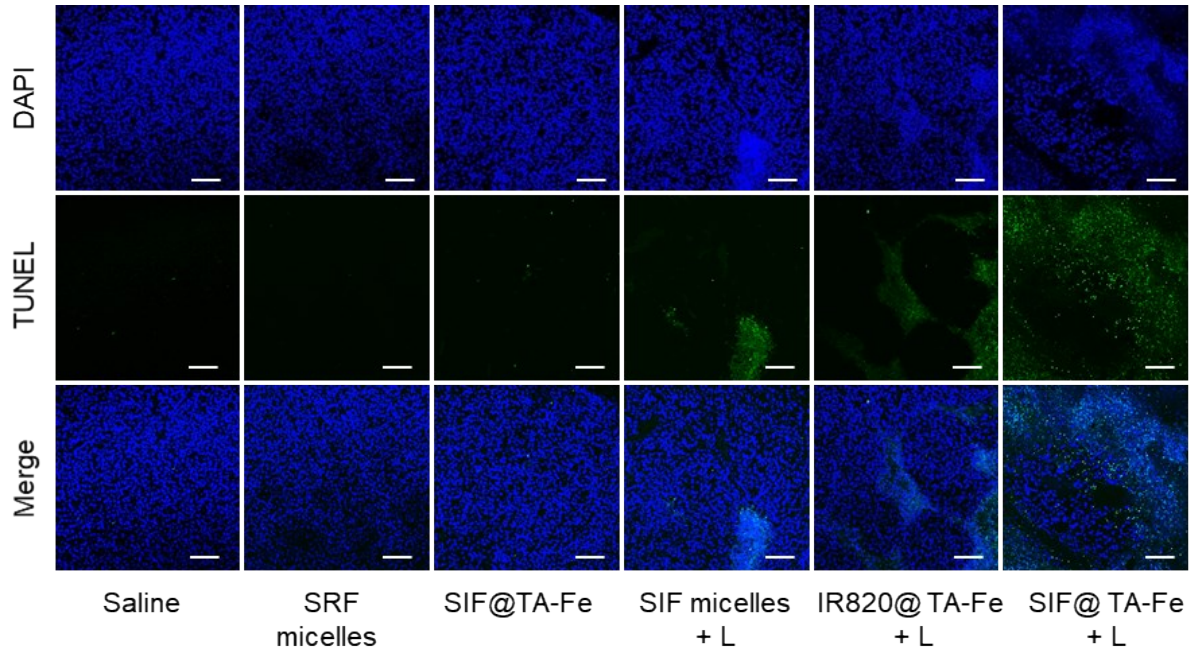

Fig. S18 Images of TUNEL-stained tumor slices from B16F10 melanoma-bearing mice on Day 3 after receiving the first treatment. Scale bar: 100 μm.

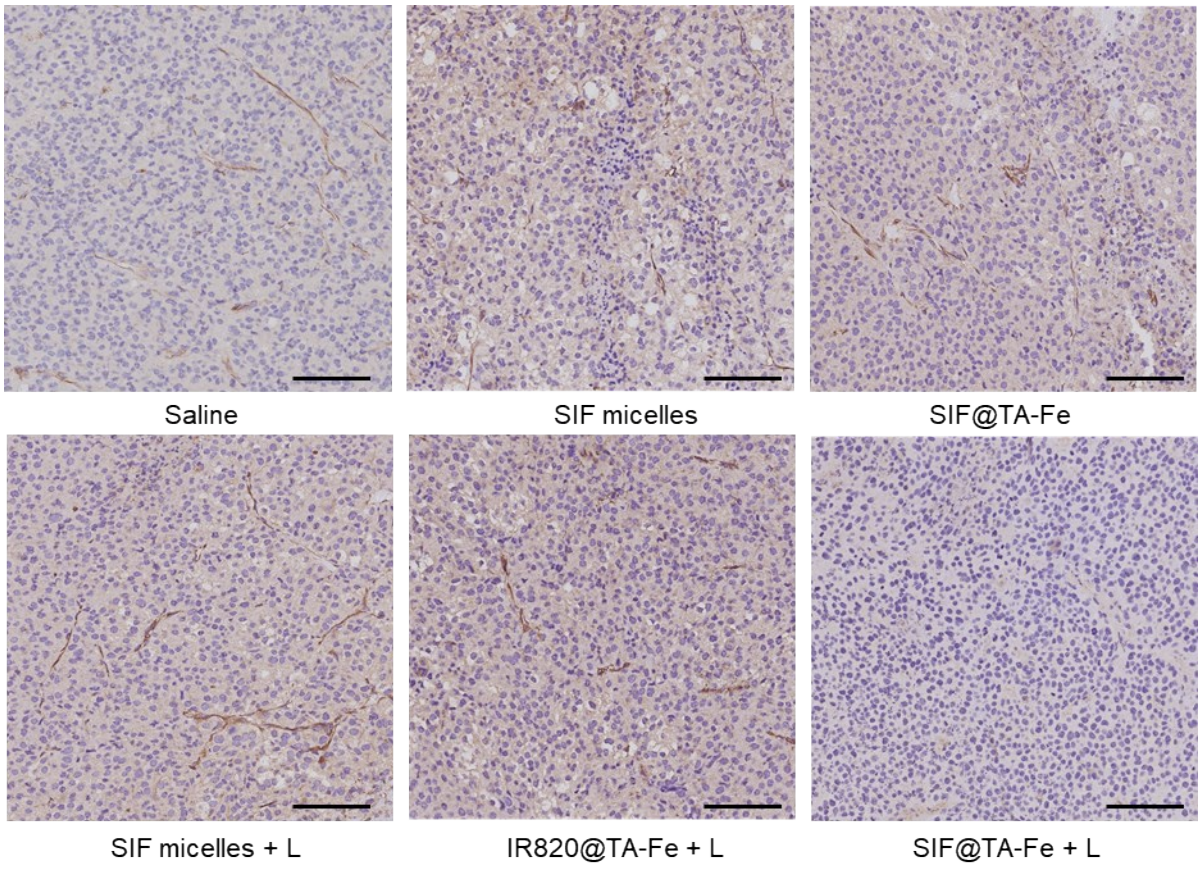

Fig. S19 Images of VEGFR2-stained tumor slices from B16F10 melanoma-bearing mice on Day 3 after receiving the first treatment. Scale bar: 100 μm.