Supporting Information

GalNAc modified FeS nanoparticles for specific chemodynamic and gas therapy against orthotopic hepatocellular carcinoma

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EXPERIMENTAL SECTION

Materials and reagents. Hexadecyl trimethyl ammonium chloride (CTAC), iron(II) sulfate heptahydrate ($FeSO_4·7H_2O$), triethanolamine (TEA), tetraethyl orthosilicate (TEOS), sodium carboxymethyl cellulose (CMC), 1,10-Phenanthroline, ammonia solution and tetrahydrofuran (THF) were purchased from Sinopharm Chemical Reagent Co., Ltd. Sodium sulfide nonahydrate ($Na_2S·9H_2O$) , Carboxyethylsilanetriol, Sodium salt (25% in water, CETS) and hydroxylammonium chloride were purchased from Shanghai Macklin Biochemical Co., Ltd. (2S,3R,4R,5R,6R)-3-Acetamido-6-(Acetoxymethyl) Tetrahydro-2H-Pyran-2,4,5-Triyl Triacetate (GalNAc-5OAc), 2-Azidoethanol, trimethylsilyl trifluoromethanesulfonate (TMSOTf) and triethylamine (TEA) were purchased from Shanghai Adamas Reagent Co., Ltd. Methylene blue and 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Tianjin Heowns Biochemical Technology Co., Ltd. Propidium iodide (PI), calcein acetoxymethyl ester (Calcein-AM) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Beyotime Biotech. The pH fluorescence probe (BCECF AM) was purchased from UElandy Biotechnology Co., Ltd. The H_2S content assay kit, Hoechst 33342 and methylene blue were purchased from Beijing Solarbio Science & Technology Co., Ltd. The mouse Hepatocarcinoma cell line (Hepa 1-6) and Hepa 1-6 cells transfected with luciferase (Hepa 1-6-Luc) were purchased from Ubigene Biosciences (Guangzhou, China). Fetal bovine serum (FBS) in cell culture medium was purchased from VivaCell. The experimental water used was Mill-Q secondary ultrapure water (18.2 $M\Omega·cm^{-1}$). All the other chemical

reagents were of analytical grade and used without further purification.

Instruments. Transmission electron microscopy (TEM) imaging was carried out on a HT7700 electron microscope (HITACHI, Japan). Zeta potential was performed on a Malvern Zeta Sizer Nano (Malvern Instruments). UV-vis absorption spectra were measured on a TU-1901 UV-visible spectrophotometer (PERSEE, Beijing). Fourier transform infrared spectroscopy (Nicolet iS50 FT-IR) was used for chemical bond measurements. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glass-calomel electrode. Absorbance in MTT assay was measured in a microplate reader (RT 6000, Rayto, USA). Confocal fluorescence imaging experiments were performed with TCS SP8 confocal laser scanning microscope (Leica, Germany). Live animal imaging system (IVIS Lumina III, US) was applied *in vivo* imaging.

Synthesis of FeS NPs. First, 2 mL of CMC (1%) was added to 22.5 mL of water, and the mixed solution was vacuumized for 1 h to completely remove O_2. Then, under the protection of N_2 atmosphere, 4 mL of $FeSO_4$ (0.043 mol/L) was added and stirred, followed by the addition of 2 mL of Na_2S (0.085 mol/L). After keeping the whole system in a vacuum environment at room temperature for 2 h, FeS NPs were separated by centrifugation at 14000 rpm.

Synthesis of FeS@MSNs. 0.065 g of CTAC and 12 mg of TEA were added to the 10 mL of FeS aqueous solution and stirred at room temperature for 1 h. Then 50 μL of TEOS was added to the mixed solution and stirred in an oil bath (85-90 ^{o}C) for 1 h. Finally, the solution was cooled to room temperature and centrifuged at 12000 rpm to

obtain FeS@MSNs.

The carboxylation of FeS@MSNs. The FeS@MSNs were dissolved in 20 mL of anhydrous ethanol and 8 mL of water, and the mixture was stirred for 10 min. Then 200 μL of ammonia was added and stirred for 30 min. Finally, 50 μL of CETS was added to the mixture and stirred overnight. The reaction mixture was centrifuged and washed several times with ethanol and water to obtain FeS@MSNs-COOH.

Synthesis of GalNAc-NH_2. Step 1: 1.17 g of GalNAc-5OAc (3 mmol, 1 eq) was dissolved in 1,2-dichloroethane (DCE, 30 mL) under 0 ℃. Then 667 μL of TMSOTf (3.9 mmol, 1.3 eq) was added dropwise. After stirring at 0 ℃ for 10 min, the reaction system was moved to a 50 ℃ oil bath and refluxed for 5 h. Then the reaction mixture was diluted with DCE and quenched with TEA. The mixture was washed with saturated $NaHCO_3$ and saturated NaCl solution. Finally, the organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The product (named as product A, yield 80%) of this step was used directly without further purification.

Step 2: Product A (2.4 mmol, 1.2 eq) and 140 μL of 2-azidoethanol (2 mmol, 1 eq) was dissolved in DCM (18 mL) under 0 ℃. And 103 μL of TMSOTf (0.6 mmol, 0.3 eq) was added dropwise. After stirring at 0 ℃ for 10 min, the reaction system was moved to a 50 ℃ oil bath and refluxed for 17 h. Then the reaction system was quenched with TEA and purified by silica gel chromatography with CH_2Cl_2/CH_3OH (v/v, 20:1) as eluent, affording GalNAc-5OAc-N_3 as a pale-yellow solid.

Step 3: 416 mg of GalNAc-5OAc-N_3 (1 mmol, 1.0 eq) and 167 mg of MeONa (0.31 mmol, 0.31 eq) was dissolved in 10 mL of MeOH (pH 9). The system was

allowed to stir at 23 ℃ for 3 h. Then ion-exchange resin was added into the solution until the pH 5-6. Finally, the resin was filtered, and the filtrate was dried to obtain GalNAc-N_3 as a pale-yellow solid.

Step 4: 58 mg of GalNAc-N_3 (0.2 mmol) and 144.26 mg of PPh_3 was dissolved in 2.5 mL of THF. Then 250 μL of H_2O was added. The reaction system was moved to a 40 ℃ oil bath and refluxed for 7 h under argon atmosphere. After dried, the product was washed with HCl (0.5 mol/L) and CH_2Cl_2 solution. Finally, the water phase was evaporated under reduced pressure to obtain GalNAc-NH_2. The GalNAc-NH_2 was used directly without further purification.

Synthesis of FeS@MSNs-GalNAc. 5.75 mg of EDC and NHS were added to 3 mL of FeS@MSNs-COOH and stirred for 30 min to activate the carboxyl group on FeS@MSNs-COOH. Then 400 μL (40 mg/mL) of GalNAc-NH_2 was added to the mixture and stirred for 7 h to connect GalNAc by amide reaction.

The Fe^{2+} Detection. 0.3 mg of FeS@MSNs was dispersed in 2 mL of different pH buffer solutions (7.4, 6.5 and 5.4) with 1,10-Phenanthroline as the indicator of ferrous ion. The supernatant was collected by centrifugation after 2 h, and the absorbance at 510 nm was measured by UV-vis spectrophotometry.

The ·OH Detection. 0.3 mg of FeS@MSNs was dispersed in 2 mL of different pH buffer solutions (7.4, 6.5 and 5.4). Then 200 μL of hydroxylammonium chloride (10%) was added to mask Fe^{3+}. Subsequently, 3 μL of MB and 10 μL of H_2O_2 (10 mol/L) were added in turn. The absorbance at 665 nm was measured by UV-vis

spectrophotometry at different time.

The H_2S Detection. FeS@MSNs were sealed in the centrifuge tubes and dispersed in PBS buffer solutions of different pH (7.4, 6.5 and 5.4) to a final concentration of 150 μg/mL. After reaction for 2 h under stirring, the supernatant was collected by centrifugation. The H_2S content in the supernatant was detected by the H_2S kit.

Cell lines and animals. Hepa1-6 cells were cultured in DMEM medium containing 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin) and were maintained at 37 °C in a 5 % CO_2 humidified incubator. Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU2024043). All animal experiments were conducted and obeyed the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, Biology Institute of Shandong Academy of Science, China.

MTT assay. First, the digested cells were evenly seeded in 96-well plates and incubated for 24 h. Then FeS@MSNs and FeS@MSNs-GalNAc with different concentrations (30, 60, 90, 120 μg/mL) were incubated in DMEM medium (pH 7.4, 6.5) for 24 h. Subsequently, the cell culture medium was sucked out and washed with PBS buffer to remove the undigested nanomaterials. Then 200 μL of MTT (0.5 mg mL^{-1}) was added and DMSO was used to dissolve formazan crystals 4 h later. The absorbance at 490 nm was measured to calculate the survival rate.

Live/dead cell staining assay. Hepa1-6 cells was planted into confocal dishes for 24

h. The fresh medium containing 120 μg/mL of FeS@MSNs and FeS@MSNs-GalNAc were added and incubated for 24 h. Then the cell culture medium was removed and the cells were washed with PBS buffer for three times. Calcein-AM and PI probes were added to the confocal dishes and incubated for 20 min. Finally, the cells were washed three times with PBS, and confocal imaging was performed using a TCS SP8 confocal laser scanning microscopy. Green fluorescence of Calcein-AM was excited at 488 nm and red fluorescence of PI was excited at 633 nm.

Intracellular ROS Measurement. Hepa1-6 cells was planted into confocal dishes for 24 h. The fresh medium containing 120 μg/mL of FeS@MSNs and FeS@MSNs-GalNAc were added and incubated for 12 h. Then cell culture medium was removed, and the cells were washed with PBS for three times. Then, the cells were treated with DCFH-DA for 20 min. Finally, the cells were washed with PBS for three times, and confocal imaging was performed using a TCS SP8 confocal laser scanning microscopy.

Intracellular H_2S Content Detection. Hepa1-6 cells was seeded in the 6-well plates and incubated for 24 h. Then fresh medium containing 120 μg/mL of FeS@MSNs and FeS@MSNs-GalNAc were added and incubated at different pH conditions for 12 h. Then the cells were washed with PBS and collected by trypsin digestion. The content of H_2S in the cells was detected by H_2S kit after cell disruption.

Intracellular pH Detection. Hepa1-6 cells was planted into confocal dishes for 24 h.

Then fresh medium containing 120 μg/mL of FeS@MSNs and FeS@MSNs-GalNAc were added and incubated at different pH conditions (7.4 and 6.5) for 12 h. The cell culture medium was removed, and the cells were washed with PBS for three times. Then the cells were treated with BCECF-AM probe for 30 min. Finally, the cells were washed three times with PBS, and confocal imaging was performed using a TCS SP8 confocal laser scanning microscopy.

Establishment of orthotopic transplantation tumor model of liver cancer. C57 mice (4-5 weeks, male) were used as animal models and fed for about one week to make their body weight reach about 20 g. Hepa 1-6-Luc cells were digested by trypsin, and then washed with PBS for three times. About every 1×10^7 of Hepa 1-6-Luc cells were dispersed in 50 μL of serum-free DMEM medium and the cell suspension was injected to mice liver to form an orthotopic transplantation tumor model.

Verification of the liver targeting of GalNAc. Firstly, 0.6 mg of IR808 was added to 3 mL ethanol solution of FeS@MSNs or FeS@MSNs-GalNAc (6 mg) and stirred for 12 h at room temperature. Then the reaction mixture was centrifuged and washed for three times with ethanol to obtain FeS@MSNs@IR808 and FeS@MSNs-GalNAc@IR808 for further use. Then, the fluorescence of FeS@MSNs@IR808 and FeS@MSNs-GalNAc@IR808 were measured. The FeS@MSNs@IR808 and FeS@MSNs-GalNAc@IR808 were dispersed in 3 mL of PBS buffer, respectively. After the solution was mixed evenly in per tube, 0.3 mL of solution was removed and centrifuged at a time. The fluorescence of supernate was measured. The precipitate

was redispersed in 0.3 mL of PBS buffer, of which the fluorescence was measured (Figure S11). C57 mice were divided into two groups: one group was intravenously injected with FeS@MSNs@IR808 NPs, and the other group was injected with FeS@MSNs-GalNAc@IR808 NPs. Fluorescence imaging was performed on mice at different time points (0, 1, 3, 6, 9, 12, 24, 36, 48 h) to observe whether GalNAc had active targeting to the liver. Then, the mice were dissected at 12 h and fluorescence imaging of the main organs was performed.

In vivo antitumor therapy. The mice were randomly divided into 3 groups 7 days later: PBS (blank control), FeS@MSNs and FeS@MSNs-GalNAc. Each mouse was injected with nanomaterials at a dose of 5 mg/kg. And the tumor sizes were observed by bioimaging at different times (day 0, 2, 6, 10, 14). Mice were euthanized at day 14, and the tumor-bearing livers were photographed. And major organs (heart, spleen, lung, kidney) and liver tissues were obtained for H&E staining. Blood samples were also collected on day 14 for blood routine and blood biochemical analysis. The body weights of mice were also recorded every other day.

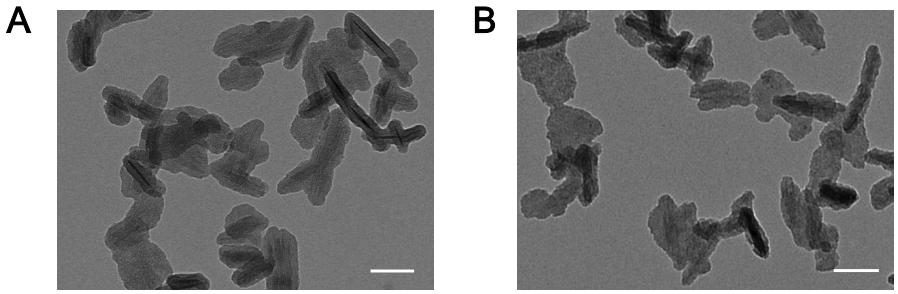

Figure S1. TEM images of FeS@MSNs (A) and FeS@MSNs-GalNAc (B). Scale bars: 100 nm.

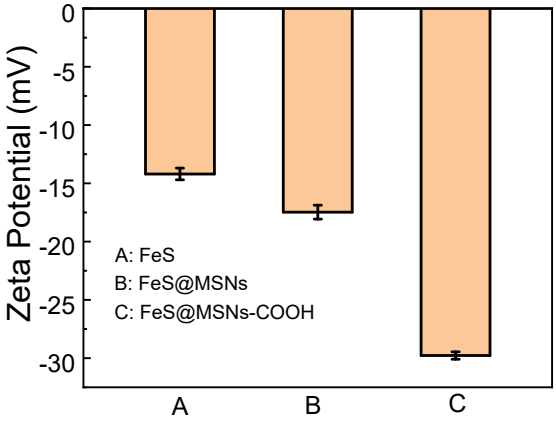

Figure S2. Zeta Potential of FeS, FeS@MSNs and FeS@MSNs-COOH.

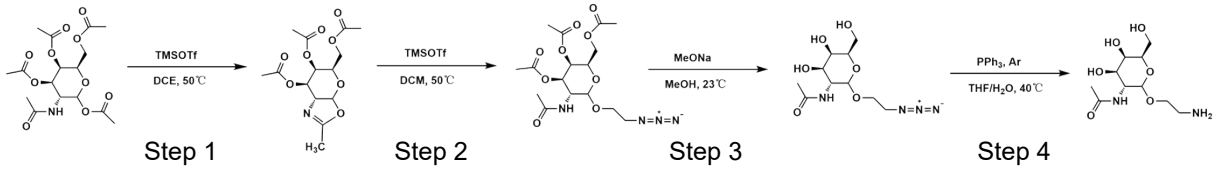

Figure S3. Synthesis steps of GalNAc-NH_2.

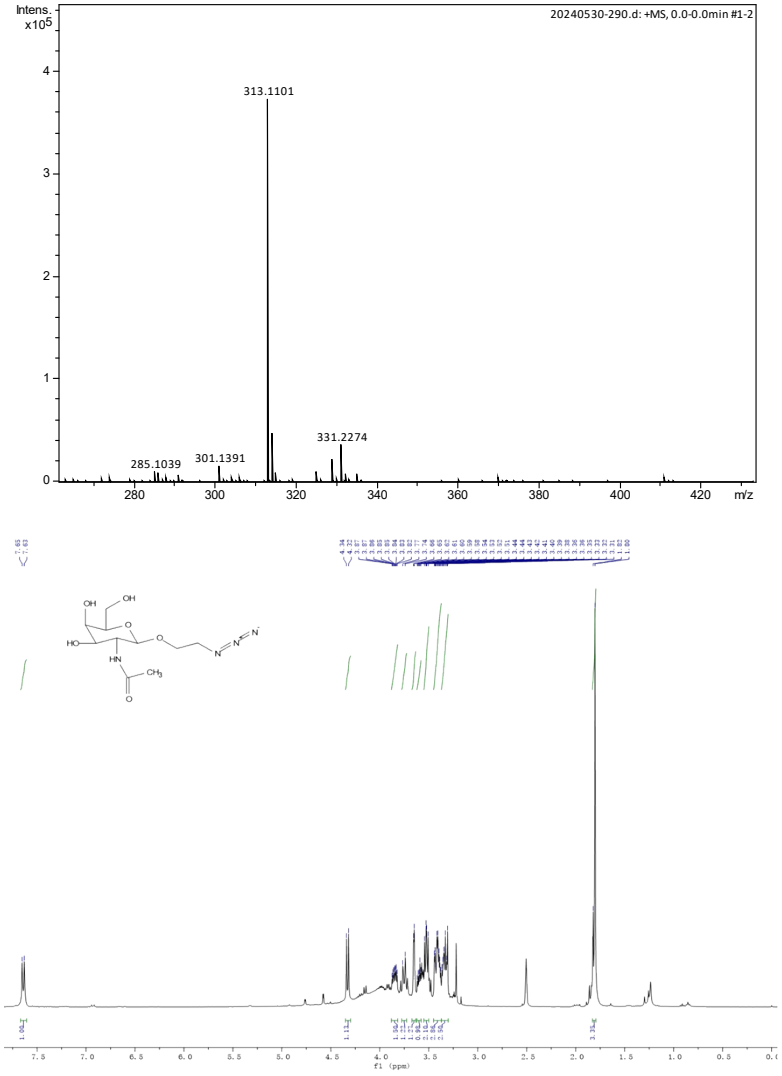

Figure S4. The MS and ^{1}H NMR Spectroscopy of GalNAc-N_3.

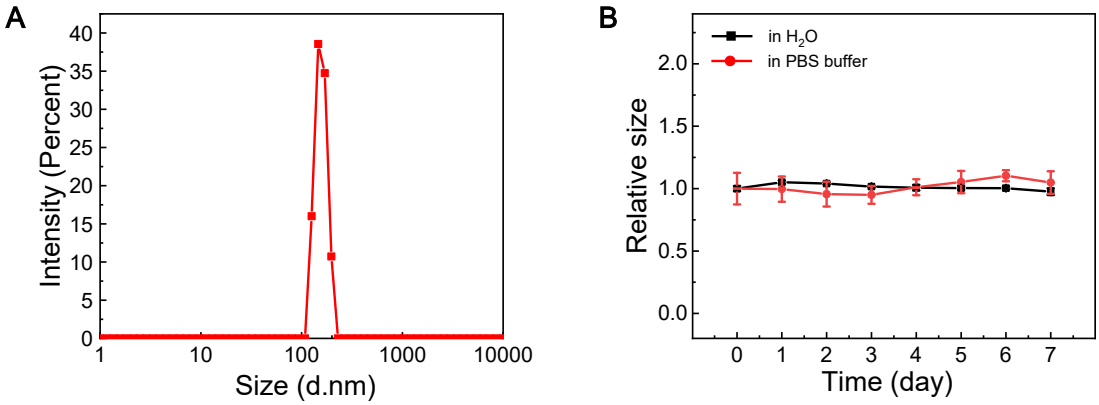

Figure S5. (A) The DLS size distribution of FeS@MSNs-GalNAc; (B)The relative size of FeS@MSNs-GalNAc in various solutions within 7 days by the DLS.

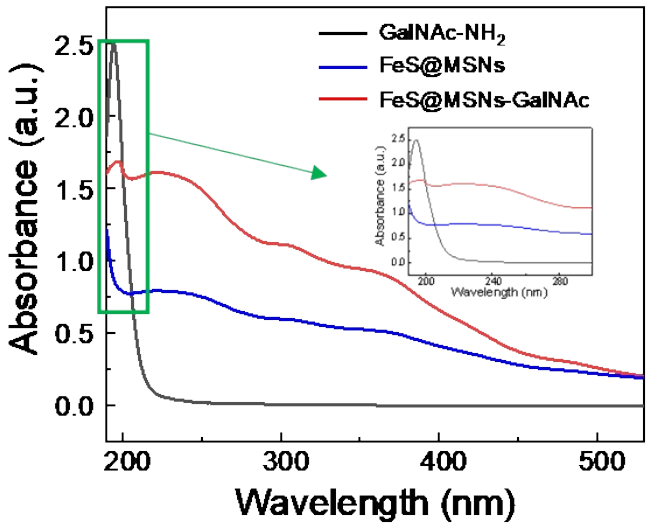

Figure S6. UV-vis spectra of GalNAc-NH_2, FeS@MSNs and FeS@MSNs-GalNAc.

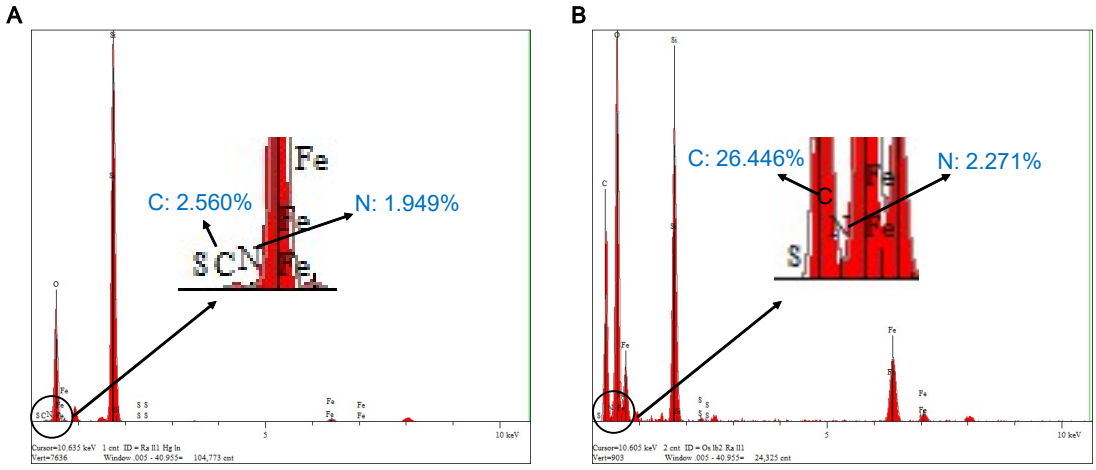

Figure S7. The EDS data of FeS@MSNs (A) and FeS@MSNs-GalNAc (B).

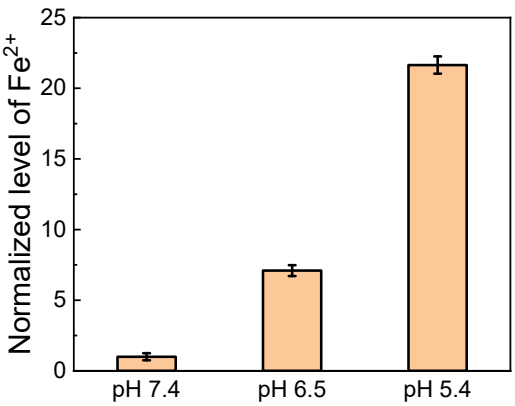

Figure S8. The detection of Fe^{2+} using ICP-AES.

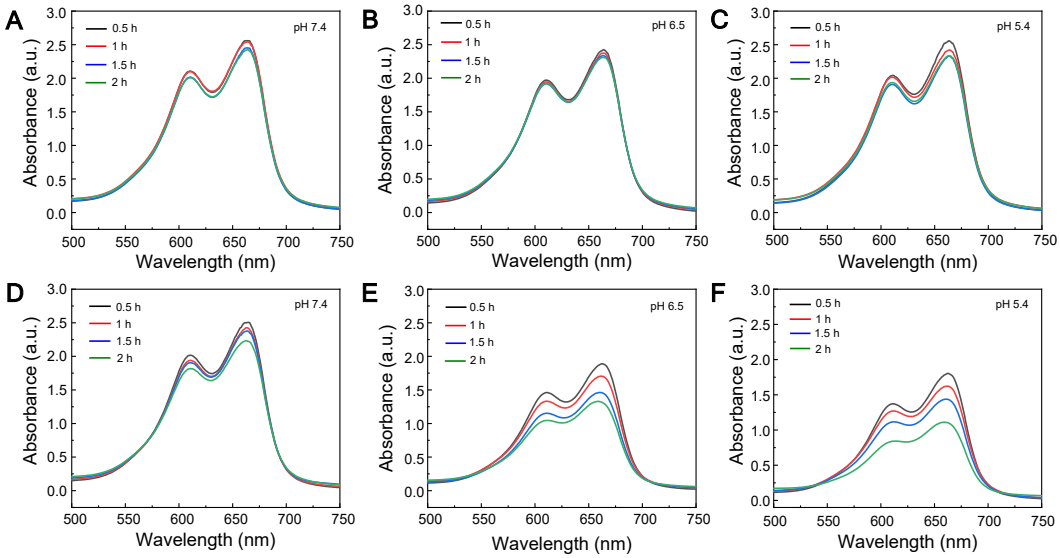

Figure S9. The detection of ·OH using MB as the probe after FeS@MSNs immersed in different pH buffers. (a) pH 7.4 buffer, (b) pH 6.5 buffer, (c) pH 5.4 buffer; and the detection of ·OH using MB as the probe when FeS@MSNs immersed in different pH buffers with H_2O_2. (d) pH 7.4 buffer, (e) pH 6.5 buffer, (f) pH 5.4 buffer.

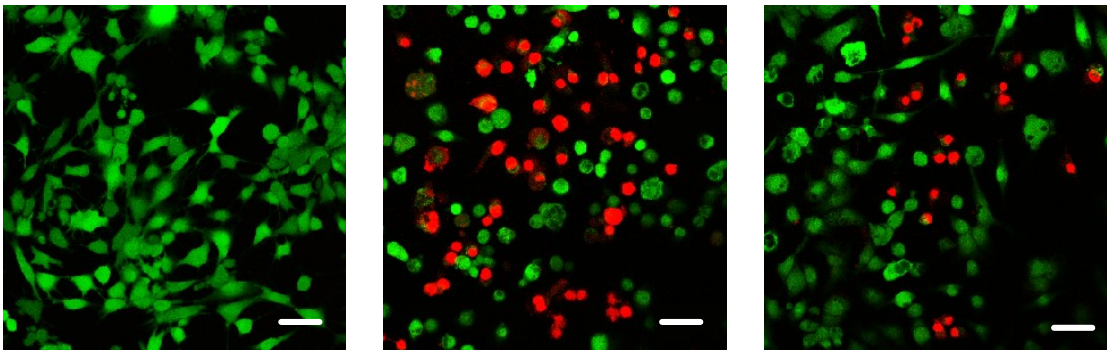

Figure S10. Live/dead cell staining assay to visualize cell viabilities of Hepa 1-6 cells under different treatments: (a) PBS, (b) FeS@MSNs-GalNAc (pH 7.4), (c) the Hepa 1-6 cells were pre-treated with the free GalNAc, then incubated with FeS@MSNs-GalNAc (pH 7.4). Scale bars: 50 μm.

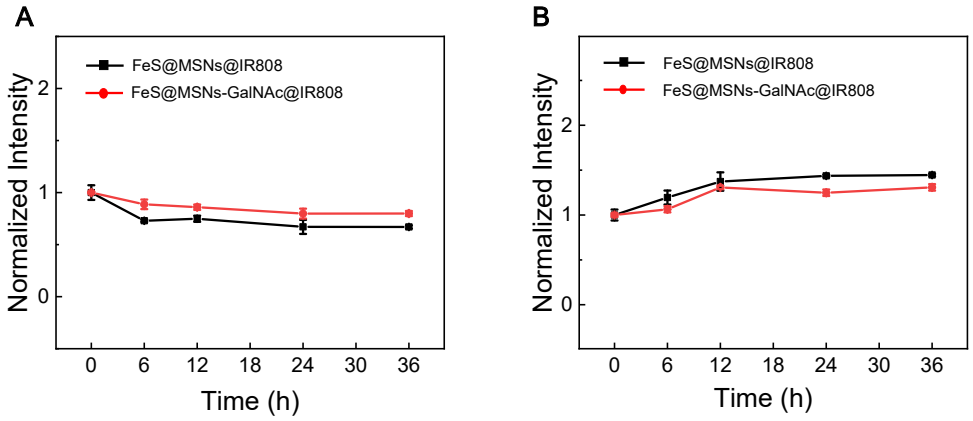

Figure S11. The normalized fluorescence signals of the precipitate (A) and supernate (B) from FeS@MSNs@IR808 and FeS@MSNs-GalNAc@IR808.

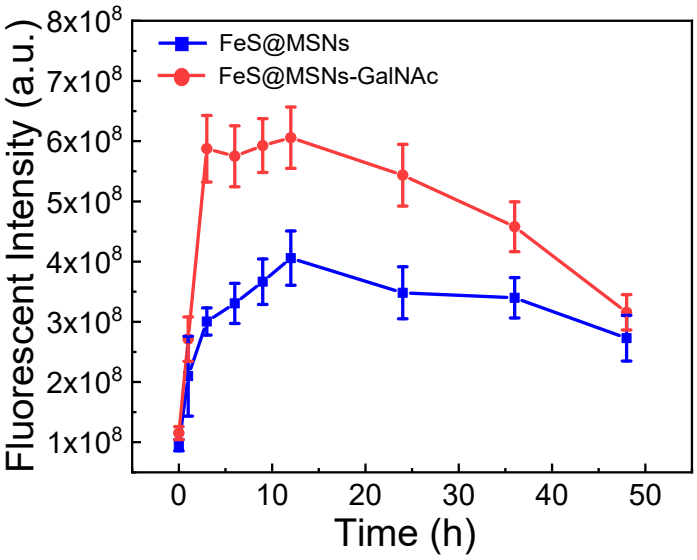

Figure S12. Corresponding quantifications of fluorescence intensity of Figure 3A.

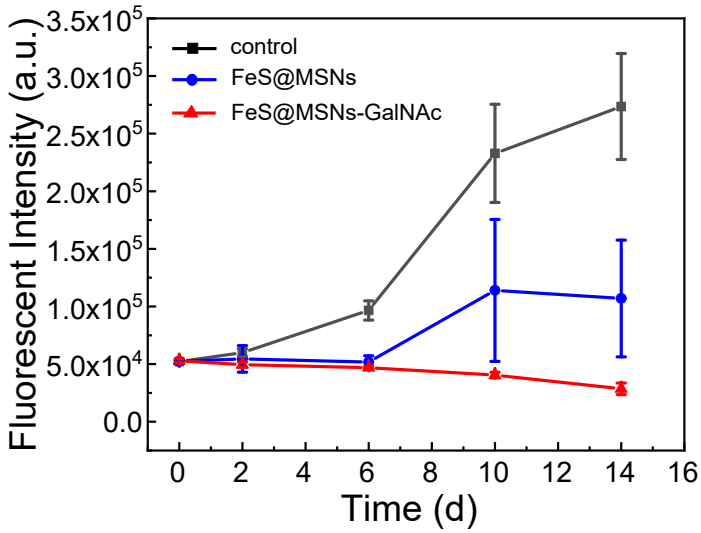

Figure S13. Time-dependent tumor growth profiles.

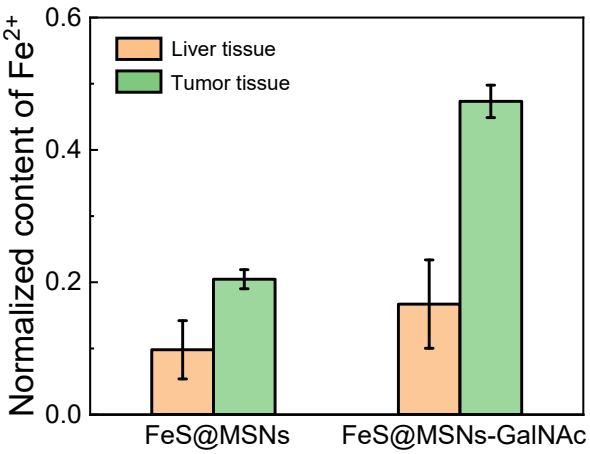

Figure S14. The normalized content of Fe^{2+}.

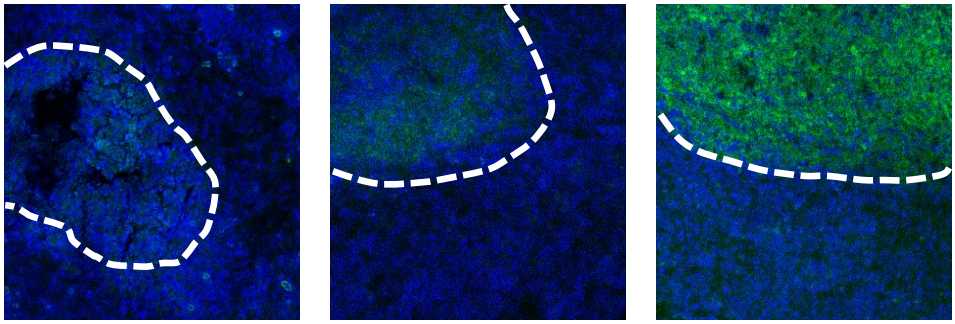

Figure S15. The ROS content of livers and tumors by the DCFH-DA as probe. Tumors: the part circled by the dotted white lines.

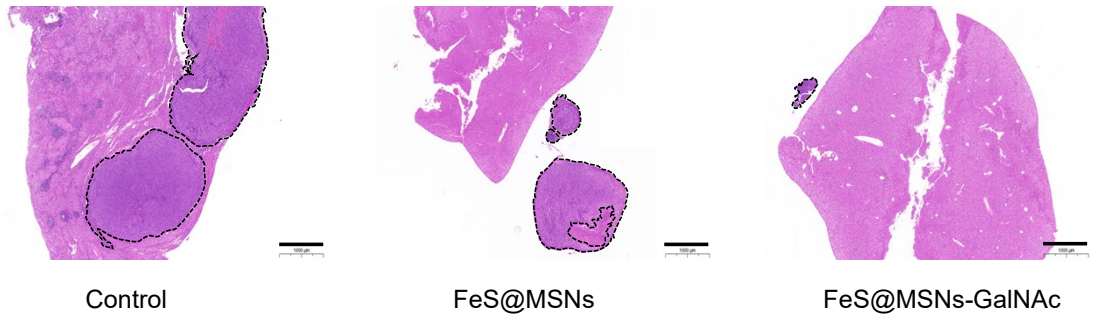

Figure S16. H&E staining images of liver tissues after various treatments. Scale bar: 1000 μm

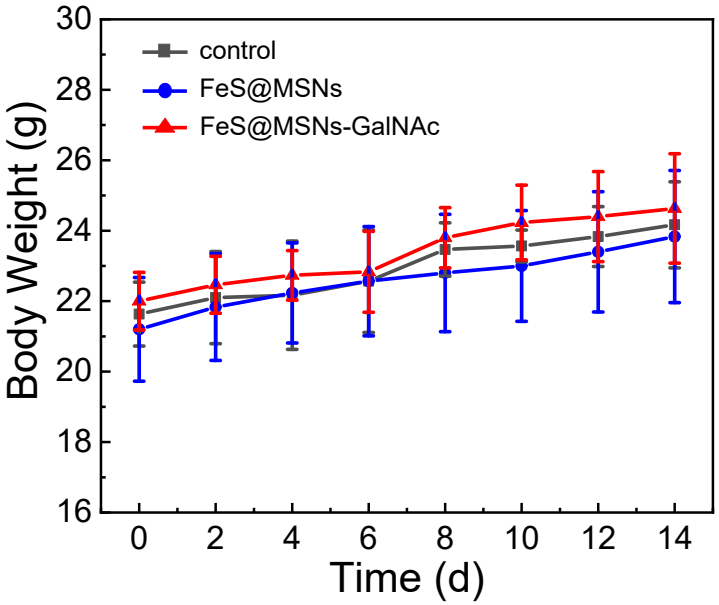

Figure S17. Body weights of C57 mice with different treatments during 14 days.

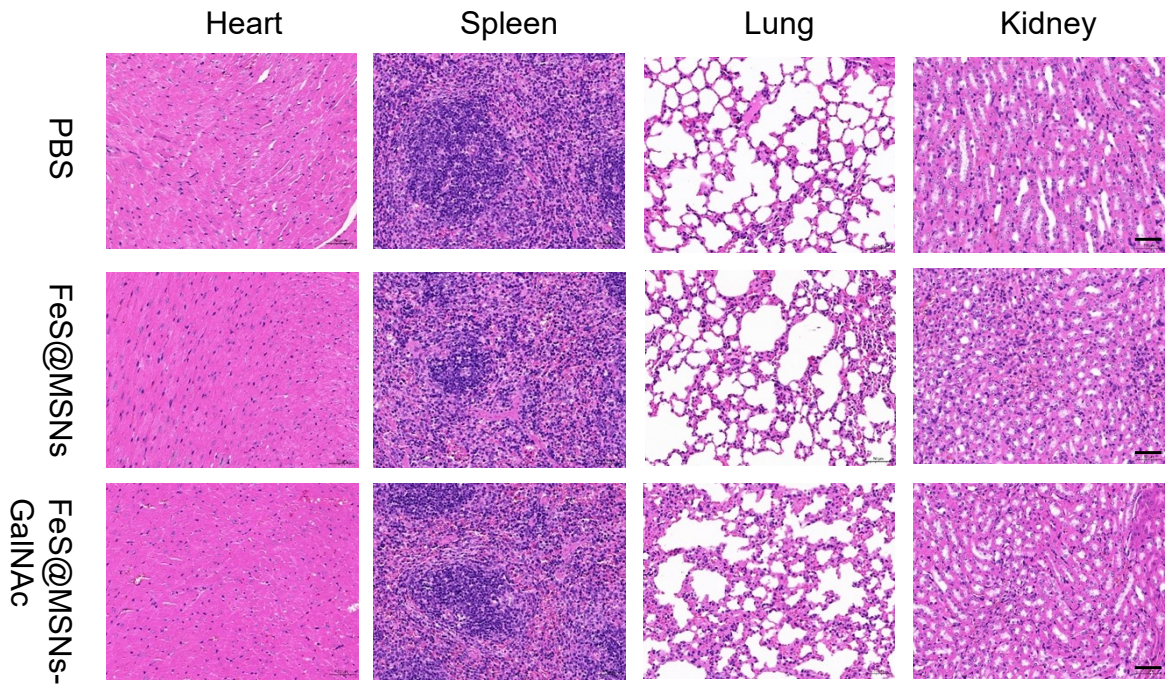

Figure S18. H&E staining of the five major organs (heart, spleen, lung and kidney) intravenously injected with different treatments after 14 days. All scale bars are 50 μm.

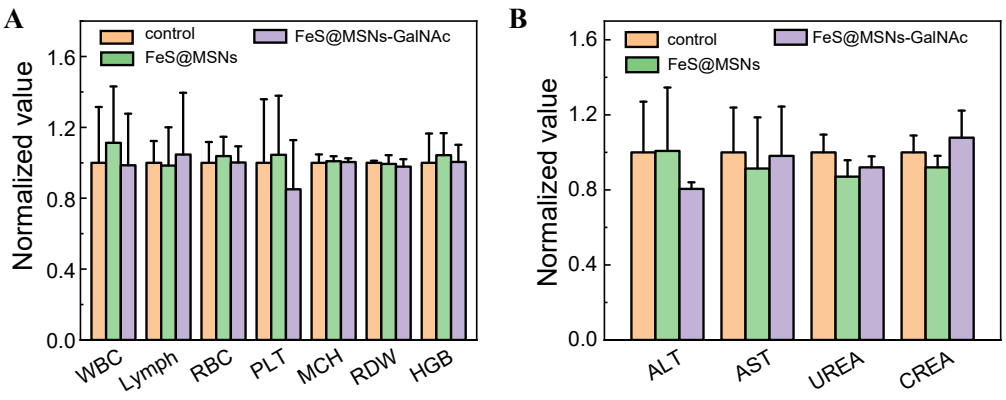

Figure S19. Blood routine (a) and biochemical indexes (b) of C57 mice with different treatments.