Electronic Supplementary Information

Hyaluronic Acid-Mediated Multifunctional Iron Oxide-Based MRI Nanoprobes for Dynamic Monitoring of Pancreatic Cancer

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Part of experimental details:

Materials

HA was purchased from Zhenjiang Dong Yuan Biotechnology Corporation (Zhenjiang, China). PEG monomethyl ether with a single carboxyl group at the end (*m*PEG-COOH, MW = 2 000) was from Shanghai Aicheng Biotechnology Corporation (Shanghai, China). FITC, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS) were supplied by J&K Chemical Ltd. (Shanghai, China). Ferrous chloride tetrahydrate ($FeCl_3 \cdot 6H_2O$> 99%), ammonia (25-28% NH_3 in water solution), triethylamine, acetic anhydride, dimethyl sulfoxide (DMSO), PEI (MW = 25 000) and all the other chemicals and solvents were purchased from Aldrich (St. Louis, MO) and used as received. The CCK8 Kit was acquired from Tokyo Dojindo (Japan). MIAPaCa-2 cells, a human pancreatic cancer cell line was obtained from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). DMEM medium, fetal bovine serum (FBS), penicillin, and streptomycin were from Shanghai Aicheng Biotechnology Corporation (Shanghai, China). The water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with a resistivity higher than 18.2 MΩ.cm. Regenerated cellulose dialysis membranes (MWCO =1000) were acquired from Fisher.

Characterization techniques: UV-Vis spectroscopy was performed using a Lambda 25 UV-Vis spectrophotometer (PerkinElmer, Boston, MA). Thermogravimetric analysis (TGA) was carried out using a TG 209 F1 (NETZSCH Instruments Co., Ltd., Germany) thermogravimetric analyzer. The samples were heated from room temperature to 650℃at a rate of 20℃/min under a nitrogen atmosphere. Zeta potential and dynamic light scattering (DLS) measurements were carried out using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, U.K.) equipped with a standard 633 nm laser. Transmission electron microscopy (TEM) was performed with a JEOL 2010F analytical electron microscope (JEOL, Japan) operating at 200 kV. TEM samples were prepared by depositing a dilute particle suspension (5 μL) onto a carbon-coated copper grid to air-dry before measurements. The Fe concentration of the particle solution was analyzed using a Leeman Prodigy Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) system (Hudson, NH03051). T_2 relaxometry was performed by a 0.5-T NMI20-Analyst NMR Analyzing and Imaging system (Shanghai Niumag Corporation, China). The samples were diluted in water with Fe concentrations in the range of 0.009-0.14 mM. The instrumental parameters were set at a

point resolution of 156 mm×156 mm, section thickness of 0.6 mm, TR of 4000 = ms, TE = 60 ms, excitation number = 1. The T_2 relaxivity was calculated by a linear fit of the inverse T_2 ($1/T_2$) relaxation time as a function of Fe concentration.

Cellular uptake: Flow cytometry was used to quantify the uptake of nHA-Fe_3O_4 NPs or HA-Fe_3O_4 NPs by MIAPa-Ca-2 cells. MIAPa-Ca-2 cells were seeded in 12-well plates at a density of 2×10^5 cells per well in 1 mL of DMEM medium and incubated at 37 ℃ and 5% CO_2. After overnight incubation to bring the cells to confluence, the medium was replaced with 1 mL fresh medium containing PBS buffer (control), nHA-Fe_3O_4 NPs or HA-Fe_3O_4 NPs at different Fe concentrations (0, 10, 20, 60, 80 and 100 μg/mL). After 4 h incubation, the cells were washed 3 times with PBS, trypsinized, centrifugated, and resuspended in 1 mL PBS before flow cytometry analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, USA). The FL1-fluorescence of 10,000 cells was measured and the measurement was repeated 3 times.

The cellular uptake of the HA-Fe_3O_4 NPs was also confirmed by confocal microscopy (Carl Zeiss LSM 700, Jena, Germany) and Prussian Blue Staining. In brief, coverslips with a diameter of 14 mm were pretreated with 5% HCl, 30% HNO_3, and 75% alcohol and then fixed in a 12-well tissue culture plate. 5×10^4 MIAPa-Ca-2cells were seeded into each well with 1 ml DMEM medium and cultured at 37℃and 5% CO_2 for 24 h to allow the MIAPaCa-2cells to attach onto the coverslips. The medium was replaced with fresh medium containing PBS buffer (control), nHA-Fe_3O_4 NPs or HA-Fe_3O_4 NPs at a Fe concentration of 60 μg/mL, and the cells were incubated at 37℃and 5% CO_2 for 4 h. After that, the cells were washed 3 times with PBS, fixed with glutaraldehyde (2.5%) for 15 min at 4℃, and counterstained with Hoechst 33342 (1 mg/mL) for 15 min at 37℃using a standard procedure. The cells on the coverslips were imaged using a 63× oil-immersion objective lens.

***In vitro* MR imaging of MIA PaCa-2 cells:** MIAPaCa-2 cells were seeded in 6-well plates at a density of 3×10^6 cells per well in 2 mL of DMEM medium and incubated at 37℃ and 5% CO_2. After incubating overnight to bring the cells to confluence, the medium was replaced with 2 mL fresh medium containing PBS buffer (control), nHA-Fe_3O_4 NPs or HA-Fe_3O_4 NPs at different Fe concentrations (5-80 μg/mL) and the cells were incubated at 37℃and 5% CO_2 for 4 h. After that, the cells were washed with PBS 3 times, trypsinized, centrifuged, and resuspended in 1 mL PBS (containing 0.5% agarose) in a 2 mL Eppendorf tube before MR imaging. T_2 MR imaging of the cell suspension in each sample tube was performed by a 0.5 T NMI20-Analyst NMR Analyzing and Imaging system (Shanghai

Niumag Corporation) using a wrist receiver coil with a CPMG sequence (TR = 6000 ms, TE = 50 ms, pointer solution =156 mm×156 mm, section thickness =0.6 mm, and number of excitation=1).

***In vivo* biodistribution and acute toxicity assessment:** The above tumor-bearing BALB/c nude mice were a euthanized 24 hour after MR scanning. Then, the heart, liver, spleen, lung, kidney, and tumor were extracted and weighed. The organs were then cut into 1-2 mm^2 pieces and digested by aqua regia (nitric acid/hydrochloric acid, v/v =1:3) for 2 days. Then Fe content in different organ pieces was determined by ICP-AES. For comparison, the mice that didn't receive an injection and the mice injected with the nHA-Fe_3O_4 NPs were used as controls.

The acute biological safety assessment was performed by the routine blood-biochemical blood testing. In brief, female SCID/Slac mice (4 weeks old, body weight ≈ 200 g) were randomly assigned to four groups (n = 5 mice in each group). After postinjection nHA-Fe_3O_4 NPs or HA-Fe_3O_4 NPs (40 mg/kg, in 100 μL saline), the blood samples and major organs were collected on 0 and 10 days. The liver function indicators including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AKP), as well as kidney function indicators involving blood urea nitrogen (BUN) and creatinine (CREA), were examined.

Statistical analysis: One-way ANOVA statistical analysis was performed to evaluate the significance of the experimental data. A p-value of 0.05 was selected as the level of significance, and the data were indicated with (*) for $p< 0.05$, (**) for $p< 0.01$, and (***) for $p< 0.001$, respectively.

Supplementary Tables:

Table S1. Zeta-potential and hydrodynamic size of the Fe_3O_4-PEI, nHA-Fe_3O_4 and HA-Fe_3O_4 NPs, respectively.

Sample	Zeta potential (mV)	Hydrodynamic size (nm)	PDI
Fe_3O_4-PEI	+32.1 ± 0.3	240.9± 3.8	0.27±0.02
nHA-Fe_3O_4 NPs	+12.2 ± 0.6	289.3±7.2	0.21±0.01
HA-Fe_3O_4 NPs	-16.6 ± 0.6	306.1±2.9	0.47±0.03

Supplementary Figures:

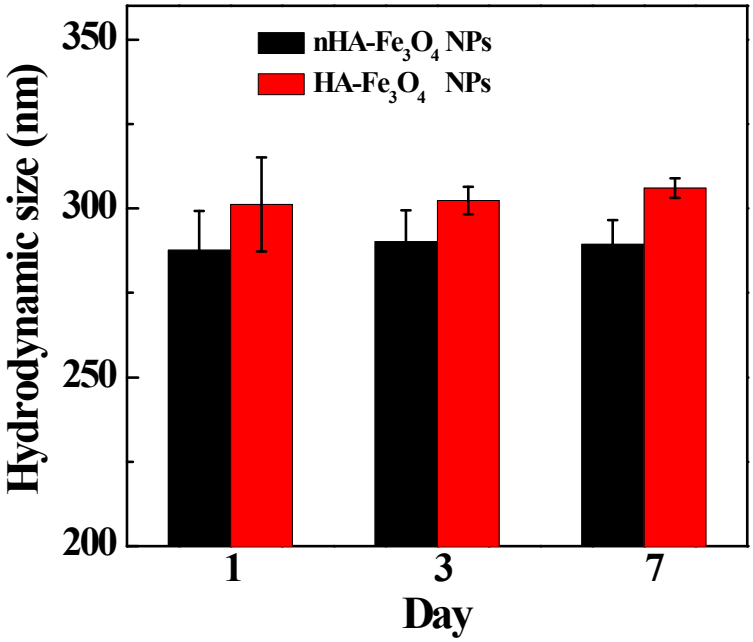

Figure S1. Hydrodynamic size of the nHA-Fe_3O_4 NPs and HA-Fe_3O_4 NPs (1 mg/mL, dispersed in water) at different time periods.

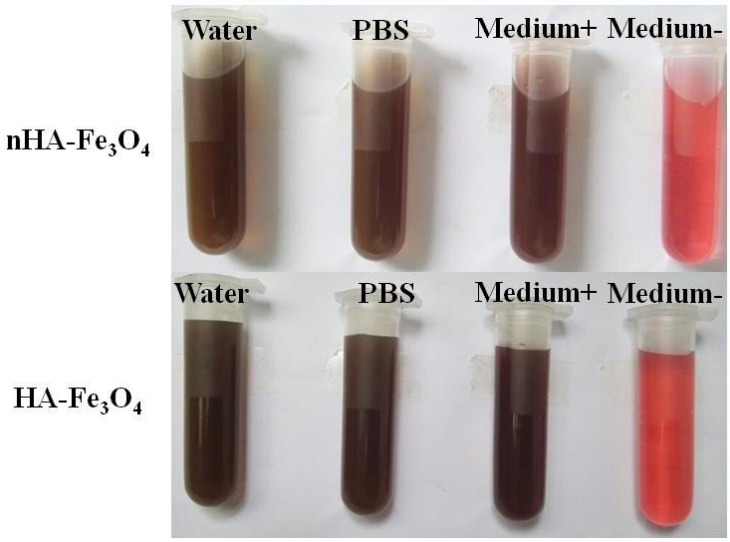

Figure S2：Photographs of the nHA-Fe_3O_4 NPs and HA-Fe_3O_4 NPs dispersed in water, PBS, FBS added cell culture medium and cell culture medium for 7days.

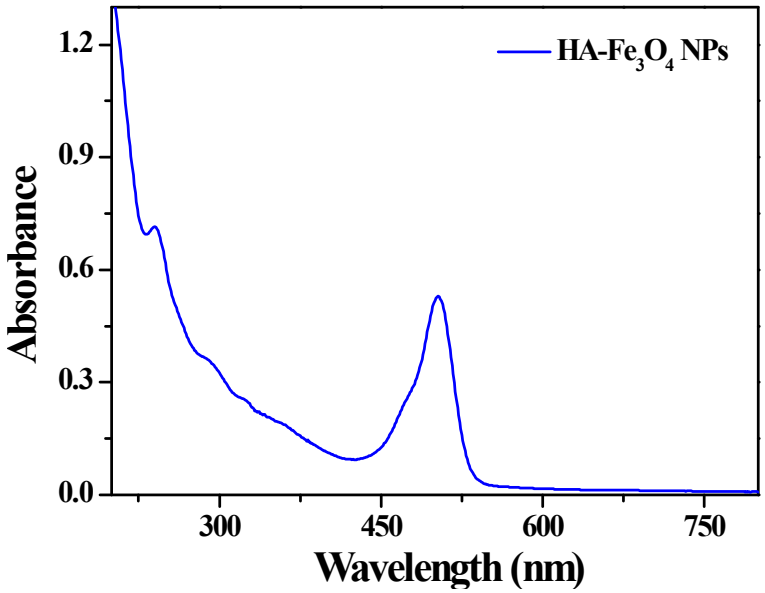

Figure S3. UV-Vis spectrum of HA-Fe_3O_4 NPs (1 mg/mL, dispersed in water).

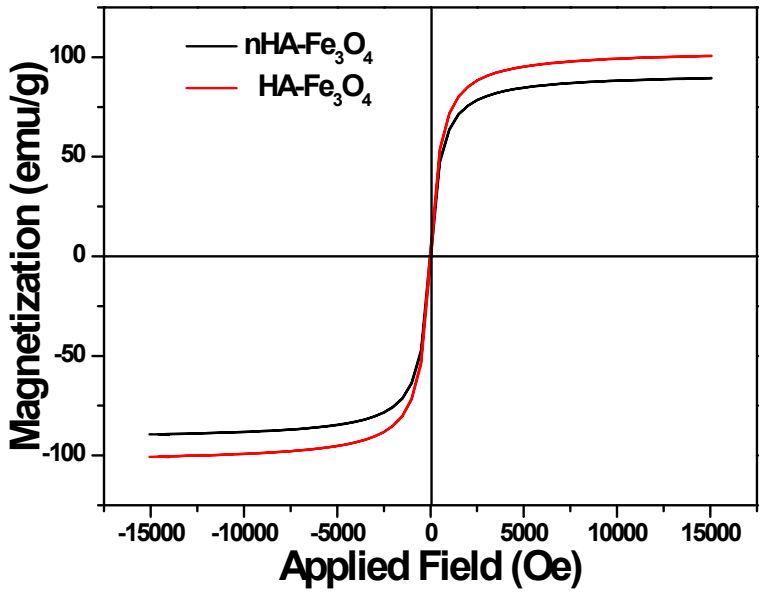

Figure S4. Normalized field-dependent magnetization curves (*M–H*) at 300 K for nHA-Fe_3O_4 and HA-Fe_3O_4 NPs, respectively.

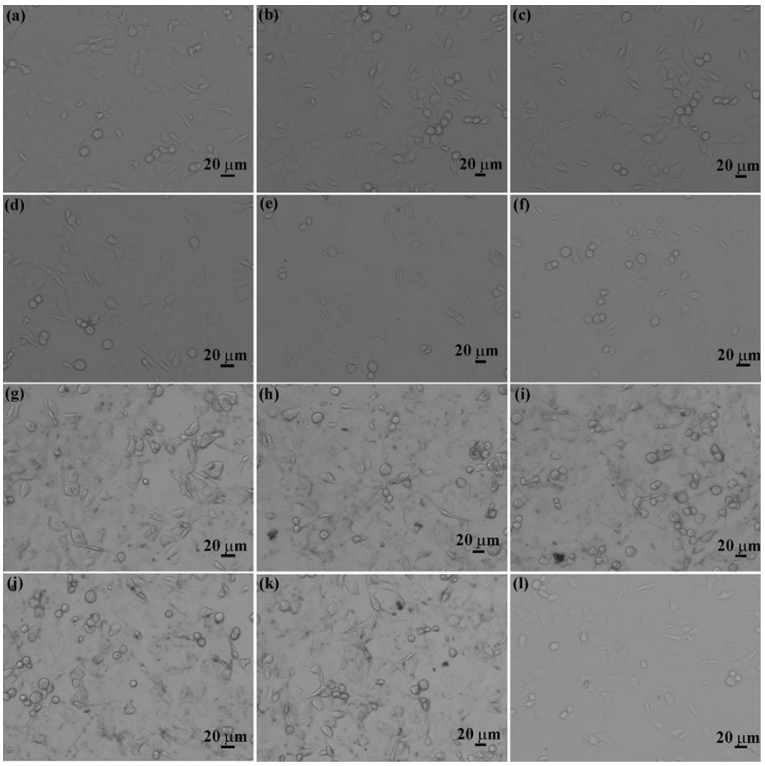

Figure S5. Micrographs of MIAPaCa-2 cells treated with PBS (a, l), nHA-Fe_3O_4 NPs at the Fe concentration of 10 (b), 20 (c), 60 (d), 80 (e), and 100 (f) μg/mL, HA-Fe_3O_4 NPs at the Fe concentration of 10 (g), 20 (h), 60 (i), 80 (j), and 100 (k) μg/mL for 24 h, respectively.

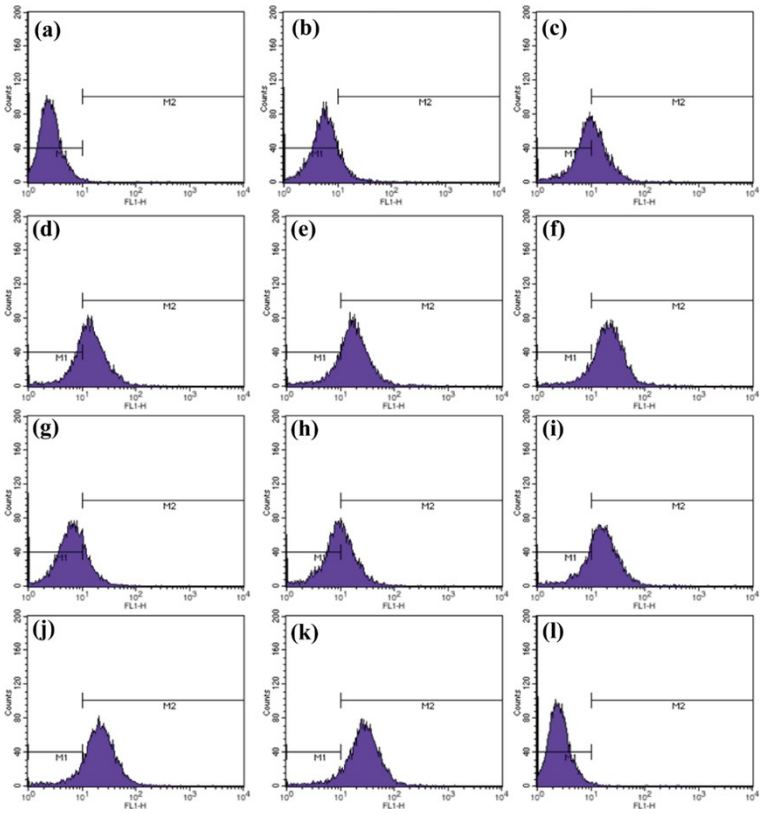

Figure S6. Flow cytometric analysis of MIAPaCa-2 cells treated with the nHA-Fe_3O_4 NPs at a Fe concentration of 0 (a), 10 (b), 20 c), 60 (d), 80 (e), and 100 (f) μg/mL or HA-Fe_3O_4 NPs at a Fe concentration of 10 (g), 20 (h), 60 (i), 80 (j), 100 (k) and 0 (l) μg/mL, respectively for 4 h. (a, I).

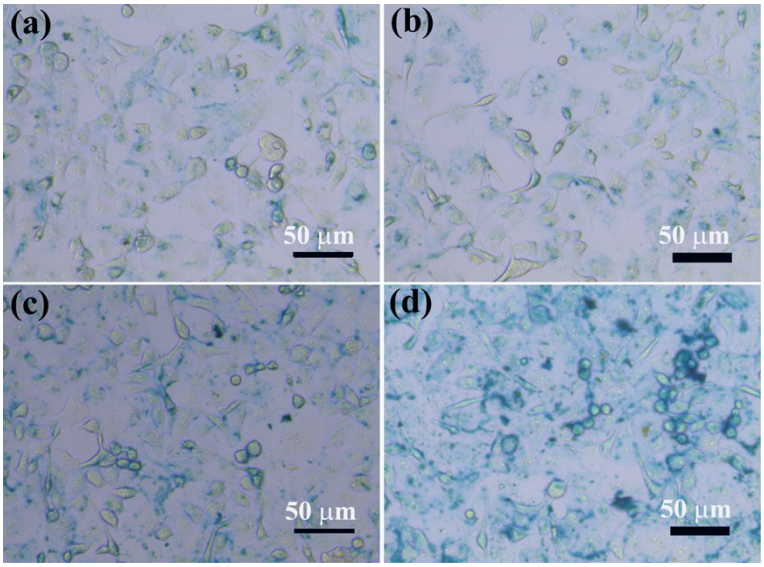

Figure S7. Prussian blue staining of MIAPaCa-2 cells after treated with the nHA-Fe_3O_4 NPs at the Fe concentration of 10 (a) and 50 (b) μg/mL or HA-Fe_3O_4 NPs at the Fe concentration of 10 (c), 50 (d) μg/mL, respectively for 4 h.

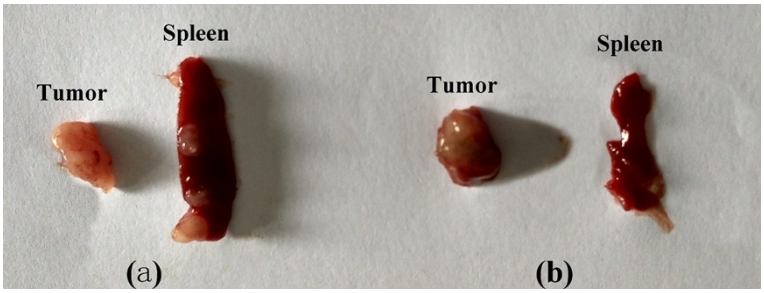

Figure S8. Photographs of the tumor, spleen of the HA-Fe_3O_4 NPs (a) and nHA-Fe_3O_4 NPs (b) mice after 7 days.

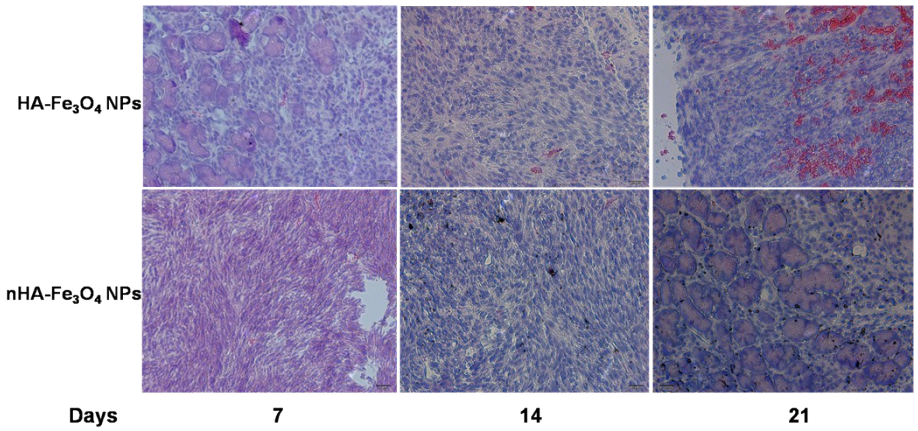

Figure S9. HE staining of tumor at 7, 14, 21days after injection of HA-Fe_3O_4 NPs and nHA-Fe_3O_4 NPs (0.6 mg Fe per mouse, in 0.3 mL PBS).

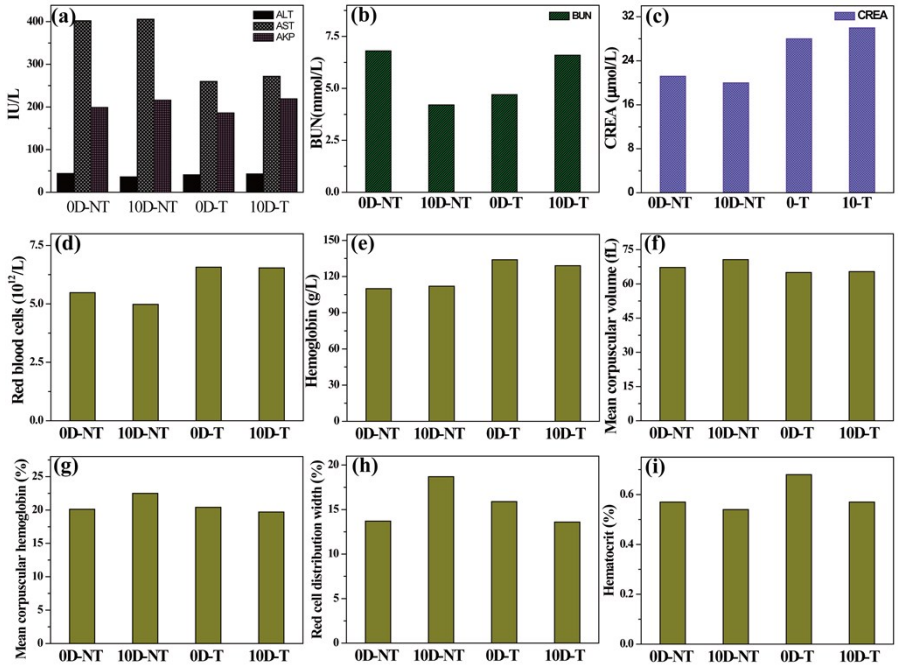

Figure S10. Short-term *in vivo* acute toxicity studies of nHA-Fe_3O_4 NPs (Nontargeted, NT) or HA-Fe_3O_4 NPs (Targeted, T) saline solution at a dosage of Fe [40 mg/Kg] and draw blood at 0 and 10 days (n=3). (a) The blood levels of ALT, AST and AKP from control and treated mice as liver function markers. (b) BUN levels and (c) CREA levels in the blood representing kidney functions. (d-i) The complete blood panel data: (d) red blood cells; (e) hemoglobin; (f) mean corpuscular volume; (g) mean corpuscular hemoglobin; (h) red cell distribution width; (i) hematocrit. The liver function indexes including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphtase (AKP), as well as kidney function indicators involving blood urea nitrogen (BUN) and creatinine (CREA), are all at the normal levels. The blood indexes in the treatment groups have no significant differences with the control group.

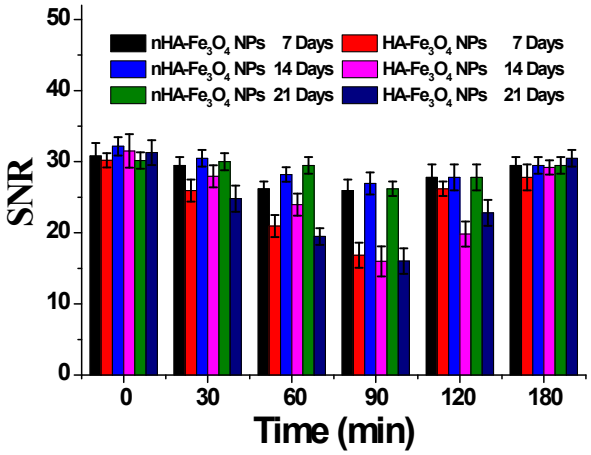

Figure S11. MR SNR of in-situ pancreatic carcinoma tumors after intravenous injection of nHA-Fe_3O_4 or HA-Fe_3O_4 NPs ([Fe] 1 mg/mL, in 200 μL saline) at different time points post-injection.