Supporting Information

# Mn-loaded apolactoferrin dots for *in vivo* MRI and NIR-II cancer imaging

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**General methods**.
All chemicals were purchased from commercial sources (such as Aldrich, Energy Chemical, TCI) and used without further purification. lactoferrin (Lf) was provided by Wuhan Heyuan biotechnology Co. Ltd. Deionized water was used in all experiments. UV-Vis absorbance of the probe was recorded on a PerkinElmer Lambda 25 UV-Vis spectrophotometer. NIR fluorescence spectrum was recorded on an Applied Nano Fluorescence spectrometer at room temperature with an excitation laser source of 785 nm.

**Characterization.**
TEM images were acquired on a JEM-2100F electron microscope operating at 200 kV. STEM image was performed on a FEI-Magellan 400 electron microscope operating at 30 kV. Standard TEM and STEM samples were prepared by dropping dilute products onto carbon coated copper grids. Dynamic light scattering (DLS) measurement was conducted on Zetasizer Nanoseries (Nano ZS90). Ultraviolet-Visible (UV-Vis) spectra were recorded on a UV-3101PC Shimadzu spectroscope. Blood indicators were measured by Chemray-240 Automated Chemistry Analyzer (Shenzhen Rayto Life and Analytical Sciences). IR spectrum were performed on IR tracer-100 (Shimadzu Fourier transform infrared spectrometer) with the scanning range from 4000 $cm^{-1}$ to 400 $cm^{-1}$. NIR-II images were performed in the NIR-II imaging system purchased from Suzhou NIR-Optics Technologies CO., Ltd. The MR imaging experiments were performed on a 7.0-T clinical MRI instrument (GE Signa 7.0T)

**Synthesis of H-dot**

***Synthesis of Apo-Lf***
200 mg lactoferrin was dissolved in 4 mL deionized water and dialyzed with 0.1 M sodium citrate solution at room temperature for 24 h. The protein solution was then dialyzed against deionized water to remove excess citric acid.

***Synthesis of $Mn^{2+}$-Apo-Lf***
50 mg of Apo-lactoferrin and 150 mg of $MnCl_2$ were dissolved in 2 mL 0.1 M $NaHCO_3$ solution, (the **Apo-Lf** concentration: manganese ion concentration = 1:3), the solution was stirred at 30° C for 12 h and then dialyzed with buffer solution for 24 h to remove excess ions not complexed with **Apo-Lf**. Afterwards, it was dialyzed with deionized water for 24 h to remove impurities.

***Synthesis of $Mn^{2+}$-Apo-Lf-PEG***
To a solution of **$Mn^{2+}$-Apo-Lf** (100 mg, 0.00125 mmol) in DMF (dry), EDCI (1.2 mg, 0.00625 mmol) and NHS (0.72 mg, 0.00625 mmol) were added, the mixture was stirred at room temperature for 2 h. Then $NH_2$-$PEG_{5000}$-$OCH_3$ (31.25mg, 0.00625 mmol) and DIPEA were added to the above reaction mixture under an argon atmosphere. The solution was stirred at 35

°C for 4 h. The crud product was dialyzed for about 24 h using dialysis bags with molecular weight cut-off (MWCO) of 10 kDa.

***Synthesis of $Mn^{2+}$-Apo-Lf-PEG-CH1055***

Compound **CH1055** (3.2 mg, 0.0033 mmol), HBTU (1.25 mg, 0.0033 mmol) were dissolved in 500 μL DMF (dry) under an argon atmosphere and the mixture was stirred at room temperature for 1 h. Then **$Mn^{2+}$-Apo-Lf-PEG** (69.3 mg, 0.00066 mmol) and DIPEA 50 μL were added into the above reaction mixture and the solution was stirred at 35 °C for 6 h. The crud product was dialyzed for about 24 h to remove impurities.

***Synthesis of H-dot***

**$Mn^{2+}$-Apo-Lf-PEG-CH1055** (50 mg) was dissolved in 2 mL of purified water. In order to form nanoparticles, 4.0 mL (99%, v/v) ethanol was added at a rate of 1 mL/min under constant stirring at room temperature. After the desolvation process, 8% glutaraldehyde solution (50 μL) was added to cross-link the desolvated **H-dot**. The cross-linking process was performed for 24 h under continuous stirring at room temperature. Afterwards, particles were purified by centrifugation steps at a speed of 16100g for 12 min twice and the solution was freeze-dried to get pale blue-green powder.

**Cell line and animal model** Human malignant glioblastoma cell line U87MG and human liver cancer cell line HepG2 were purchased from the China Center for Type Culture Collection (CCTCC). U87MG cells and HepG2 cells were cultured (37° C, 5% $CO_2$) in DMEM medium containing 10% fetal bovine serum and 1% penicillin streptomycin. The cells were routinely harvested by treatment with a trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.25 %). The U87MG and HepG2 tumor models were established by intra-tibial injection of these cells (~$5 \times 10^6$ in 80 μL of PBS) into the right leg of six-week-old female Balb/c nude mice (Beijing Vital River Laboratory Animal Technology Co., Ltd.) under anesthesia using isoflurane. The tumor bearing mice were imaged when the tumor volume reached 400-800 $mm^3$ (about 4-6 weeks after inoculation). All animal studies were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee and approved by The Institutional Animal Care and Use Committee (IACUC), Wuhan University Center for Animal Experiment, Wuhan, China.

**Cell cytotoxicity assay** *In vitro* cytotoxicity was assessed by the standard MTT assay. A number of 5000 U87MG or HepG2 cells per well were seeded in a 96-well plate with DMEM media (100 μL per well) overnight. To each well, 100 μL solution of Lf, **$Mn^{2+}$-Apo-Lf-PEG** and **H-dot** at concentration of 0, 0.25, 0.5 1, 1.5 and 2 mg/mL in DMEM medium was added and incubated for 24 h. Then 20 μL MTT solution (5 mg/mL in PBS buffer solution) was added to per well and cells were incubated for 4 h at 37°C. After that, the medium was removed, 150 μL DMSO per well was added to dissolve the intracellular purple formazan crystals. At last, Absorbance at 490 nm was measured to calculate the viability of cell growth. The following formula was used to calculate the viability of cell growth: Cell viability (%) = (mean of Absorbance value of treatment group /mean of Absorbance value of control) × 100.

***In vivo* NIR-II fluorescence imaging of tumors** 200 μL portions of **H-dot** containing 1 mg or 2 mg was injected intravenously into tumor bearing nude mice. NIR-II fluorescence signals were acquired using a two-dimensional InGaAs array (Suzhou Optics) and thus fluorescence images were collected. Excitation light was provided by the 808 nm diode laser. The emitted light from animals was filtered through a 1000 nm long pass filter for NIR-II imaging coupled with an InGaAs camera.

**MR imaging** The MR imaging experiments in water solutions were performed on a 7.0-T clinical MRI instrument (GE Signa 7.0T) and the pulse sequence used was a T1-weighted with the following parameters: TR/TE = 800/11 ms; FOV: 2.0 cm; NEX: 1; SI: 1.00 / 1.00 mm; FA: 180 deg. *In vivo* MR imaging experiments were performed on a 7.0-T clinical MRI instrument (GE Signa 7.0T) and the pulse sequence used was a T1-weighted with the following parameters: TR/TE = 800/11 ms; FOV: 3.0 cm; NEX: 1; SI: 0.50 / 0.50 mm; FA: 180 deg.

***Ex vivo* biodistribution analysis** For tumor imaging, at 48 h after injection of **H-dot** into U87MG (or HepG2) tumor-bearing BALB/c mice, mice (n = 3) were sacrificed, then the major organs and tumor tissues were collected for imaging study. *Ex vivo* organs and tumor tissues were imaged in the NIR-II imaging system which used for *in vivo* fluorescent imaging (3.5 W, 100 ms, LP 1000).

***In vivo* pharmacokinetics and biodistribution of H-dot** Female nude mice were used for pharmacokinetics and biodistribution studies with 3 mice for each experiment. All mice were intravenously injected with **H-dot** in PBS buffer (200 μL, 2 mg). At time points of 0.25, 0.5, 1, 3, 6, 12, 24, 48 and 72 h p.i., 20 μL blood were collected using a capillary tube stuck into the corner of one eye, and then diluted with heparin sodium solution. The concentration of the **H-dot** in blood was estimated by calculating the $Mn^{2+}$ concentration in blood using graphite furnace atomic absorption spectrometry. Biodistribution was performed after 48 h p.i. with different concentrations of **H-dot** (200 μL, 2 mg or 200 μL, 4 mg). Main organs including heart, liver, spleen, lung and kidney were collected. The organs were weighed and then dissolved in 5 mL digest solution ($HNO_3$ : $HClO_4$ = 2:3). The samples were heated to 260 °C for 5 h. The reaction stopped when the solution became clear and then cooled down to room temperature. Each of the resulting solutions was diluted by deionized water to 3 mL, and subsequently analyzed by graphite furnace atomic absorption spectrometry to determine the concentration of $Mn^{2+}$ in each sample. The calculation formula of biodistribution is: % ID/g= (The amount of Mn atom in organs) / (The amount of Mn atom in injected solution × the mass of organs) × 100%.

**Measure of blood toxicity** Each mouse was injected into 100 μg/g **H-dot**. The blood was taken from mice eyeball at 24 h and 144 h respectively into the 1.5 mL EP tubes. After the blood was clearly stratified, it was centrifuged at a temperature of 4 ℃ with a rotation speed of 3000 RPM for 10 min. The serum was then taken and analyzed by Chemray-240 Automated Chemistry Analyzer to determine the blood hepatic toxicity of ALT, AST, APL, T-bill and renal toxicity creatinine and BUN.

**Histological analysis** Major organs and tumors were taken from the tumor-bearing mice were taken from the tumor-bearing mice and fixed with EDTA/formalin solution. After embedment and section, tissue samples were further stained with hematoxylin and eosin and subsequently imaged using a NIKON Eclipse ci microscope with 40× and 200× magnification.

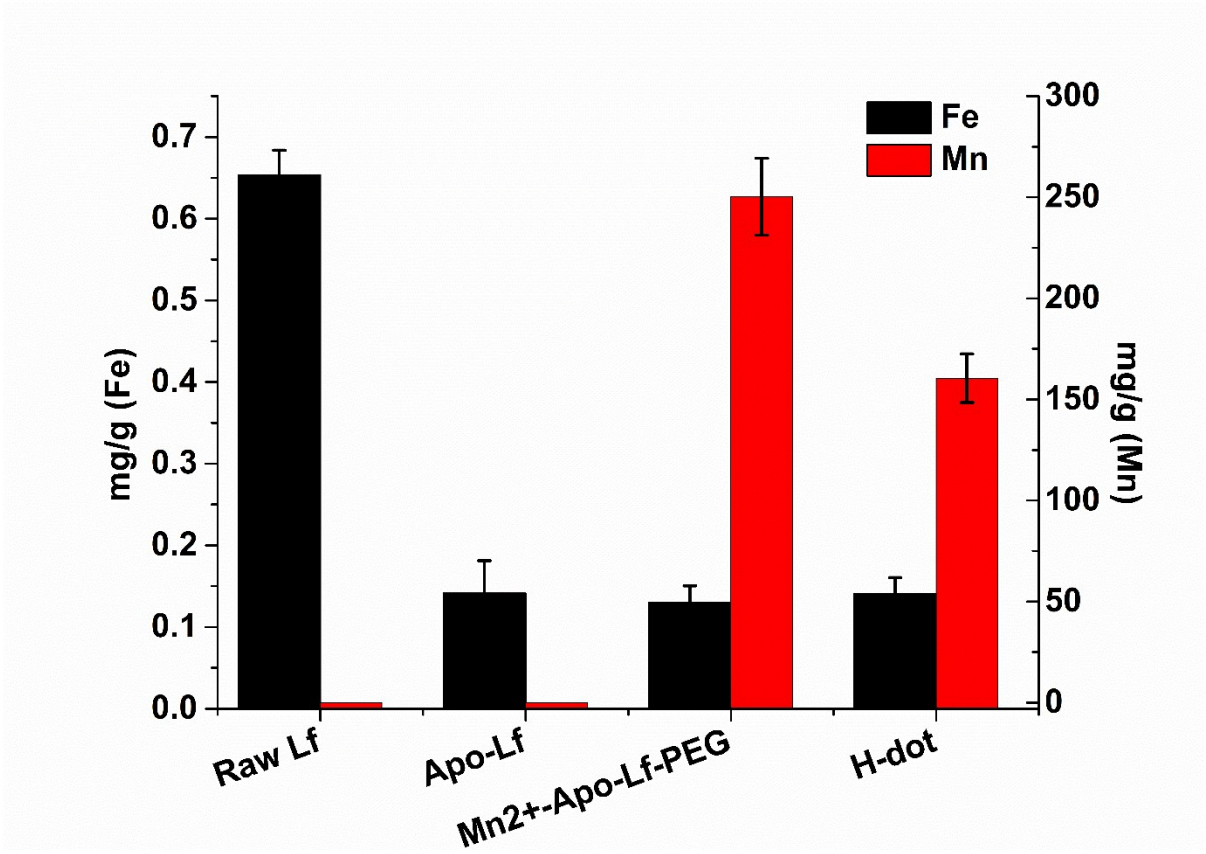


**Fig. S1:** Different ion concentrations in different compounds. (Ordinate unit means $m_{ion}$ : $m_{compound}$).

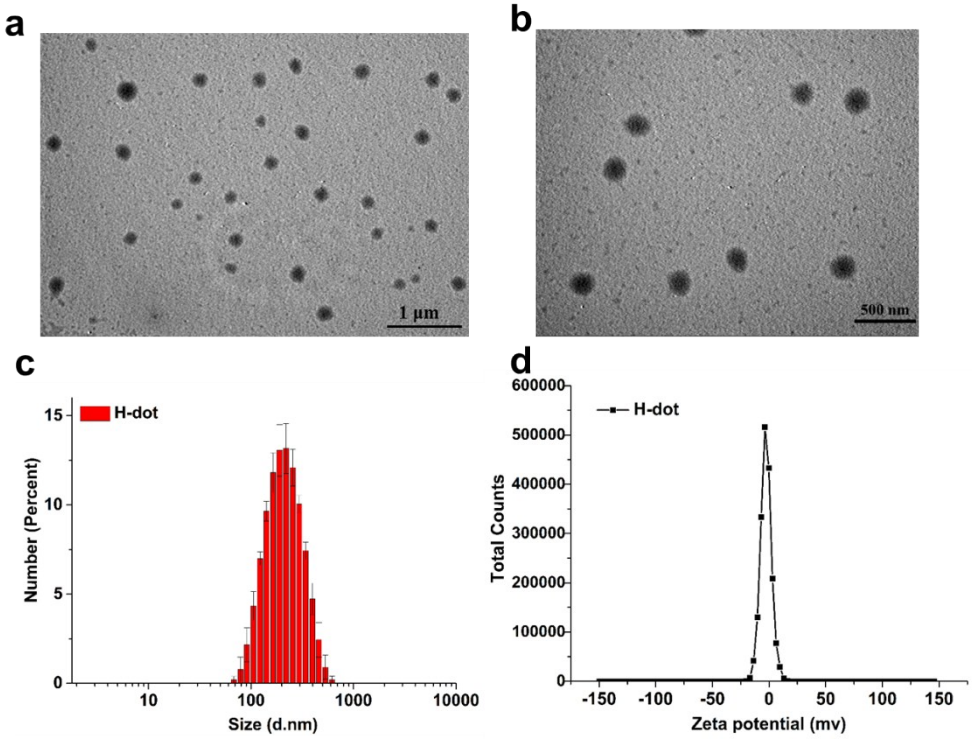


**Fig S2:** (a) and (b) TEM images of **H-dot**; (c) hydrodynamic particles size distribution and (d) zeta potential of **H-dot**.

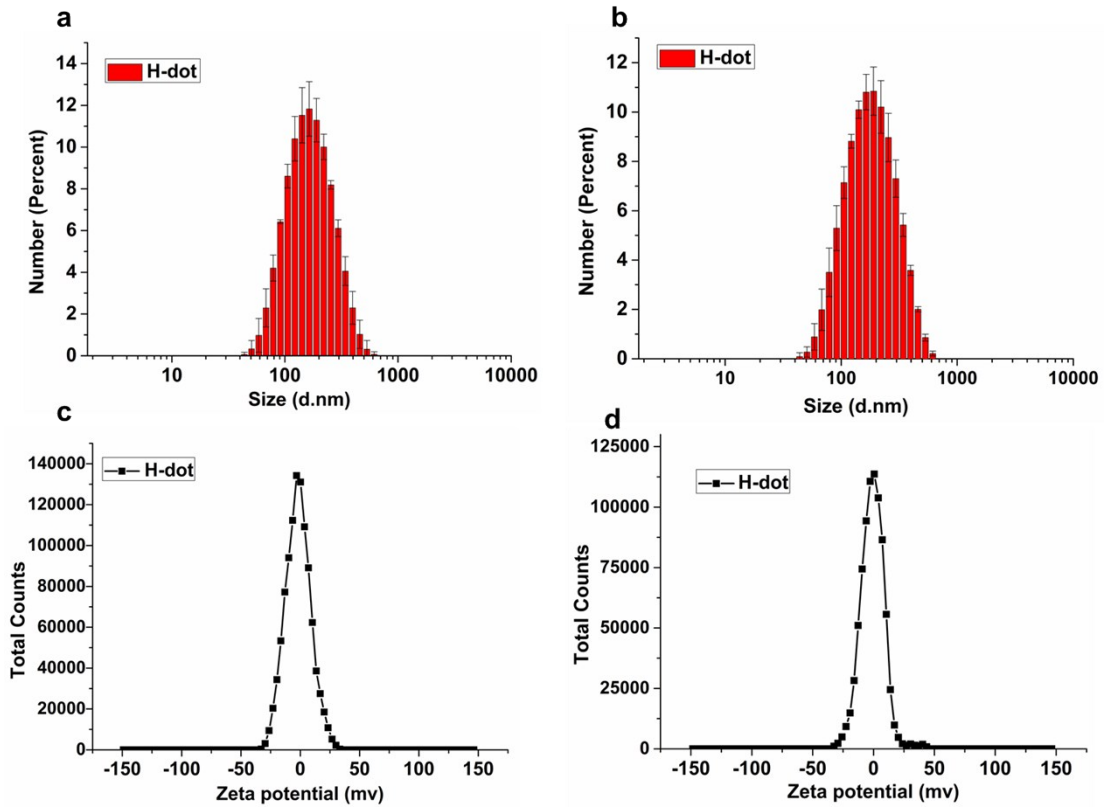


**Fig. S3:** (a) and (b) the size distribution of **H-dot** in PBS and DMEM medium based on dynamic light scattering measurement (DLS) after 6 h (3 measurements, hydrodynamic size ~134.5 nm and ~143.2 nm); (c) and (d) Zeta potential of **H-dot** in PBS and DMEM medium (-1.56 mV and -0.9 mV) after 6 h.

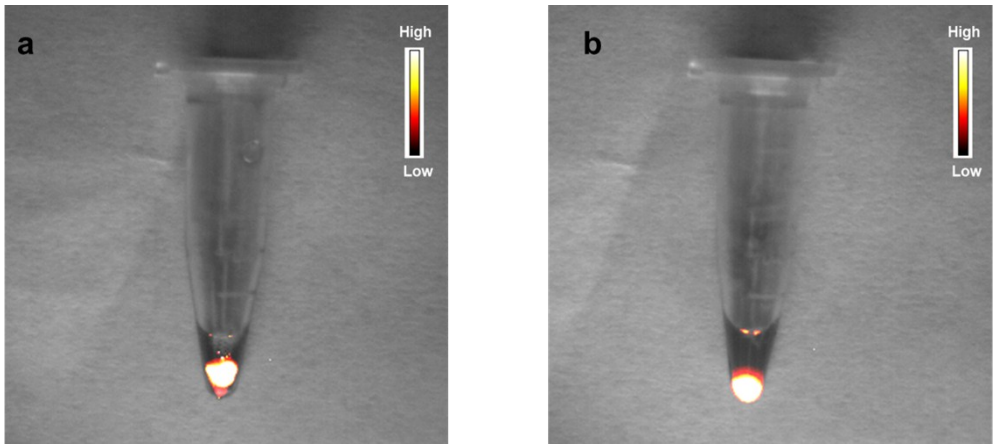


**Fig. S4:** Representative *in vitro* cellular uptake image (80 mW $cm^{-2}$, 808 nm excitation, 1000 nm LP and 50 ms) of (a) HepG2 and (b) U87MG cells incubated with **H-dot** (100 µg/mL) for 6 h.

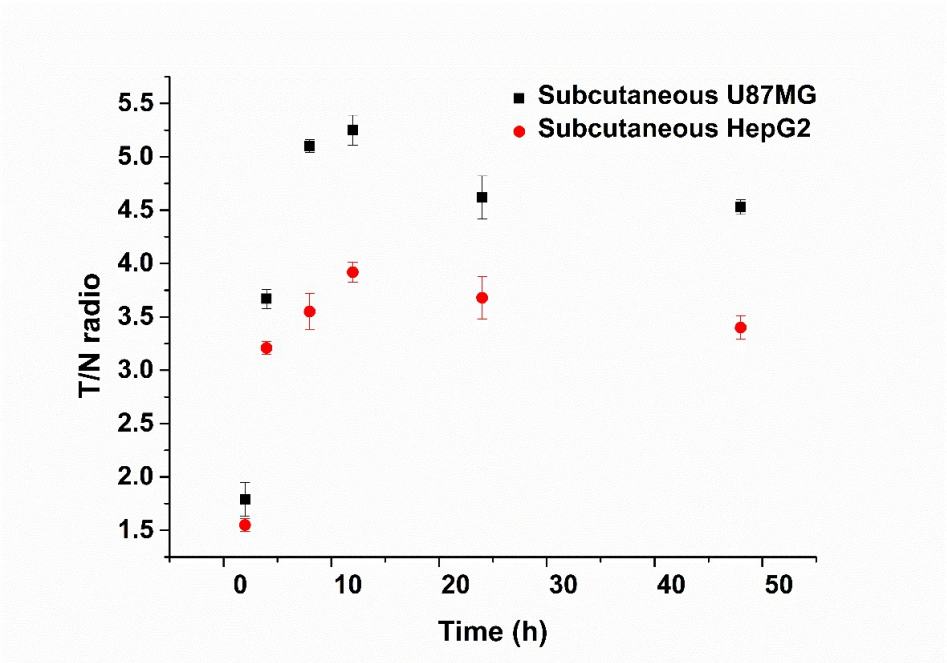


**Fig. S5:** Tumor signals/ Normal tissue signals (T/N) ration of subcutaneous U87 xenografts and HepG2 xenografts in NIR-II imaging.

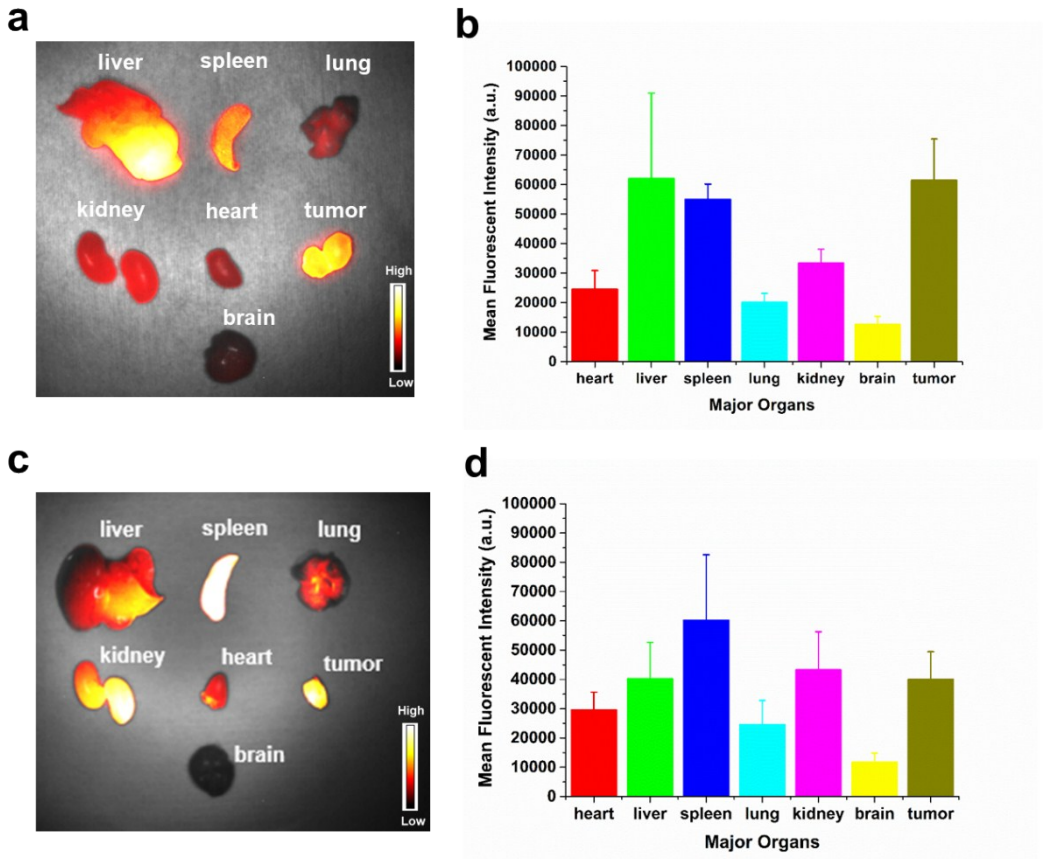


**Fig. S6:** The biodistribution of **H-dot** in (a) U87MG tumor-bearing mice and (c) HepG2 tumor bearing mice at 48 h under 808 nm excitation (1000 LP and 100 ms exposure time). Quantitative analysis of mean fluorescence intensity for the ex vivo images of different organs in Figure S6b and S6d.

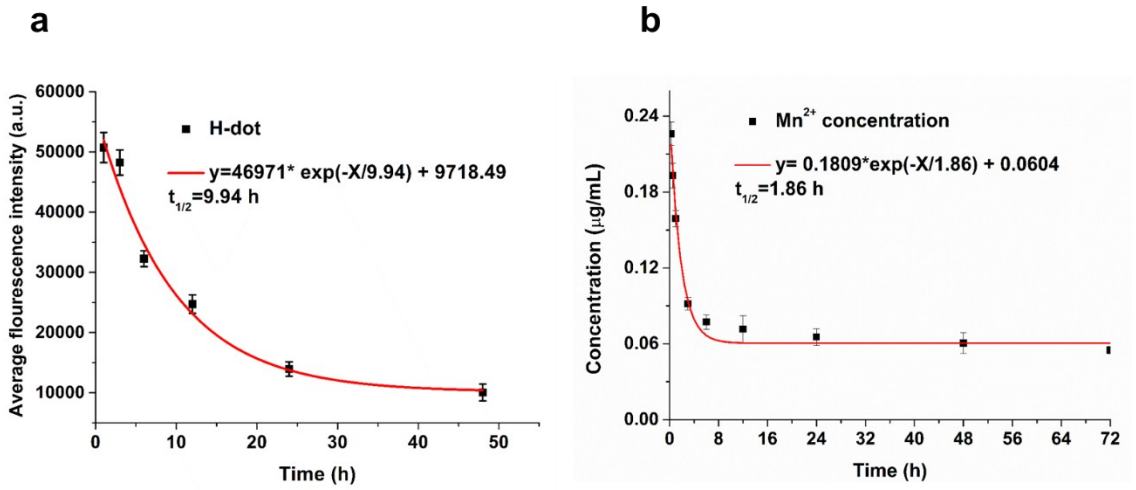


**Fig. S7**: (a) Blood circulation half-life curve of **H-dot** in mice. The circulation half-life is determined to be about 9.94 h by fitting the data (1, 3, 6, 12, 24, 48 h) to a first-order exponential decay (n = 3). (b) Blood circulation half-life curve of $\mathbf{Mn^{2+}}$ concentrations mice. The circulation half-life is determined to be about 1.86 h by fitting the data (0.25, 0.5, 1, 3, 6, 12, 24, 48, 72) to a first-order exponential decay (n = 3).

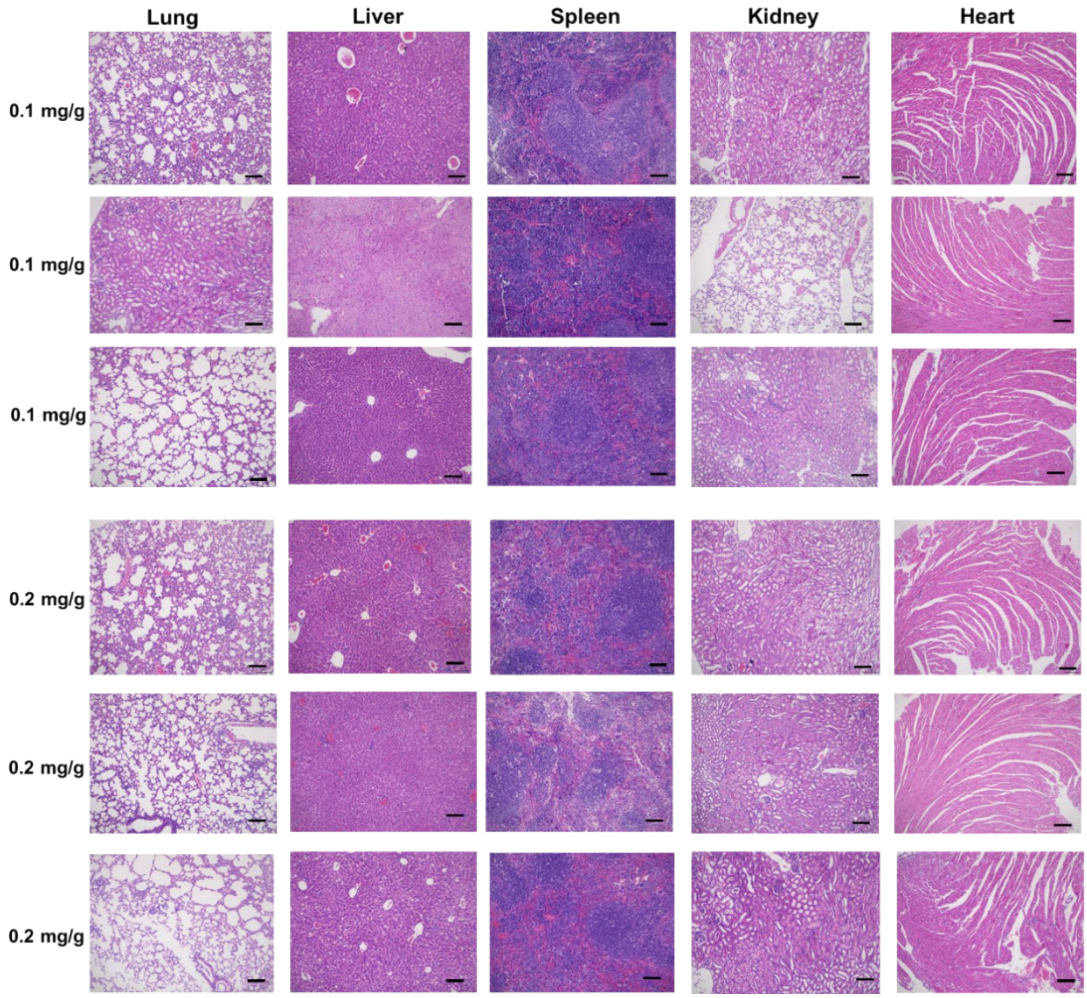


**Fig. S8:** Safety evaluation of **H-dot**: HE staining of different organs in nude mice (n = 3) after 48 h injection of **H-dot** with different dosage ($m_{\mathbf{H\text{-}dots}}$ : $m_{mice}$= 0.1mg/g or 0.2 mg/g). Scale bar: 100 μm.