Electronic Supplementary Information

A facile approach to fabricate self-assembled magnetic nanotheranostics for drug delivery and imaging

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Experimental

Material and Characterizations

Irinotecan hydrochloride (IR) was purchased from AK Scientific Inc. (Mountain View, CA). LysoTracker® Green DND-26 was purchased from Invitrogen-Molecular Probes (Eugene, OR). Annexin V staining and Propidium iodide solution were purchased from Biolegend (San Diego, CA). Dicyclohexylcarbodiimide (DCC), 4-Dimethylaminopyridine (DMAP), lauric acid, iron oxide magnetic nanoparticles and all other chemicals were purchased from Sigma-Aldrich. Cell culture medium and fetal bovine serum were purchased from Mediatech, Inc. (Manassa, VA). Culture dishes and plates were from VWR (Radnor, PA). The purified prodrugs were analyzed by Bruker UltraFlextreme MALDI-TOF-MS and 600 MHz Avance III NMR

Spectrometer (Bruker, German). The mean diameter and polydispersity of nanoparticles were measured by dynamic light scattering (DLS Malvern Zeta Sizer, Nano-ZS, Worcestershire, UK). Transmission electron microscopy (TEM) was performed on a Philips CM120 TEM (FEI Company, Hillsboro, OR, U.S.A) with 120kV acceleration voltage. For confocal microscopy, the stained HT-29 cells were visualized under a DeltaVision deconvolution microscope. Magnetic Resonance Imaging (MRI) was obtained using a Bruker Biospec 7T MRI scanner.

Synthesis of Irinotecan-lauric acid Prodrug

Lauric acid (0.4 mmol) and DCC (0.5 mmol) were dissolved in dried dichloromethane (CH_2Cl_2) (10 mL) and the mixture was stirred at 0 ^{O}C for 30 min until plenty of white precipitate was observed. Then, the solution of irinotecan hydrochloride (0.2 mmol) and DMAP (0.08 mmol) in 10 mL dried CH_2Cl_2 was added. The resulting mixture was stirred at room temperature while the esterification reaction was monitored by TLC. After filtering off the white precipitates, the resultant mixture was subjected to silica gel 100–200 mesh column chromatography using 3% methanol-dichloromethane as eluent to obtain the irinotecan-lauric acid prodrug (LA-IR).

Preparation of SPIO@IR Nanoparticles

Briefly, with 5 mg LA-IR dissolved in. Then the tetrahydrofuran solution was slowly added into 1 mL deionized water dropwise under magnetic string and kept on stirring for overnight till the tetrahydrofuran was completely evaporated. This yielded a stable solution of nano-formulated SPIO@IR prodrug with a final concentration of 5 mg/mL of LA-IR.

Iron Quantification of Particles

The iron concentrations in the SPIO@IR and hydrophilic SPIO were determined using the 1,10-phenanthroline colorimetric method. In detail, 40 uL sample was transferred into a tube, followed by the addition of 20 uL of concentrated hydrochloric acid. The solution was incubated for 1h at room temperature to dissolve the SPIO and to yield ferrous and ferric chloride. Then, 100 uL of 100 mg/mL hydroxylamine hydrochloride solution was added, to reduce Fe (III) to Fe (II), followed by the addition of 500 μL of 1,10 phenanthroline 3 mg/ml to form the orange red complex of

tris(1,10-phenanthroline) iron(II). Finally, the samples were diluted to 1800 μL using ammonium acetate 500 mM buffer (pH 4.5) , which was also used in the reagents' solutions. The absorbance of the samples was measured at 510 nm using a Shimadzu UV-1800 spectrometer (Shimadzu Inc., Kyoto, Japan). The concentration of iron (II) was calculated using a calibration curve obtained using ammonium ferrous sulfate solution in HCl 0.01 M in a concentration range of 0.1-10 μg/mL.

Stability Evaluations of SPIO@IR

SPIO@IR were diluted to 50 μM/mL (IR concentration) by aqueous solution, then stored at ambient temperature. The time-dependent size distributions were monitored by DLS to evaluate the stabilities of SPIO@IR.

MRI Relaxivity Measurements of SPIO@IR

MR imaging was performed using a 7 T Bruker BioSpec MRI Scanner at room temperature. Samples were then positioned coaxially and measured in parallel to the external magnetic field to determine r_2 relaxivities (in mM^{-1} s^{-1}), which represent the MR sensitivity of the SPIO@IR NPs. The paramagnetic nature of iron oxide locally induces a magnetic field inhomogeneity, leading to an enhanced MR signal decay in gradient echo images. To determine the r_2 relaxivity, transverse images were obtained using a 2D multiple gradient echo MR sequence with 15 echoes with a first echo time of 15 ms and an echo spacing of 15 ms. The imaging parameters used were 800 ms repetition time with a 8 cm × 8 cm field of view (FOV), 1 mm slice thickness, and 256 × 256 image matrix. Finally, the r_2 relaxivity values of SPIO@IR (expressed in mM^{-1} s^{-1}) were calculated through the slope of the curve of $R_2(1/T_2)$ relaxation rates as a function of the iron concentration in mM.

Accumulated drug releasing of SPIO@IR

SPIO@IR solution was prepared to determine the accumulated drug release profile. The initial LA-IR concentration in SPIO@IR was set as 200 μg/mL. SPIO@IR was first loaded into dialysis cartridges (Pierce Chemical Inc.) with a MWCO of 3500 Da, the cartridges were then submerged into 4000 mL aqueous solution at different pH values (7.4 and 5.0) at ambient temperature under moderate-speed stirring. The LA-IR remained in the dialysis cartridge was drawn with a micro-syringe at various time

points, and quantitatively measured by UV-vis spectrometer at 359 nm. Each value was reported as the means for each triplicate sample.

Cytotoxicity Studies

The HT-29 human colon cancer cell line was incubated in a humidified 5% CO_2 incubator at 37 ^{O}C in ATCC-formulated McCoy's 5a Medium Modified supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin. The PZ-HPV-7 prostatic epithelial cell line was incubated in Keratinocyte Serum Free Medium. HT-29/PZ-HPV-7 cells were first seeded into 96-well plates at a density of 5000 cells per well and cultured. After 24 hrs, cells were incubated in medium containing different concentrations of IR, SPIO@IR and the equivalent doses of hydrophilic SPIO for 48 hrs, and washed with PBS. Growth inhibition was measured by using MTS assay. CellTiter 96 Aqueous Cell Proliferation Reagent, which is composed of MTS and an electron coupling reagent PMS, was added to each well per the manufacturer's instructions. The cell viability was determined by measuring the absorbance at 490 nm with a microplate reader (SpectraMax M2, Molecular Devices, USA). Untreated cells served as a control. Results were shown as the average cell viability $[(OD_{treat}-OD_{blank})/(OD_{control}-OD_{blank})\times100\%]$ of triplicate wells.

Apoptosis analysis

20 μmol/mL of IR, SPIO@IR and the equivalent doses of hydrophilic SPIO were incubated with HT-29 cell for 24 hrs. Cells were washed with PBS and replaced with fresh cell culture medium. Before analysis with flow cytometry (Becton Dickinson, San Jose, CA), cells were stained with Annexin V/Propidium iodide per manufactory's manual. The data was analyzed by Flowjo.

Subcellular Localization of Prodrug Nanoparticle and Imaging

HT-29 cells were seeded into a 35 mm dish with a glass bottom, incubated at 37 ^{O}C for 4 h, then treated with 50 μmol/L IR, SPIO@IR and the equivalent doses of hydrophilic SPIO for 4h at 37 ^{O}C. Cells were then stained with LysoTracker® Green DND-26 according to the manufacture's instruction to label lysosomes. Cells were then washed gently with PBS for two times and imaged using a confocal laser

scanning microscopy with DAPI channel for IR and FITC channel for LysoTracker® Green DND-26.

Cellular uptake study

Cellular uptake of IR, SPIO, and SPIO@IR was further investigated quantitatively by flow cytometry. For flow cytometry, 10^5 cells per well were seeded into 6-well plates, then cultured with IR, SPIO, and SPIO@IR at a final concentration of 50 μmol/L. Cells were harvested and analyzed using the FACScan. Mean value of Violet-1 channel was determined as the average fluorescence intensity of irinotecan in cells.

***In vitro* MRI on HT-29 Cells**

In vitro T_2 relaxation times were calculated on HT-29 cells that were incubated with IR, SPIO@IR and the equivalent doses of hydrophilic SPIO with Fe concentration at 20 μg/mL. Cells were washed with PBS three times, digested with 0.25% trypsin, centrifuged for 3 min, and resuspended in agarose (1mL, 1.0%) using an Eppendorf tube. MR imaging was performed on a 7.0 T MR system. T_2 map images were performed using the following parameters: TR=1000 ms, TE=15-225 ms, slice thickness=1 mm, a 8 cm×8 cm FOV, and an image matrix of 256×256. Quantitative T_2 relaxation times were reconstructed within the T_2 signal intensities measured within the region of interest (ROI) fitting to the equation: $S(t) = S_0 \times e^{(-t/T2)}$.

Animal and Tumor Models

Nude mice with 6-8 weeks of age were ordered from Harlan (Livermore, CA) for the establishment of tumor-bearing mouse model. All animal procedures were performed under requirements of institutional guidelines and according to the protocols approved by the Use and Care of Animals Committee at University of California, Davis. HT-29 cells in a 200 μL mixture of PBS suspension and Matrigel (1:1 v/v) were injected subcutaneously into the left flank of nude mice.

***In vivo* MRI on HT-29 Cells**

Nude mice bearing HT-29 colon cancer xenograft were scanned on a 7 T MRI equipped with a high-resolution animal coil, before and after injection of SPIO@IR and hydrophilic SPIO via tail vein (time points: pre, 2h, 4h, 6h, and 24h). Mice received 300 μL of SPIO@IR and hydrophilic SPIO at the same Fe concentration. All

mice were imaged under the T2WI spin-echo sequence: TR/TE (800/40 ms), matrix (256*256), FOV (8 cm×8 cm), slice thickness (1 mm), slice spacing (1.1 mm). For T2-mapping, the MRI parameters were TR/TE (1000/15-225 ms) matrix (256*256), FOV (8 cm×8 cm), slice thickness (1 mm), slice spacing (1.1 mm). Quantitative T2 maps were reconstructed and measured using Paravision 4 software.

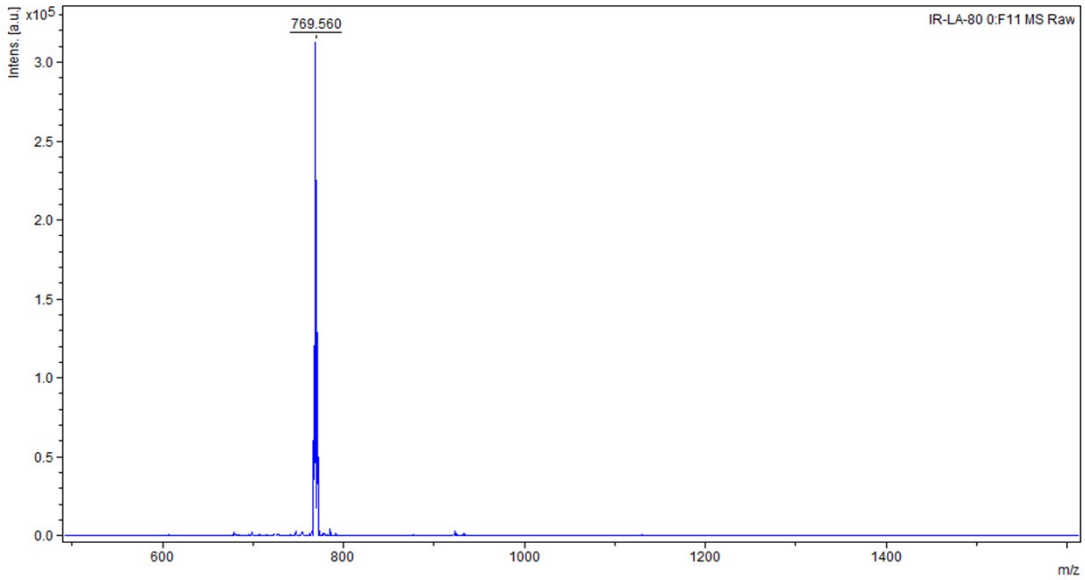

Figure S1. The molecular weight of LA-IR measured by MALDI-TOF Mass Spectrometry.

HNMR (600 MHz, DMSO-d6) 8.16 (dd, 1 H, J1=9.0 Hz, J2=2.4 Hz), 8.0 (s, 1 H), 7.68 (d, 1 H, J=9.6 Hz), 7.01 (s, 1 H), 5.50 (d, 2 H, J=3.0 Hz), 5.35 (d, 2 H, J=6.6 Hz), 4.39 (s, 1 H), 4.21 (s, 1 H), 3.43 (s, 3 H), 3.21 (q, 2 H, J=7.2 Hz), 2.96 (s, 3 H), 2.55 (m, 1 H), 2.21 (m, 4 H), 1.84 (m, 5 H), 1.74 (s, 2 H), 1.59 (m, 2 H), 1.30 (m, 10 H), 1.12 (m, 12 H), 0.94 (t, 3 H, J=7.2 Hz), 0.83 (t, 3 H, J=7.2 Hz).

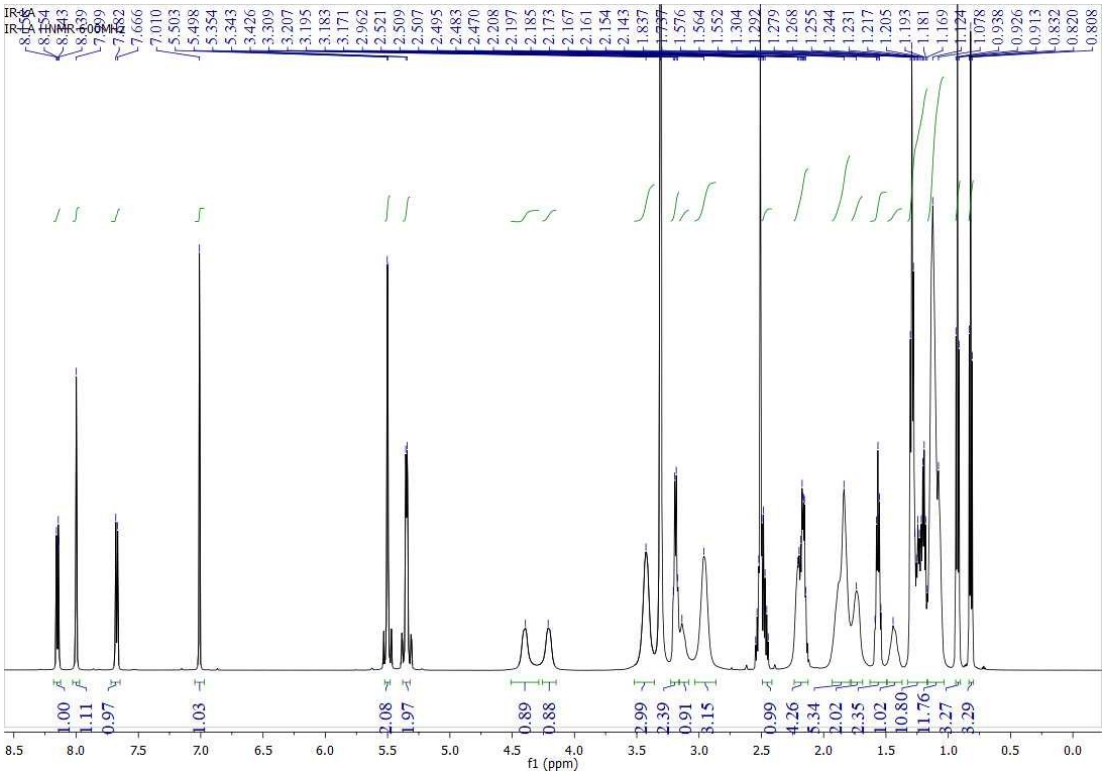

Figure S2. ^{1}H NMR spectrum of LA-IR by using DMSO-d6 as a solvent.

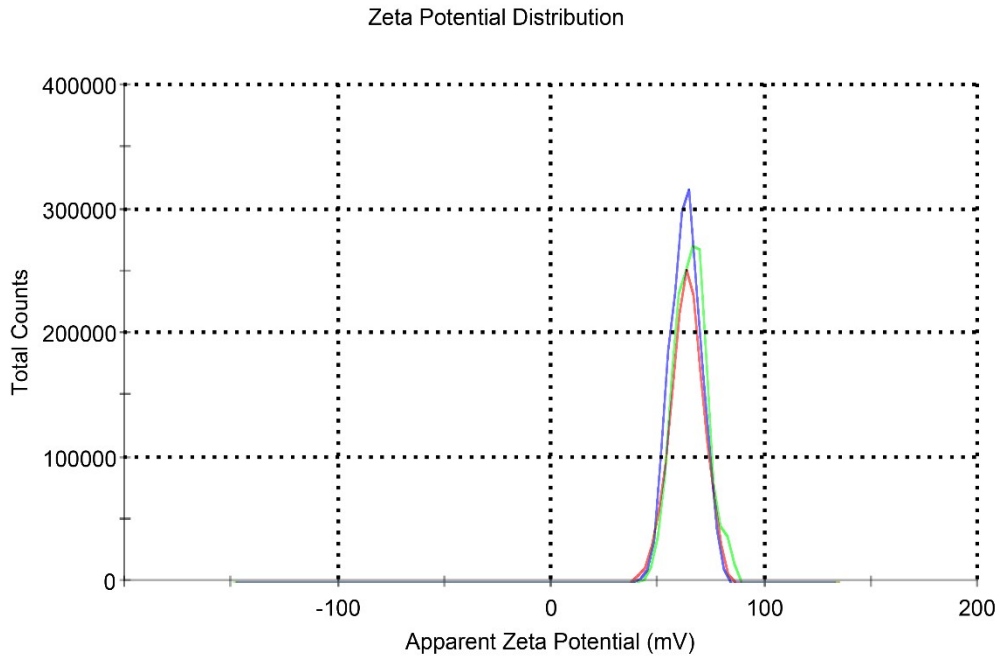

Figure S3. Zeta potential of SPIO@IR measured by DLS.

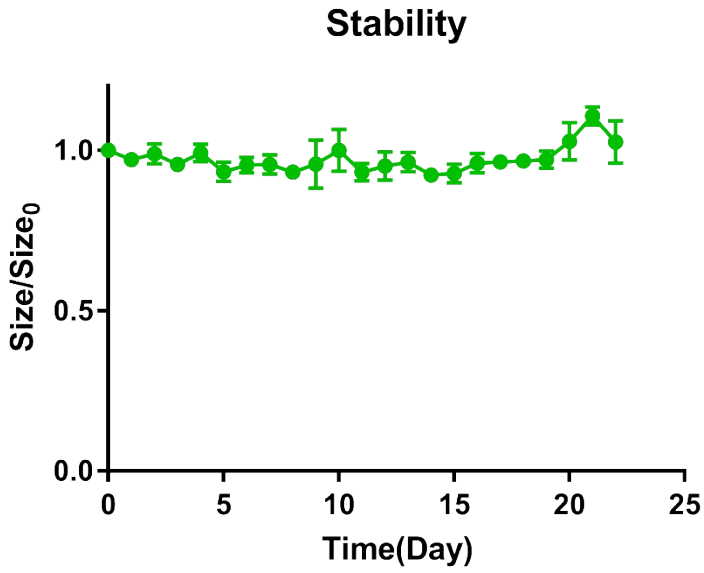

Figure S4. Stability study *via* monitoring the size changes of SPIO@IR in aqueous solution measured by DLS.

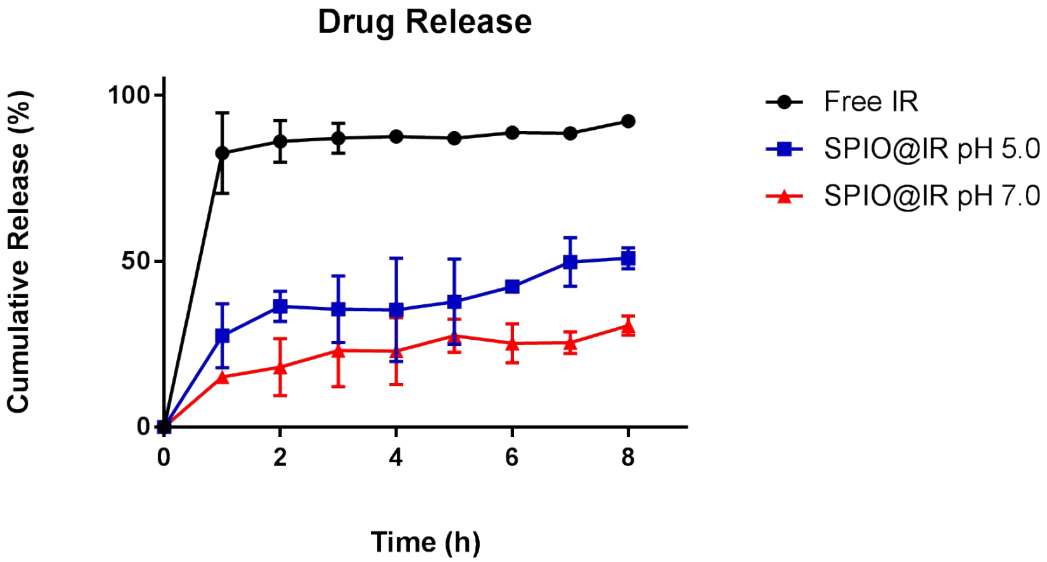

Figure S5. Accumulated drug release of SPIO@IR compared with free IR.

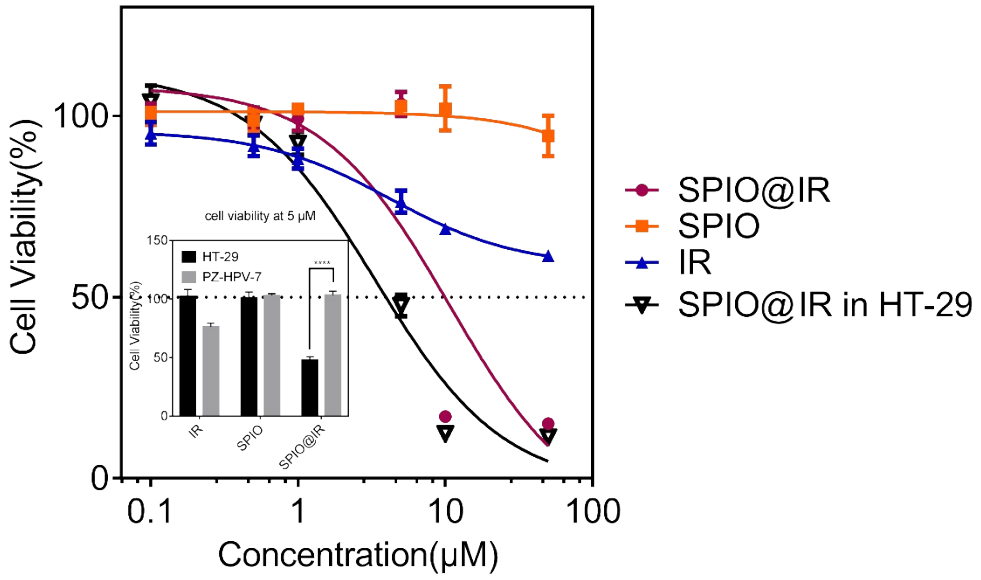

Figure S6. *In vitro* cytotoxicity of HT-29 cell line and PZ-HPV-7 cell line treated with hydrophilic SPIO, free IR, and SPIO@IR. The insert figure was the viability of the cells treated with hydrophilic SPIO, free IR and SPIO@IR at the equivalent IR concentration of 5 μM.

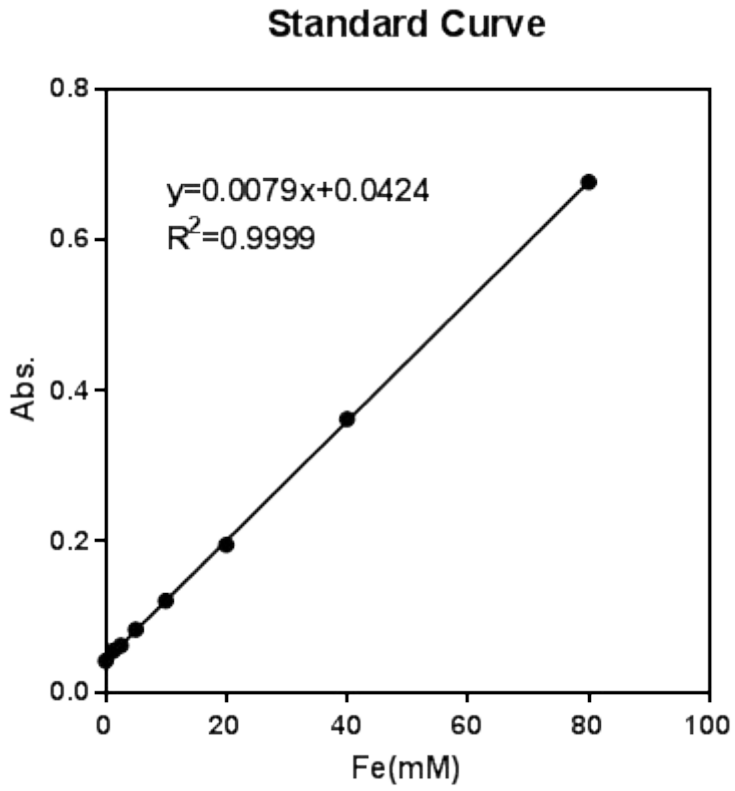

Figure S7. Standard curve of 1,10-phenanthroline colorimetric method to determine the iron concentration.

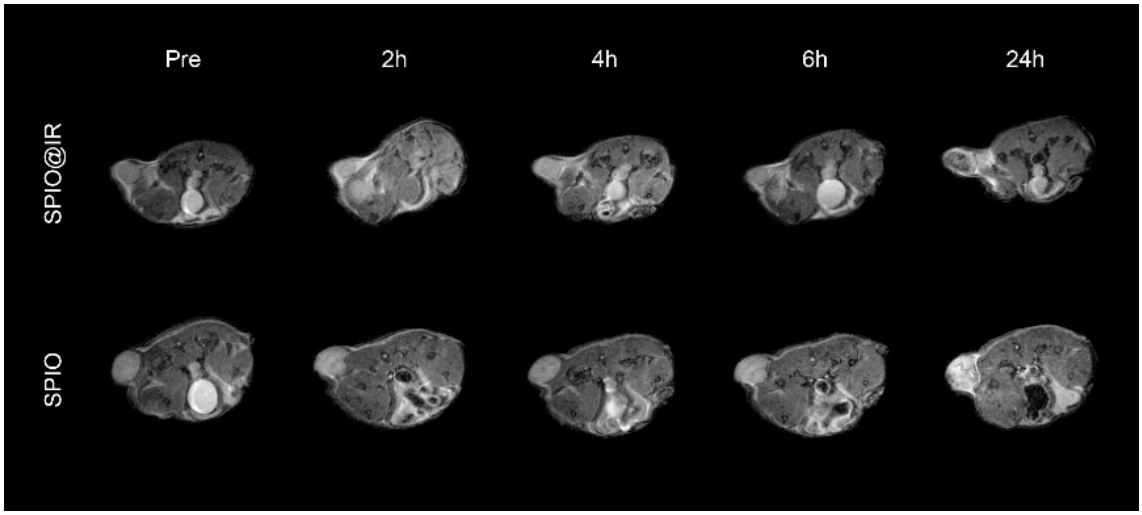

Figure S8. T_2-weighted imaging of colon cancer (HT-29) bearing mice pre-injection and at different time points post-injection of SPIO@IR and hydrophilic SPIO.

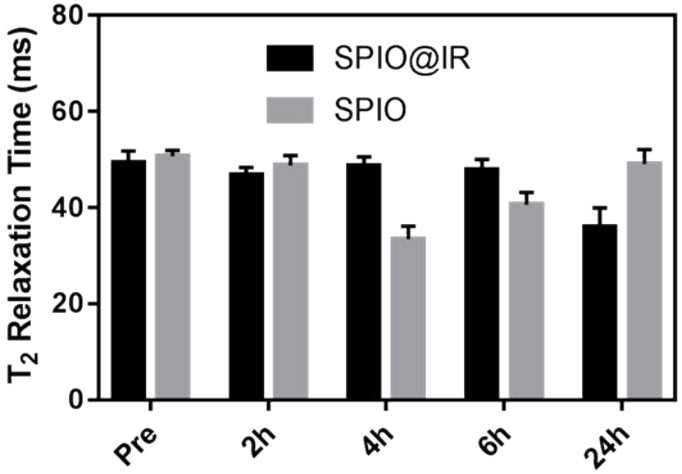

Figure S9. T_2 relaxation time in tumor area pre-injection and at different time points post-injection of SPIO@IR and hydrophilic SPIO.