Electronic Supplementary Material (ESI) for Nanoscale Advances.

Ultrasmall Mn-doped Iron Oxide Nanoparticles with Dual Hepatobiliary and Renal Clearances for T_1 MR Liver Imaging

Sanghoon Lee,[a] Arim Byun,[a] Juhee Jo,[b] Jong-Min Suh,[c] Jeasang Yoo,[c] Mi Hee Lim,[c] Ji-wook Kim,*[b] Tae-Hyun Shin*[b] and Jin-sil Choi*[a]

[a]Department of Chemical and Biological Engineering, Hanbat National University 34158 Daejeon, Republic of Korea.

[b]Inventera Inc., Seoul 06588, Republic of Korea

[c]Department of Chemistry, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 34141, Korea.

Supplementary Methods

Synthesis of ultrasmall superparamagnetic iron oxide nanoparticles (USPIOs)

USPIOs were synthesized using a similar method as that employed for UMIOs. PAA (0.2606 g, 2.28 mmol) was mixed with 25 mL distilled water as the solvent. Then, the mixture was bubbled with Ar gas for 40 min followed by heating to 55 °C. To prepare an Fe solution, $FeCl_3 \cdot 6H_2O$ (0.27 mmol) and $FeCl_2 \cdot 4H_2O$ (0.1395 mmol) were dissolved in 1 mL HCl (1 M). The Fe salt solution was injected into the PAA solution while maintaining a temperature of 55 °C. Thereafter, 3.5 mL NH_4OH was introduced into the resulting solution, and the reaction was conducted at 55 °C for 20 min. The resulting solution was purified three times by centrifugation with acetone and five times using Centricon (10 K) to obtain USPIOs.

Electron paramagnetic resonance (EPR) spectroscopy

X-band EPR spectra were obtained by a Bruker EMXplus EPR spectrometer (Bruker BioSpin, Silberstreifen, Rheinstetten, Germany) equipped with an ER 4141VT digital temperature control system (Bruker BioSpin) and an ER 4119HS cavity (Bruker BioSpin) using a capillary cell (d = 0.006 mL/cm). The EPR spectra of the samples were recorded with the following experimental parameters: microwave frequency, 9.38 GHz; microwave power, 2 mW; modulation amplitude, 3 G; time constant, 163.84 ms; sweep time, 120 s; number of scan, 4; room temperature.

Measurements of T_1 and T_2 and calculation of T_1 and T_2 relaxivity coefficients (r_1 and r_2)

T_1 and T_2 were measured under 3.0 Tesla MRI scanner with respective inversion recovery pulse sequence and Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. In brief, T_1 signal was measured with varying inversion time (*TI*) of 50, 150, 300, 600, 900, 1500, 2500, and 5000 ms, followed by exponential fitting using the equation provided below. For T_2 measurements, scans were performed with various echo times (*TE*) of 15 ~ 480 ms with interval of 15 ms, and the corresponding T_2 signal was fitted exponentially using the equation below.

$$T_1 \text{ signal intensity } = M_0\left(1 - e^{\frac{-TI}{T_1}}\right)$$

$$T_2 \text{ signal intensity } = M_0 e^{\frac{-TE}{T_2}}$$

where, M_0 is constant, *TI* is inversion time, T_1 is T_1 relaxation time, *TE* is echo time, and T_2 is T_2 relaxation time.

The r_1 and r_2 values were determined by plotting $1/T_1$ and $1/T_2$ against the concentration (metal) of contrast agents. The slope of each plot was calculated as r_1 and r_2 (Figure S6).

Stabilities of UMIOs under different pH conditions and in saline solutions

Stabilities of UMIOs were assessed by measuring the hydrodynamic sizes of UMIOs under different

pH conditions (citrate buffer for pH = 3–5, phosphate buffer for pH = 6–8, and carbonate–bicarbonate buffer for pH = 9) and in saline solution. All buffer concentrations were 10 mM.

Hemolysis assay

The hemolysis assay was conducted following the procedure outlined by Adem Yildirim et al. with some modifications. Fresh mouse blood was obtained through cardiac puncture and stabilized using 0.5 M EDTA. Subsequently, 0.5 mL of the blood sample was mixed with 1 mL of phosphate-buffered saline (PBS) and centrifuged at 3,000 rcf for 5 minutes to isolate red blood cells (RBCs). These RBCs underwent five additional washes with 2.5 mL of PBS and were ultimately diluted to a volume of 5 mL with PBS. For the experimental setup, 0.2 mL of the diluted RBC suspension was mixed with 0.8 mL of the UMIO, and final concentrations were adjusted to 19, 29, 39, 59, and 78 μg/mL (test group, 39 μg/mL representing the estimated UMIO concentration in the blood when injected into mice). As controls, PBS and distilled water were employed as the negative and positive control groups, respectively. Following incubation at 37°C for 3 hours, all samples were centrifuged for 5 minutes at 3,000 rcf. Subsequently, 100 μL of the supernatant from each sample was transferred to a 96-well plate, and the absorbance was measured at 577 nm. The background was corrected using UMIO solutions at each concentration, as there is absorption at 577 nm for UMIO. The hemolytic degree was quantified using the hemolysis ratio, calculated with the following formula

$$\text{Hemolysis ratio (\%)} = (OD_{test} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control}) \times 100.$$

In vitro cytotoxicity assay

Human lung (A549 cells) and liver (HepG2) cancer cell line was purchased from American Type Culture Collection (USA) and grown in DMEM supplemented with 10% FBS and 1% Pen Strep under a humidified atmosphere with 5% CO_2 at 37 °C. Cytotoxicity of UMIOs were analyzed via the CCK-8 assay. A549 cells were cultured in a 96-well plate at a density of 5×10^4 cells per well and incubated at 37 °C under 5% CO_2 for 24 h. After removing the culture medium, UMIOs were added to each well at various concentrations (80, 150, 400, 800, and 1250 μg Fe /mL in the culture medium) followed by overnight incubation under the same abovementioned conditions. Subsequently, the culture medium was replaced with 110 μL fresh medium containing 10 μL CCK-8 assay solution, and the plate was further incubated for 4 h. Finally, the absorbance of each well was measured at 450 nm using the multi-mode microplate reader to determine the extent of cell viability.

Supplementary Tables

Table S1. Chemical composition of UMIOs

	Mn (atomic ratio)	**Fe (atomic ratio)**
ICP-OES	0.38	0.62
XPS	0.40	0.60
EDX	0.36	0.64

Table S2. Clinical observations in mice after administration of UMIOs at dosages of 7.5 and 15 mg_{Fe+Mn}/kg. N indicates that the mice appear normal.

Dosage (mg_{Fe+Mn}/kg)	**Animal No.**	**Clinical observations**						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
7.5	M1	N	N	N	N	N	N	N
	M2	N	N	N	N	N	N	N
	M3	N	N	N	N	N	N	N
15	M4	N	N	N	N	N	N	N
	M5	N	N	N	N	N	N	N
	M6	N	N	N	N	N	N	N

Supplementary Figures

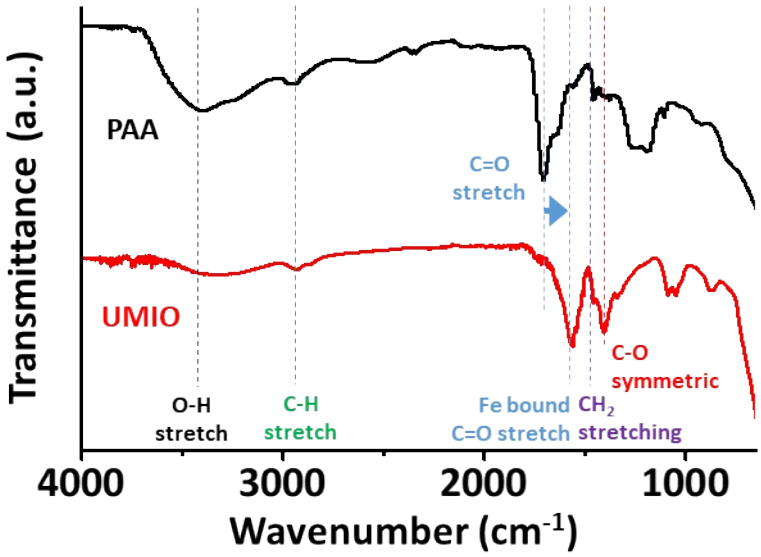

Figure S1. IR spectrograph of PAA and UMIOs

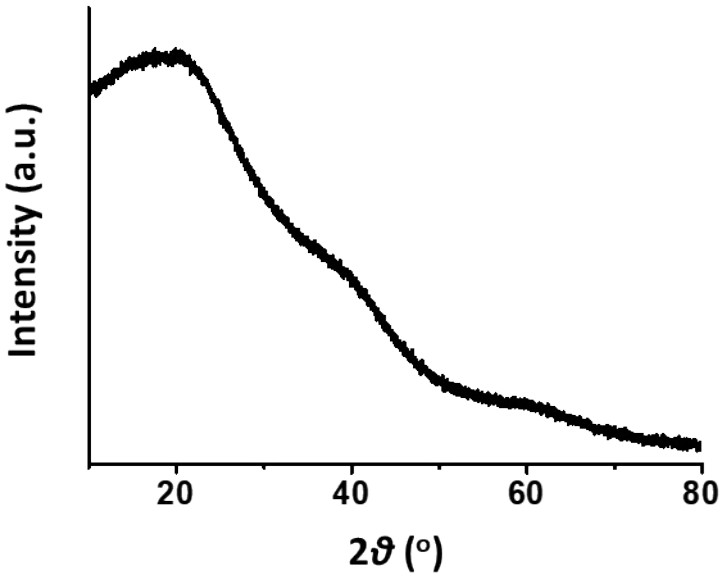

Figure S2. XRD graph of UMIOs.

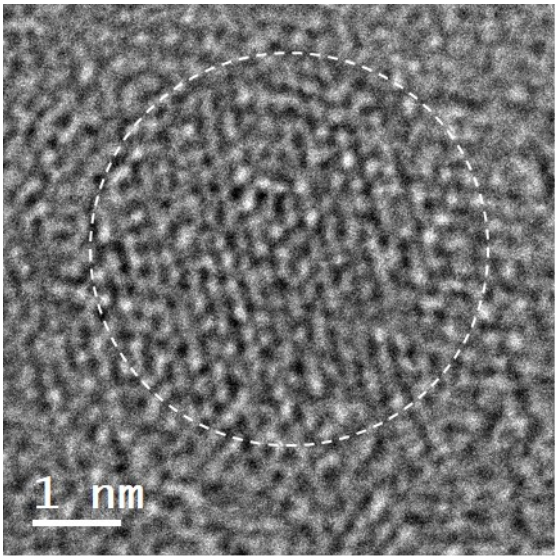

Figure S3. HR-TEM image of UMIO.

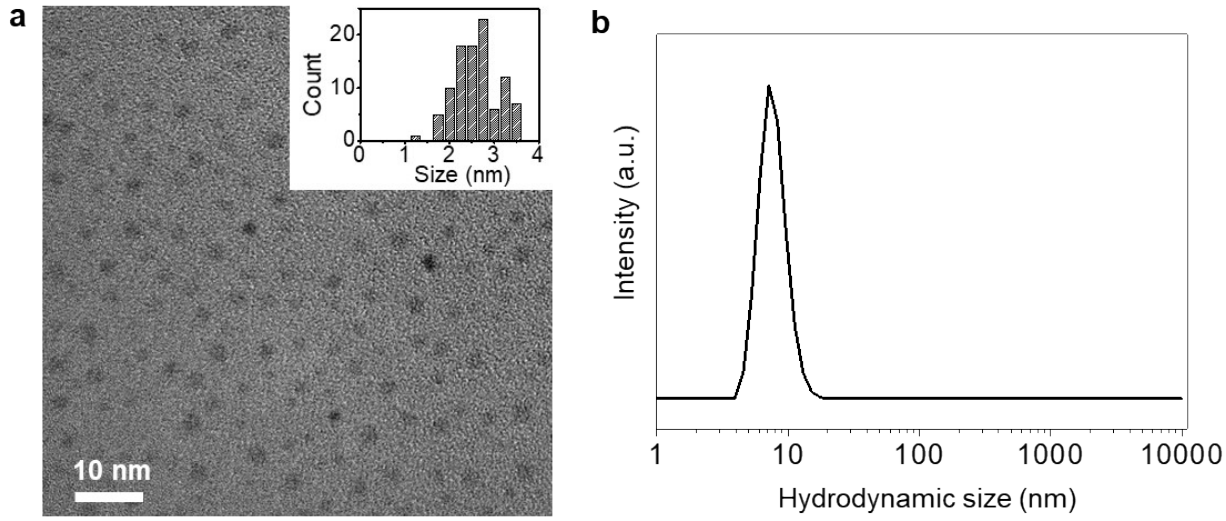

Figure S4. (a) TEM image and size histogram (inset) of USPIOs. (b) Hydrodynamic sizes of USPIOs in aqueous solution.

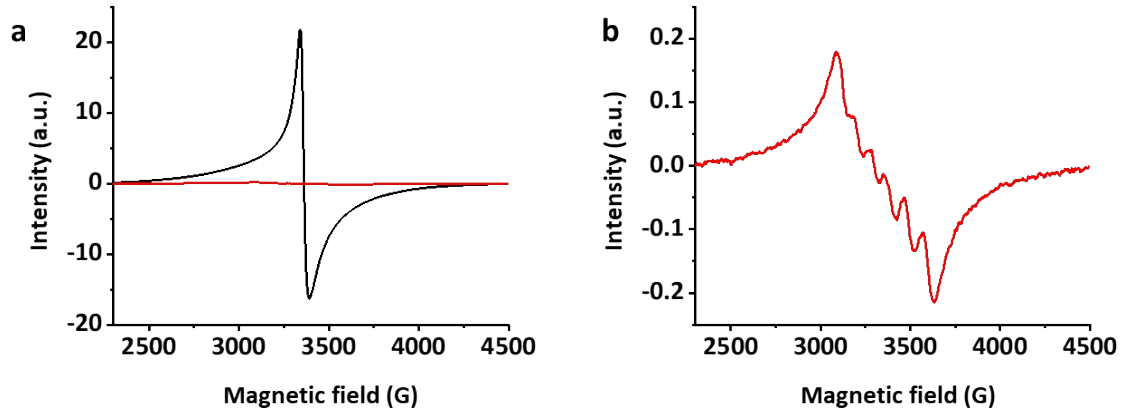

Figure S5. (a) EPR graph of UMIOs (red) and USPIOs (black) and (b) magnified EPR graph of UMIOs.

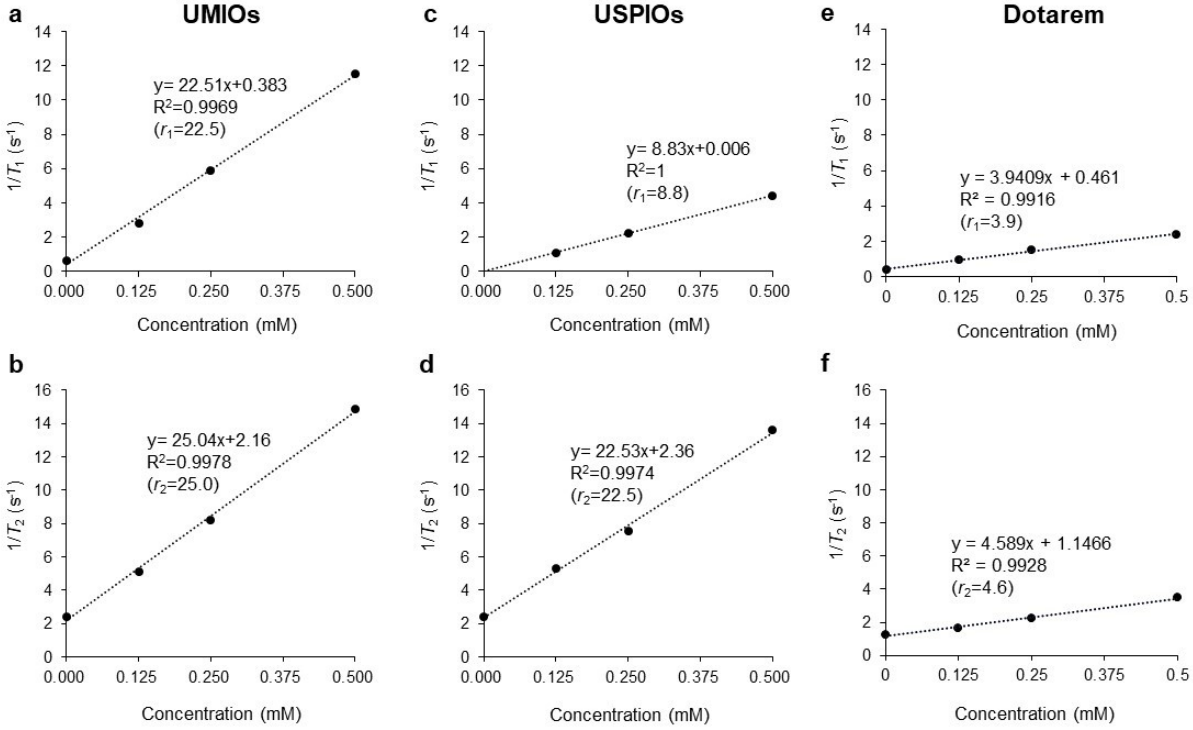

Figure S6. T_1 and T_2 relaxivity coefficients (r_1 and r_2) calculation. Plots of $1/T_1$ (a,c,e) and $1/T_2$ (b,d,f) versus metal (Fe+Mn for UMIOs, Fe for USPIOs, Gd for Dotarem) concentration. The slopes of the plots (a,c,e) and (b,d,f) determine the respective r_1 and r_2 for each contrast agents.

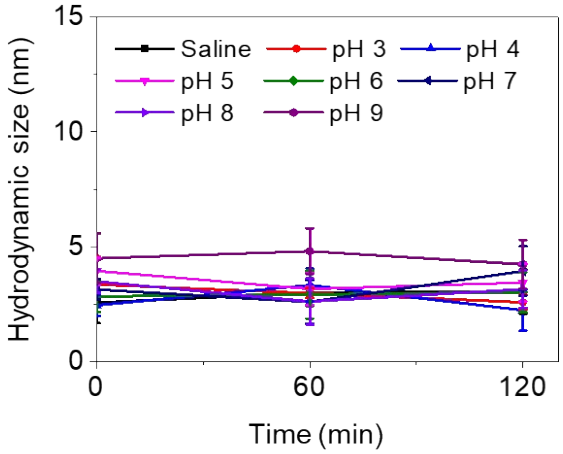

Figure S7. Colloidal stabilities of UMIOs measured in saline and buffer solutions with pH = 3–9.

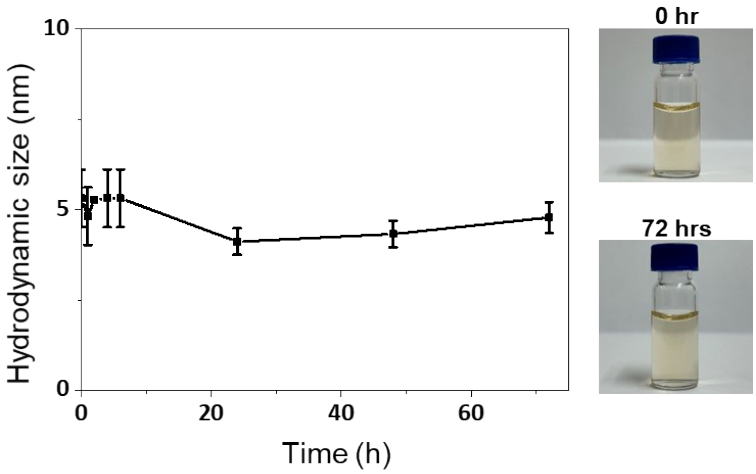

Figure S8. Colloidal stabilities of UMIOs measured in physiological saline.

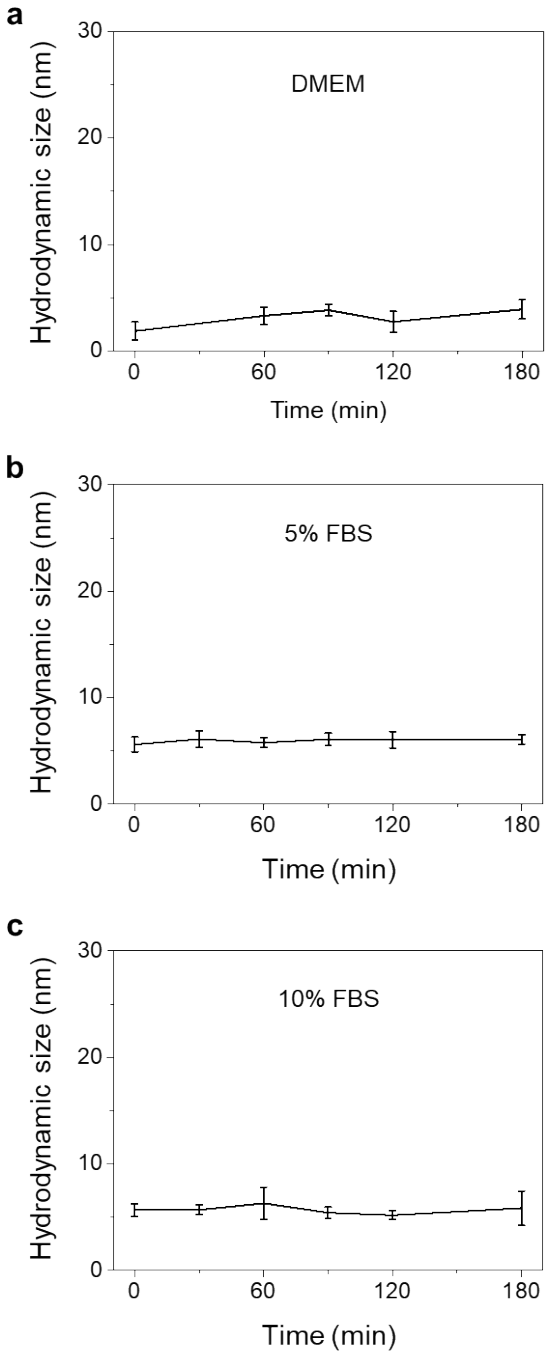

Figure S9. Colloidal stabilities of UMIOs measured in (a) DMEM cell media, (b) 5% FBS, and (c) 10% FBS.

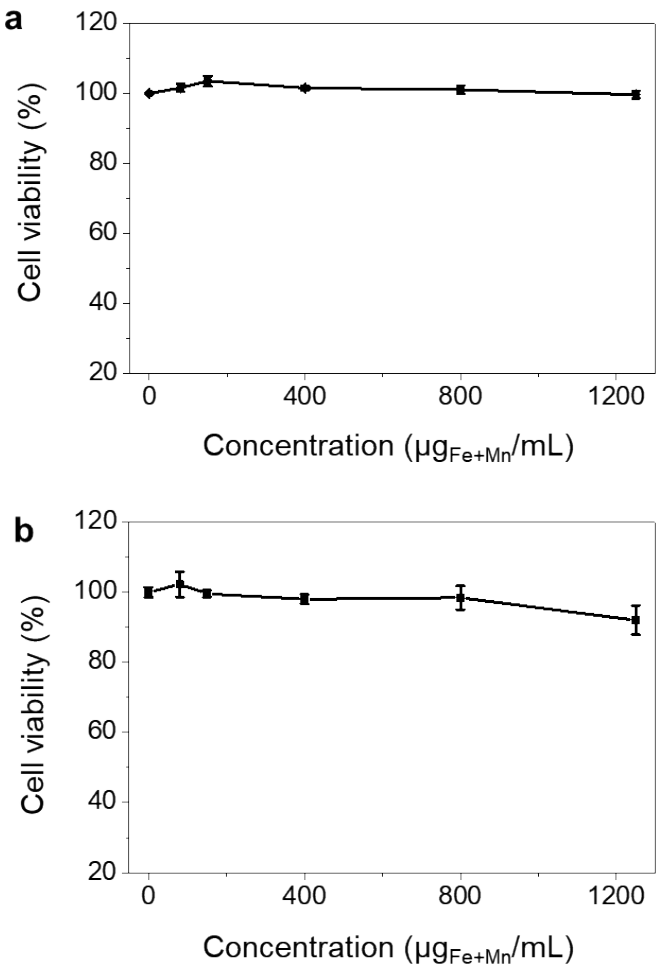

Figure S10. Cell viabilities of (a) A549 (lung cancer cell line) and (b) HepG2 (liver cancer cell line) cells treated with varying concentrations of UMIOs.

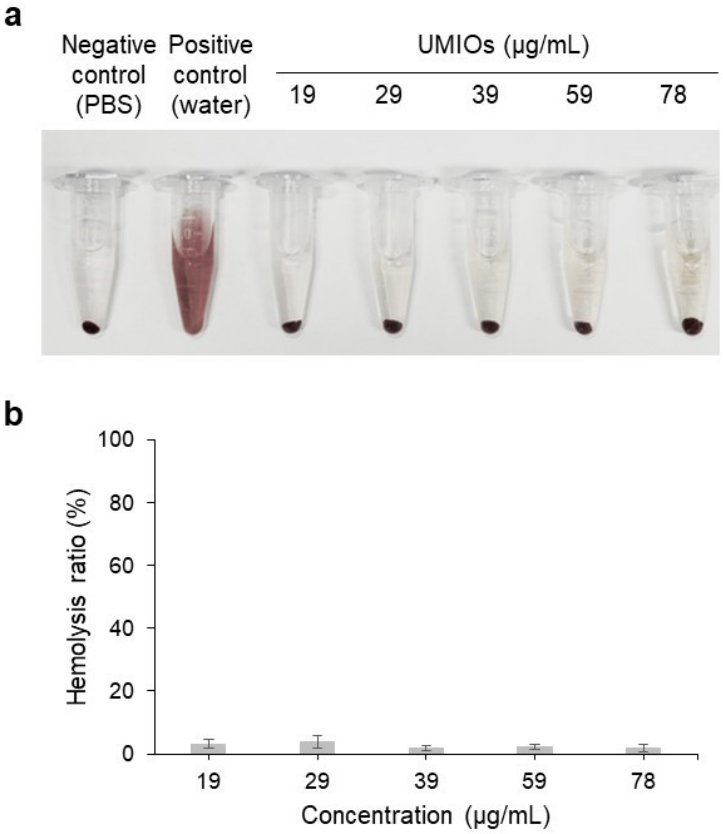

Figure S11. Hemolysis assay of UMIOs. (a) A photo of hemolysis assay samples consisting of a negative control, positive control, and UMIOs at varying concentrations. (b) Hemolysis ratios of UMIOs samples.

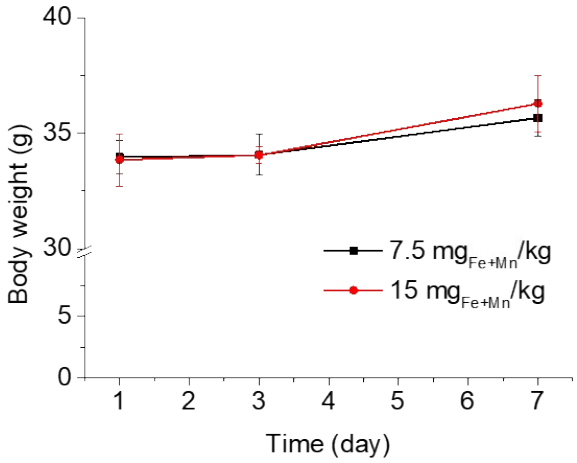

Figure S12. Changes in the body weights of mice after the administration of UMIOs at dosages of 7.5 and 15 mg$_{Fe+Mn}$/kg.

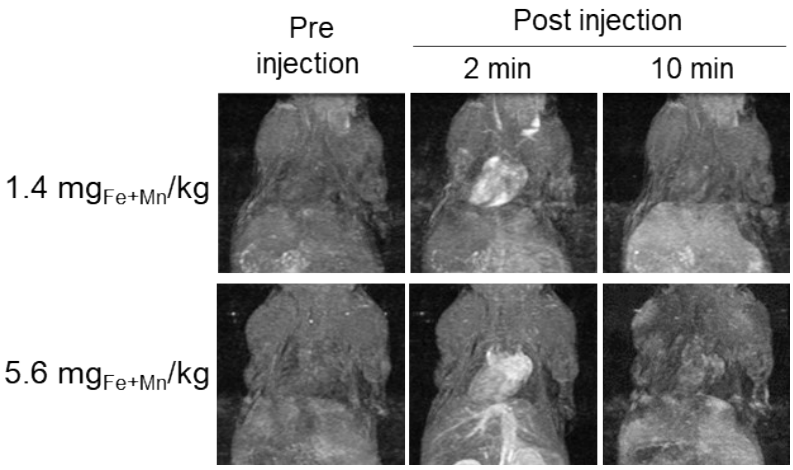

Figure S13. T_1 MR images of mice injected with UMIOs at dosages of 1.4 and 5.6 mg$_{Fe+Mn}$/kg.

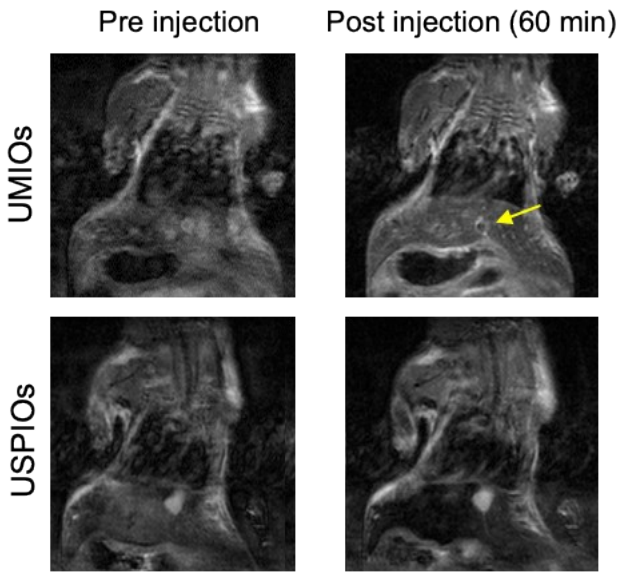

Figure S14. T_2 MR images of mice obtained at pre-injection and 60 minutes post-injection of respective UMIOs and USPIOs (dosage: 2.8 mg_{metal}/kg). Yellow arrow indicates UMIOs condensation in the gallbladder.

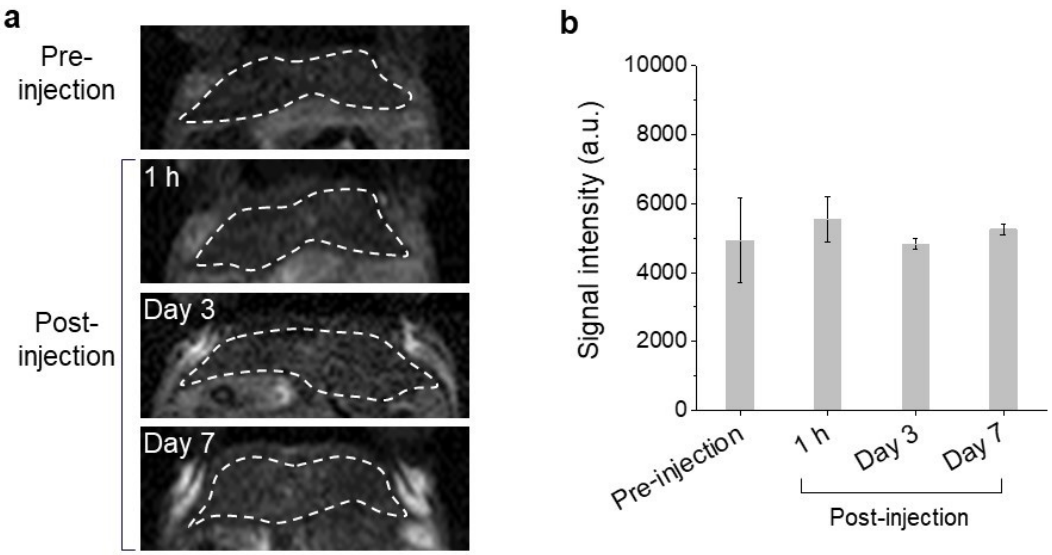

Figure S15. T_2 MR images (a) and signal intensity measured in the livers of mice injected with UMIOs, acquired at pre-injection, 1 h, day 3, and day 7 post-injection. The dotted line indicates the region of interest in the liver for the signal intensity measurement.