

Supporting Information for:

Micellar Hybrid Nanoparticles for Simultaneous Magneto-Fluorescent Imaging and Drug Delivery

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Experimental Section

For the micellar hybrid nanoparticle (MHN) synthesis, 100 μL (MHN1), 300 μL (MHN3), or 500 μL (MHN5) of TOP-coated CdSe/ZnS or CdSe_xTe_{1-x}/ZnS quantum dots (QD, Invitrogen, CA, USA) in chloroform (2 mg/mL), and 100 μL of oleic acid-coated magnetic iron oxide nanoparticles (MN, prepared using a previously reported method [1]) in chloroform (2 mg/mL), were mixed with 200 μL of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-PE or PEG-phospholipids, Avanti Polar Lipids, Inc., AL, USA) in chloroform (10 mg/mL). For the drug-incorporated MHN, 100 μL of doxorubicin (DOX, Sigma-Aldrich Chemicals, MO, USA) in chloroform containing triethylamine (TEA) (molar ratio 1:1=TEA:DOX, 1 mg/mL) was also added. After complete evaporation of the chloroform, the dried film was hydrated by adding 1 mL water at 75 °C and the synthesis vessel was placed in an ultrasonic bath for 5 min to obtain an optically clear suspension. The suspension was first filtered through a 0.1 μm membrane.

The MHN and micellar MN (MMN) were then selectively collected by trapping on a magnetic column (Miltenyi Biotec, Bergisch Gladbach, Germany) and rinsing with phosphate buffered saline (PBS) three times. Only the MHN and MMN were trapped on the magnetic column due to their magnetic properties, while micellar QD (MQD), DOX micelles, and empty micelles passed through the column. The MHN and MMN were eluted from the column after removal of the magnet, using 1 mL PBS. The eluted solution containing the MHN and MMN was then centrifuged at 14,000 rpm for 10 min and the supernatant, containing smaller individual MMN, was discarded. The MHN were

re-suspended in deionized water or PBS solution.

The MMN or MQD shown in Figure 1e and 1f were prepared by encapsulating either hydrophobic MN or QD alone with PEG-phospholipids. For the MMN preparation, 100 μL of MN in chloroform (0.2 mg/mL) were mixed with 200 μL of PEG-phospholipids in chloroform (10 mg/mL). After complete evaporation of the chloroform, the dried film was hydrated by adding 2 mL of water at 75 $^{\circ}\text{C}$ and the synthesis vessel was placed in an ultrasonic bath for 5 min to obtain an optically clear suspension. The suspension was first filtered through a 0.1 μm membrane. The MMN were then selectively collected by trapping on the magnetic column and rinsing with PBS three times to remove empty micelles. For the MQD preparation, 100 μL of QD in chloroform (0.2 mg/mL) were mixed with 200 μL of PEG-phospholipids in chloroform (10 mg/mL). After complete evaporation of the chloroform, the dried film was hydrated by adding 2 mL water at 75 $^{\circ}\text{C}$ and the synthesis vessel was placed in an ultrasonic bath for 5 min to obtain an optically clear suspension. The suspension was first filtered through a 0.1 μm membrane. The MQD were then selectively collected by rinsing on a centrifuge filter (100,000 MWCO, Millipore) three times with PBS to remove empty micelles.”

To determine the amount of DOX incorporated into the MHN, DOX-incorporated MHN were disrupted in 0.5 M HCl-50% ethanol overnight and the fluorescence intensity of DOX loaded in MHN was compared with a standard curve of DOX fluorescence in the same solution.

For transmission electron microscope (TEM) imaging, an aliquot of MMN, MQD, or MHN dispersed in water was dropped onto the carbon film covering a 300-mesh copper minigrid (Ted Pella, Inc., CA, USA), which was then gently wiped off after approximately 1 min and air-dried. For negative staining, the grid was incubated with pH 13 1.3% phosphotungstic acid for an additional 1 min. TEM images were obtained using a Hitachi H-600A transmission electron microscope. Hydrodynamic size of MMN, MQD or MHN was obtained using a Zetasizer ZS90 dynamic light scattering machine (Malvern Instruments, Worcestershire, UK).

The photoluminescence (PL) spectra of MMN, MQD or MHN were obtained using a 450 nm excitation source with an Acton 0.275-m monochromator, 480-nm cutoff filter, and a UV-enhanced liquid nitrogen-cooled, charge-coupled device (CCD) detector (Princeton Instruments, NJ, USA). The collection optics consisted of a 2.54 cm diameter microscope objective lens coupled to fiber-optic cable.

For NIR fluorescence imaging and MRI T₂ mapping, MMN, MQD, or MHN serially diluted in PBS, and cells incubated with/without MHN for 2 h were placed in a 386-well plate, containing 95 µl total sample/well. The tumors injected with PBS or MHN were placed in flat plastic plate. The optical images were obtained in the Cy5.5 channel (excitation at 680 nm/emission at 720 nm) or the Cy7 channel (excitation at 760 nm/emission at 800 nm) with a NIR fluorescence scanner (LI-COR biosciences, NE, USA). The MRI was performed using a 7 cm bore, Bruker (Karlsruhe, Germany) 4.7 T magnet. R₂ is longitudinal relaxation rate equal to the reciprocal of the T₂ relaxation time (R₂=1/T₂) and it is calculated with a T₂-weighted MRI map. The fluorescence and MR images were analyzed using the OsiriX program (Apple). For magnetic measurement, freeze-dried MMN or MHN were placed in gelatin capsules and the capsules were inserted into transparent plastic drinking straws. The measurements were performed at 298 K using a MPMS2 superconducting quantum interference device (SQUID) magnetometer (Quantum Design, CA, USA). The samples were exposed to direct current magnetic fields in stepwise increments up to 0.5 Tesla. Corrections were made for the diamagnetic contribution of the capsule and straw. The magnetic data were used to quantify the amount of MN in the MHN.

KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK (F3) peptide has been shown to bind preferentially to blood vessels and tumor cells in various tumors [2, 3]. The peptide was synthesized using Fmoc chemistry in a solid-phase synthesizer, and purified by preparative HPLC. The sequence and composition of the peptide was confirmed by mass spectrometry. For further conjugation, an extra cysteine residue was added to the N-terminus. For conjugation with F3, 5% of the PEG-PE used in the MHN synthesis was

replaced with 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Maleimide (Polyethylene Glycol)2000] (maleimide PEG-PE, Avanti Polar Lipids, AL, USA). 200 µg of F3 was reacted with 2 mg maleimide-activated MHN in PBS. After incubation for 30 min at room temperature, the F3-modified MHN sample was purified on a desalting column (Pall, NY, USA).

For *in vitro* studies, MDA-MB-435 human carcinoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin-streptomycin. For fluorescence microscopy, the cells (3000 cells per well) were seeded into 8-well chamber slides (Lab-Tek) overnight. The cells were then incubated with 50 µg of MHN or F3-MHN per well for 2 h (for intracellular targeting) and 50 µg of DOX-loaded F3-MHN (0.093 mg DOX per mg MHN) or 4.5 µg of free DOX (equivalent with DOX amount for DOX-loaded F3-MHN) physically mixed with 50 µg of F3-MHN per well for 30 min, 2 h, and 24 h (for intracellular drug delivery) at 37 °C in the presence of 10% FBS. For the intracellular targeting study, the cells were observed without any fixation using an inverted fluorescence microscope (Nikon, Tokyo, Japan). For the intracellular drug delivery study, the cells were fixed with 4% paraformaldehyde for 20 min, mounted in Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA), and observed with a fluorescence microscope. For endosome staining, the cells incubated with DOX-incorporated F3-MHN for 30 min were fixed with 4% paraformaldehyde for 20 min, and permeabilized and blocked with the solution containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS for 30 min, incubated with 2 µg/mL endosome marker (EEA1 antibody, Abcam) for 1 h, and then with 2 µg/mL AlexaFluor[®] 488 conjugated goat anti-mouse IgG antibody for 1 h at room temperature. The nuclei stained with DAPI were observed in blue channel (excitation at 360 nm/emission at 460 nm). The DOX fluorescences were observed in Cy3 channel (excitation at 540 nm/emission at 580 nm). The QD fluorescence of MHN(705) were observed in Cy5.5 channel.

For cytotoxicity test, MDA-MB-435 human carcinoma cells were incubated with

free DOX, MHN, DOX-loaded MHN, and DOX-loaded F3-MHN with different concentrations (equivalent amount of DOX and MHN, $n = 3$) for 4 h, rinsed with cell medium three times, and then incubated for an additional 44 h. The cytotoxicity of various formulations of MHN was evaluated using MTT assay (Invitrogen). Cell viability was expressed as the percentage of viable cells compared with controls (cells treated with PBS).

To quantify blood half-life, MHN(800) in PBS (100 μ L) were intravenously injected into nude BALB/c mice ($n = 3$) at a dose of 3 mg/kg. Heparinized capillary tubes (Fisher) were used to draw 15 μ L of blood from the periorbital plexus at different times after intravenous injection. The extracted blood samples were immediately mixed with 10 mM EDTA (in PBS) to prevent coagulation. The blood extracted at different times was imaged in a 96-well plate in Cy7 channel using the NIR fluorescence scanner and the blood half-life was calculated by fitting the fluorescence data to a single-exponential equation using a one-compartment open pharmacokinetic model [4].

To determine if the MHN are dissociated during *in vivo* circulation, ~ 0.5 mL blood was extracted from the mouse 1 h after intravenous injection of MHN (10 mg/kg) and immediately mixed with ~ 0.5 mL of 10 mM EDTA (in PBS) to prevent coagulation. The MHN were recovered from the blood mixture by rinsing on the magnetic column 5 times with PBS. Their size and shape were observed using TEM with negative staining by pH 13 1.3% phosphotungstic acid (Note that the TEM used here can detect the micelle coating layer as well as MN and QD).

To quantify *in vivo* tumor accumulation, MDA-MB-435 human carcinoma tumors were subcutaneously implanted bilaterally into the hind flanks of nude BALB/c mice. Tumors were used when they reached ~ 0.5 cm in size. All animal work was reviewed and approved by Burnham Institute for Medical Research's Animal Research Committee. The MHN (or PBS control) samples were intravenously injected into mice ($n = 2\sim 4$) with a dose of 10 mg/kg. For real-time observation of tumor uptake, mice were imaged under anesthesia in Cy7 channel using the NIR fluorescence scanner, both pre- and 20 h post-

injection of MHN(800). To determine biodistribution, mice were sacrificed 20 h after MHN(800) injection by cardiac perfusion with PBS under anesthesia, and the organs were dissected and imaged in Cy5.5 or Cy7 channel using the NIR fluorescence scanner.

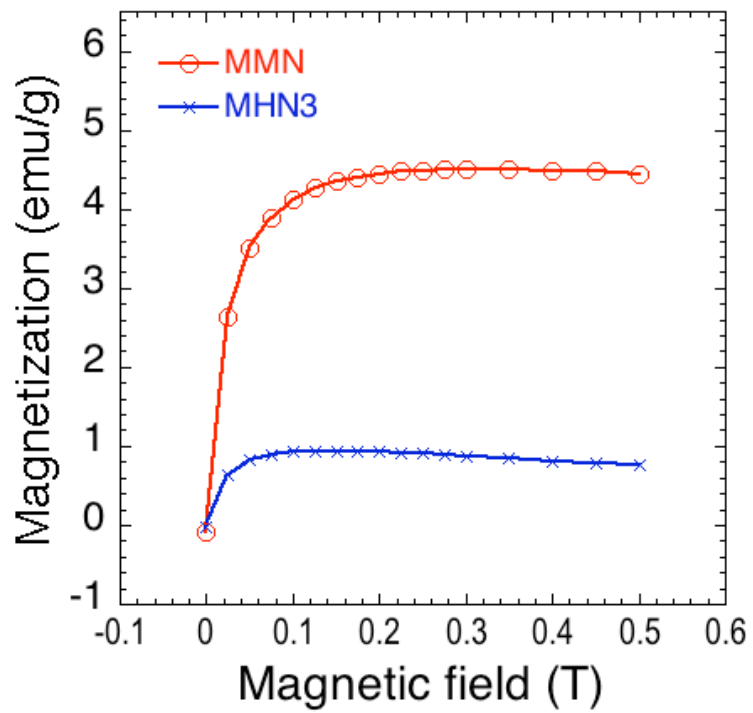


Figure S1. SQUID magnetization curves for MMN and MHN3 samples. The magnetization values are normalized by the total mass of particles in each sample.

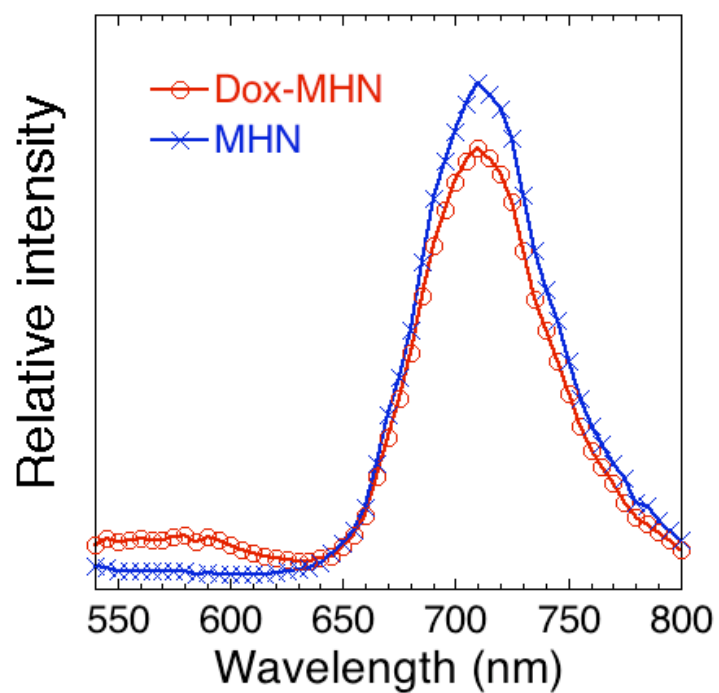


Figure S2. Fluorescence spectra of micellar hybrid nanoparticles (MHN) and doxorubicin-loaded MHN (DOX-MHN), obtained using 480 nm excitation. The weak fluorescence observed in the wavelength range 540-630 nm for the DOX-MHN sample is attributed to intrinsic fluorescence from DOX.

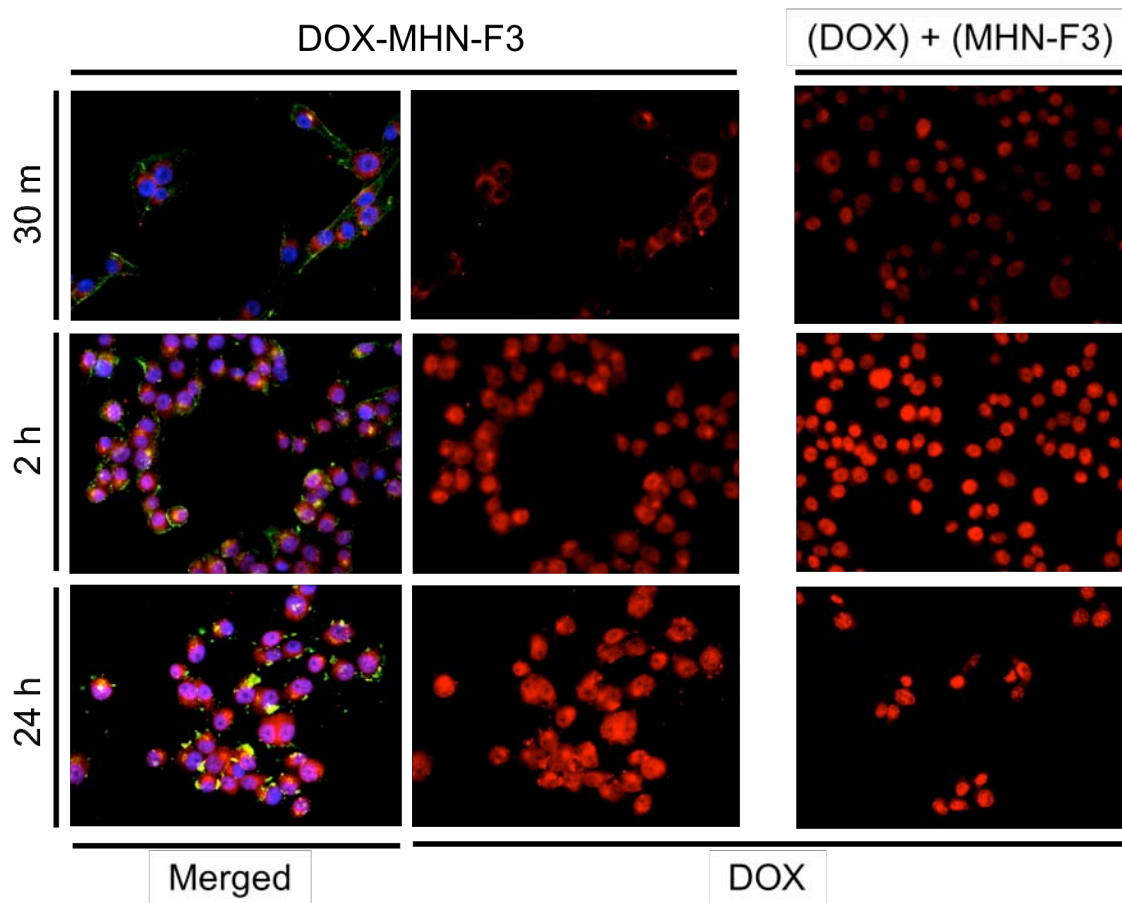


Figure S3. Targeted intracellular drug delivery of doxorubicin (DOX)-incorporated F3-MHN (DOX-MHN-F3) into MDA-MB-435 human carcinoma cells at multiple time points. The left and middle panels are for DOX (red)-incorporated F3-MHN (green). Nuclei are stained with DAPI (blue). The right panels are for free DOX that is physically mixed with F3-MHN (not incorporated into the MHN).

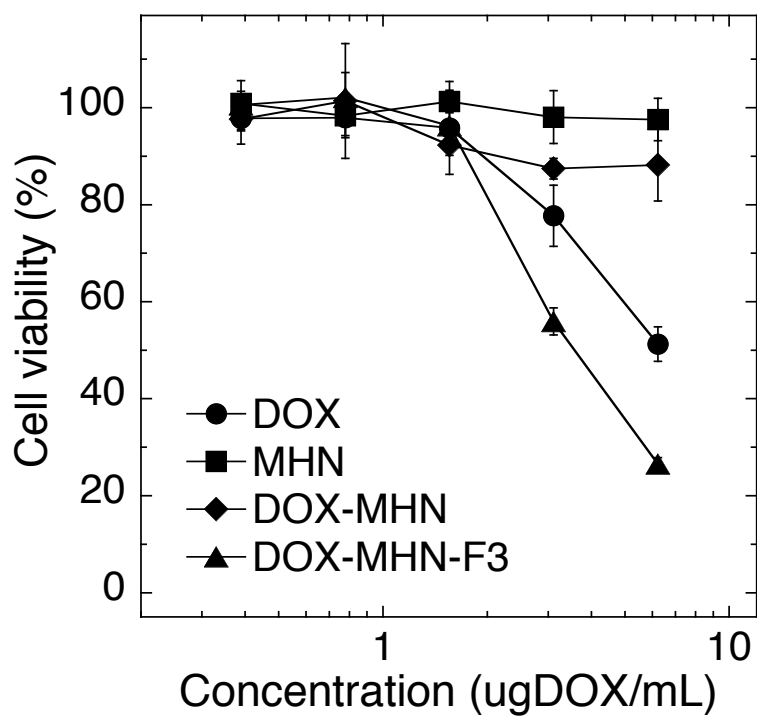


Figure S4. Cytotoxicity of various formulations of MHN by MTT assay. MDA-MB-435 human carcinoma cells are treated with free DOX, MHN, DOX-incorporated MHN (DOX-MHN), and DOX-incorporated F3-MHN (DOX-MHN-F3) for 4 h. The amounts of DOX and MHN used here are equivalent for all formulations (~0.093 mg of DOX per mg of MHN).

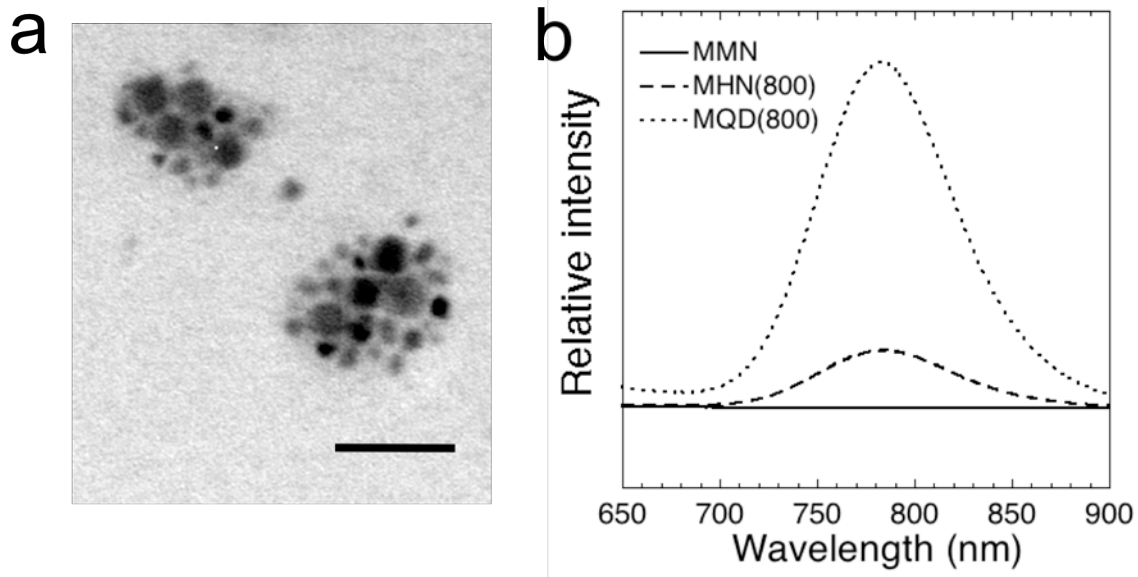
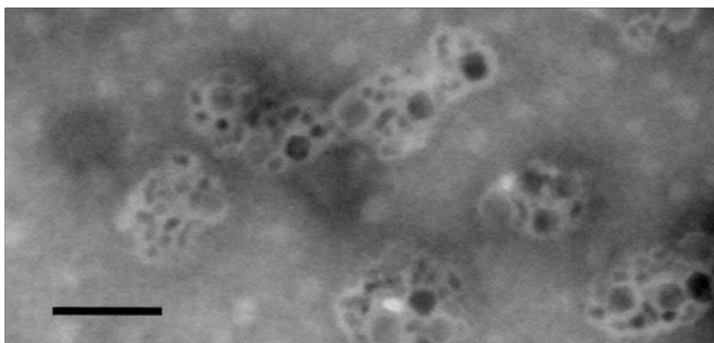
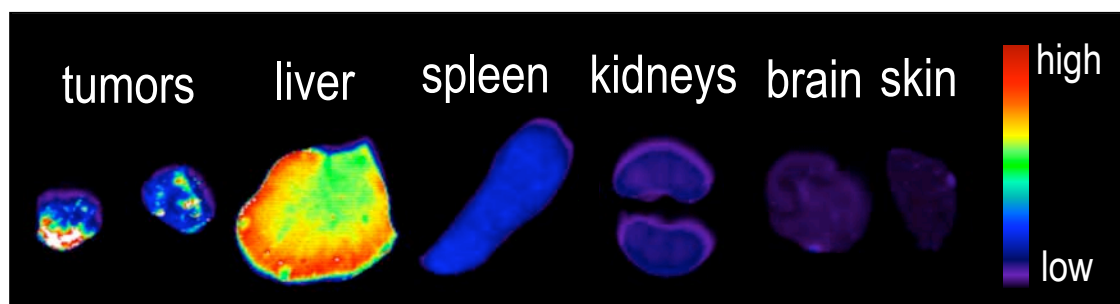


Figure S5. (a) TEM image of MHN composed of MN and QD emitting at 800 nm [MHN(800)]. Scale bar is 50 nm. (b) Photoluminescent spectra of MMN, MQD(800), and MHN(800), obtained with 450 nm excitation.

a



b



c

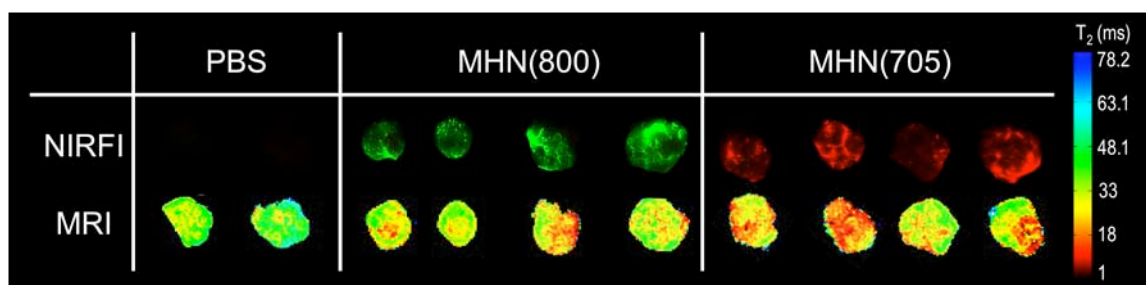


Figure S6. a) TEM image of MHN recovered from the blood circulation in mouse 2 h after intravenous injection (negative staining by 1.3% phosphotungstic acid). Scare bar is 50 nm. b) Biodistribution of MHN(800) 20 h after injection with a dose of 10 mg/kg. The organs were imaged in the Cy7 channel using a NIR fluorescence imaging (NIRFI) system. c) *ex vivo* NIRF and MR images of tumors harvested from mice 20 h after injection of either MHN(800) (green in NIRF images), or MHN(705) (red in NIRF images). MHN(800) or MHN(705) doses for the injections were 10 mg/kg. The control corresponds to tumors injected with equivalent volumes of phosphate-buffered saline (PBS). MR images obtained in T_2 -weighted mode; the color map for the T_2 values is indicated at the far right of the Figure.

References

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