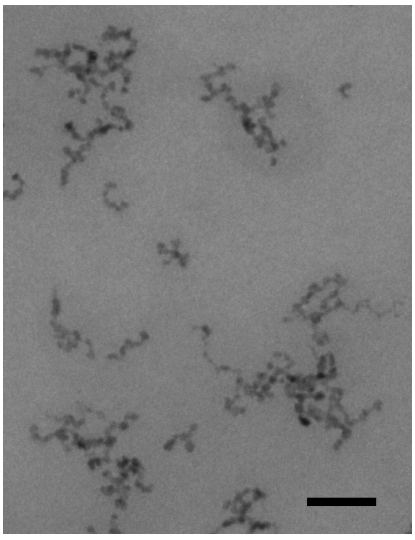
Supporting information for:

Magnetic Iron Oxide Nanoworms for Tumor Targeting and Imaging

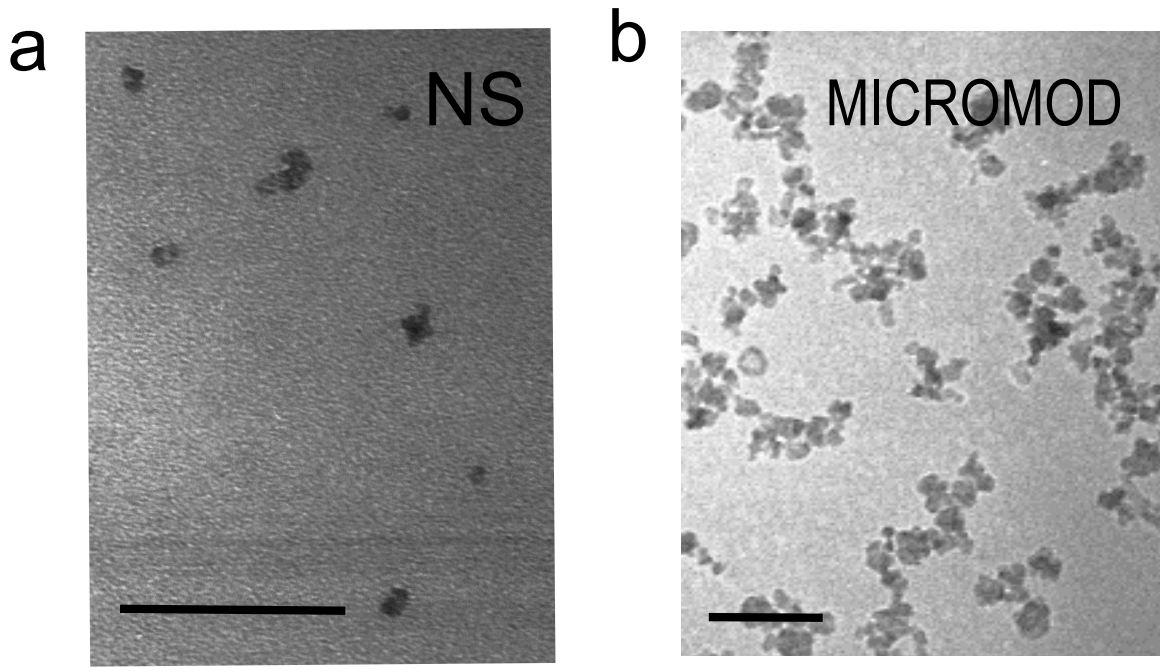
By *Ji-Ho Park, Geoffrey A. von Maltzahn, Lianglin Zhang, Michael P. Schwartz, Erkki Ruoslahti, Sangeeta N. Bhatia, and Michael J. Sailor**

Supplementary figures

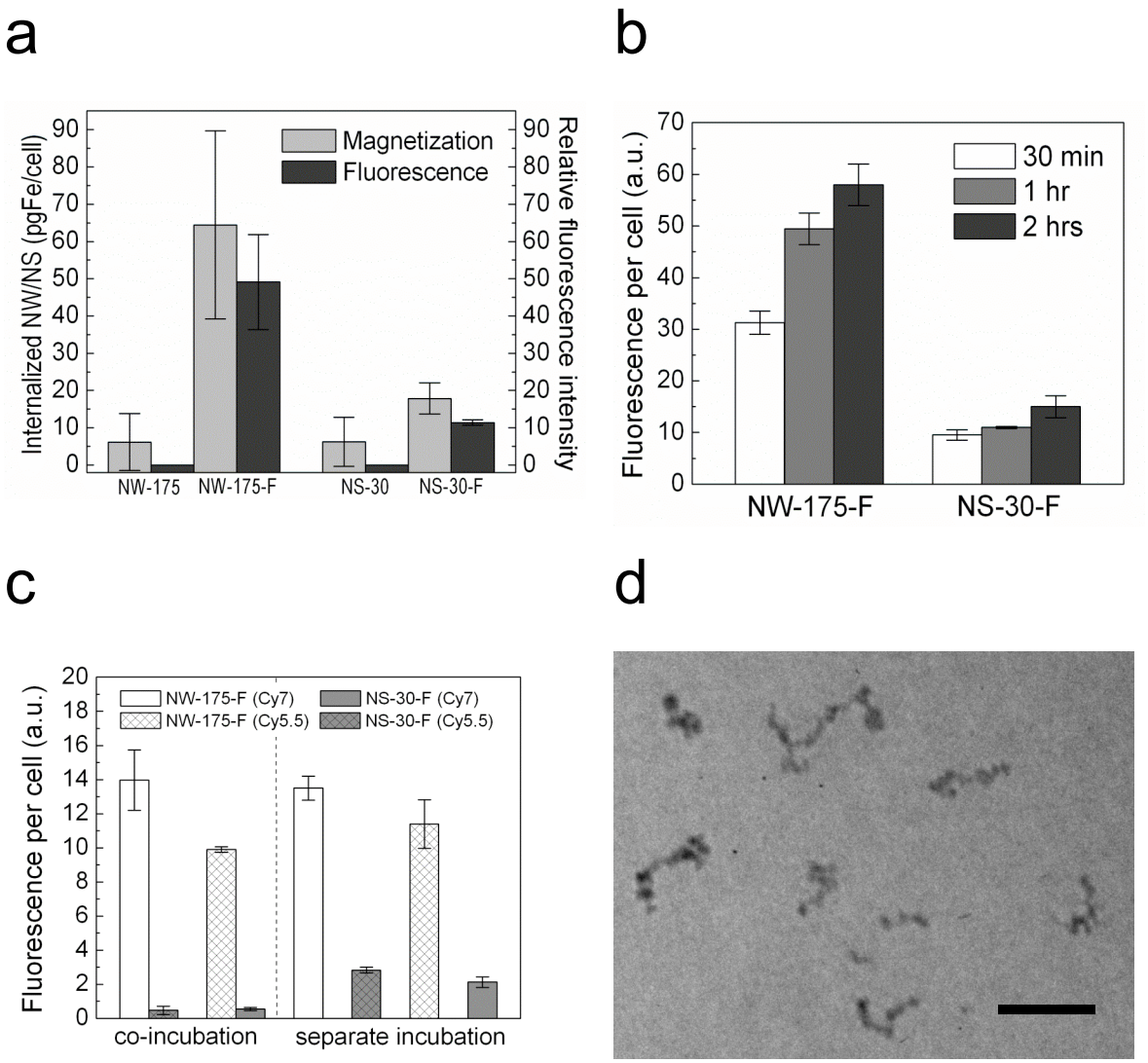
property	40kDa Dextran
size (nm)	97.1 ± 44.5
zeta potential (mV, at pH 7)	- 4.9
saturation magnetism (emu/gFe)	77.4
blood half-life (min)	~ 480



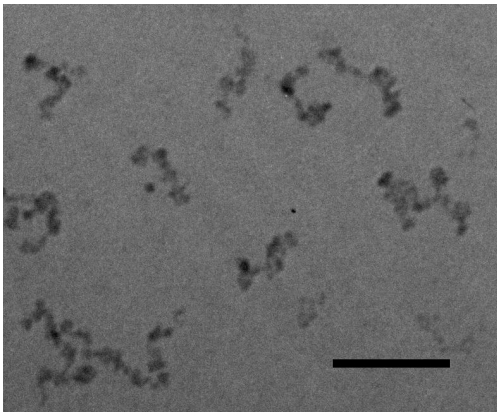
Supplementary Figure 1. Physical and biological properties of large, highly-branched NW prepared with high molecular weight dextran (MW 40,000, Sigma). Scale bar in the TEM image is 50 nm. These samples were not tested for their tumor-homing properties in the present study.



Supplementary Figure 2. Transmission Electron Microscope (TEM) images showing the shape and size of (**a**) NS and (**b**) commercial dextran-coated IO nanoparticles (Micromod). The shape and size of the NS made in this work are similar to iron oxide nanoparticles with a cross-linked dextran coating reported previously (CLIO). These materials display a spherical morphology with a relatively narrow size distribution*[15, 16, 18, 32]*. Micromod samples appear as clusters of IO cores, rather than the chain-like structures observed with the NW prepared in this study. Scale bar is 50 nm.



Supplementary Figure 3. (**a**) Comparison of fluorescence intensity and magnetization with the cells internalized with NW-175-F or NS-30-F after 2 hrs of incubation with MDA-MB-435 cells in vitro. (**b**) Quantification of fluorescence intensity in MDA-MB-435 cells incubated with Cy7-labeled NW-175-F or NS-30-F for the indicated times. (**c**) Comparison of cellular internalization of NW-175-F and/or NS-30-F incubated together or separately for 2 hrs. (**d**) Transmission Electron Microscope (TEM) image showing the shape and size of the NW recovered after internalized in the cells for 24 hrs. Scale bar is 50 nm. All error bars show the standard deviation for three or more samples.



Supplementary figure 4. Transmission Electron Microscope (TEM) images showing the shape and size of the NW recovered after circulating in the blood stream for 24 hrs. Scare bar is 50 nm.

Supplementary methods

Nanoworm and nanosphere preparation

0.63 g of FeCl3◊6H2O and 0.25 g FeCl2◊4H2O were mixed with 4.5 g dextran in 10 mL of deionized (Millipore) water at room temperature. This acidic solution was neutralized by the dropwise addition of 1 mL concentrated aqueous ammonia under vigorous stirring and a steady purge of nitrogen, and it was then heated at ~70 oC for 1 hr. After purification by centrifuge filtering column (100,000MWCO, Millipore), the magnetic colloid was crosslinked in strong base (5M aqueous NaOH solution) with epichlorohydrin (Sigma) and filtered through a 0.1 μm pore diameter membrane (Millipore). NW with a size range of 50~80 nm were separated using a MACS® Midi magnetic separation column (Miltenyi Biotec). Nanosphere (NS) with a size range of 25~35 nm were prepared as described[2]. Micromod IO nanoparticles (50 nm nanomag-D-SPIO with amines) were obtained from Micromod Partikeltechnologie GmbH, Rostock, Germany. Feridex IO nanoparticles were obtained from Berlex, NJ, USA.

Size and shape determination using TEM, AFM and DLS

For transmission electron microscopy (TEM) imaging, an aliquot of NW, NS, or Micromod nanoparticles 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. TEM images were obtained using a Hitachi H-600A transmission electron microscope. More than 80% of the nanostructures synthesized with 20-kDa dextran displayed chain-like shapes in the TEM images. For atomic force microscopy (AFM) imaging, an aliquot of NW in water was dropped onto a glass microscope slide (Fisher, US) and then dried in air. AFM images were obtained with a Nanoscope IIIa multimode atomic force microscope (Digital Instruments, US) operating in intermittent contact (tapping) mode. For the dynamic light scattering (DLS) measurement, 50 mL of a diluted solution of NW or NS was transferred into a quartz cuvette and hydrodynamic size measurements were obtained using a Malvern (Worcestershire, UK) Zetasizer ZS90. Aggregation of IO cores observed in the TEM images did not appear to be due to a drying effect in the preparation of the TEM samples, as the particle sizes derived from the TEM images were well correlated with hydrodynamic diameter measurements by DLS for both the NW and NS. (Note that TEM only images the IO cores and not the dextran coating).

Magnetic measurement

The SQUID (Superconducting Quantum Interference Device) magnetometry provides a direct measure of the total number of magnetic IO nanoparticles in a sample, as it measures the magnetization of a sample rather than the total iron content or the fluorescence intensity from

a molecular tag. The SQUID measurements are thus more relevant to MRI imaging applications, because the magnetization data correlates with T2[5]. The SQUID technique has the additional advantage that it can be performed on cells, cell extracts, or on whole organs, and little sample workup is needed.

A solution of the NW or NS sample was frozen and lyophilized to dryness in gelatin capsules. The capsules were inserted into the middle of transparent plastic drinking straws. The measurements were performed at 298 K using a Quantum Design (CA, USA) MPMS2 superconducting quantum interference device (SQUID) magnetometer. The samples were exposed to direct current magnetic fields in stepwise increments up to one Tesla. Corrections were made for the diamagnetic contribution of the capsule and straw.

MRI T2 mapping

MRI T2 mapping of NW or NS samples was performed using a 7 cm bore, Bruker (Karlsruhe, Germany) 4.7 T magnet. Samples were serially diluted with aqueous PBS (Mediatech) in a 384-well plate, containing 95 ml total sample/well. 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.

Targeting peptide and dye conjugation

For near-infrared (NIR) fluorescence imaging, Cy7-labeled NW or NS were prepared by reacting aminated NW (500 mg Fe) or NS (900 mg Fe) in PBS buffer with 6 mg of Cy7-NHS ester (GE Healthcare Bio-Sciences) in DMSO (Sigma) for 1 hr to have same fluorescence per iron atom for both NW and NS (one Cy7 dye per one IO core). The remaining free amines were used for conjugation with the targeting peptides. 500 mg Fe of Cy7-labed NW or NS were first reacted with 200 mg of Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC, Pierce Chemicals) or 2 mg NHS-PEG(5kDa)-MAL (Nektar) in PBS solution for 1hr and then purified using a desalting column (GE Healthcare Bio-Sciences). 200 mg of targeting peptide with a free terminal cysteine was then added to the 500 mg Fe NW or NS sample in PBS solution. After incubation for 2 h with mild shaking at room temperature, the sample was purified with a centrifuge filter (100,000 MWCO, Millipore), and then re-suspended in PBS solution.

In vitro cell internalization

For cell internalization studies, MDA-MB-435 human breast 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. To obtain co-incubation and separate incubation data on NW-F and/or NS-F, the cells were co-incubated with Cy7-labeled NW-F3 (20 mgFe) and Cy5.5-labeled NS-F3 (20 mgFe) or Cy5.5-labeled

NW-F3 (20 mgFe) and Cy7-labeled NS-F3 (20 mgFe), or incubated separately with Cy7-labeled NW-F3 (20 mgFe), Cy5.5-labeled NS-F3 (20 mgFe), Cy5.5-labeled NW-F3 (20 mgFe), or Cy7-labeled NS-F3 (20 mgFe) per well for 2 hrs at 37 °C in the presence of 10% FBS. The cells were rinsed three times with cell media and then imaged simultaneously in both the Cy5.5 (680 nm excitation/700 nm emission) and Cy7 channels with a NIR fluorescence scanner (LI-COR biosciences). The total number of attached Cy7 or Cy5.5 dye molecules was controlled to yield the same fluorescence intensity on a per-iron basis for both types of particles. The relative fluorescence of the images (each well) was analyzed using the ImageJ (NIH) or OsiriX (Apple) programs. All error bars show the standard deviation for three or more samples.

To quantify the internalized amount of NW or NS, the cells were carefully detached from each well using trypsin-EDTA, and centrifuged into a pellet. The pellets were freeze-dried in gelatin capsules, and then analyzed for magnetization using SQUID[6]. All error bars show the standard deviation for three or more samples.

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 10 mg (total Fe content) of Cy7-labeled peptide-conjugated NW or NS per well for 3h at 37 oC in the presence of 10% FBS. After incubation, the slides were rinsed three times with PBS, fixed with 4% paraformaldehyde, and then washed three times with PBS and mounted in Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). The slides were observed with a fluorescence microscope (Nikon, Tokyo, Japan).

To determine if the shape of NW are changed by cell uptake or not, the internalized NW were recovered from the cells 24 hrs after incubation. The cells incubated with NW for 24 hrs were rinsed three times with culture medium, carefully detached from the well using trypsin-EDTA, and centrifuged into a pellet. The pellet was dissolved in 10% SDS (in PBS) for 10 min and the NW isolated from the cells were rinsed using centrifugation and magnetic column. Their size and shape was analyzed using DLS and TEM.

Blood half-life and biodistribution

To quantify the in vivo circulation times of NW or NS samples in Nude BALB/c mice, NW or NS in PBS (100 mL) were intravenously injected into nude BALB/c mice at a dose of 3 Fe/kg body mass mass (n = 3 for each NW or NS formulation). Heparinized capillary tubes (Fisher) were used to draw 15 mL (for fluorescence) or 70 ml (for magnetization) of blood from the periorbital plexus at different times after intravenous injection. The extracted blood samples were immediately mixed with 10 mM EDTA to prevent coagulation. For Cy7-labeled NW or NS formulations, blood extracted at different times was imaged in a 96-well plate in Cy7 channel (762 nm excitation/800 nm emission) with a NIR

fluorescence scanner (LI-COR biosciences, NE, USA). The images were analyzed using the ImageJ (NIH) or Osirix (Apple) programs. For non-labeled NW or NS samples, blood samples extracted at different times were immediatly freeze-dried in gelatin capsules, and then analyzed for magnetization using SQUID[6]. All error bars show the standard deviation for three or more animals.

To determine if the shape of NW are changed during circulation, the NW were extracted from the blood stream 24 hrs after intravenous injection and rinsed completely 5 times on the magnetic column with PBS solution, and their size and shape was analyzed using DLS and TEM.

For the mouse biodistribution studies, unmodified NW in PBS (100 mL) were intravenously injected into Nude BALB/c mice at a dose of 3 mg Fe/kg body mass (n = 3 for both the PBS controls and the NW samples). The animals were sacrificed 24 hrs after injection by cardiac perfusion with PBS under anesthesia, and the blood, brain, heart, kidney, liver, lung, lymph node, skin and spleen were collected. Organs and blood were immediately weighed, freeze-dried in gelatin capsules, and then analyzed for magnetization using SQUID[6]. Percentages of injected dose of the NW per wet weight of each organ were further corrected by subtracting magnetization of the PBS-injected organs (controls) from magnetization of the particles-injected organs.

In vivo passive tumor homing

MDA-MB-435 human breast carcinoma cells (2 x 10) were injected into the mammary fat pad or subcutenously injected into nude BALB/c mice, respectively. 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. Cy7-labed NW or NS were intravenously injected into mice (n = 3~4 for each formulation) with a dose of 1 mg Fe/kg body mass. For real-time observation of tumor/liver uptake, animals were imaged under anesthesia in Cy7 channel using the BonSai fluorescence-imaging system (Siemens, PA, USA) 6 hrs, 24 hrs or 48 hrs after injection.

Supplementary References

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