Supplementary Information

Tumor-homing hyaluronic acid nanoparticles for tumor-specific optical/MR dual imaging in tumor-bearing mice

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Experimental Section.

Materials. Sodium hyaluronate ($M_w = 2.344 \times 10^5$), purchased from Lifecore Biomedical (Chaska, MN, USA), was dialyzed against distilled water and lyophilized before use. Ethylenediamine, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), 5β-cholanic acid, adipic acid dihydrazide (ADH), 1-hydroxybenzotriazole (HOBt), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), 1-octadecene, oleic acid, tetrahydrofuran (THF), potassium ferrocyanide(II) trihydrate, and formaldehyde were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The near-infrared fluorescence dye, Cy5.5, was purchased from Amersham Biosciences (Piscataway, NJ, USA). The water used for synthesis and characterization was purified by distillation, deionization, and reverse osmosis (Milli-Q Plus). All other chemicals were of analytical grade and were used without further purification.

Preparation of HNP-SPIONs. HA-CA conjugates, capable of being self-assembled into nanoparticles in aqueous solution, were synthesized by chemical conjugation of CA to the backbone of HA through amide formation as described in a previous report.[15] To observe the real-time biodistribution of HNPs, HA-CA conjugates were labeled with Cy5.5 in the presence of EDC and HOBt.[17] The amount of Cy5.5 in the conjugate was fixed at 3.2 wt. %, as determined with a UV/VIS spectrophotometer at 680 nm. Oleic acid-coated SPIONs (OA-SPIONs) of 6.8 nm diameter were synthesized by thermal decomposition as described in a previous report.[2] Briefly, 5.4 g of ferric

chloride hexahydrates (96.69 mmol) and 18.25 g of sodium oleate (59.94 mmol) were dissolved in a solution that contains 40 ml of ethanol, 30 mL of distilled water, and 70 mL of hexane. The mixed solution was heated to 70 °C for 4 h. The iron-oleate complex in the upper organic layer was then washed with 30 mL of distilled water. The hexane remaining in the organic layer was evaporated. Then, 19.44 g of iron-oleate was dissolved in 2.62 mL of oleic acid and 126.74 mL of 1-octadecene in a 500 ml round bottom flask. The mixture was heated to 320 °C, until the mixture turns black and kept for 40 min at this temperature for nanoparticle growth. The resulting solution was washed with an ethanol/acetone solvent and centrifuged at 2000 rpm for 15 min. It was then re-dispersed in hexane and washed 5 times to remove any remaining organic solvent.

The OA-SPIONs were encapsulated into the Cy5.5-conjugated HNPs (Cy5.5-HNPs) via dialysis. In brief, OA-SPIONs (1, 3, and 5 mg) were first dissolved in 0.5 mL of THF. Then, 10 mg of Cy5.5-HNPs were dissolved in a mixed solvent of THF (5 mL) and deionized water (5 mL). The solution containing OA-SPIONs was slowly mixed with the solution containing Cy5.5-HNPs. The resulting mixture was sonicated for 15 min using a Branson Sonifier 450 (Danbury, CT, USA) and dialyzed against deionized water for 24 h (MWCO = 12K-14K) to remove the residual organic solvent. The final product was filtered using a syringe filter (0.8-μm pore size) to remove large aggregates, and then lyophilized. The SPION-encapsulated Cy5.5-HNPs are designated as HNP-*x*-SPION, where *x* indicates the feed amount of OA-SPIONs. The detailed structure of the HNP-*x*-SPION is shown in **Scheme 1**.

Characterization of HNP-SPIONs. The average hydrodynamic radius of the HNP-SPIONs was determined by electrophoretic light scattering (ELS 8000, Otsuka Electronics, Osaka, Japan). HNP-SPIONs were suspended in PBS (pH 7.4) and subjected to particle size analysis. The angle was fixed at 90°C for size measurements. The shape and morphology of the HNP-SPIONs were observed using a transmission electron microscope (TEM, Philips CM30, Philips Research Laboratories, Eindhoven,

The Netherlands) with an accelerating voltage of 200 keV. The samples were prepared by coating the HNP-SPIONs suspensions onto a copper grid (200 mesh size). The iron content of the HNP-SPIONs was determined using an inductively coupled plasma (ICP) mass spectrometer (Direct Reading Echelle ICP, Teledyne Leeman Labs, Hudson, NH, USA). The samples were digested with 70% HNO_3, incubated at 90°C for 1 h, and diluted to 5 mL with pure water. The blanks and five standard iron solutions (0.01, 0.1, 1, 10, and 100 ppm) were used to determine the iron concentration of the HNP-SPIONs and its iron contents were normalized to the iron concentration of pure water.

NIRF and T_2-weighted MR phantom study for HNP-SPIONs. To evaluate their optical properties, HNPs and HNP-SPIONs were dispersed and sonicated in PBS, and the solutions with various concentrations of the samples were prepared by dilution in PBS. The solutions were then transferred into a 96-well plate and placed directly in a Kodak Image Station. The fluorescent images were obtained with a 12-bit CCD camera (Kodak Image Station 4000 MM, New Haven, CT, USA) equipped with a special C-mount lens and Cy5.5 band pass emission filter (680 nm to 720 nm; Omega Optical, Brattleboro, VT, USA). The *in vitro* MR contrast effect of the sample was measured by a phantom study. MR phantom images of the HNPs and HNP-SPIONs were obtained at 1.5 T by T_2-weighted fast spin echo sequence. The detailed experimental conditions were as follows: pulse repetition time/echo time (TR/TE) = 5000 ms/16 ms, DFOV = 110 mm, slice thickness = 1.5 mm, matrix = 256 x 192. An eight-channel head coil was used.

Magnetization and T_2 relaxivity studies. A superconducting quantum interference device (SQUID) magnetometer (MPMS-5, Quantum Design, San Diego, CA, USA) was used to investigate the magnetic properties of the HNP-SPIONs and SPIONs. The magnetization hysteresis was obtained by varying H between +10000 Oe and −10000 Oe. The measurements were carried out at 300 K.

The T_2 relaxivity of the HNP-SPIONs in PBS was measured using a clinical 1.5-T MRI scanner (Siemens 1.5T Sonata, Erlangen, Germany). HNP-SPIONs were dissolved in PBS at concentrations of 0.007, 0.015, 0.03, 0.06, 0.12, and 0.24 mM. T_2-weighted images were acquired at 25 °C in PBS using the following parameters: 5000 ms of TR and TEs ranging from 16 to 64 ms. To determine T_2 relaxation times, a circular region was selected and the T_2 relaxation times were obtained. The transverse relaxation rates with increasing Fe concentration were analyzed by linear regression analysis. The T_2 relaxivity (r_2, $mM^{-1}s^{-1}$) of the HNP-SPIONs was calculated from the slope of the linear plot of $1/T_2$ versus Fe concentration.

***In vitro* cellular uptake of HNP-SPIONs**. SCC7 cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Gelatin-coated cover slips were placed in 12-well plates containing RPMI-1640. SCC7 cells were seeded in each well at density of 5 x 10^4 cells per well. After 48 h of incubation, HNP-SPIONs (25 μg Fe/well) were added to the 12-well plates. Two hours later, each well was washed with cold PBS, treated with 2 mL/well of 4% paraformaldehyde solution for 10 min, and then washed twice with cold PBS. The cells were then stained with Prussian blue. Two milliliters of a 1:1 mixture of 4% potassium ferrocyanide (II) trihydrate and 4% HCl was added to each well. The cells were incubated for 30 min in a CO_2 incubator, and each well was then washed three times with cold PBS. The cells were subsequently stained with nuclear fast red for 5 min. Each cover slip was placed on a slide, treated with PermaFluor Mounting Medium, and dried for 2 h. The Prussian blue staining results were assessed using a light microscope (BX51, Olympus, Tokyo, Japan) and fluorescence was observed (excitation: 673 nm, emission: 692 nm) using a fluorescent microscope (Axioskop2FS, Carl Zeiss, Inc., Thornwood, NY, USA).

***In vivo* biodistribution and tumor-targeted imaging of HNP-SPIONs**. Tumor-bearing mice were prepared by subcutaneously injecting a suspension of 1×10^6 SCC7 cells in physiological saline (100 μL) into C3H/HeN mice (six weeks old, 20–25 g). When the tumors grew to approximately 150–200 mm^3, HNP-SPIONs were intravenously injected into the mice via their tail veins at a dose of 6 mg Fe/kg body mass. HNPs were also intravenously injected into the mice as a control. After the intravenous injections, biodistribution of both HNPs and HNP-SPIONs was evaluated by positioning the mice on an animal plate heated to 36 °C in the eXploreOptix System (Advanced Research Technologies Inc., Montreal, Canada). During imaging, the laser power and count time settings were optimized at 3 μW and 0.3 s per point, respectively. The excitation and emission spots were raster-scanned in 1-mm steps over the region of interest to generate emission wavelength scans. A 670-nm pulsed laser diode was used to excite the Cy5.5 molecules. The NIR fluorescence emissions at 700 nm were collected in a fast photomultiplier tube (Hamamatsu, Japan) and detected with a time-correlated single photon counting system (Becker and Hickl GmbH, Berlin, Germany). To compare the distributions of HNPs and HNP-SPIONs in tissues and tumors, the mice were sacrificed one day after the intravenous injection. The excised tissues, including the livers, spleens, lungs, kidneys, hearts, and tumors, were examined using a Kodak Image Station. The accumulation of HNPs and HNP-SPIONs in the tumor regions were quantified and recorded as total photons per centimeter squared per steradian (p/s/cm^2/sr) for each tumor (n = three mice per group).

***In vivo* MR imaging**. Tumor-bearing mice were prepared by subcutaneously injecting SCC7 cells into their left flanks. When the tumors grew to approximately 150–200 mm^3, HNP-SPIONs were injected intravenously through the tail veins of the mice. MR imaging was performed with a 1.5-T MRI system (Siemens 1.5T Sonata, Erlangen, Germany). The experimental conditions were as follows: TR/TE = 12 ms/3.5 ms, DFOV = 60 mm, slice thickness = 1.5 mm; a wrist coil was used. The mice were placed at the same axial distance within the scanner. They were imaged pre-injection

and at 1, 3, and 24 h post-HNP-SPIONs injection. The signal intensity was measured in defined regions of interest, which were in comparable positions for each tumor.

***Ex vivo* histology studies**. The tumors and muscles were excised, stored in 4% paraformaldehyde, dehydrated with a graded ethanol series, and embedded in paraffin. The paraffin tissue was cut into 6-μm slices. The paraffin-embedded histological slices were stained with Prussian blue to detect HNP-SPIONs. Prussian blue staining was used to confirm the presence of iron in the histological sections of the tumors and muscles. The samples were deparaffinized and dehydrated and then immersed in a solution of 4% w/v potassium ferrocyanide and 4% hydrochloric acid (v/v) in water for 10 min. The slides were rinsed with water and images were obtained with a microscope. To observe fluorescence signals, the tumors and muscles were dissected and fixed in a 4% formaldehyde solution for 10 min at room temperature. The tumors and muscles were frozen in OCT compound (Miles Inc., Elkhart, IN, USA) with liquid nitrogen and sectioned into 10-μm slices using a cryostat (CM1850; Leica Microsystems GmbH, Wetzlar, Germany). Fluorescence was observed (excitation: 673 nm, emission: 692 nm) using a fluorescent microscope (Axioskop2FS, Carl Zeiss, Inc., Thornwood, NY, USA).

Statistical analysis. All data are expressed as the mean ± s.e. of at least triplicate experiments. Data for in vivo characteristics were calculated by using the region of interest (ROI) function of the eXplore Optix system software (Advanced Research Technologies Inc., Montreal, Canada). All date processing was performed using the ORIGIN 8.0 statistical software program (OriginLab Corp., U.S.A.).

Table S1. Physicochemical characteristics of HNP-*x*-SPIONs

Sample	SPION Content [a] (wt %)	Loading efficiency [a] (%)	Size (nm) [b]
HNP	-	-	250.2 ± 12.59
HNP-10%-SPION	8.64	86.42 %	348.8 ± 10.01
HNP-30%-SPION	20.87	69.58 %	307.7 ± 4.09
HNP-50%-SPION	33.70	67.40 %	310.2 ± 7.09

[a] Loading content and loading efficiency of SPIONs determined by ICP; [b] Particle size measured using electrophoretic light scatering (ELS).

Fig. S1. Size distribution of HNP-*x*-SPIONs confirmed with ELS. Inset shows the TEM image