A simple method for the synthesis of hyaluronic acid coated magnetic nanoparticles for highly efficient cell labeling and *in vivo* imaging

Supporting Information

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Content

I. Materials and instrumentation

All chemical were reagent grade and were used as received from the manufacturer. Ethyl-(3,3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), iron (III) acetylacetonate, 1,2-hexadecanediol, benzyl ether, nuclear fast red solution, and fetal bovine serum (FBS) were from Sigma-Aldrich. Potassium ferrocyanide [$K_4Fe(CN)_6$] trihydrate was purchased from Mallinckrodt. Adipic dihydrazide and n-hexanol were purchased from Acros Organics. Oleylamine was purchased from Fluka. CellTiter 96® Aqueous One solution, containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) and phenazine ethosulfate (PES), was purchased from Promega. Ultrafiltration membranes were purchased from Millipore and dialysis tubings were obtained from BioDesign Inc. Hyaluronic acid (31 kDa) was purchased from Lifecore Biomedicals. Phosphate buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), fluorescein isothiocyanate (FITC), sodium pyruvate (100 mM), glutamine, and Penicillin-Streptomycin (Pen Strep) mixture were from Invitrogen. B16F10 and SKOV-3 cell lines were obtained from American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% FBS, 2% Pen Strep mixture, 1% and sodium pyruvate. Transmission electron microscopy (TEM) images were collected on a JEM-2200FS operating at 200 kV using Gatan multiscan CCD camera with Digital Micrograph imaging software. Thermogravimetric analysis (TGA) was carried on a Thermal Advantage (TA-Instruments-Waters LLC) TGA-Q500 series and the samples were burned under nitrogen. The hydrodynamic diameter and zeta potential were assessed on Malvern Zetasizer Nano zs instrument. FACS experiments were conducted on a BD Vantage SE flow cytometer.

II. Experimental procedures

1. Synthesis of oleic acid coated-iron oxide nanoparticles (OA-IONPs)

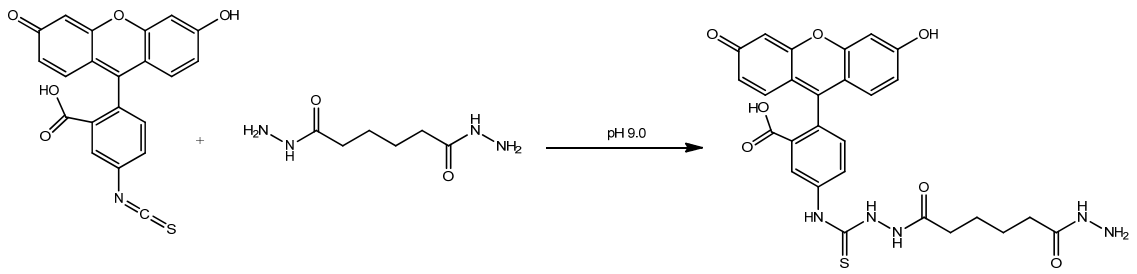
Iron (III) acetylacetonate [$Fe(acac)_3$] (0.71 g; 2 mmol), 1,2-hexadecanediol (2.58 g; 10 mmol), oleic acid (1.69 g; 6 mmol), oleyl amine (1.61 g; 6 mmol), and benzyl ether (40 ml) were mixed together and stirred under a flow of nitrogen. The mixture was heated to 200^{o}C for 2 hours followed by refluxing (>300^{o}C) for 1 hour. The black mixture was cooled to room temperature and ethanol (50 ml) was added. The iron oxide nanoparticles were separated by an external magnet, and washed three times with ethanol to remove excess starting material. The iron oxide nanoparticles were dispersed in hexane (50 ml) and the dispersion was placed on an external magnet to remove undispersed magnetic material. The supernatant containing the nanoparticles was centrifuged at 6000 rpm to remove the non magnetic large particulates. 300 mg of OA-coated iron oxide nanoparticles dispersed in 50 ml hexane were collected (6 mg/ml). The nanoparticles had a core size of 6 nm as determined by TEM, and hydrodynamic diameter of 9.5 nm as determined by DLS.

To synthesize the cobalt ferrite nanoparticles (CFNP), cobalt acetylacetonate [$Co(acac)_2$] and $Fe(acac)_3$ were reacted at 1:2 ratio under the same experimental conditions used to generate IONPs. Briefly, $Fe(acac)_3$ (0.71 g; 2 mmol), $Co(acac)_2$ (0.25 g;1 mmol), 1,2-hexadecanediol (2.87 g; 15 mmol), oleic acid (2.54 g; 9 mmol), oleyl amine (2.42 g; 9 mmol), and benzyl ether (60 ml) were mixed together and stirred under a flow of nitrogen. The mixture was heated to 200^{o}C for 2 hours, and then to reflux for 1 hour. Following cooling to room temperature and addition of ethanol, the CFNPs were collected on an external magnet, washed with ethanol, and resuspended in hexane. TEM images showed CFNPs with core size of 10 nm.

2. Coating the IONPs with hyaluronic acid (HA) via ligand exchange

OA-IONPs (25 mg) were dissolved in toluene (15 ml) and added to a solution of HA (25 mg; 31 kDa) in water (30 ml) containing potassium hydroxide (1N; 0.5 ml). The two phase system was refluxed for 24 hours under rapid stirring to allow the mixing between the organic layer containing the NPs and the aqueous layer containing HA. The aqueous layer containing the HA-coated IONPs (HA-IONPs) was collected from a separatory funnel, and purified by ultrafiltration (MWCO 100,000) to remove the excess HA and KOH. The purified HA-IONPs were diluted with water to a final volume of 50 ml, and placed on an external magnet to remove large particulates. The hydrodynamic diameter of HA-IONPs was ~ 50-55 nm.

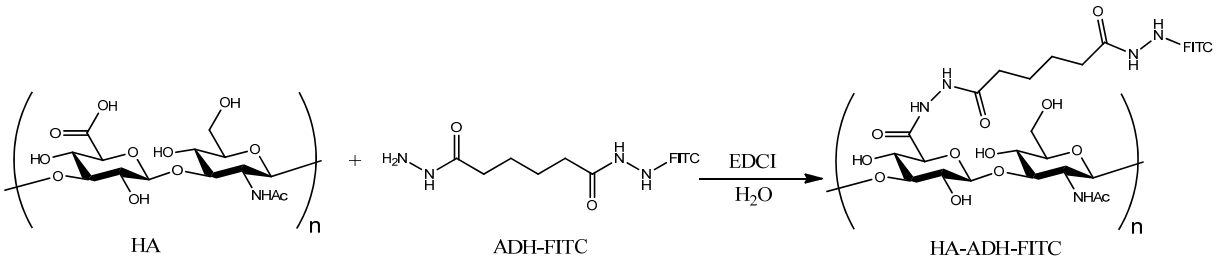
3. Synthesis of adipic dihydrazide-fluorescein isothiocyanate (ADH-FITC)



FITC ADH ADH-FITC

Adipic dihydrazide (0.5 g; 2.9 mmol) was dissolved in DI water (20 ml) and the pH was adjusted to 9 by dropwise addition of 0.1 N sodium hydroxide (NaOH) solution. FITC solution, prepared by sonicating FITC (0.2 g; 0.51 mmol) in 50 ml of bicarbonate/ carbonate buffer (50 mM; pH 9), was added dropwise. The mixture was stirred at room temperature for 1 hour. The desired product was precipitated by adjusting the solution's pH to ~3.7 via the dropwise addition of 1 M aqueous HCl solution. The precipitate was collected by centrifugation during which it was washed 4 times with water. The desired product was collected as a yellow solid in 86% yield following drying on a lyophilizer.

4. Synthesis of FITC-ADH-HA conjugate



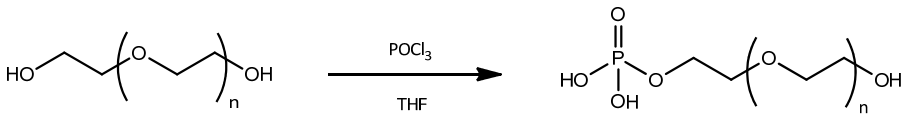
HA (31 kDa) was dissolved in DI water (25 ml) and FITC-ADH (250 mg) was added. The pH of the solution was adjusted to 4.5-4.8 by the dropwise addition of 0.1 N aqueous HCl solution. EDCI (5 mg) was added and the reaction was stirred at room temperature for 3 hours during which the pH was constantly adjusted to 4.5-4.8. The pH of the reaction mixture was then brought to 7 by adding 0.1 N aqueous NaOH solution. The mixture was transferred to a dialysis bag (MWCO 3500) and dialyzed against DI water for 8 hours. Water was then evaporated on a high vacuum pump. The percentage of FITC on HA was found to be 1.7% (w/w) as determined by UV-vis (Absorbance λ=488 nm) (Figure S1a).

5. Synthesis of FITC-HA-IONPs

HA-FITC (70 mg) was dissolved in DI water (50 ml), and KOH (0.1 N; 0.5 ml) was added. OA-IONPs (70 mg) dispersed in toluene (25 ml) were added to the aqueous HA solution. The mixture was refluxed for 24 hours during which the NPs transferred from the toluene organic phase to the aqueous phase indicating that OA was displaced by HA. The aqueous layer was collected on a separatory funnel, and subjected to sonication for 2 hours. The mixture was then filtered on a 0.22 μm membrane to remove large aggregates. The filtered solution was then

purified by ultrafiltration to remove the excess HA and KOH. 55 mg of FITC-HA-IONPs, dispersed in 60 ml of water, were collected. The NPs were characterized (Figure S1b).

6. Synthesis of polyethylene glycol phosphate conjugate



PEG PEG-Phosphate

PEG(1500) (1.5 g, 1 mmol) was dissolved in anhydrous THF (50 ml) and the solution was degassed under nitrogen for 20 minutes. The mixture was cooled to 0°C, and $POCl_3$ (0.17 g, 1.1 mmol) was added dropwise while stirring vigorously. The mixture was stirred at room temperature for 4 hours after which it was quenched by the addition of water. The desired product was extracted by chloroform. The organic layer was collected and dried on anhydrous sodium sulfate, and the volatiles were evaporated under reduced pressure. The resulting residue was dried on a high vacuum pump. PEG-phosphate (1.3 g) was collected as a white solid. The success of the reaction was assessed by ^{31}P-NMR (Figure S7). (U. I. Tromsdorf, O. T. Bruns, S. C. Salmen, U. Beisiegel and H. Weller, *Nano Lett.*, 2009, **9**, 4434-4440.)

7. Synthesis of PEG-coated iron oxide nanoparticles

PEG-phosphate (140 mg) was dissolved in DI water (50 ml) and KOH (0.1 N, 0.5 ml) was added. OA-IONPs (70 mg) in toluene (25 ml) were added to the aqueous PEG solution. The heterogeneous mixture was refluxed for 24 hours. The aqueous layer containing the PEG-IONPs was collected from a separatory funnel, and sonicated for 2 hours. The mixture was filtered through a 0.22 μm membrane to remove large aggregates, and the filtrate was purified by

ultrafiltration (MWCO 100,000). The polyacrylic acid coated iron oxide nanoparticles were prepared in a similar manner.

8. Colloidal Stability of the IONPs in PBS and 10% FBS containing PBS

HA-IONPs, FITC-HA-IONPs, PEG-IONPs, and Feridex were dispersed in PBS or 10% FBS containing PBS solution to a final concentration of 0.5 mg-NPs/ml. The hydrodynamic diameter and PDI were monitored over a period of 1 month during which the samples were stored at 4^{o}C (Figure S4).

9. Determining the r_2 values for the various MNPs*

Five different dilutions of each MNP were prepared to a final volume of 5 ml in 15 ml-centrifuge tubes (Corning). The tubes were placed on a polystyrene tube holder. All MRI experiments were carried out on a GE 3T Signa® HDx MR scanner (GE Healthcare, Waukesha, WI). To evaluate the r_2 characteristics of the nanoparticles, the following parameters were used: Head coil, 2D fast spin echo, flip angle = 90°, eight echo times (TEs) = 8.0 ms, 16.0 ms, 23.9 ms, 31.9 ms, 39.9 ms, 47.9 ms, 55.8 ms and 63.8 ms, time of repetition (TR) = 500 ms, receiver bandwidth (rBW) = ± 31.2 kHz, field of view (FOV) = 16 cm, slice thickness = 3 mm, number of slices = 2, acquisition matrix = 256 × 256, number of excitation (NEX) = 1, and scan time = 2 min 10 sec. To evaluate the r_2* characteristics of the nanoparticles, the following parameters were used: Head coil, 3D fast spoiled gradient recalled echo sequence, flip angle = 15°, 16 TEs = 2.1 ms, 4.6 ms, 7.0 ms, 9.4 ms, 11.8 ms, 14.3 ms, 16.7 ms, 19.1 ms, 21.5 ms, 24.0 ms, 26.4 ms, 28.8 ms, 31.2 ms, 33.7 ms, 36.1 ms, and 38.5 ms, TR = 41.9 ms, rBW = ± 62.5 kHz, FOV = 16 cm, slice thickness = 1.5 mm, number of slices = 16, acquisition matrix = 256 × 256, NEX = 1, and scan time = 1 min 55 sec.

10. Qualitative evaluation of the uptake of HA-IONPs, Feridex and HA-DESPION by SKOV-3 ovarian cancer cell line using the Prussian blue stain

SKOV-3 (5 x 10^5 cells/well) cancer cells were attached overnight in 6-well plates in FBS containing DMEM. The media was removed and the cells were washed with PBS twice. HA-IONPs, HA-DESPION, and Feridex were added at a series of concentrations (21, 10.5, 5.25, 2.62, and 1.31 µg-Fe/ml). The cells were incubated for 18 hours after which the supernatant was removed and the cells were washed three times with PBS to remove the unbound particles. The cells were fixed with 10% neutral formalin (1 ml/well) for 15 min, and then washed with PBS twice. To each well was added 1 ml of a 1:1 mixture of 4% potassium ferrocyanide (II) trihydrate and 4% HCl solution (in PBS) and the cells were incubated in the dark for 30 min. The staining solution was removed, and the cells were washed with PBS and counterstained with nuclear fast red (0.4 ml/well) for 3 min. The supernatant was removed and the cells were washed with water to remove the staining solution. Images were collected on an inverted light microscope.

11. Quantifying the uptake of HA-IONPs, Feridex and HA-DESPIONs by SKOV-3 ovarian cancer cell line using the Prussian blue stain.

SKOV-3 cancer cells (5 x 10^5 cells/well) were cultured in FBS-containing DMEM overnight at 37^{o}C and 5% CO_2. The media was removed and nanoparticles (21, 10.5, 5.25 µg-Fe/ml, 2 ml) were added in serum free media. The cells were incubated for 18 hours after which the nanoparticles were removed, and the cells were washed with PBS (3 times). The cells were trypsinized, collected by centrifugation (2500 rpm, 4^{o}C, 5 min), and washed twice with PBS. The pelleted cells were lysed in 6 N aqueous HCl solution (750 µl) for 2 hours. The lysate was collected by centrifugation and the debris was discarded. 4% [K_4FeCN_6] trihydrate (750 µl) was added. The solution was incubated at room temperature for 15 minutes, and the

absorbance of the solution was then measured UV-vis spectroscopy. The Prussian blue dye has a λ_{max} of 700 nm.

12. Evaluating HA-IONPs and Feridex uptake by SKOV-3 cancer cells using MRI

SKOV-3 cells (5 x 10^6 cells/plate) were cultured in a 10 cm cell culture plate in FBS-containing DMEM overnight. After removing the media and washing the cells with PBS, serial dilutions of HA-IONPs or Feridex in serum-free DMEM with the same amounts of iron were added (21, 10.5, 5.25, and 2.62 µg-Fe/ml), and the cells were incubated at 37^{o}C for 18 hours. The nanoparticles were removed and the cells were washed with PBS three times. The cells were trypsinized using 0.25 % Trypsin/EDTA (2 ml/plate). FBS-containing DMEM (8 ml/plate) was added and the cells were transferred to 15 ml centrifuge tube and pelleted by centrifugation (2500 rpm, 4^{o}C, 5 minutes). The supernatant was removed and the cells were washed with PBS three times. After the last wash, the cells were resuspended in 2% agarose gel (5 ml), and the tubes were placed at room temperature for 5 min and then at 4^{o}C till the time of MRI. To evaluate the T2 effect of cellular uptake of the nanoparticles, the following parameters were used: Wrist coil, 2D fast spin echo, flip angle = 90°, eight TEs = 8.5 ms, 17.1 ms, 25.6 ms, 34.2 ms, 42.7 ms, 51.3 ms, 59.8 ms and 68.4 ms, TR = 500 ms, rBW = ± 31.2 kHz, FOV = 10 cm, slice thickness = 3 mm, number of slices = 2, acquisition matrix = 256 × 256, NEX = 1, and scan time = 2 min 10 sec. To evaluate the T2* effect of cellular uptake of the nanoparticles, the following parameters were used: Wrist coil, 3D fast spoiled gradient recalled echo sequence, flip angle = 15°, 16 TEs = 2.5 ms, 5.1 ms, 7.7 ms, 10.2 ms, 12.8 ms, 15.3 ms, 17.9 ms, 20.5 ms, 23.0 ms, 25.6 ms, 28.1 ms, 30.7 ms, 33.3 ms, 35.8 ms, 38.4 ms and 40.9 ms, TR = 41.9 ms, rBW = ± 62.5 kHz, FOV = 16 cm, slice thickness = 1.5 mm, number of slices = 16, acquisition matrix = 256 × 256, NEX = 1, and scan time = 3 min 49 sec.

13. Assessing biocompatibility of HA-IONPs using MTS cell viability assay

SKOV-3 cancer cells (5000 cells/well) were cultured in serum-containing DMEM in a 96 well-plate and incubated at 37^{o}C for 24 hours. The media was removed and the cells were washed with PBS. Serial dilutions of HA-IONPs or Feridex were added (198, 99, 49.5, 24.75, 12.38 µg-Fe/ml; 200 µl/plate), and the plate was incubated at 37^{o}C for 24 hours. The test reagents were removed and the cells were washed with PBS twice. Serum-containing DMEM (200 µl/well) was added and the plate was incubated at 37^{o}C for 48 hours. MTS reagent (20 µl/well) was added and the plate was incubated at 37^{o}C for 3 hours in the dark. A brown color appeared in the wells containing live cells. The absorbance was measured on a plate-reader (BioRad) at 490 nm.

14. Competitive inhibition of HA-IONPs uptake by B16F10 melanoma cells

B16F10 melanoma cell line was use in this study since SKOV-3 cell line was not available at the time the assay was run. The cells (2 x 10^5 cells/well) were attached overnight at 37^{o}C and 5% CO_2. The media was removed and the cells were washed with PBS twice. HA polymer (20 mg) in DMEM (1 ml) was added to cells, followed by incubation for 2 hours. FITC-HA-IONPs (final concentration 270 µg-NP/ml) was added and the cells were incubated for another 2 hours. The supernatant was removed and cells were washed with PBS three times. The cells were collected by centrifugation, resuspended in serum-containing DMEM (300 µl), and transferred to FACS tubes. Propidium iodide (3.3 µl) was added before each measurement.

15. Evaluating the biodistribution of HA-IONPs in C57BL/6 mice using MRI

All animal experiments were in accordance with the guidelines and the approval of Institutional Animal Care and Use Committee., Michigan State University. C57BL/6 mice were anesthetized

by intraperitoneally injecting a cocktail of ketamine (80 mg/kg) and xylazine (10-16 mg/kg). The mice were restrained in a tube restrainer and transferred to a wrist coil containing a warm wrap to preserve the mice body temperature on the MRI scanner. Pre-injection images were collected for comparison. HA-IONPs (4.63 mg-Fe/ml, 50µl) were administered via retro-orbital injection, and the mice were imaged. The presence of the HA-IONPs was assessed in aorta, kidneys, and liver. To evaluate the T_2* effect of the uptake of the nanoparticles *in vivo*, the following parameters were used: Wrist coil, 3D fast spoiled gradient recalled echo sequence, flip angle = 15°, echo times (TEs) = 11.3 ms, time of repetition (TR) = 26.6 ms, receiver bandwidth (rBW) = ± 7.8 kHz, field of view (FOV) = 3 cm, slice thickness = 1 mm, number of slices = 36, acquisition matrix = 256 × 256, frequency direction = anterior/posterior, number of excitation (NEX) = 4, and scan time = 16 min 21 sec.

III. Figures and Tables

Table S1. Hydrodynamic diameters, polydispersity indices (PDI), zeta potential, and TGA data for HA-IONPs, FITC-HA-IONPs, and PEG-IONPs

	Hydrodynamic diameter (nm)	PDI	Zeta Potential (mV)	Weight loss by TGA (%)	Relaxivity (mM^{-1} sec^{-1})
HA-IONPs	54	0.17	-50	67	340
FITC-HA-IONPs	68	0.13	-60	66	314
PEG-IONPs	56	0.17	-49	23	334

Figure S1. UV-vis spectra of FITC-HA and FITC-HA-IONPs.

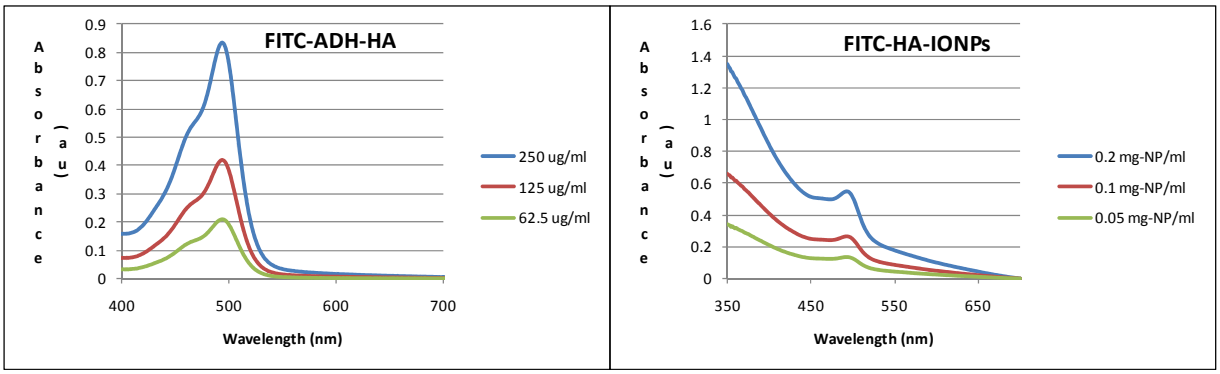
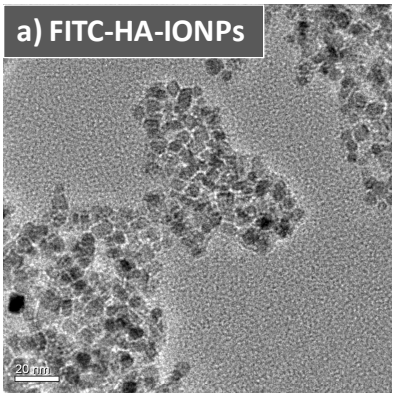
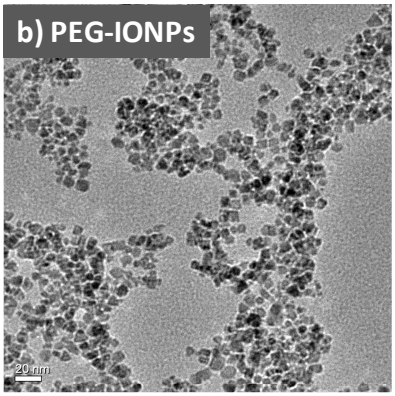
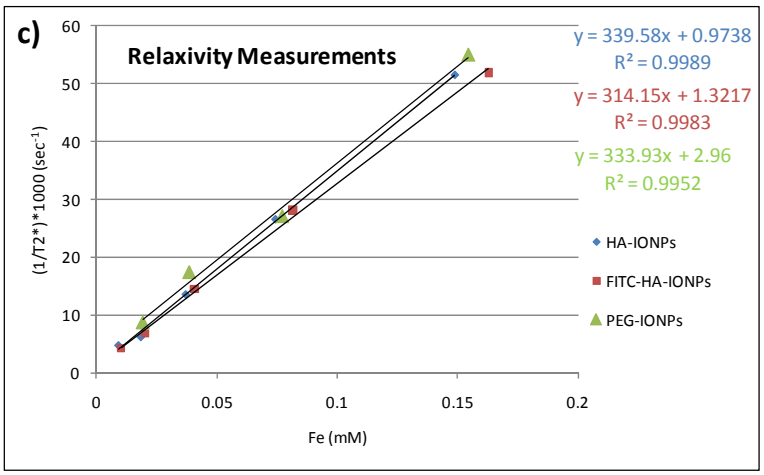


Figure S2. Characterization of IONPs. TEM images of (a) FITC-HA-IONPs and (b) PEG-IONPs. (c) Relaxivity measurements for HA-IONPs, FITC-HA-IONPs and PEG-IONPs; and (d) TGA for OA-IONPs, HA-IONPs, FITC-HA-IONPs and PEG-IONPs.







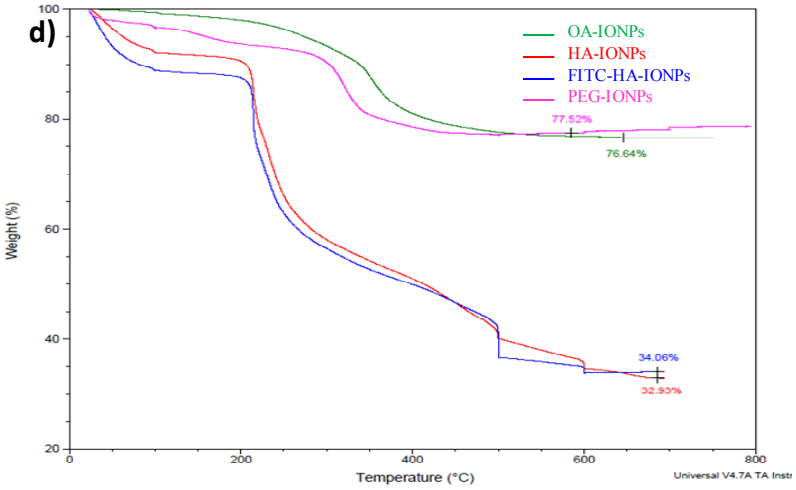


Figure S3. Colloidal stability assay. Average PDI of HA-IONPs, PEG-IONPs, FITC-HA-IONPs, and Feridex upon incubation with PBS or 10% FBS containing PBS. Average PDI was obtained by averaging PDIs measured on days 6, 18, 24, and 30 post incubation. Feridex showed a much higher increase of PDI values.

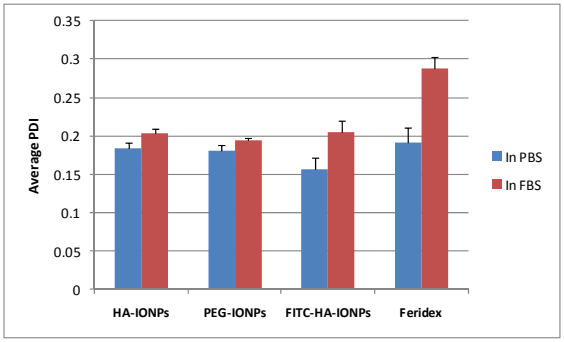


Figure S4. Characterization of CFNPs. (a) TEM image of HA-CFNPs; (b) Relaxivity of HA-CFNPs and (c) polyacrylic acid coated CFNPs (AA-CFNPs); (d) TGA of OA-CFNPs, AA-CNFPs and HA-CFNPs.

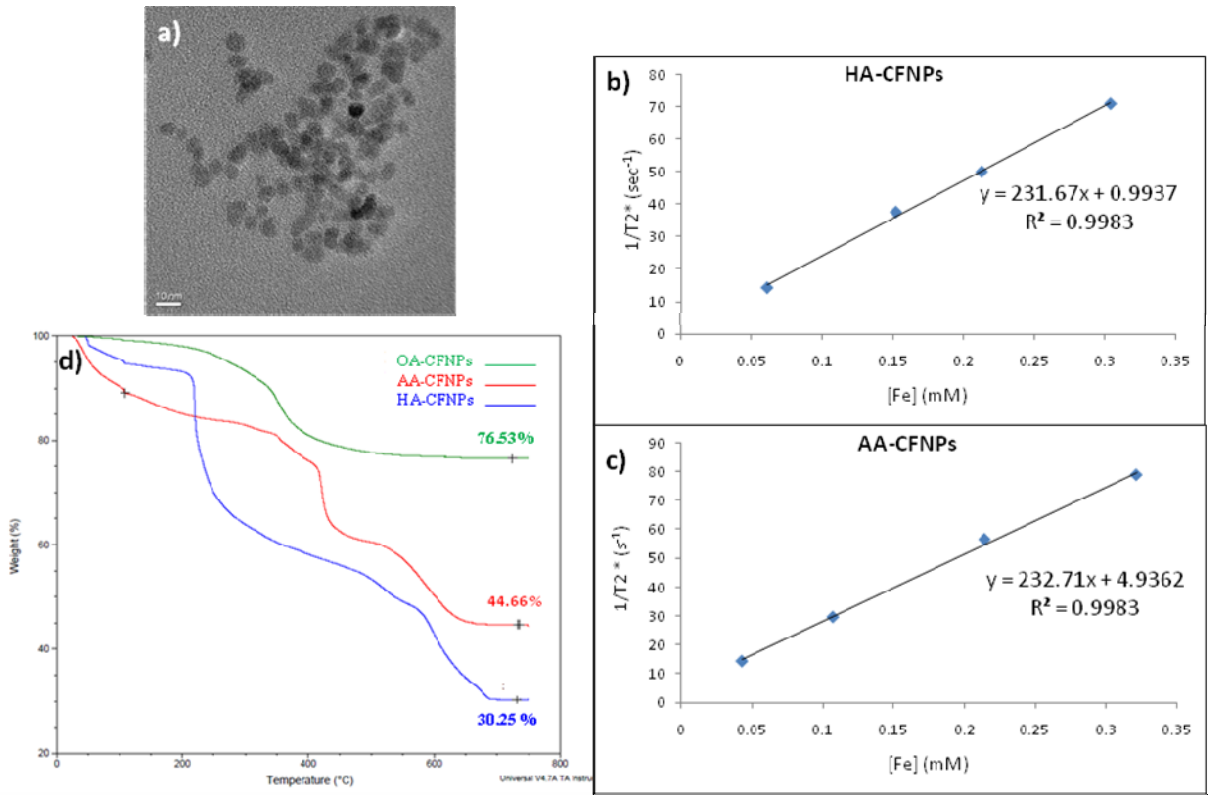


Figure S5. Assessing uptake of HA-IONPs and Feridex using MRI. (a) T2* weighted images of SKOV-3 cells upon incubation with various concentrations of HA-IONPs and Feridex after removing unbound NPs. (b) T2* values of SKOV-3 cells upon incubation with various concentrations of HA-IONPs and Feridex after removing unbound NPs. Dose dependent loss in T2* signal was observed. (c) Linear correlation between relaxation rate 1/T2* and concentration of iron.

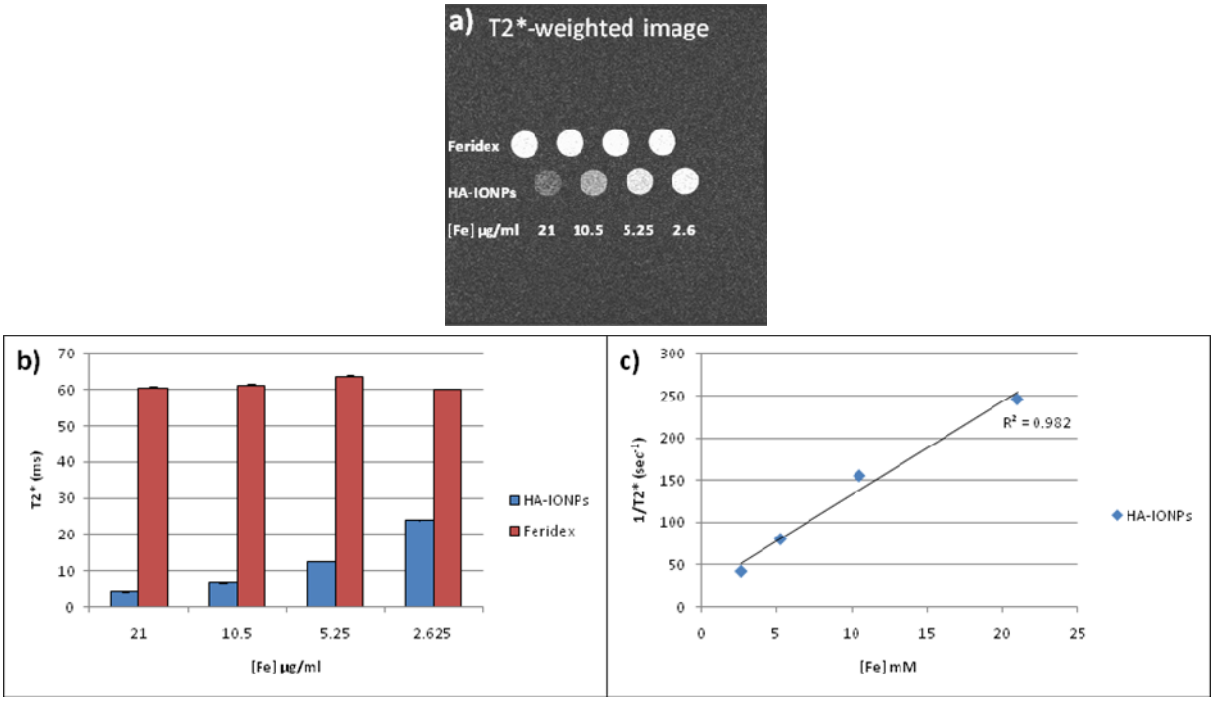


Figure S6. Competitive inhibition of B16F10 melanoma cell uptake of HA-IONPs by free HA. Significant reduction in NPs uptake was observed upon co-incubation with free HA polymer.

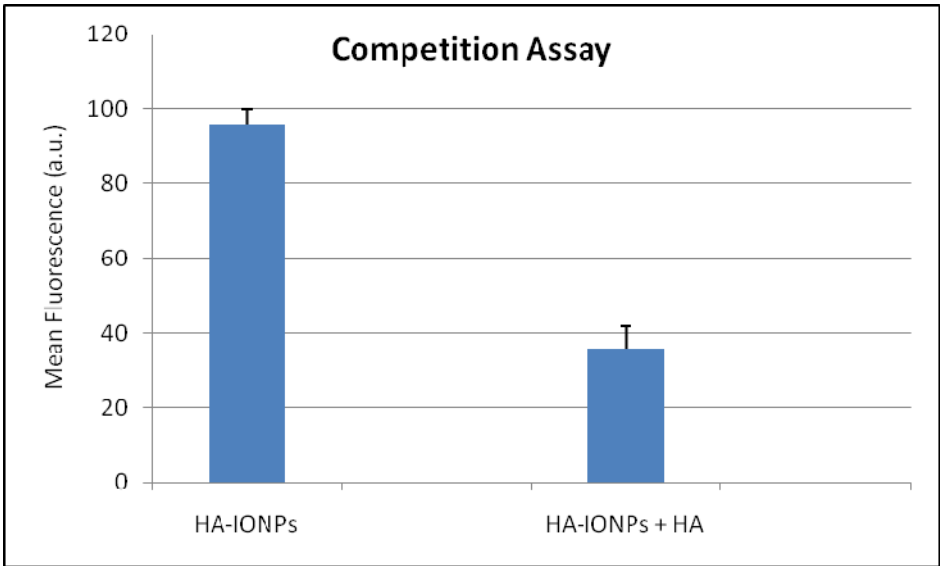


Figure S7. ^{31}P-NMR of PEG-phosphate confirming the successful introduction of phosphate group onto PEG.