ADVANCED MATERIALS

Synthesis of Complexable Fluorescent Superparamagnetic Iron Oxide Nanoparticles (FL SPIONs) and Its Cell Labeling for Clinical Application

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EXPERIMENTAL SECTION

All chemicals were purchased from Sigma-Aldrich unless specified otherwise. TEXAS RED® and fluorescein were supplied as lysine fixable dextran conjugates (molecular weight 3 kDa, Invitrogen, Carlsbad, CA). Ferumoxides (Feridex IV®, Berlex Laboratories, Wayne, NJ) with an average hydrodynamic size of 150 nm and concentration of iron at 11.2 mg/mL was used as the SPION. Human cervical carcinoma (HeLa) cells (CCL-2, ATCC, Manassas, VA) were used for cell labeling. All cells were grown in the recommended media growth factors at 37˚C and 5% CO_2. Cells were allowed to grow until 70~90% confluence in culture flasks prior to cell labeling.

Synthesis of Fluorescent SPION. A suspension of 2.5 mL Ferumoxides (FE) was oxidized by sodium periodate (1mg sodium periodate/mg Fe) at 4˚C in the dark room overnight,[1] resulting in the oxidation of the hydroxyl groups on the dextran to aldehyde groups. After reaction, the aldehyde FE was purified by running the solution through a PD 10 column (GE Healthcare, Piscataway, NJ) filled with sephadex G-25 twice. Lysine fixable fluorescein (Fluo) or TEXAS RED® (Texas) fluorescent dye conjugated dextran was reacted with the aldehyde FE at room temperature (the ratio was 0.5 mg dye/mg Fe). One hour later sodium cyanoborohydride ($NaBH_3CN$, 50 mM) was added to the solution and allowed to react overnight at 4˚C in the dark room. The following day the fluorescent dye conjugated FE SPION (FL FE) solution was passed four times through PD 10 columns or until the column showed a clear separation. Dye conjugation efficiency (dye/iron) was obtained from the absorbance of maximum peak of dye (Fluorescein 494 nm, TEXAS RED® 595 nm) using the extinction coefficient (fluorescein $68000 M^{-1}cm^{-1}$, TEXAS RED® $80000 M^{-1}cm^{-1}$ respectively). Briefly, the baseline absorbance of FL FE solutions was calculated from standard FE solutions based on the iron concentration. The difference in absorbance between the FL FE solution and the standard FE solution was measured, and the dye conjugation efficiency was determined. Fluorescent ferumoxides was filtered through a 0.22 μm mesh prior to cell labeling and submitted for bacterial culture to assure sterility. Successful conjugation of fluorescent markers to iron oxide nanoparticles is dependent on the stability during synthesis of the nanoparticles and fluorescent markers. The stability of nanoparticles was measured by stability index (SI) = aggregation amount/initial amount.

Biophysical properties of nanoparticles and complexes. FL FE-Pro complexes in water were prepared at different protamine sulfate with a fixed 100 μg/mL concentration of Fe, and checked by sight and microscopy a day later. The surface charge of nanoparticles or of complexes was measured by a zeta potential (ZP) analyzer (ZetaPALS, Brookhaven Instruments, Long Island, NY), reported in millivolts (mV). Nuclear magnetic resonance (NMR) relaxometry was performed to determine relaxation parameters (i.e., $1/T_1$, and $1/T_2$) of nanoparticles or of complexes (in 4% gelatin) as previously described using a 1.0 T (42.6 MHz) at 23˚C.[2] The hydrodynamic diameter was analyzed by dynamic light scattering (ZetaPALS, Brookhaven Instruments, Long Island, NY). Each sample was measured three times and the average diameter was reported. For transmission election microscopy (TEM) analysis, the nanoparticles were deposited on evaporated thin carbon films approximately 5 nm in thickness, which had been floated onto lacey carbon films covering 200 mesh copper grids (EM Sciences, Fort Washington, PA). The nanoparticles were imaged with an FEI CM120 transmission electron microscope (FEI Inc., Hillsboro, OR) operating at an accelerating voltage of 120 kV. Micrographs were recorded digitally using a Gatan Biofilter GIF (Gatan Inc., Warrendale, PA) equipped with a 1024 x 1024 pixel CCD camera. Elastic bright-field images were recorded in zero-loss mode and inelastic dark-field images were recorded at an energy loss of 30±10 eV in order to visualize the organic component of the nanoparticles.

Formation of FL FE-Protamine sulfate complex for Cell Labeling. Fluorescent ferumoxides (FL FE) (iron concentratioin, 100 μg/mL) was mixed together with 6μg/mL protamine sulfate (Pro, 10 mg/mL, molecular weight 4.2 kDa, American Pharmaceuticals Partner, Schaumburg, IL) with RPMI media without additives and allowed to complex for 5 minutes. The complex solutions of FE and Pro (FE-Pro) or the complex solutions of FL FE and Pro (FL FE-Pro) were added to HeLa cells that were grown in 24-well plates at density of $2x10^5$ cells/mL. Two hours later (or maximally overnight) fresh RPMI media with additives was added to each well at a predetermined amount to reach a final volume of media to contrast agent of 2 mL per well (i.e., iron concentration 50 μg/mL). Control unlabeled HeLa cells were maintained under the same media conditions without the addition of contrast agents. All cells were incubated overnight at 37º C with 5% CO_2. The following day the media was removed and cells were trypsinized and washed three times in PBS containing 10 U Heparin/mL (American Pharmaceuticals Partner, Schaumburg, IL, USA) and filtered through a 100 micron cell strainer.

Cellular viability and proliferation capacity. Cell viability was determined by a trypan blue exclusion test. Cell proliferation capacity was determined by the MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) cell proliferation assay (CellTiter 96® Aqueous One Solution, Promega, Madison, WI) at different times. Briefly, after cell labeling a specific number of unlabeled cells, FE-Pro labeled and FL FE-Pro labeled cells (5000 cells) were grown in 96-well plates for 24 hours and 72 hours. 20 μL of MTS reagent was added to the cells in 96-well plates and the

cells were further incubated for 3 hours. Proliferation and viability of unlabeled, FE-Pro labeled and FL FE-Pro labeled cells were recorded by measuring the absorbance at 490 nm using UV-visible spectrophotometer (UV-1601, Shimadzu, Japan). Pulse chase experiments were performed with HeLa cells to rule out any long-term effects on cell proliferation. HeLa cells were incubated with FE-Pro and FL FE-Pro complexes for 24 hours and washed three times in heparinized PBS, and then incubated in fresh culture media for 9 days. Cell viability of unlabeled, FE-Pro labeled and FL FE-Pro labeled cells was performed as described above.

Determination of mean iron concentration per cell. Iron concentration was assayed by a variable-field relaxometer (Southwest Research Institute, San Antonio, TX) and UV-visible spectrophotometer as previously described.[3-5] For relaxometry, 500 µL of an iron standard or a solution of 500 µL $1x10^5$ cells/mL in PBS was added to 500 µL 10N hydrochloric acid and allowed to digest overnight before running the relaxometry. The NMR relaxation rate $1/T_2$ (s^{-1}) was measured at 1.0 T (42.6 MHz) at 23˚C.

Cytology. For microscopy, cells were washed as described above, fixed with 4% paraformaldehyde and cytospin slides were prepared. For fluorescent microscopy, slides were then allowed to air dry, washed in distilled water in the dark, allowed to dry again and cover-slipped with VectaShield with DAPI (Vector Laboratories, Burlingame, CA). To minimize auto fluorescence, exposure times were based on the signal intensity from unlabeled control cells and three fluorescent images were obtained using DAPI, FITC and Cy3 filters. Z-stacking was used to obtain overlapping images. Prussian blue stains were done on cytospin slides with a 30 minute incubation using a 1:1 ratio of 20% potassium ferrocyanide and 20% hydrochloric acid to detect the presence of SPION in cells. The slides were then washed in de-ionized water and counterstained with Nuclear Fast Red (Scytek, Logan, UT). The Prussian blue stained cytospin slides were evaluated for iron staining with a Zeiss microscope (Axioplan Imaging II; Zeiss, Oberkochen, Germany) at x 40/0.75 objective lens and Axiovision 4 software (Zeiss). FE-Pro labeling efficiency was determined using images of the Prussian blue stained FL Fe-Pro labeled cells captured by Axioplan Imaging II at 100x magnification using x100/1.3 (oil) immersion objective lens. The images were imported into the Image J program of NIH to obtain cell counts of labeled versus unlabeled cells.

Flow cytometry. The fluorescent properties of one of the agents, fluorescein dextran conjugated FE (Fluo FE), was analyzed not only by fluorescent microscopy, but also for fluorescent labeling efficiency and detection properties by flow cytometry (FACS Calibur, BD BioSciences, San Jose, CA). The TEXAS RED® dextran conjugated FE (Texas FE) could not be analyzed on the FACS Calibur due to excitation and emission filter limitations. Unlabeled cells, Fe-Pro labeled cells, and Fluo FE-Pro labeled cells were analyzed by FACS for comparison of fluorochrome detection.

MRI at 3 Tesla. A phantom was made from a cylindrical glass tube, 6 cm in diameter, filled with distilled water. Plastic vials with 5.0×10^5 FE-Pro labeled, FL FE-Pro labeled or unlabeled cells were suspended in 1 mL 2% agarose gel. The sealed vials were embedded in the middle of the glass cylinder on a plastic rack. MRI was performed on a 3T clinical MR scanner (Acheiva, Philips Medical System, Best, The Netherlands) using a dedicated 7 cm solenoid receive only RF-coil (Philips Research Laboratories, Hamburg, Germany). T_2 weighted images were acquired with a spin echo sequence with TR = 1000 ms, TE = 20 ms. T_2* weighted images were acquired with a gradient echo sequence with TR = 1000 ms, TE= 35 ms, FA = 25°. All images were acquired with a field of view = 100 mm × 100 mm, data matrix = 256 × 256, slice thickness = 1 mm, and number of excitations = 2.

References

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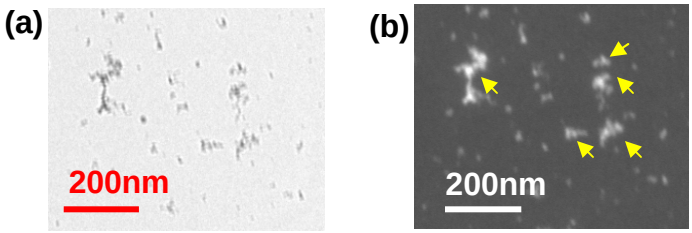
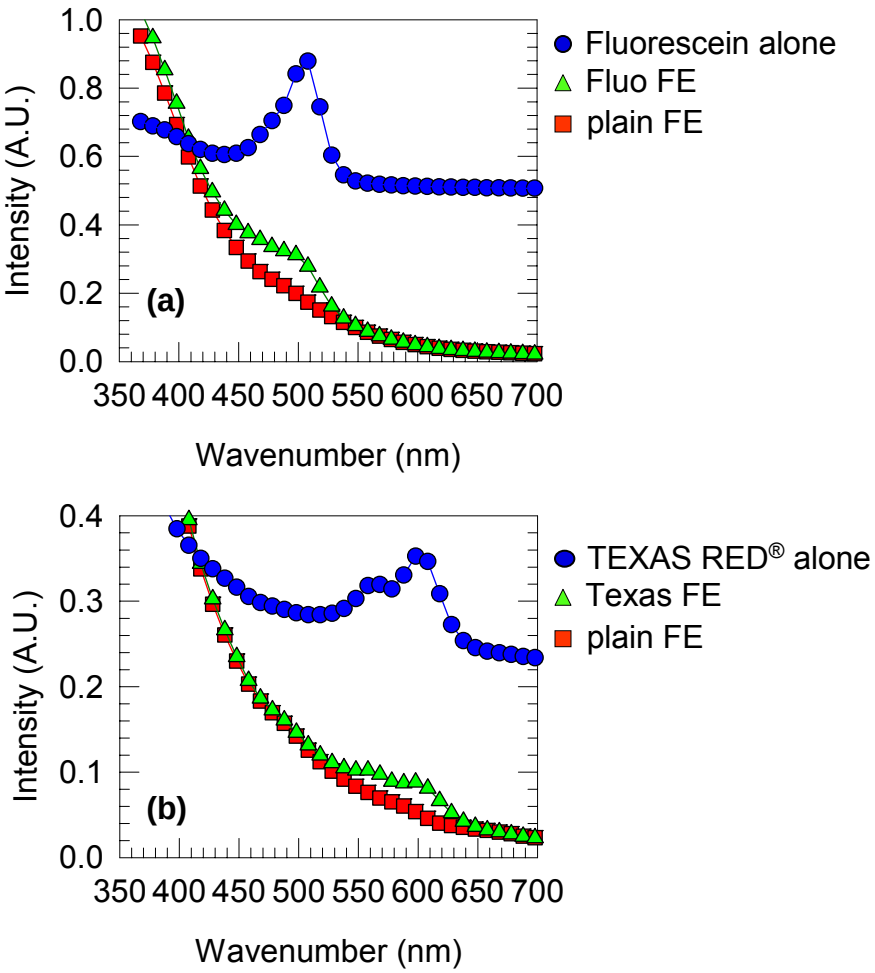
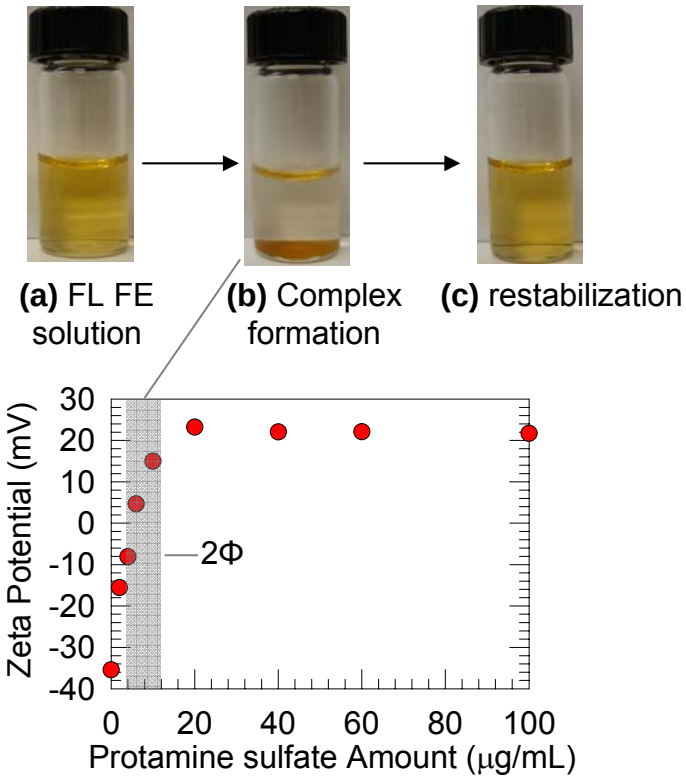


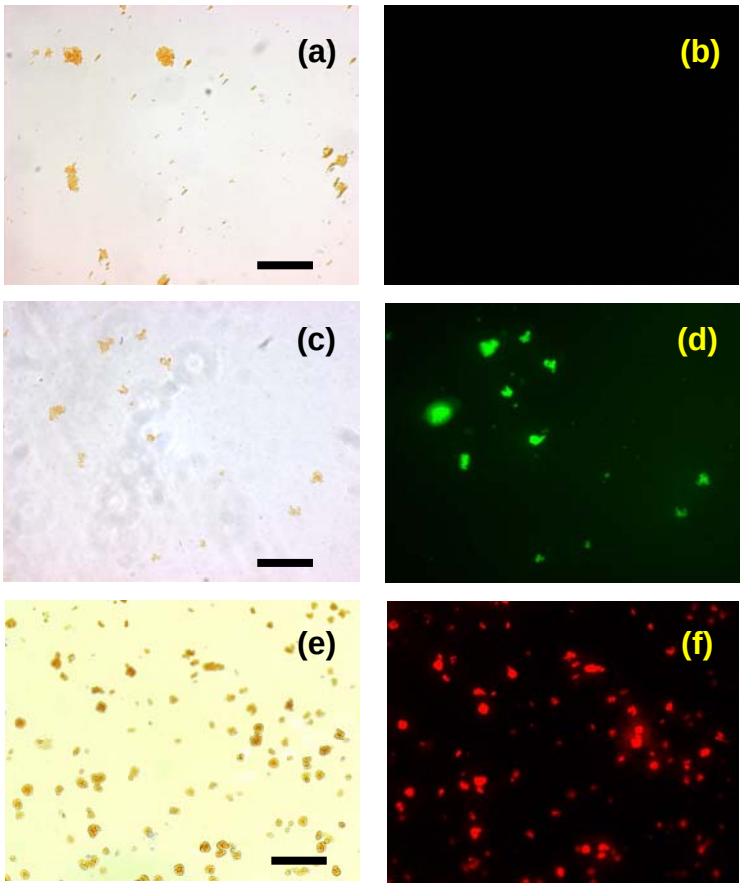
Supporting Figure 1. TEM images of a representative fluorescent dye conjugated ferumoxides (FL FE). (a) elastic bright field image of the nanoparticles iron core; and (b) inelastic dark field image of the nanoparticles iron core and dextran coat. Arrows indicate branched aggregates of smaller dense nanoparticles.



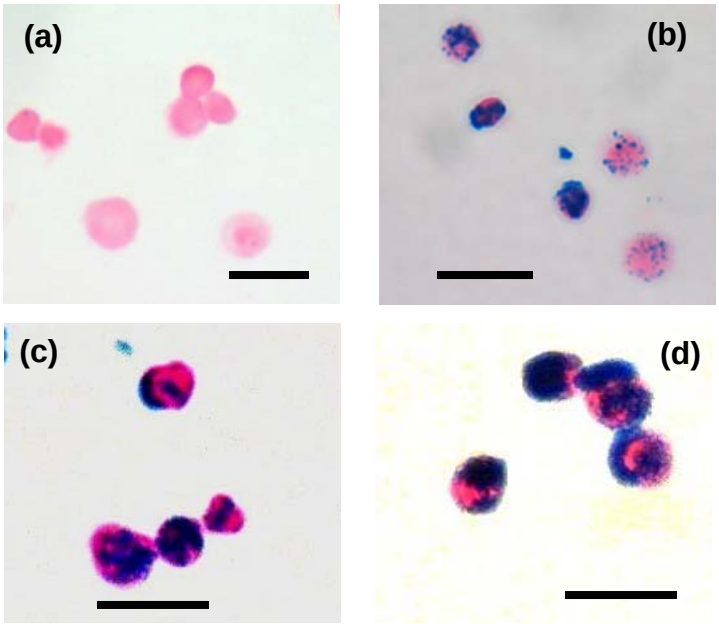
Supporting Figure 2. Absorption spectra of fluorescent dye conjugated ferumoxides (FL FE). (a) fluorescein dextran conjugated FE (Fluo FE); and (b) TEXAS RED® dextran conjugated FE (Texas FE): fluorescein or TEXAS RED® dextran (top, blue circle) (shifted upward on the Intensity scale by 0.5 A.U. for figure a. and by 0.21 A.U. for figure b. to minimize overlap), Fluo FE or Texas FE (middle, green triangle), and plain FE (bottom, red square).



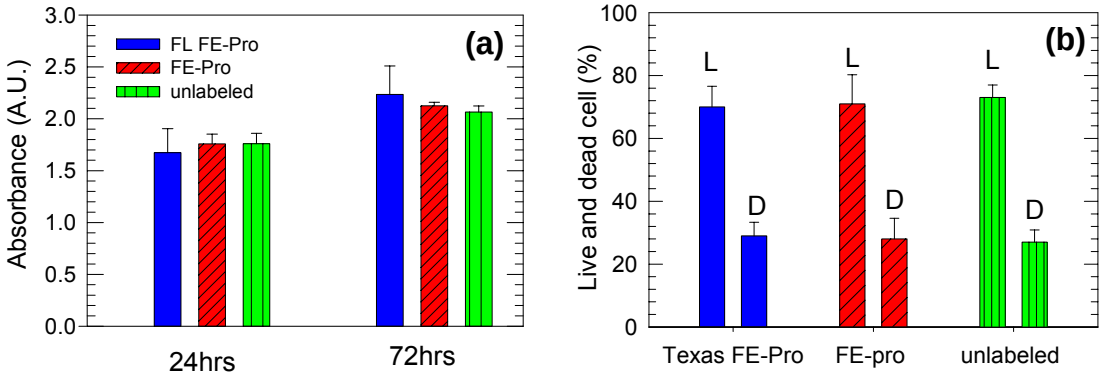
Supporting Figure 3. Photographs and zeta potentials of the FL FE-Pro complex in water at different protamine sulfate concentrations with a fixed 100 μg/mL concentration of Fe. At levels of protamine sulfate (a) below 5 μg/mL the FL FE stays in solution, (b) it begins to complex and precipitate out of solution between 5-10 μg/mL while in the second phase (shaded area), but (c) it is restabilized with excess protamine sulfate above 10 μg/mL. The zeta potential is stable at 20 mV once the solution has been restabilized.



Supporting Figure 4. Representative optical and fluorescent microscope images of complexes. (a) optical image and (b) fluorescent image of control FE-Pro complexes; (c) optical image and (d) fluorescent image of Fluo FE-Pro complexes under FITC filter; (e) optical image and (f) fluorescent image of Texas FE-Pro complexes under Cy 3 filter. X 20 Scale bar represents 5 µm.



Supporing Figure 5. Comparison of Prussian Blue staining images with (a) control unlabeled HeLa cells; (b) FE-Pro labeled HeLa cells; (c) Fluo FE-Pro labeled HeLa cells; and (d) Texas FE-Pro labeled HeLa cells. All nuclei were counterstained with nuclear fast red. Scale bar represents 10 μm.



Supporting Figure 6. Cell viability test: (a) MTS assay comparison of FL FE-Pro labeled HeLa cells, FE-Pro labeled HeLa cells, and unlabeled HeLa cells after 5000 cells were grown in 96-well plates for 24 hours and 72 hours. The absorbance was measured at 490nm for proliferation and viability of the cells, the results indicate no significant difference at either time point; (b) pulse chase experiment comparison of live cells (L) and dead cells (D) percentage with FL FE-Pro labeled , FE-Pro labeled, and unlabeled cells after cells were grown in 96-well plates for 9 days. Results demonstrate no significant differences.