Published in final edited form as:
Adv Mater. 2008 July 2; 20(13): 2512–2516. doi:10.1002/adma.200800223.

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

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Keywords

fluorescent probes; biotechnology; hybrid materials; nanotechnology; imaging agents

Superparamagnetic iron oxide nanoparticles (SPIONs) have been used for labeling mammalian cells providing the basis for cellular MRI in experimental models.[1-4] Several methods have been developed to facilitate the uptake of SPION by endocytosis in cells.[1-5] The advantage of one particular SPION, ferumoxides, is that it is an food and drug administration (FDA) approved MRI contrast agent for hepatic imaging that is presently being used off-label for clinical cellular imaging studies.[6] By mixing ferumoxides (FE) contrast agent, a molecule with a negative zeta potential, with protamine sulfate (Pro), a polycation that is an FDA approved drug for the treatment of heparin anticoagulation overdose, a self-assembled complex forms that has been used to efficiently and effectively label cells.[3] The FE-Pro complex does not require any complex synthesis or modification of the SPION, and it has been proven less toxic than other chemical conjugation methods.[7]

**This research was supported by the Intramural Research Program of the Clinical Center at the National Institutes of Health. The authors would like to acknowledge the help of Dr. Leapman for TEM analysis.((Supporting Information is available online from Wiley InterScience or from the author)).

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The FE-Pro complex is taken up by macropinocytosis and as a result the SPIONs are located in endosomes in the cell.[5] MRI of SPION labeled cells results in a T_2 and T_2* shortening of the surrounding water protons and cells appear as hypointense (dark) voxels on T_2* weighted images. However, it may be difficult to distinguish SPION labeled cells from endogenous iron (i.e., hemorrhage or hemosiderin) that can also cause decrease signal intensities on T_2* weighted images in tissues. This limitation makes it difficult to obtain quantitative information on the numbers of SPION labeled cells that can possibly be used to optimize cellular therapy.[8] To enhance the features of SPION and the ability to differentiate labeled cells from tissue macrophages loaded with iron, the introduction of fluorescent marker onto the nanoparticles has been explored.[9-11] The conjugation of fluorescent markers onto magnetic nanoparticles would offer additional sensitivity in the detection of cells within tissues using noninvasive optical imaging or with ex-vivo fluorescent microscopy.

Creating stable fluorescent magnetic nanoparticles for experimental and potential clinical applications is difficult because these agents can easily become unstable precipitating out of aqueous solution during synthesis. These fluorescent magnetic nanoparticles are also potentially toxic to cells in culture. In the current study, a simple fluorescent conjugation strategy that did not require cross-linking of the dextran coating[12] was developed to generate stable nanostructures that can be applied to synthesize a variety of stable fluorescent SPIONs. To achieve this goal, ferumoxides was conjugated with fixable fluorescent dextrans (FL FE) such as fluorescein (Fluo) and TEXAS RED® (Texas). This FL FE conjugation strategy demonstrated in vitro stability and following sterilization by filtration, protamine sulfate was complexed to the FL FE by electrostatic interactions to enhance cellular uptake for cell tracking by MRI and fluorescent imaging.

Ferumoxides with an average hydrodynamic size of 150 nm and concentration of iron at 11.2 mg/mL was oxidized by sodium periodate,[13] resulting in the oxidation of the hydroxyl groups on the dextran to aldehyde groups. After the reaction, lysine fixable fluorescein (Fluo) or TEXAS RED® (Texas) fluorescent dye conjugated dextran was reacted with the aldehyde FE at room temperature. The ratio of the concentration of the aldehyde FE to fixable fluorescent dextran, and sodium cyanoborohydride were optimized to prevent destabilization (i.e., precipitation) and increase dye conjugation efficiency. The conjugation efficiency of FL FE determined by UV-visible spectrophotometer was 1.4±0.2 TEXAS RED® mmole/mole iron (i.e., 2600 dyes per crystal) for Texas FE and 3.4±0.5 fluorescein mmole/mole iron (i.e., 6400 dyes per crystal) for Fluo FE. Table 1 summarizes the physico-chemical properties of the contrast agents used in this study. Dextran (3kDa) has a hydrodynamic diameter of 3.1 nm [14] and therefore when conjugated to ferumoxides the hydrodynamic size of the SPION was not altered (see Table 1). The hydrodynamic size of FL FE (145-154 nm) was similar to FE (157 nm) and no significant change in nanoparticle size occurred during the synthesis as determined by TEM measurements (see Supporting Information). The spectrum of Fluo FE demonstrates a fluorescein peak at 494 nm, while the Texas FE has a peak at 595 nm. Neither Fluo FE nor Texas FE spectra overlapped the spectra of the FE control solution (Supporting Information).

Fluorescent ferumoxides complexes have a similar zeta potential (Fluo FE −32 mV, Texas FE −28.9 mV) to ferumoxides in solution (Table 1). The FL FE and Pro self-assembled complexes had the same physico-chemical properties as FE-Pro complexes[3] (Supporting Information). The zeta potentials of both FL FE and FL FE-Pro suggest that the addition of the fluorochrome did not alter the surface properties of ferumoxides. Complexation between FL FE and Pro occurred very sharply at ratios between 100 μg/mL of iron to 5-10 μg/mL of protamine sulfate. These ratios of FL FE:Pro result in a zeta potential close to 0 mV and the complex precipitates out of solution (see Supporting Information). Increasing the concentration of Pro to >10 μg/mL re-stabilized FL FE (see Supporting Information) and the hydrodynamic diameter remained

almost the same size at concentrations of Pro equal to 20 μg/mL, (168.3±3.4 nm). The behavior of this complex implies that protamine sulfate completely coats the FL FE surface at a near zero zeta potential value and that any excess protamine sulfate in solution, produces a net positive charge resulting in a repulsion between the nanoparticles. When protamine sulfate was complexed to FL FE, at a ratio of FL FE:Pro 100 μg/mL: 6 μg/mL, there was a shortening effect of the R_2 relaxation rate, which then increased as the concentration of protamine increased to 20 μg/mL (see Table 2). Shortening of R_1 and R_2 relaxation rates of FE-Pro, Fluo FE-Pro and Texas FE-Pro complexes was dependent on the concentration of protamine in solution as previously reported when ferumoxides alone are complexed to protamine sulfate [3] and clearly demonstrated that at a protamine dose of 20 μg/mL there was relatively little effect on the NMR relaxivities of the FL FE-Pro compared to FE-Pro alone (Table 2). The decrease of R_1 and R_2 relaxation rates when the zeta potential of the FL FE-Pro complex was approximately equal to zero results from shielding of the water from the paramagnetic sites on the surface of the crystal.[3] The return to higher R_1 and R_2 relaxation rates at higher protamine sulfate concentration (i.e., at restabilization condition) is inferred due to return of similar size of ferumoxides in solution. We speculate that when high concentrations of protamine sulfate are used to form the FL FE-Pro complex there may be an alteration to the dextran coat that changes the dipole-dipole interaction and outer sphere effects of local water molecules thus increasing R_1 and R_2 of the complex in solution. FE-Pro complexes did not exhibit any fluorescence whereas Fluo FE-Pro complexes demonstrated fluorescent signal when observed under a FITC filter (see Supporting Information). Texas FE-Pro complexes also showed strong fluorescent signal using the Cy 3 filter. The fluorescent microscopy suggests that dye conjugation onto ferumoxides was achieved. Our hypothetical explanation for stability and complex formation of the FL FE nanoparticle is as follows. When the fixable lysine or amine groups of the fluorescent dextrans are conjugated on the aldehyde functionalized SPION surface, fractions of the conjugated dextran are then exposed on the FL FE surface. The hydroxyl group of conjugated dextran serves as a polymeric steric stabilizer. The dextran stabilized FL FE can complex with the polycationic protamine sulfate via electrostatic interactions.

The average iron content per cell for FL FE-Pro labeled HeLa cells after overnight incubation was as follows: Texas FE-Pro labeled cells 29.9.0±4.5 pg/cell (i.e., about 1.8×10^5 nanoparticles/cell, 4.5×10^8 dyes/cell); Fluo FE-Pro labeled cells = 31.2±4.5 pg/cell (i.e., about 1.8×10^5 nanoparticles/cell, 1.1×10^9 dyes/cell); and FE-Pro labeled cell = 28.0±4.4 pg/cell (i.e., 1.6×10^5 nanoparticles/cell). These results suggest that the cell labeling efficiency with FL FE-Pro complexes was equivalent to FE-Pro complexes. Of note, both FL FE and HeLa cells have negative zeta potentials [15] and therefore the nanoparticles usually requires either modifications to the surface charge or cells need to be incubated with high concentrations of iron in media to facilitate uptake into endosomes. We also found that negative FE alone cannot be taken up any types of cells [3] and that cell labeling occurrs at complexation condtion (100 μg/mL of iron to 5-10 μg/mL of protamine sulfate) most efficiently even though unstable, which suggests that cell endocytosis can be mediated by optimal size of self-assembled complex and charge of compelx. Prussian blue staining of FL FE-Pro and FE-Pro labeled cells indicates that cell labeling efficiency of FE-Pro, Fluo FE-Pro and Texas FE-Pro labeled cells was > 90% (data not shown). Cell labeling efficiency was dependent on several factors: cell type, cell concentration, media and media supplements, ratio of FE to Pro, and incubation time. [3] Cell proliferation capacity by MTS and pulse chase experiments showed no significant differences between unlabeled, FE-Pro and FL FE-Pro labeled cells (Supporting Information). Trypan blue dye exclusion test also showed no significance in viability for unlabeled, FE-Pro and FL FE-Pro labeled cells (Supporting Information).

The labeling efficiency for FL FE-Pro was evaluated by flow cytometry (FACS Calibur, BD BioSciences). Figure 1 compares control unlabeled cells and FE-Pro labeled cells, with Fluo

FE-Pro labeled cells. Unlabeled and FE-Pro labeled cells did not exhibit fluorescence by FACS analysis. Greater than 91% of Fluo FE-Pro labeled cells were positive by FACS analysis, similar to the results obtained with Prussian blue staining of the cells. Figure 2 contains fluorescent images of unlabeled cells, FE-Pro labeled cells, Fluo FE-Pro labeled cells, and Texas FE-Pro labeled HeLa cells with nuclei stained with DAPI (blue). Unlabeled and FE-Pro labeled cells did not exhibit fluorescence (Figure 2a & b). Fluo FE-Pro (Figure 2c green) and Texas FE-Pro (Figure 2d red) labeled cells exhibited intracytoplasmic fluorescence with no evidence of nuclear incorporation of nanoparticles. These results indicate that cells were successfully labeled with both Fluo FE-Pro complexes and Texas FE-Pro complexes. These results also suggest it is possible to conjugate other dyes with magnetic nanoparticles. Figure 3 contains T_2 and T_2* weighted images obtained at 3 Tesla demonstrating the T_2 and T_2* shortening of FE-Pro or FL FE-Pro labeled HeLa cells. The FE-Pro and FL FE-Pro labeled cells appear as hypointense regions in the agar compared to the homogenous appearance of the agar containing unlabeled cells. Therefore, in the current study both qualitative MR imaging and fluorescent microscopy showed that FL FE-Pro complexes can be used to label cells with the same efficiency as FE-Pro complexes.

In summary, a simple straightforward conjugation approach was developed to attach fluorescent dyes to ferumoxides. By modifying dextran coated SPIO nanoparticles with lysine or amine fixable fluorescent dextran we were able to use previously reported complex formation conditions to magnetically label cells. No toxicity was observed in cells labeled with the FL FE-Pro complex. FACS and fluorescent microscopy also demonstrated that FL FE-Pro effectively labeled cells and may facilitate automatic cell sorting of the fluorescently labeled cells. Magnetic labeling of cells with FL FE-Pro complexes will provide the ability to use multimodality approaches to monitor cells by MRI and correlate findings on pathology using fluorescent microscopy.

EXPERIMENTAL SECTION

Details are described in Supporting Information. TEXAS RED® and fluorescein were supplied as lysine fixable dextran conjugates (molecular weight 3kDa, Invitrogen). Ferumoxides (Feridex IV®, Berlex Laboratories) was used as the SPION. Human cervical carcinoma (HeLa) cells (CCL-2, ATCC) were used for cell labeling because HeLa cells were commonly used before conducting other cell line.

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. After reaction, the aldehyde FE was purified by running the solution through a PD 10 column (GE Healthcare) 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 (NaBH3CN, 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). The surface charge and hydrodynamic diameter of nanoparticles or of complexes was measured by a zeta potential (ZP) analyzer (ZetaPALS, Brookhaven Instruments). 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.[16]

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) 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 2×10^5 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).

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.[1,17,18]

Cytology

For microscopy, cells were washed, 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.

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). 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) using a dedicated 7 cm solenoid receive only RF-coil (Philips Research Laboratories).

Acronyms

FL SPIONs	Fluorescent Superparamagnetic Iron Oxide Nanoparticles
SPIONs	(Superparamagnetic iron oxide nanoparticles)
FE	ferumoxides
Pro	protamine sulfate
FL	fluorescent
Fluo	fluorescein
Texas	TEXAS RED®

DAPI

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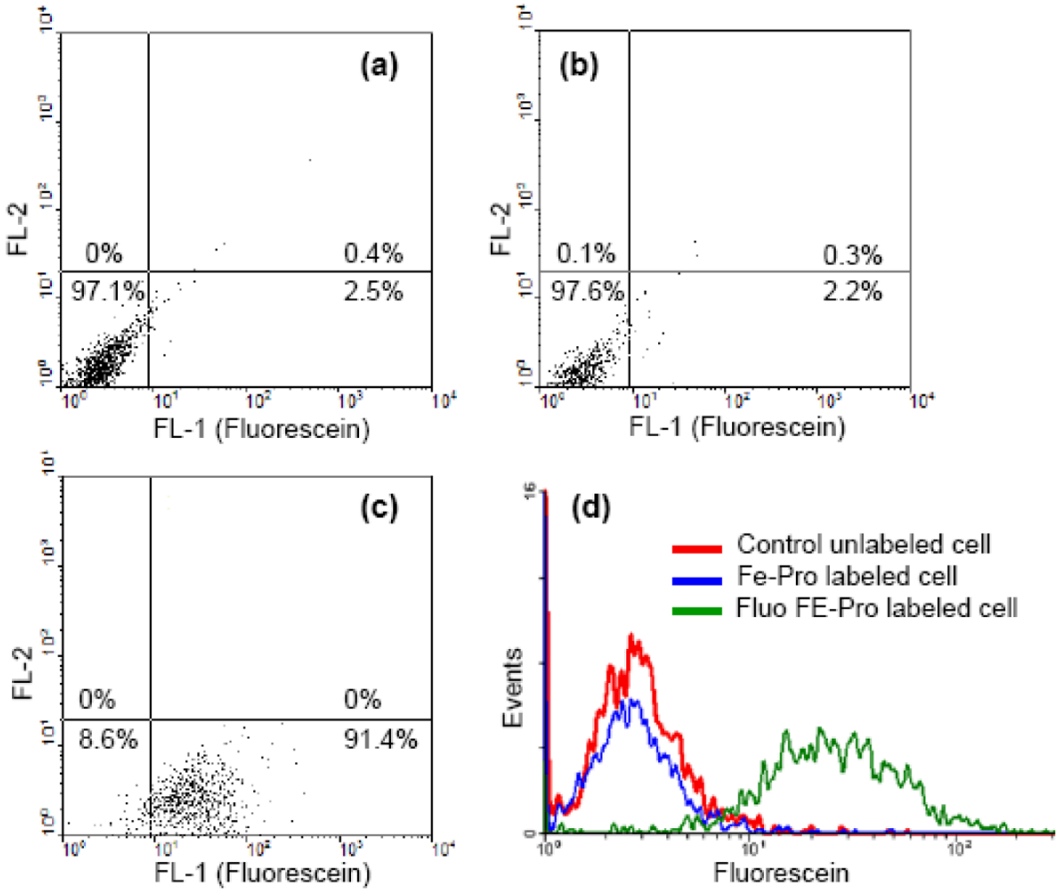

Figure 1.

Flow cytometry results of (a) control unlabeled HeLa cell; (b) FE-Pro labeled HeLa cell; (c) Fluo FE-Pro labeled HeLa cell; and (d) histogram overlay, FL-1 is the fluorescent channel for fluorescein and FL-2 is the fluorescent channel for PE (Phycoerythrin). Figures C. shows a shift in the cell population of the Fluo FE-Pro labeled cells to the right with 91.4% of the cells positive for fluorescence in the fluorescein channel.

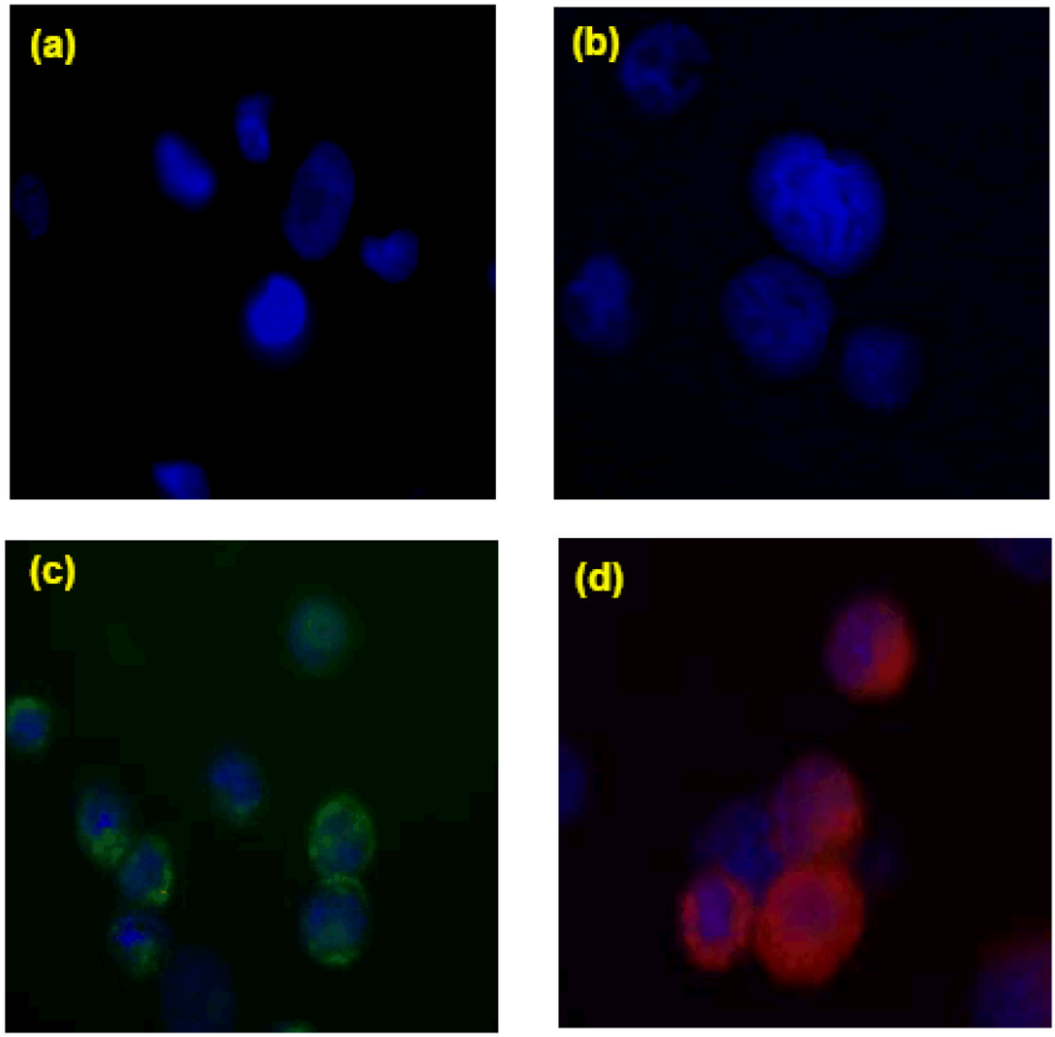

Figure 2.
Comparison of fluorescent microscopy images with (a) control unlabeled HeLa cells; (b) FE-Pro labeled HeLa cells; (c) Fluo FE-Pro labeled HeLa cells; and (d) Texas FE-Pro labeled HeLa cells. All nuclei were labeled with DAPI.

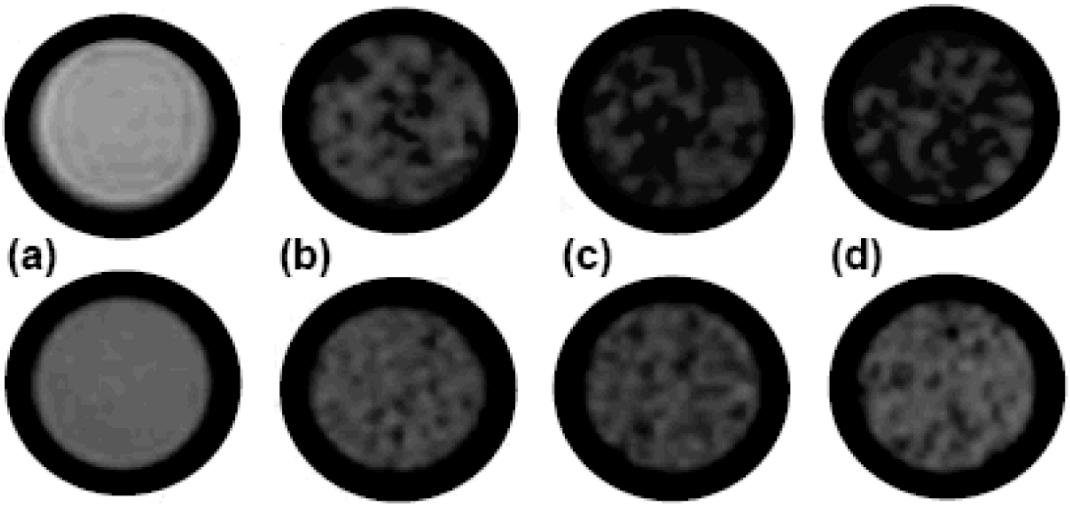

Figure 3.

MR images from FL FE-Pro complexes labeled cells suspended in an agar gel (top row) T_2* weighted sequence image and (bottom row) T_2 weighted sequence image.(a) unlabeled HeLa cells; (b) FE-Pro labeled HeLa cells; (c) Fluo FE-Pro labeled HeLa cells; (d) Texas FE-Pro labeled HeLa cells. Each gel contains cell concentrations of 5×10^5 cells/mL.

Table 1

Physico-chemical properties of fluorescent ferumoxides (FL FE)

Sample	Hydrodynamic diameter (nm)	Zeta Potentials, mV	R_1 [$s^{-1}mM^{-1}$] *[a]*	R2 [$s^{-1}mM^{-1}$] *[b]*	R_2/R_1
Ferumoxides (FE)	157.3±1.2	−32.6±0.4	12.2±1.6	162.1±22.5	13.3±0.1
Fluo FE	145.9±1.0	−32.0±0.7	12.4±1.7	121.9±3.9	9.9±1.0
Texas FE	153.9±3.1	−28.9±1.3	12.1±2.2	133.6±16.1	11.2±1.5
Texas Red Dextran		4.3±1.9			
Fluorescein Dextran		−7.6±1.4			

[a] T_1 Relaxation rate

[b] T_2 Relaxation rate

Adv Mater. Author manuscript; available in PMC 2009 July 2.

Table 2

NMR Relaxivities of FE-Pro complex in a gel

Type of Complex	Ratio μg/mL	R_1 $[s^{-1}mM^{-1}]$ *[a]*	R_2 $[s^{-1}mM^{-1}]$ *[b]*
FE: Pro	100:0	10.2±0.8	187.6±16.0
FE: Pro	100:6	1.8±0.4	8.5±0.8
FE: Pro	100:20	10.1±1.1	284.2±41.4
Fluo FE: Pro	100:0	12.2±1.0	127.6±10.9
Fluo FE: Pro	100:6	6.3±1.4	119.5±11.5
Fluo FE: Pro	100:20	8.2±0.9	225.0±32.8
Texas FE: Pro	100:0	9.7±1.1	137.3±11.7
Texas FE: Pro	100:6	3.8±0.8	34.1±3.3
Texas FE: Pro	100:20	10.3±1.2	178.7±26.1

[a] T_1 Relaxation rate

[b] T_2 Relaxation rate