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The cell labeling efficacy, cytotoxicity and relaxivity of copper-activated MRI/PET imaging contrast agents

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Abstract

A new class of nanoparticle-based dual-modality positron emission tomography/magnetic resonance imaging (PET/MRI) contrast agents has been developed. The probe consists of a superparamagnetic iron oxide (SPIO) or manganese oxide core coated with 3,4-dihydroxy-d,l-phenylalanine (dl-DOPA). The chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) was conjugated to DOPA termini. The DOTA modified nanoparticles allow chelation of copper for PET imaging. These surface functionalized nanoparticle-based probes have been characterized by various analytical techniques. The cell-labeling efficacy, cytotoxicity and relaxivity of these nanoparticles have been evaluated and compared with the same properties of one of the most commonly utilized MRI contrast agents, Feridex[®]. Evidently, this new nanoparticle has a great potential for use in cell tracking with MRI and PET in the absence of transfecting agent. It is noteworthy that there is a sharp increase in r_2 relaxivity of these nanoparticles on coordination with Cu²⁺ ions. Thus these iron oxide nanoparticles can also be explored as the smart magnetic resonance (MR) sensor for the detection of micromolar changes in copper concentration for neurodegenerative diseases such as Alzheimer's disease, Menkes and Wilson's diseases, amyotrophic lateral sclerosis and prion diseases.

Keywords

Nanoparticle; Magnetic resonance imaging; Positron emission tomography; Multimodal imaging probe; Relaxivity; Stem cell labeling

1. Introduction

Molecular imaging plays a very important role in molecular or personalized medicine, which has been described as the future of patient management and healthcare [1]. Molecular imaging enables visualization of the biological targets and understanding its complexities for diagnosis and treatment of the disease. The molecular abnormalities are traced by different imaging techniques for the understanding of the disease process. An accurate and real-time imaging of biological targets provides a thorough understanding of the fundamental biological processes and helps to diagnose various diseases successfully [2] and [3]. It is difficult to obtain all the necessary information about the biological structure and function of an organ by any single imaging modality among all the existing imaging techniques. Therefore attempts are being made to fuse the advantages of different imaging techniques by combining two or more imaging modalities while eliminating or reducing their disadvantages. Thus dual or multimodal imaging methods are used to enhance the quality of the images to achieve proper visualization of the organs and a better reliability of the collected data. Multimodal contrast agents are becoming increasingly important for biomedical applications. A variety of combinations of different modalities including MRI–optical, PET–near-infrared optical fluorescence (NIRF) and PET–CT have been reported [1], [4], [5], [6], [7] and [8].

The fusion of PET and MRI in a single contrast agent has proved to be beneficial as it gives images of high sensitivity and high resolution [1]. For example, the low sensitivity of magnetic resonance imaging (MRI) is often found insufficient for detection of tissue injury, assessment of tissue/organ function and more recently for tracking of implanted stem cells. To overcome this limitation of low sensitivity, a number of contrast agents have been developed, however, these contrast agents often have to be administered in a high concentration in order to obtain clinically useful images [9], [10], [11] and [12]. On the contrary positron emission tomography (PET) has the advantage of high sensitivity and isotropism *i.e.*, ability to detect and quantify the exogenous radioactive isotopes accurately [13]. However, the low spatial resolution of PET in comparison to MRI often makes PET imaging insufficient to obtain reliable biological information [14], [15] and [16].

Nanoparticles are the ideal scaffolds that allow the integration of several different imaging modalities onto a single platform. For example, a superparamagnetic nanoparticle can act as a magnetic resonance imaging modality, higher effective concentrations of a second imaging probe such as a fluorophore or radionuclide can be incorporated into the nanoparticle shell and the introduction of a targeting moiety on the surface of the nanoparticle allows the specific labeling of the region of interest *in vivo*. The usefulness of the multi-component nanoparticle probes is not limited to diagnostic purposes only. These hybrid nanoparticles can be used as probes for biological processes such as cell trafficking and gene delivery under *in vitro* and *in vivo* conditions when conjugated with the biologically active species such as stem cells and viruses [17] and [18]. When target specificity and tissue regeneration capabilities are imparted by endocytosis of the magnetic nanoparticles into the stem cells, the composite nanoparticles can be used as dual-function probes possessing both *in vivo* MRI

trafficking and stem cell functions [19]. However, if the nanoparticles are to be exploited in these important research areas, their surfaces must be modified such that they can be readily taken up by the cells.

Metal oxide nanoparticles are valuable probes for multimodal detection because their diameters and surface functional groups can be easily fine tuned and may act as contrast enhancement probes in MRI (*e.g.* Fe₃O₄ and MnO). Recently, nanoparticle-based PET agents have been developed for multimodal imaging, however, only a few combined PET/MRI probes have been reported to date, while the majority of the bi-modal probes have been developed for use in optical imaging in combination with either PET or MRI. [1], [20], [21], [22], [23] and [24]. Chen and coworkers reported nanoparticle-based probe for dual-modality PET/MRI, by using MnMEIO as MRI agent and ¹²⁴I for PET [25]. There is increasing interest in the development of nontraditional positron-emitting radionuclides [26] and [27]. With respect to nanoparticle-based PET probes, copper-based radionuclides are particularly interesting and offer a varying range of half-lives and positron energies. In addition, there is a multitude of well-established ligands that can strongly coordinate with copper ions and can be incorporated onto the surface of a nanoprobe and provide *in vivo* stability [3]. There have been reports of attempts to build a scanner with the capabilities to simultaneously image MRI and PET [14], [29], [30] and [31].

We have recently reported potential MRI/PET dual contrast agents by suitable surface modification of iron oxide nanoparticles by silica shell and a ligand to bind Cu²⁺ ions. These nanoparticles can be used for cell labeling and interestingly upon binding with Cu²⁺ ions to the surface anchored ligands, these nanoparticles exhibited significant lowering of the *T*₂ relaxivity of the iron oxide nanoparticles. This lowering of the *T*₂ relaxivity upon Cu²⁺ ion binding onto the nanoparticles offers a potential mean for the detection of Wilkinson's disease, which is caused by excess Cu²⁺ ion storage in the liver [32]. Though we were able to control the *T*₂-weighted MRI contrast through coordination with Cu²⁺, the *T*₂ values were lower than anticipated, presumably due to dampening of the water relaxation within the silica shell. To remedy this deleterious effect we have eliminated the silica shell and have bound the ligands directly to the nanoparticle surface. Specifically, to utilize the potential benefits of multimodal functionality in biomedical applications, we have designed and fabricated multifunctional *T*₁ (MnO) and *T*₂ (Fe₃O₄) based nanoparticles (Scheme 1). The magnetic nanoparticles have been derivitized with 3,4-dihydroxy-D,L-phenylalanine and subsequently conjugated with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) to bind with a nuclear tracer, ⁶⁴Cu, yielding the bi-modal imaging probes for PET and MRI. As demonstrated in Scheme 1, the free carboxylate group of 3,4-dihydroxy-D,L-phenylalanine molecule remains available for further modifications such as attachment of a target specific moiety or fluorescent tag. Herein we report the development of a new class of MRI/PET agents and the preliminary result of their MRI and cell labeling studies. It is important to highlight that the strategies leading to the effective endocytosis of nanoparticles into cells in the absence of transfecting agents is critical for any real applications. In this regard we have also introduced ligands that are capable of fine-tuning the surface charge of the nanoparticles in order to facilitate endocytosis without the aid of a

transfecting agent. In addition the relaxivities of these nanoparticle-based contrast agents increase sharply upon binding of Cu^{2+} ions with the DOTA moieties conjugated with dl-DOPA on the surface of the nanoparticles providing the opportunity to explore them as a new class of chemosensors for molecular imaging of copper biology in living systems.

Scheme 1

2. Experimental procedures

2.1. Materials

The reagents and solvents were obtained from the commercial sources: iron (II) chloride tetrahydrate (99%), iron (III) chloride hexahydrate (97%), diethylene glycol (99%), sodium hydroxide (97%; 20–40 mesh beads), oleic acid (OA, 90%), 3,4-dihydroxy-dl-phenylalanine (dl-DOPA), (3-carboxypropyl)trimethylammonium chloride (CTAC), 1-octadecene, 1-hexadecene, were purchased from Aldrich and used without further purification. 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono(N-hydroxysuccinimide ester) (DOTA–NHS-ester) was purchased from Macrocyclics.

X-ray diffractometry (XRD) measurements were performed with a Bruker AXS X-ray system equipped with a graphite monochromator ($\text{Cu K}\alpha$ radiation, λ 1.54056 Å). A transmission electron microscope (TEM) Philips CM20 FEG operating at 200 kV was used to determine particle size and morphology. The dynamic Light scattering (DLS) measurements of all nanoparticles were performed using the NanoSight NTA. NMR studies were performed on a Varian Gemini-300 spectrometer. FT-IR spectra were obtained on a Perkin–Elmer 1600 spectrometer using KBr pellets. XPS spectrum for Cu was recorded on a Physical Electronics 5600 multi-technique system. UV–visible

spectra were recorded on a Cary 5000 instrument. To determine the metal content of the nanoparticles, ICP analyses were carried out using Varian Vista-Pro ICP-ES CCD spectrometer at the chemistry department of the University of Ottawa. The magnetic susceptibility measurements for the nanoparticles were obtained using a Quantum Design SQUID magnetometer MPMS-XL housed at the chemistry department of the University of Ottawa. This magnetometer works between 1.8 and 400 K for dc applied fields ranging from -7 to 7 T. Magnetic susceptibility measurements were performed at 300 K and 2 K. The magnetic data were corrected for the sample holder and the diamagnetic contributions. The surface modifications by ligand exchange are shown in Scheme 2.

Scheme 2. Schematics of the ligand exchange procedure.

2.2. Synthesis of the nanoparticles and ligand exchange

Magnetite nanoparticles were prepared as describe previously by hydrolysis of chelated metal alkoxide complexes at an elevated temperature dissolved in diethylene glycol as the chelating alcohol [33]. Organic soluble two different MnO nanoparticles with uniform diameters of 7 and 20 nm were synthesized by the thermal decomposition of Mn-oleate complex using 1-hexadecene and 1-octadecene as the solvents, respectively [28]. Water-dispersible and biocompatible nanoparticles were prepared through ligand exchange processes. Specifically, a dispersion of the as synthesized nanoparticles (Fe_3O_4 or MnO) in hexane (10.0 mL, 0.5 mg/mL) was mixed with a solution of dl-DOPA (40.0 mg) in buffer (5.0 mL, pH 5.5): EtOH (10:1 mL, v/v) and a mixture of dl-DOPA (40.0 mg) and (3-carboxypropyl)trimethylammonium chloride (10.0 mg) in buffer (5.0 mL, pH 5.5): EtOH (10:1 mL, v/v) in separate experiments. These different reaction conditions lead to nanoparticles with increasingly more positive charge on their surfaces as the (3-carboxypropyl)trimethylammonium chloride is introduced. The biphasic mixture was stirred vigorously at room temperature for 12 h. The water-soluble nanoparticles were obtained by separating the aqueous phase from the hexane layer. The resultant aqua dispersion of the nanoparticles was dialyzed for 24 h in dialysis tubing (MWCO 3500) against water to remove excess ligands. These nanoparticles are

denoted as $\text{Fe}_3\text{O}_4\text{-DOPA}$, $\text{Fe}_3\text{O}_4\text{-DOPA}(+)$, $(\text{MnO})7\text{-DOPA}$, $(\text{MnO})7\text{-DOPA}(+)$, $(\text{MnO})20\text{-DOPA}$, $(\text{MnO})20\text{-DOPA}(+)$ and used for further conjugation with DOTA. Water was removed from the aqua dispersion of the nanoparticles by centrifugation and decantation. To remove trace amounts of water from nanoparticles they were redispersed in DMF, centrifuged and the supernatant was decanted. This process was repeated thrice. Finally the dl-DOPA modified nanoparticles were dispersed in DMF and used for further DOTA conjugation.

2.3. Conjugation of DOTA with the dl-DOPA modified nanoparticles

The reactive NHS ester of DOTA (15 mg) was added to a dispersion of the dl-DOPA modified nanoparticles (20 mg) in DMF (10.0 mL). The resulting mixture was stirred over vortex for 12 h. The DOTA conjugated nanoparticles were separated by centrifugation washed thrice with water (5.0 mL) and dialyzed the resultant nanoparticles overnight in dialysis tubing against water to remove excess NHS ester of DOTA. These nanoparticles are denoted as $\text{Fe}_3\text{O}_4\text{-L}$, $\text{Fe}_3\text{O}_4\text{-L}(+)$, $(\text{MnO})7\text{-L}$, $(\text{MnO})7\text{-L}(+)$, $(\text{MnO})20\text{-L}$, $(\text{MnO})20\text{-L}(+)$ (L = dl-DOPA conjugated DOTA).

2.4. Binding of Cu^{2+} with the nanoparticles

A stock solution of 0.01 mM cupric acetate was prepared in 0.1M sodium acetate (pH 7.2). This cupric acetate solution (100 μL) was mixed with a dispersion of $\text{Fe}_3\text{O}_4\text{-L}$, $\text{Fe}_3\text{O}_4\text{-L}(+)$ (1.15 mg) in sodium acetate buffer (1.0 mL) and stirred for 1 h. The resulting $\text{Fe}_3\text{O}_4\text{-L}$, $\text{Fe}_3\text{O}_4\text{-L}(+)$ nanoparticles coordinated with Cu^{2+} ions were magnetically confined from the solution by placing an external magnet next to the reaction vessel. The $(\text{MnO})7\text{-L}$, $(\text{MnO})7\text{-L}(+)$, $(\text{MnO})20\text{-L}$, $(\text{MnO})20\text{-L}(+)$ nanoparticles were coordinated to Cu^{2+} under identical conditions, though the nanoparticles were purified through centrifugation. After the supernatant solution was discarded, the nanoparticles were washed five times with 10 mL portions of deionized water to remove any non-coordinated Cu^{2+} ions.

2.5. Measurement of relaxivity

Relaxation times of the *in vitro* phantoms with different concentrations of nanoparticles were determined using a 3.0 T MR system (MAGNETOM Trio, Siemens Healthcare, Erlangen, Germany). For measurements of T_1 relaxation times of the phantoms, a spin-echo sequence with an echo time of 7 ms and 14 repetition-times (TR) was used. The 14 TRs were 100, 300, 500, 700, 900, 1100, 1300, 1500, 1700, 2000, 23000, 2600, 2900, and 3200 ms. For measurements of their T_2 relaxation times, a multiple spin-echo sequence with a TR of 2200 ms and viable TEs (TE = 7, 17, 27, 40, 60, 80, and 100 ms) was used. For T_2^* measurements, a spoiled gradient-echo pulse sequences with TE of 5, 7, 9, 11, 13, 15, 17, 19, 21, 23 and 25 ms were performed. Common acquisition parameters were: field of view = 151 $\times$ 230 mm, matrix = 168 $\times$ 256, spatial resolution = 0.9 $\times$ 0.9 mm². The flip angle of the spoiled gradient-echo sequence was 15°.

2.6. Cell culture and stem cells labeling with the contrast agents

Rat adipose-derived stem cells (ASC) were isolated from subcutaneous fat tissue in abdominal and inguinal regions of Lewis rats. Briefly, excised adipose tissue was washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and blood cells, and then it was finely minced and digested with 2 mg/mL collagenase in 37 °C water bath for 20 min. After filtration through 70 µm filters and centrifugation at 1000 g for 10 min, the cell pellet was re-suspended in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 15% fetal bovine serum (FBS) (Thermo Scientific Inc. USA), 100 I.U./mL penicillin (Thermo Scientific Inc. USA), 100 µL/mL Streptomycin (Thermo Scientific Inc. USA), 2 mmol L-glutamine (Thermo Scientific Inc. USA), 1 mmol sodium pyruvate (Thermo Scientific Inc. USA) and 1% MEN/NEAA (100X) (Thermo Scientific Inc. USA). The purified ASCs were seeded in 24-well plate at 3×10^4 /well. New nanoparticles were then added to the cell cultures at a concentration of 10, 20 or 30 µg/mL with 1.5 µg/mL poly-L-lysine (Sigma–Aldrich Co, USA) as a transfecting agent. ASCs were cultured in the conditions for 48 h. The labeled cells were assessed using Prussian blue staining technique.

3. Results and discussion

3.1. Synthesis of the nanoparticles

The iron oxide nanoparticles were synthesized by utilizing a green synthesis approach developed by Caruntu et al., in which iron (II) and iron (III) salts are chelated to diethylene glycol and reduced at high temperature to obtain highly crystalline, non-aggregated nanoparticles [33]. Oleic acid capped iron oxide nanoparticles were obtained by adding oleic acid at the last step of the synthesis. Manganese oxide (MnO) nanoparticles with two different particle sizes 7 nm and 20 nm, dispersed in non-polar organic solvent (*n*-hexane and chloroform), were synthesized by the thermal decomposition of Mn-oleate complex in 1-hexadecene and 1-octadecene as the solvent, respectively [28].

3.2. Crystal structure and morphology of the nanoparticles

The powder X-ray diffractometry (XRD) analysis of the as synthesized SPIO shows that the nanoparticles are highly crystalline and the XRD pattern matches well with the literature values of cubic crystalline bulk magnetite (Fe₃O₄) (JCPDS file No. 19-629). No other secondary iron oxide phases could be traced in the XRD pattern. The PXRD data are presented in the supporting information (Fig. S-1). The refined lattice parameter derived from this PXRD data is 8.38 Å, which is essentially identical to that of the lattice parameter reported for the bulk Fe₃O₄ (8.396 Å) [34]. The size of the Fe₃O₄ nanocrystals calculated using Scherrer's formula from the most intense peak of the XRD pattern was 5.5 ± 1 nm [35], which also agrees well with the average SPIO diameter calculated from the transmission electron microscopy (TEM) images (Fig. S-2). We used TEM images to compare the morphologies of the as prepared magnetite nanoparticles and the corresponding surface modified magnetite nanoparticles. The

TEM images of the magnetite nanoparticles made by following the reported procedures of Caruntu et al. [33] indicated a narrow size distribution and the monodispersed nature. The TEM images of surface modified Fe₃O₄-L nanoparticles indicated that the morphology and crystallinity of these nanoparticles remain unchanged (Fig. S-2). The powder X-ray diffractometry (XRD) of the manganese oxide nanoparticles (7 nm and 20 nm) (Fig. S-3) showed that the nanoparticles are highly crystalline and the XRD pattern corresponds to the literature values of cubic MnO (JCPDS file No. 07-0230). The presence of no other secondary oxide phases could be traced in the XRD pattern [28] and [36]. The sizes of the MnO nanocrystals were calculated using Scherrer's formula [35] from the most intense peak of the XRD pattern. The sizes of the two types of MnO nanoparticles were found to be 7 ± 1 nm and 20 ± 1 nm and these values agree well with the average diameter of the MnO nanoparticles calculated from the TEM images. The TEM images (Fig. S-4, 5) of both the types of the MnO nanoparticles indicated that these nanoparticles were uniform, well dispersed and highly crystalline in nature. The aqua dispersion of both the types of the nanoparticles did not show any indication of degradation or aggregation when stored over several months. These nanoparticles are antiferromagnetic in nature (see Supporting Information) thus they do not exert the susceptibility artifacts in MRI as observed with the SPIO-based T_2 contrast agents.

Both the Fe₃O₄ and MnO nanocrystals were coated with hydrophobic ligands (*i.e.*, oleic acid) and are only soluble in organic solvents *n*-hexane, Chloroform *etc.* However, for their use as intracellular imaging probes, it was necessary to appropriately modify the nanocrystal surface with hydrophilic ligands to promote solubility in aqueous media and simultaneously to provide a moiety for radioactive metal ion binding. Water-dispersible and biocompatible nanoparticles were prepared according to a reported method with some modifications [36], [37], [38], [39] and [40]. The ligand exchanges of these nanoparticles were carried out as shown in Scheme 2. The dispersion of different nanoparticles in *n*-hexane were agitated, in separate experiments, with an aqueous solution of dl-DOPA or a mixture of dl-DOPA and (3-carboxypropyl)trimethylammonium chloride to result dl-DOPA modified nanocrystals with neutral zwitterionic surface and mixed dl-DOPA: (3-carboxypropyl)trimethylammonium modified nanocrystals with positively charged surface, respectively.

In aqueous medium, the (3-carboxypropyl)trimethylammonium chloride ligands are attached to the nanoparticle surface (Scheme 2) with the carboxylate end, while the appended ammonium cation introduces a positive charge and hydrophilicity. Thus manifestation of two different surfaces, neutral and positive could be achieved by simple manipulation of the addition of surface modifying agents. Excess of the surface modifying ligands were removed from the isolated nanoparticles by repeated dialysis. dl-DOPA is a derivative of phenylalanine and abundantly present in the mussel adhesive protein *Mytilus edulis*, [41] and considered to have high affinity binding group to stabilize iron oxide nanoparticles in water [42] and physiologic buffers via M–O (M = Fe, Mn) bonding [43], [44], [45] and [46]. In addition dl-DOPA can provide the opportunity of conjugating DOTA on the surface of the nanoparticles through amide bond formation. The spectroscopic study by Rajh [46] suggested that bidentate enediol

ligands such as DOPA convert the under-coordinated Fe surface sites back to a bulk-like lattice structure with an octahedral geometry for oxygen-coordinated iron, which is expected to result in tight binding of DOPA to iron oxide. DOTA is a well-established ligand to form stable complex with metal ions like Cu(II), Gd(III) and In(III) [47]. DOTA derived ligands have widely been utilized to coordinate radioactive metal ions to use as PET imaging agent [48]. For the controlled incorporation of ^{64}Cu , highly efficient Cu(II) binding ligand, DOTA was chosen. The amide linkage formation via active ester group of mono (N-hydroxysuccinimide ester) of DOTA was selected for the coupling reaction with the free amine group of the dl-DOPA on the surface of the nanoparticles. The dl-DOPA modified nanoparticles were reacted with mono-(N-hydroxysuccinimide ester) of DOTA, where one of the carboxylic acid groups of DOTA is activated as succinimidyl ester. The reaction led to conjugation of DOTA with the amine group of dl-DOPA, attached to the surface of the nanoparticles. The excess un-reacted DOTA molecules were removed by dialysis.

The DOTA conjugation with the dl-DOPA, anchored on the surface of the nanoparticles was proved indirectly by ^1H NMR analysis (Fig. S-6). The absence of free DOTA in the solution phase of a dispersion of the nanoparticles in D_2O was indicated by the diffusion ordered spectroscopy (DOSY) analyses. In the DOSY measurements the resonances of the free and conjugated surface-bound ligands can be easily identified based on the respective diffusion dimensions [49]. The DOTA conjugated nanoparticles (MnO)₇-L and (MnO)₂₀-L were dispersed in D_2O and the ^1H NMR spectrum was recorded. The peak due to the methylene protons of DOTA conjugated with the surface-bound dl-DOPA appears as a broad singlet at δ 1.80 ppm based on the correlation of the resonance chemical shifts and the diffusion coefficients. Further, the rapid diffusion of this signal also indicates that the DOTA is attached to the paramagnetic nanoparticle surface through conjugation with the amine group of the surface anchored dl-DOPA molecules.

DOTA is able to coordinate with Cu^{2+} to form thermodynamically stable complex. Coordination between the surface anchored DOTA and Cu^{2+} was also confirmed by UV-vis and X-ray photoelectron spectroscopy (XPS) (Figs. S-7–9). The DOTA anchored nanoparticles were reacted with 0.01 mM aqueous cupric acetate solution and the UV-visible spectra of the supernatants were checked after the removal of the Cu^{2+} bound nanoparticles by magnetic separation or centrifuge technique. The lowering of the intensity of the $d-d$ transition band of the supernatants in comparison to the Cu^{2+} control solution prior to binding with DOTA modified nanoparticles, allows the quantification of Cu^{2+} binding to the surface of the nanoparticles. XPS measurements were also employed to characterize the Cu^{2+} binding to the nanoparticles. As highlighted in Figs. S 7 and S 8 the peaks at 951 eV and 931 eV correspond to copper bound to the ligand on the nanoparticles surface.

The dynamic light scattering (DLS) measurements of the nanoparticles revealed the hydrodynamic diameter of the nanoparticles in aqueous dispersion. The hydrodynamic diameter data of the nanoparticles before and after Cu^{2+} coordination are collected in the Table S-1. The diameters of the nanoparticles measured by the DLS study are

higher compared to the diameter of the nanoparticles obtained from the TEM measurements. Such a phenomenon of difference in the mean diameters obtained by DLS and TEM has already been observed with other nanomaterials by Bootz et al. [50]. The larger colloidal particle size in aqueous dispersion than in the dry state or in the non-polar organic dispersion is essentially due to the highly hydrated ligand and surface molecule layer on the nanoparticles. The molecules at the surface of the nanoparticles, described here, bear large numbers of polar groups *viz* carboxylate, amide, tertiary amine and quaternary ammonium ions. All these functional groups are capable of forming hydrogen bonding with the solvent water molecules or being solvated by water molecules through polar interaction causing an extensive hydration of the nanoparticles. Thus the z-average diameters of the nanoparticles obtained by the DLS measurements are higher. As the TEM only visualizes the iron oxide cores, the particle size obtained from the TEM measurements in a dried state or in a non-polar organic dispersion is expected to be lesser than the values obtained from the DLS measurements [51], [52], [53] and [54]. Our interest for acquiring the DLS data for these nanoparticles was to understand the interaction among the nanoparticles after Cu^{2+} coordination. The DLS data of the diameter of the nanoparticles before and after Cu^{2+} coordination do not alter substantially indicating that after the Cu^{2+} coordination there is no inter-nanoparticle associative interaction or conjugation.

3.3. Magnetic measurement

Magnetic measurements of the Fe_3O_4 nanoparticles on an SQUID magnetometer indicate that the nanoparticles are superparamagnetic at room temperature (Fig. 1). The Fe_3O_4 nanoparticles, both before and after surface modification, showed superparamagnetic behavior at room temperature. This indicates that the thermal energy can overcome the anisotropy energy barrier of a single particle and the net magnetization of the particle assemblies in the absence of an external field is zero [55]. It can be seen that these nanoparticles are superparamagnetic at 300 K and ferromagnetic at 2 K, with a coercivity of 225 Oe, 60 Oe, and 150 Oe, for the as synthesized Fe_3O_4 and $\text{Fe}_3\text{O}_4\text{-L}$ and $\text{Fe}_3\text{O}_4\text{-L-Cu}$, respectively, while the saturation magnetizations (M_s) for these nanoparticles are 51.3 emu g^{-1} , 38.3 emu g^{-1} and 33.3 emu g^{-1} , respectively (Fig. 1). Due to the surface modification of the nanoparticles with the ligands which are bound directly to the iron ions at the surface of the nanoparticles, a surface spin disorder is caused, which induces significant changes in the magnetic properties of the nanocrystalline materials [56], [57], [58], [59], [60] and [61]. Hence $\text{Fe}_3\text{O}_4\text{-L}$ and $\text{Fe}_3\text{O}_4\text{-L-Cu}$ has lower saturation magnetization values than as the as prepared Fe_3O_4 nanoparticles. The saturation magnetization values at 300 K are systematically lower than those measured at 2 K, presumably, due to the thermal fluctuations of the magnetic moments of the nanoparticles at higher temperatures [61].

Fig. 1. The magnetic hysteresis loops of the as synthesis iron oxide nanoparticles, surface modified Fe_3O_4 (L) and Fe_3O_4 (L)-Cu.

The Fe_3O_4 -L-Cu NPs show slightly reduced magnetization compared to the Fe_3O_4 and Fe_3O_4 -L NPs. This may be caused by the presence of Cu^{2+} ions bound to the DOTA on the surface of the Fe_3O_4 -L-Cu NPs. Apparently no virtual change in the magnetic properties due to the presence of the paramagnetic Cu^{2+} ions was expected, as observed earlier [56]. The paramagnetic moment from the Cu^{2+} ion is evidently outweighed by the strong magnetization induced by the iron oxide core [62]. The blocking temperature T_B shifted from 24 K to 20 K after the surface modification (Fig. S 10). The lowering of T_B was observed in the literature when the nanocrystals are chemically coated with a thin layer of nonmagnetic material. The T_B of the nanoparticles was suppressed to a lower temperature compared to that of the approximately similar sized uncoated nanoparticles [63]. The (MnO)₇-L samples showed the antiferromagnetic behavior at 2 K [64] and [36]. While the (MnO)₇-L-Cu shows ferromagnetic behavior with the coercivity of 1795 Oe. The observed blocking temperature T_B was 34.5 K for both (MnO)₇-L and (MnO)₇-L-Cu NPs (Fig. S 11, 12).

3.4. Relaxivity

The relaxation properties of the superparamagnetic nanoparticles were verified by measuring the spin–spin relaxation times (T_2) of the water protons in the dispersion of the nanoparticles in 1% agarose gel. The superparamagnetic Fe_3O_4 nanocrystals shortened the water proton spin–spin relaxation times, by perturbing the magnetic relaxation processes of the protons in the surrounding water molecules resulting in a decrease in the MRI signal intensity and consequent darkening of the MR images [65] and [66]. Relaxivities were calculated using the relaxation times and the

concentrations obtained from inductively coupled plasma atomic emission spectroscopy (ICP-AES) measurements with each type of nanoparticles. For the as synthesized iron oxide nanoparticles the r_2 and r_2^* relaxivities were determined to be $54 \text{ mm}^{-1} \text{ s}^{-1}$ and $148 \text{ mm}^{-1} \text{ s}^{-1}$, respectively. Compared to the Feridex ($r_2 = 148 \text{ mm}^{-1} \text{ s}^{-1}$), a commercially available iron-oxide based MR contrast agent used in the clinics, the surface modified $\text{Fe}_3\text{O}_4\text{-L}$ and $\text{Fe}_3\text{O}_4\text{-L}(+)$ nanoparticles exhibited much less r_2 relaxivities (52 and $66 \text{ mm}^{-1} \text{ s}^{-1}$, respectively). Thus the r_2 relaxivities of $\text{Fe}_3\text{O}_4\text{-L}$ remain essentially identical to that of the unmodified Fe_3O_4 NPs, while the r_2 relaxivities of $\text{Fe}_3\text{O}_4\text{-L}(+)$ increases slightly (from 55 to $66 \text{ mm}^{-1} \text{ s}^{-1}$). However, the r_2^* relaxivity decreased substantially upon surface modification. The r_2^* relaxivity is related to the T_2 relaxation time as $1/T_2^* = 1/T_2 + 1/T_2'$, with T_2' being a time constant arising from magnetic field inhomogeneity [67]. The r_2^* relaxivities of the neutral surface $\text{Fe}_3\text{O}_4\text{-L}$ ($115 \text{ mm}^{-1} \text{ s}^{-1}$) and the positively charged surface $\text{Fe}_3\text{O}_4\text{-L}(+)$ ($136 \text{ mm}^{-1} \text{ s}^{-1}$) are smaller than the same for the as synthesized iron oxide nanoparticles ($148 \text{ mm}^{-1} \text{ s}^{-1}$) and Feridex ($215 \text{ mm}^{-1} \text{ s}^{-1}$) indicating that the surface modification and DOTA conjugation decrease the magnetic field inhomogeneity contribution towards the r_2^* relaxivity. This decrease in the magnetic field inhomogeneity contribution towards the r_2^* relaxivity may be attributed to the nature of the charge of the newly added surface on the original nanoparticles, however, the exact nature of the interaction causing this effect is still unknown. It is noteworthy that both the r_2 and r_2^* relaxivities for $\text{Fe}_3\text{O}_4\text{-L}(+)$ is higher than $\text{Fe}_3\text{O}_4\text{-L}$. The surface of the $\text{Fe}_3\text{O}_4\text{-L}(+)$ nanoparticles are modified with (3-carboxypropyl)trimethylammonium chloride in addition to DOTA conjugated di-DOPA resulting in a positively charged surface. Thus it appears that the positively charged iron oxide nanoparticles are having higher relaxivities compared to their neutral counterparts. The similar behavior is consistent with the observations from previous work we had carried out with positively charged and neutral silica coated iron oxide nanoparticles, though it is unclear exactly why this effect occurs [32]Fig.2.

Fig. 2. r_1 and r_2 values of the iron oxide nanopartilces with and without copper and Feridex®.

The relaxation properties after binding of Cu^{2+} with the ligand on the surface modified Fe_3O_4 nanoparticles was also studied to evaluate the potential of these nanoparticles as MRI/PET dual agents. The $\text{Fe}_3\text{O}_4\text{-L}$ and $\text{Fe}_3\text{O}_4\text{-L}(+)$ nanoparticles were mixed with increasing known concentrations of aqueous Cu^{2+} in order to obtain the $\text{Fe}_3\text{O}_4\text{-L-Cu}^{2+}$ and $\text{Fe}_3\text{O}_4\text{-L}(+)\text{-Cu}^{2+}$. As described earlier, excess Cu^{2+} ions were removed through repeated centrifugation and decantation washing cycles with deionized water. The relaxivities of these Cu^{2+} coordinated iron oxide nanoparticles were measured and clear trend was found. The first observation demonstrated that as Cu^{2+} is anchored to the nanoparticles, the r_2 relaxivity increased from $52 \text{ mm}^{-1} \text{ s}^{-1}$ to $167 \text{ mm}^{-1} \text{ s}^{-1}$ for $\text{Fe}_3\text{O}_4\text{-L}$ vs. $\text{Fe}_3\text{O}_4\text{-L-Cu}^{2+}$ while from $66 \text{ mm}^{-1} \text{ s}^{-1}$ to $198 \text{ mm}^{-1} \text{ s}^{-1}$ for $\text{Fe}_3\text{O}_4\text{-L}(+)$ vs. $\text{Fe}_3\text{O}_4\text{-L}(+)\text{-Cu}^{2+}$. These relaxivity data are presented in [Table S-2](#). The shortening of the T_2 relaxation time of the water proton is known to originate from the induction by the paramagnetic moment of the superparamagnetic iron oxide nanoparticles in the presence of an external magnetic field. The effect is eventually distributed to the bulk by the diffusion of the water molecules from the closer proximity of the nanoparticles to the outer sphere environment of the nanoparticles, *i.e.*, the bulk water [68].

The enhanced r_2 relaxivities upon Cu^{2+} coordination may be attributed to the geometry around the Cu^{2+} ion on the surface of the nanoparticles and the modified surface obtained after Cu^{2+} binding. DOTA binds with the Cu^{2+} ion through the nitrogen atoms of the tetra-aza macrocyclic ring. The acetate coordination is labile in aqueous media and the water molecules can establish an association–dissociation equilibrium with the Cu^{2+} ions coordinated by the DOTA moieties on the surface of the nanoparticles. This dynamic association–dissociation equilibrium facilitates the propagation of the magnetic induction of the nanoparticles to the bulk water molecules. Further, the interface between the surface of the superparamagnetic nanoparticles and the aqueous phase is the closest possible approach of the water molecules towards the iron oxide surface. When the surfaces of both the $\text{Fe}_3\text{O}_4\text{-L}$ and $\text{Fe}_3\text{O}_4\text{-L}(+)$ are anchored by Cu^{2+} ions through binding with the DOTA moieties on the modified surface, the molecular weight and the surface area are higher than the same for the bare iron oxide nanoparticles. The larger interface between the copper anchored magnetic nanoparticles and the bulk water lead to a favorable interaction of the Cu^{2+} anchored iron-oxide nanoparticles with the bulk water resulting in higher relaxivity.

Further, the binding of the Cu^{2+} by DOTA on the surface of nanoparticle will relieve steric congestion around the iron oxide surface. This will affect the dynamic association–dissociation equilibrium between the Cu^{2+} ions on the nanoparticles surface and the water molecules in the intimate vicinity resulting in a shorter residence lifetime, τ_m , of coordinated water. The high degree of cooperative interaction facilitates the induction of the paramagnetic moment of the superparamagnetic iron oxide nanoparticles to the bulk water and consequently a larger r_2 relaxivity. The enhanced r_2 relaxivities obtained after Cu^{2+} coordination to the ligands of the nanoparticles is essentially not due to clustering of the nanoparticles. From DLS data the difference in the hydrodynamic diameter of the nanoparticles before and after Cu^{2+} coordination is not too high (approximately $\pm 20\text{--}30 \text{ nm}$). This observation proves that there is no cluster formation among the nanoparticles or conjugation between nanoparticles. The

increased relaxivity values after the Cu^{2+} coordination is purely due to the coordination of the Cu^{2+} ions to the DOTA moiety of the ligand on the nanoparticle surface. The increase in r_2 relaxivity is also reflected in the T_2 -weighted phantom images as shown in [Fig. 3](#). The negative contrast of the MR images was increased for copper coordinated nanoparticles compared to the nanoparticles with no coordinated Cu^{2+} ions. The enhanced r_2 and r_2^* relaxivities were observed upon Cu^{2+} binding for both the types of the iron oxide nanoparticles and the relaxivity values are comparable to the same for Feridex. The relaxivity data are summarized in [Table S-2](#).

Fig. 3. T_2 and T_2^* images of nanoparticles, 10 $\mu\text{g/mL}$ in 1% agarose gel, acquired with a 3T Siemens MR system using a gradient-echo sequence with echo time of 60 ms and 10 ms respectively.

Further, the variation of the relaxivities as a function of the amount of Cu^{2+} bound onto the surface of the $\text{Fe}_3\text{O}_4\text{-L}$ nanoparticles were monitored to understand the trends of the signal enhancement in the MR images. In separate experiments, 20 μL , 40 μL , 60 μL and 80 μL , 0.01 mM Cu^{2+} solutions were allowed to bind with 1.15 mg $\text{Fe}_3\text{O}_4\text{-L}$ nanoparticles in 1.0 mL 0.01 M sodium acetate buffer at pH 7.2. It was observed that $\text{Fe}_3\text{O}_4\text{-L}$ nanoparticles treated with 20 μL 0.01 mM Cu^{2+} solution has more colloidal stability. The colloidal stability of the nanoparticles decreased with increased amount of added 0.01 mM Cu^{2+} solution. The r_2 relaxivity increased when 20 μL 0.01 mM Cu^{2+} was bound with 1.15 mg of $\text{Fe}_3\text{O}_4\text{-L}$ compared to the r_2 relaxivity of the bare $\text{Fe}_3\text{O}_4\text{-L}$ nanoparticles. This higher r_2 relaxivity practically remained unchanged when 40 μL and 60 μL 0.01 mM Cu^{2+} was bound with 1.15 mg of $\text{Fe}_3\text{O}_4\text{-L}$. A further increase in the r_2 relaxivity was observed when 80 μL 0.01 mM Cu^{2+} was bound with 1.15 mg of $\text{Fe}_3\text{O}_4\text{-L}$ nanoparticles ([Table S-3](#)). The enhanced r_2 relaxivities were reflected in the phantom MR images obtained with $\text{Fe}_3\text{O}_4\text{-L}$ bound with different amount of Cu^{2+} ions ([Fig. 4](#)). As these contrast agents lead to change in r_2 values in response to Cu^{2+} ions, they can also be utilized as the chemosensors. It will also be useful to understand the physiological or pathological consequences of the presence of copper in the biological

system and for the diseases like Wilson's disease [69] by using MR-based molecular imaging.

Fig. 4. T_1 and T_2 weighted phantom images of iron oxide nanoparticles with increased concentrations of Cu^{2+} and without Cu^{2+} .

When the radio isotope of Cu^{2+} will be made to bind with the $\text{Fe}_3\text{O}_4\text{-L}$ or $\text{Fe}_3\text{O}_4\text{-L}(+)$, the combined effect of the presence of radio isotope of Cu^{2+} and the higher r_2 relaxivities of $\text{Fe}_3\text{O}_4\text{-L-Cu}^{2+}$ and $\text{Fe}_3\text{O}_4\text{-L}(+)\text{-Cu}^{2+}$ are expected to enable them as the suitable candidates for MRI/PET dual agents. This is evident from the contrast in the phantom images obtained with the $\text{Fe}_3\text{O}_4\text{-L}$ before and after Cu^{2+} binding as shown in Fig. 4.

In contrast to the iron oxide based nanoparticles, manganese oxide (MnO) nanoparticles were shown to give brighter signal in the T_1 weighted MR images indicating that MnO nanoparticles are potential T_1 contrast agents [2]. To explore the ability of the MnO nanoparticles of different diameters to be used as MRI contrast agents, relaxivities of water protons in the dispersion of the MnO nanoparticles in 1% agarose gel were determined. In general MnO-L exhibit higher r_1 relaxivities, but the MnO nanoparticles of 7 nm particle size exhibit higher r_1 relaxivity (16.4 and 17.8 $\text{mm}^{-1} \text{s}^{-1}$ for $(\text{MnO})7\text{-L}$ and $(\text{MnO})7\text{-L}(+)$, respectively) compared to the MnO nanoparticles of 20 nm particle size (10.8 and 11.5 $\text{mm}^{-1} \text{s}^{-1}$ for $(\text{MnO})20\text{-L}$ and $(\text{MnO})20\text{-L}(+)$, respectively). The relaxivity data have been collected in Table S-4. The relaxivity data are also reflected in the brightness of the phantom images obtained with the MnO nanoparticles, as shown in Fig. 5. The higher relaxivities of the smaller nanoparticles may be attributed to the larger surface area/volume ratios of these nanoparticles. That is, the larger surface area of the MnO nanoparticles of smaller size exposes large number of MnO units to the water molecules in the immediate vicinity of the nanoparticles and thereby facilitating a more efficient shortening of the T_1 relaxation time of water protons and hence the higher r_1 relaxivity. Consequently the relatively small surface area of the larger MnO nanoparticles leads to lower relaxivity.

Fig. 5. T_1 and T_2 weighted phantom images of the MnO nanoparticles with and without copper.

To explore the MnO nanoparticles as MRI/PET dual agent copper coordination with to MnO-L was carried out. Upon Cu^{2+} coordination, the r_1 relaxivities actually decrease. The decreased r_1 relaxivities upon Cu^{2+} ion binding suggests that there is a lowering of the net paramagnetic moment of the coordinated nanoparticles. This may be caused by the anti-parallel alignment of the magnetic spin of the paramagnetic Cu^{2+} ions on the surface of the nanoparticles and the magnetic spin of the MnO units at the surface of the nanoparticles. The changes in the relaxivities are also reflected in the brightness of the phantom images obtained with these nanoparticles as shown in [Fig. 5](#).

3.5. Cell labeling

The Fe_3O_4 nanoparticles, reported in this study, show promising MR signal enhancement as contrast agents, particularly those based on Fe_3O_4 -L. Cell labeling studies with superparamagnetic iron oxide particles (SPIO) has emerged as a powerful tool to monitor trafficking of transplanted cells by magnetic resonance imaging. Such studies are valuable for tracking cells used in regenerative medicine *in vivo*. Generally, intracellular labeling is carried out with the use of a transfecting agent such as Lipofectamine, Super-Fect, or polylysine (PLL) [69]. These transfecting agents are not approved for clinical use as they cannot be used in humans without the threat of potentially dangerous side effects. As such, it is important to develop the surface chemistry that will facilitate effective endocytosis into the cells without the use of transfection agents. Herein, we evaluate how well our nanoparticles can be endocytosed by adipose-derived stem cells in the presence as well as in absence of the transfecting agent, PLL and elucidate how the presence of a positively charged dilutant ligand affects the nanoparticle uptake. A similar study was also performed with the commercially available SPIO (Feridex) for comparison. Cell viability experiments were conducted on the rat adipose-derived stem cells (ASC). The labeled cells were assessed using prussian blue staining technique for the detection of intracellular iron. This investigation included Fe_3O_4 -L, Fe_3O_4 -L- Cu^{2+} , Fe_3O_4 -L(+) and Fe_3O_4 -L(+)- Cu^{2+} and Feridex over the dosages ranges of 20–80 $\mu\text{g mL}^{-1}$ of the nanoparticles, with the 0 $\mu\text{g mL}^{-1}$ dosage being the control.

Surface modification of the nanoparticles is a general strategy utilized to enhance the cellular uptake of the nanoparticles. The differential behavior concerning the uptake into

the stem cells between the two surface modified nanoparticles and Feridex was established. The cell labeling efficiencies were evaluated by following the standard method [70], [71] and [72]. A strong Prussian blue staining was observed when the cells were incubated with the Feridex plus transfecting agent. When Feridex was used for the cell labeling in the absence of transfecting agent, there was very little nanoparticle uptake as evidenced by the lack of blue staining within the cells, indicating little intracellular uptake. The presence of many blue particles in the cells can be observed when labeling was carried out with the $\text{Fe}_3\text{O}_4\text{-L}$ and $\text{Fe}_3\text{O}_4\text{-L}(+)$ both in the presence as well as in the absence of the transfecting agent (Fig. 6). Thus the efficiency of the surface modified nanoparticles for the cell labeling is higher compared to Feridex in absence of the transfecting agent.

Fig. 6. Stem cells were incubated with nanoparticles with and without PLL as transfecting agent and cellular labeling were visualized histochemically by the Prussian blue reaction.

Feridex being a dextran modified SPIO does not bear any charge on its surface, while $\text{Fe}_3\text{O}_4\text{-L}$ and $\text{Fe}_3\text{O}_4\text{-L}(+)$ bear carboxylic acid groups and carboxylic acid groups in combination with positively charged quaternary ammonium chloride groups, respectively. Presumably the presence of the ionic surface on the surface modified nanoparticles render them a better cell labeling agents compared to Feridex in absence of the transfecting agents. The presence of PLL as the transfecting agent does not exhibit any beneficial effect on the cell labeling efficiency of either $\text{Fe}_3\text{O}_4\text{-L}$ or $\text{Fe}_3\text{O}_4\text{-L}(+)$. This observation may be attributed to the fact that under the physiological conditions (pH 7.4), the carboxylic acid group mostly remain in the deprotonated form, *i.e.*, negatively charged and as reported earlier the negatively charged surface of the nanoparticles facilitate the phagocytosis of the nanoparticles and hence the cell labeling [72]. When Cu^{2+} ions are coordinated to the DOTA on the surface of the nanoparticles, the carboxylate groups of DOTA moiety would no longer remain free resulting in a significant change in surface charges. Upon Cu^{2+} binding, the surface charges on the $\text{Fe}_3\text{O}_4\text{-L}$ and $\text{Fe}_3\text{O}_4\text{-L}(+)$ nanoparticles become less negative and essentially positive, respectively. The result of changes in surface charge affects the cellular uptakes of $\text{Fe}_3\text{O}_4\text{-L-Cu}$, $\text{Fe}_3\text{O}_4\text{-L}(+)\text{-Cu}$ by the stem cell. $\text{Fe}_3\text{O}_4\text{-L-Cu}$ has less cell labeling compared

to $\text{Fe}_3\text{O}_4\text{-L}(+)\text{-Cu}$ in absence of transfecting agent. The decreased number of free carboxylic groups and the absence of positively charged quaternary ammonium chloride groups on the surface of $\text{Fe}_3\text{O}_4\text{-I-Cu}$, lower its cell labeling efficiency. The presence of Cu^{2+} on the surface of $\text{Fe}_3\text{O}_4\text{-I-Cu}$ nanoparticles helps the endocytosis of the nanoparticles in the ASC cells to take place however, the extent of cell labeling is definitely lowered compared to the same for $\text{Fe}_3\text{O}_4\text{-L}$. The reactive carboxylic acid groups ($-\text{COOH}$) present on the nanoparticle surface are expected to provide the opportunity for further functionalization of the surface. It is also expected that a variety of other bio-molecules may be immobilized onto the nanoparticle surface to enhance specific cell recognition for use in targeting studies.

The contrast agents showed an efficient cellular uptake and high cell labeling efficiency for the ACS stem cells. All the surface modified nanoparticles both under Cu^{2+} bound and Cu^{2+} unbound state are suitable agents for labeling at a low concentration without using a transfecting agent. In contrast, Feridex is not so effective for cell labeling in the absence of a transfecting agent. This offers the opportunity to omit transfecting agents from the labeling protocol for the surface modified nanoparticles [73].

4. Conclusions

In summary, the hydrophilic and biocompatible multimodal probes, consisted of a monocrystalline superparamagnetic iron oxide (SPIO) or manganese oxide core and stable in water or physiological environment, have been developed. Surface modifications and subsequent DOTA conjugation enable the fine-tuning of the surface charge of these nanoparticles. The relaxivity studies indicate a relaxivity enhancement upon Cu^{2+} binding. These properties enable the nanoprobe as potent for dual PET/MRI, intracellular labeling without transfecting agent and *in vivo* MR/PET tracking. We are currently investigating the targeting abilities of these nanoprobe, the scope of these nanoparticles as T_2 CEST agents for dual PET/MRI, application to disease detection and stem cell treatment.

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Appendix.

Figures with essential color discrimination. Fig. 1 and Fig. 6 in this article is difficult to interpret in black and white. The full color image can be found in the online version, at [doi:10.1016/j.biomaterials.2010.10.013](https://doi.org/10.1016/j.biomaterials.2010.10.013).

Appendix. Supplementary material

Supplementary data related to this article can be found online at [doi:10.1016/j.biomaterials.2010.10.013](https://doi.org/10.1016/j.biomaterials.2010.10.013).

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