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## Preparation, Characterization and Tests of Incorporation in Stem Cells of Superparamagnetic Iron Oxide

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**Abstract.** Superparamagnetic iron oxide nanoparticles (SPIONs) have been produced and used as contrast-enhancing agents in magnetic resonance imaging (MRI) for diagnostic use in a wide range of maladies including cardiovascular, neurological disorders, and cancer. The reasons why these SPIONs are attractive for medical purposes are based on their important and unique features. The large surface area of the nanoparticles and their manipulation through an external magnetic field are features that allow their use for carrying a large number of molecules such as biomolecules or drugs. In this scenario, the present work reports on the synthesis and characterization of SPIONs and *in vitro* MRI experiments to increase their capacity as probes for MRI applications on stem cells therapy. Initially, the SPIONs were prepared through the co-precipitation method using ferrous and ferric chlorides in acidic solution. The SPIONs were coated with two thiol molecules such as mercaptosuccinic acid (MSA) and cysteine (Cys) (molar ratio SPIONs:ligand = 1:20), leading to the formation of a stable aqueous dispersion of thiolated nanoparticles (SH-SPIONs). The SH-SPIONs were characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), transmission electron microscopy (TEM), and vibrating sample magnetometry (VSM). The results showed that the SH-SPIONs have a mean diameter of 14 nm and display superparamagnetic behavior at room temperature. Preliminary tests of incorporation of SH-SPIONs were evaluated stem cells. The results showed that the thiolated nanoparticles have no toxic effects for stem cells and successfully internalized and enhance the contrast in MRI.



## 1. Introduction

Nanostructured materials have been proposed for an increasing number of biomedical applications. Among these materials, superparamagnetic iron oxides nanoparticles (SPIONs), in particular magnetite ( $\text{Fe}_3\text{O}_4$ ), have attracted the attention of the scientific community [1-2]. SPIONs have been effectively applied in several biomedical applications, such as in diagnosis [3], drug delivery [4-5] and hyperthermia [6]. These nanoparticles are interesting because present two fundamentals characteristics. The first feature is the presence of magnetic monodomains, i.e. these nanoparticles show superparamagnetic behavior at room temperature and act as a single magnetic domain. Individual nanoparticle has a large constant magnetic moment and behave as a giant paramagnetic atom, rapidly responding to applied magnetic fields with negligible residual magnetism and coercivity [7], therefore superparamagnetism is a term that describes an analogy of the small magnetic moment of the paramagnetic atom with large magnetic moment of the nanoparticle. Another peculiarity is the large surface area on which it is possible to functionalize molecules of interest, such as biomolecules. Furthermore these system present low toxicity, they can be guided *in vivo* to specific target by an external magnetic field. SPIONs are of comparable size to important biological systems; display low sedimentation rates and improved thorough tissues [4]. The majority of studies *in vitro* explores physicochemical characteristics of the nanoscale particles, such as shape, size, and surface coating. However, studies have shown that the surface of the nanoscale particles changes after of the interaction with the surrounding environment [8-9].

Coprecipitation of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  salts in alkaline conditions has been used for the synthesis of magnetite nanoparticles ( $\text{Fe}_3\text{O}_4$ ) [10]. The coating of these systems is of great importance with regard to cell incorporation [11-12]. Usually, coated magnetic nanoparticles have been found to be nontoxic, whereas the simple particles present some toxic effects, such as oxidative stress [13].

The effects of SPIONs coated with biocompatible ligands containing functional groups such as COOH and SH as well as sizes of these nanoparticles are critical determinants in toxicological studies [14].

Cell labeling with SPIO substances is an increasingly common method for *in vivo* cell separation as well as detection by magnetic resonance image (MRI) [15]. Considering interest in biomedical applications of SPIONs, the present paper reports the synthesis and characterization of water soluble magnetic iron oxide nanoparticles and first *in vitro* MRI experiments. The other aim of this work is to optimize SPIONs analyses for MRI applications on stem cells therapy. SPIONs were prepared through the co-precipitation of iron salts, followed by titration with ammonium hydroxide and the suspension formed was separated magnetically. In order to functionalize their surfaces with biocompatible molecules, the SPIONs were coated with two molecules with two hydrophilic molecules containing thiol groups: mercaptosuccinic acid (MSA) and cysteine (Cys) in a molar ratio iron: ligand 1:20 giving stable aqueous dispersion of thiolated nanoparticles (SH-SPIONs). SH-SPIONs were characterized by X-ray diffraction (XRD), quantification of thiols group on the surface of particle by titration with DTNB and transmission electron microscopy (TEM). The magnetization analysis was carried out by isothermal magnetic measurements in applied field. Preliminary studies of incorporation into stem cells and MRI were also accomplished.

## 2. Methods

### 2.1. Synthesis of $Fe_3O_4$ nanoparticles

SPIONs were synthesized by using a co-precipitation method, as previously reported [10]. In brief, 4.0 mL of  $FeCl_3 \cdot 6H_2O$  and 1.0 mL of  $FeCl_2 \cdot 4H_2O$  (molar ratio 2:1), prepared in 1.0 mol/L HCl, were mixed and stirred, while a volume of 50 mL of  $NH_4OH$  (0.7 mol/L) was added as precipitator. At this stage, the black suspension formed was decanted magnetically followed by the addition of thiolated (MSA and Cys) ligands in molar ratio  $Fe_3O_4$ : ligand 1:20. This mixture was then stirred for 1 hour. The dispersion was centrifuged and the new precipitate was washed several times with ethanol leading to a SPIONs covered with MSA and Cys.

### 2.2. Cell preparation and SPIONs up-take

Stem cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (complete medium). Cells were plated in 6 well plates and treatment was performed at 80–90% confluence in serum free medium with 50  $\mu g/ml$  of SH-SPIONs in water. A negative control without treatment was carried out. After 1, 3, 6 and 20 h, cells were harvested by scrapping them from the plate for optic microscopy staining and magnetic resonance.

### 2.3. Determination of the amount of thiol groups on the surface of SPIONs

The quantity of thiol groups ( $-SH$ ) on the surface of the nanoparticles was measured by the DTNB reaction, based on the absorbance at 412 nm ( $\epsilon = 14,150 \text{ molL}^{-1}\text{cm}^{-1}$ ) of the 2-nitro-5-thiobenzoate anion ( $TNB^{2-}$ ) generated in the reaction of  $-SH$  groups with DTNB [16]. SH-SPIONs were added to 3.0 mL of DTNB (0.01  $\text{molL}^{-1}$ ) in PBS buffer (pH 7.4) containing 1  $\text{mmolL}^{-1}$  of ethylenediaminetetraacetic acid. After 5 min of incubation, the suspensions were filtered by centrifugal ultrafiltration using a Microcon centrifugal filter device containing ultrafiltration membranes (MWCO 10-kDa molar mass cut-off filter, Millipore, Billerica, MA, USA). The supernatant was placed into a quartz cuvette, and the intensity of the absorption band at 412 nm was measured in an Uv-Vis Spectrophotometer (Agilent, model 8553, Palo Alto, CA, USA). The experiments were carried out in triplicates.

### 2.4. X-ray diffraction (XRD)

The diffractograms were obtained with approximately 200 mg of powdered MSA- $Fe_3O_4$  or Cys- $Fe_3O_4$  onto a glass substrate of 2×2 cm. The measurements were performed in reflection set-up; with a conventional X-ray generator (CuK $\alpha$  radiation of 1.5418 Å and a graphite monochromator) coupled to a scintillation detector. The angular scanning performed on all samples ranged from 20 to 80° with 0.05° step-width at 5 s per angle.

### 2.5. Transmission Electron Microscopy (TEM)

Photomicrographies of the nanoparticles were obtained using a Philips CM200 transmission electron microscope (TEM) with an energy dispersive spectrometer, operating at 160 kV. The samples for observation were suspended in water, and then a drop of the supernatant dispersion was placed on an amorphous carbon film supported by a copper grid.

### 2.6. Vibrating Sample Magnetometry (VSM)

The magnetic curves of the SH-SPIONs were calculated at room temperature, and the apparatus was calibrated to a Ni pattern. The magnetization measurements were carried out on a known quantity of powdered sample, which slightly pressed and conditioned in cylindrical Lucite holders.

### 2.7. Magnetic Resonance Image (MRI)

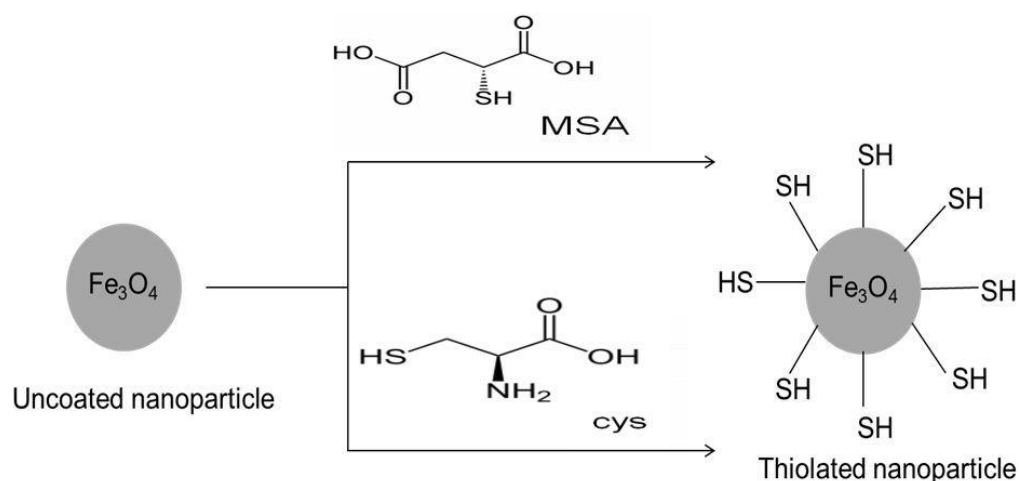
The MRI scan of labeled cells was performed using a 2T scanner (Elscent, Prestige, Haifa, Israel) at FCM, UNICAMP. It was used as a head coil and acquired two different pulse sequences. The parameters of gradient recall-echo T2 was: TR=500 ms, TE=10 ms, flip angle=18, 2 number of excitations, matrix=256×190; and the fast spin-echo T2 was: TR=4800 ms, TE=128 ms, flip angle=170, 1 number of excitation, matrix=256×256. Both sequences had field of view adjusted of the size of plaque and the thickness of slice was 3 mm. MRI experiments were carried out adding nanoparticles dispersed in agarose and also cells before and after nanoparticles incorporation.

## 3. Results and Discussion

The mixture of Fe<sup>2+</sup> and Fe<sup>3+</sup> salts in acidified aqueous solution led to the formation of Fe<sub>3</sub>O<sub>4</sub>, according to equation 1



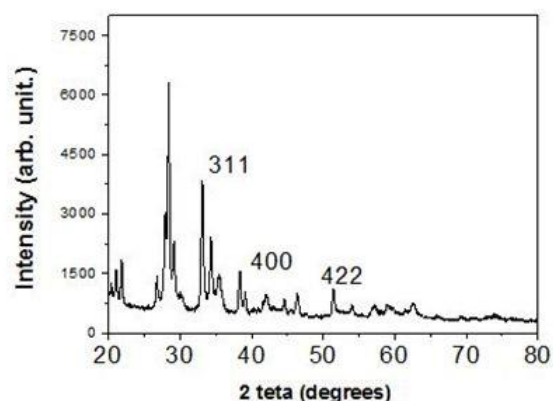
Fig. 1 shows the schematic representation of the formation of thiolated Fe<sub>3</sub>O<sub>4</sub> nanoparticles coated with mercaptosuccinic acid (MSA) or cysteine (Cys). It had been used an excess of thiolated ligands to total covering of the Fe<sub>3</sub>O<sub>4</sub> surface.



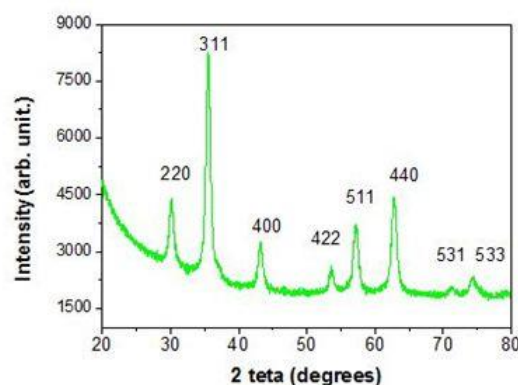
**Figure 1.** Schematic representation of the formation of thiolated Fe<sub>3</sub>O<sub>4</sub> nanoparticles coated with mercaptosuccinic acid (MSA) or cysteine (Cys).

The amounts of free thiol (-SH) groups on the surface of SH-SPIONs were determined by the reaction of the thiolated nanoparticles with a thiol-specific reagent, DTNB [16]. Values of 28 ± 8 μmol SH/g of MSA-SPIONs and 12 ± 8 μmol SH/g of Cys-SPIONs were found.

Fig. 2 shows the X-ray diffractograms from two synthesized samples: SPIONs coated with MSA and coated with Cys, respectively, along with their characterization as the inverted spinel structure [17]. The diffraction measurements demonstrated that the crystallographic structure of SH-SPIONs corresponds to magnetite ( $\text{Fe}_3\text{O}_4$ ) (JCPDS 20–596). Crystallite sizes from 12 to 14 nm were obtained from Scherrer's equation [18] by taking into account the (311) Bragg's reflection.



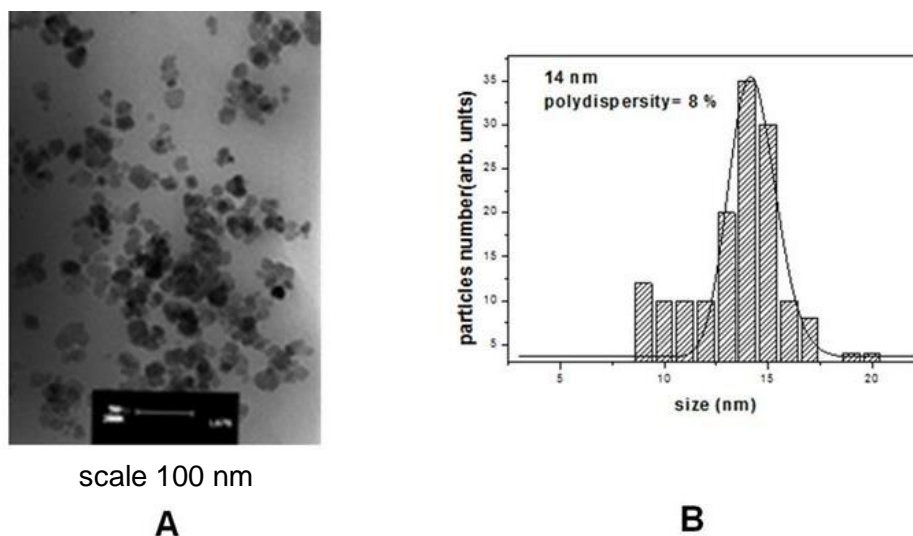
■ XRD: X-ray powder diffraction from a representative sample of a thiolated  $\text{Fe}_3\text{O}_4$  nanoparticle coated with cysteine, displaying the Bragg peak reflections of magnetite.



■ XRD: X-ray powder diffraction from a representative sample of a thiolated  $\text{Fe}_3\text{O}_4$  nanoparticle coated with MSA, displaying the Bragg peak reflections of magnetite.

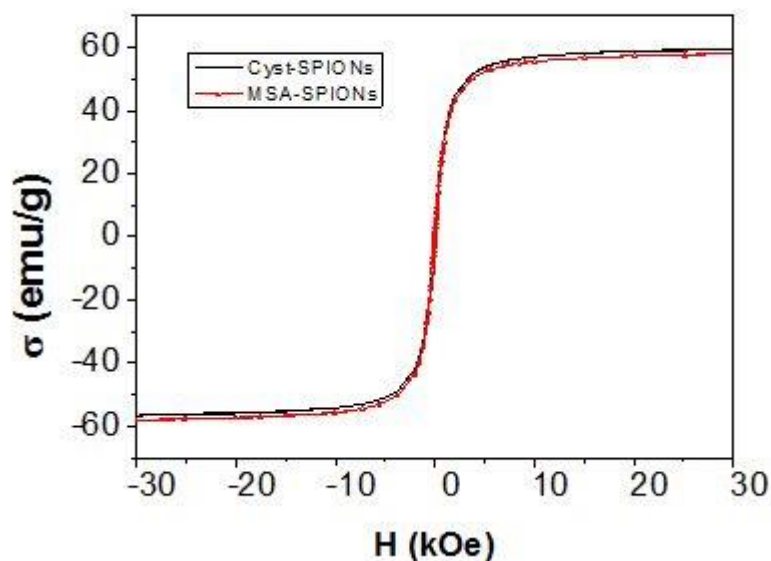
**Figure 2.** X-ray powder diffractograms from a representative sample of SH-SPIONs coated with MSA and Cys, displaying the Bragg peak reflections of magnetite.

The morphologies and sizes distribution of the SH-SPIONs were analysed by transmission electron microscopy (TEM). Fig. 3(A) shows a representative image from sample Cys-SPIONs. It can be observed that the nanoparticles exhibit spherical continuous, however they present aggregation. It was also to observe in some particles a dark-core light-shell type of contrast. Such a kind of contrast may be associated to differences in the electronic density between iron core and the coating suggesting that the nanoparticles are formed by a Fe-oxide shell with an inner oxygen-poor core. This core-shell morphology is in agreement with the formation of  $\text{Fe}_3\text{O}_4$  [19]. The histogram of the size distribution showed in Fig. 3(B) revealed a narrow distribution with a mean diameter of 14 nm and polydispersity of 8%.



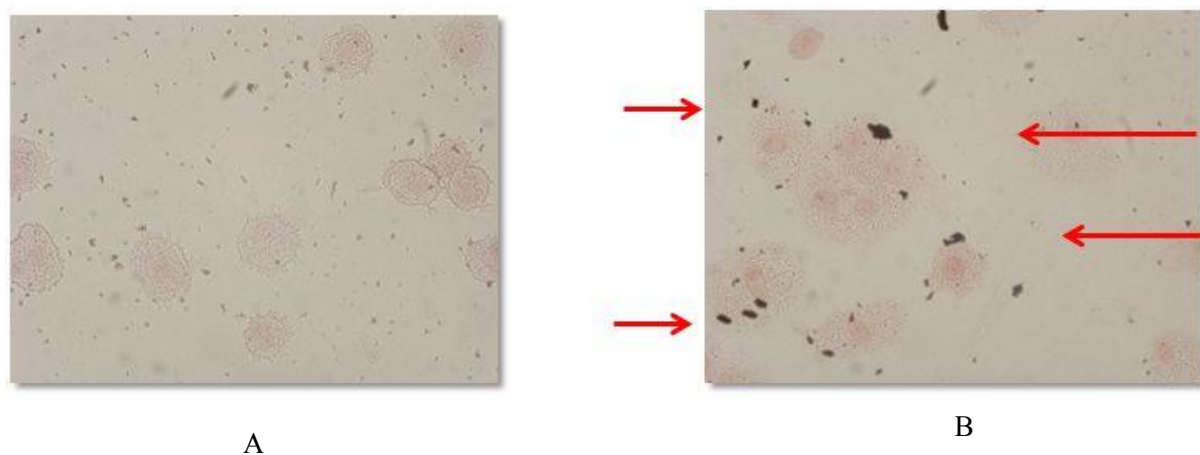
**Figure 3.** (A) Transmission Electron Microscopy (TEM) of Cyst-SPIONs; (B) sizes distribution of Cys-SPIONs.

Finally, magnetic characterizations were also carried out to observe the magnetic behavior of samples. All SH-SPIONs exhibited the typical superparamagnetic behaviour (Fig. 4) expected for magnetic nanoparticles in this size range. The coercive field is approximately 0 Oe. Values of saturation magnetization around 60 emu/g were found, compatible with that found for Fe<sub>3</sub>O<sub>4</sub> magnetite.



**Figure 4.** Magnetic curves of samples Cys-SPIONs (black) and MSA-SPIONs (red)

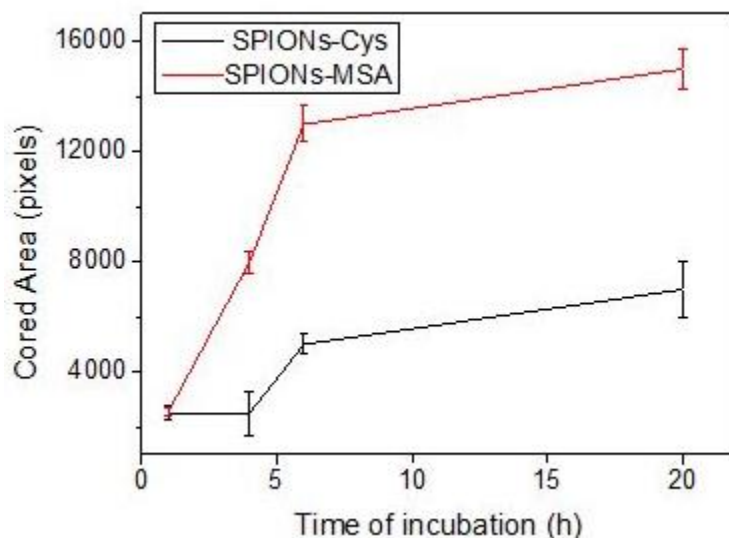
Preliminary tests *in vivo* of incorporation were performed. Fig. 5 shows the incorporation of Cyst-SPIONs sample (0.05 mg/mL) for 3 hours in stem cells. Fig 5 (A) represents control cells and the arrows (Fig. 5 B) show the incorporation of aggregated Cys-SPIONs in cells. Results not shown showed that thiolated nanoparticles have no toxic effects for cells [20].



**Figure 5.** Incorporation of Cys-SPIONs in stem cells for 3 hours. (A) control cells; (B) Cyst-SPIONs (0.05 mg/mL) incorporated in stem cells.

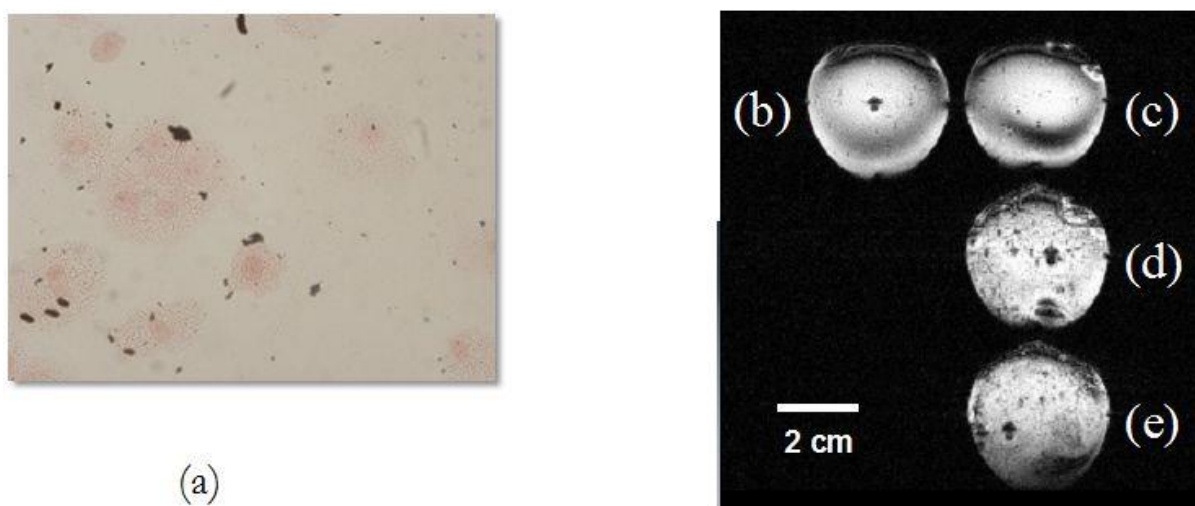
Figure 6 shows the incorporation curves of SH-SPIONs (0.05mg/mL) up to 20 hours. The curves were obtained by Prussian blue technique, where the Fe (III) reacts with a composition of 2% HCl acid mixed and 2%  $K[Fe(CN)_6]$  producing  $Fe_4[Fe(CN)_6]_3$ , the so called Prussian blue, which is an insoluble blue reaction product deposited inside the cell at the location of the Fe (III) ions. The blue stained area/cell (in pixels) was determined by thresholding as an estimate of the number of nanoparticles phagocytized. It can be seen that the major nanoparticle up-take by the cells occurs in the first 3 hours of incubation, for both samples. It can be seen that the SPIONs containing MSA has a larger incorporation area. This fact suggests an influence of amine group of cysteine molecule in phagocytosis mechanism. Further testing will be performed to elucidate the mechanism of internalization of the particles.





**Figure 6.** Incorporation curves of SH-SPIONs (0.05 mg/mL) in stem cells up to 20 hours: MSA-SPIONs (red curve); Cys-SPIONs (black curve)

Initial essay by magnetic resonance image (MRI) of stem cells containing nanoparticles with MSA incorporated for 3 hours (Fig. 7 a) revealed an increase of negative contrast (Fig. 7 d and e). The control wells in agarose (Fig. 7 b and c) showed no similar pattern of signal loss or reduction. Adjustments in T2-weighted pulse sequence and combination with post-processing imaging techniques (texture analysis) can further improve detection of labelled cells with these particles using clinical MR scanners.



**Figure 7.** (a) Incorporation curves of MSA-SPIONs (0.05 mg/mL) in stem cells for 3 hours; (b) and (c) agarose; MRI tests of MSA-SPIONs (0.05 mg/mL) in stem cells for 3 hours (d) and (e).

#### 4. Conclusions

This work describes the preparation and characterization of SH-SPIONs and their impact as contrast agents. These systems investigated were crystalline and presented low polydispersity with superparamagnetic behavior. The advantages of this new approach are the low toxicity of SH-SPIONs in stem cells and the increase of the contrast in MRI. SH-SPIONs act as diagnostic are extremely attractive for diverse biomedical applications.

#### 5. Acknowledgements

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