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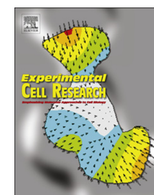

Research Article

Potential stem cell labeling ability of poly-L-lysine complexed to ultrasmall iron oxide contrast agent: An optimization and relaxometry study

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ABSTRACT

For non-invasive stem cells tracking through MRI, it is important to understand the efficiency of *in vitro* labeling of stem cells with iron oxide with regard to its relaxation behavior. In this study, we have carried out a pilot study of labeling mice mesenchymal stem cells (mMSCs) with ultrasmall superparamagnetic iron oxide (USPIO) entrapped with poly-L-lysine (PLL) in different ratios and incubated with different times. Our results demonstrated that 50:1.5 μg/ml of iron oxide and PLL at an incubation time of 6 h with 10% serum concentration are sufficient enough for effective labeling. Optimized labeling showed that >98% of viability and <3% toxicity were observed at a total iron content of 11.8 pg/cell. *In vitro* relaxometry study showed that almost a 6.6 fold reduction in transverse relaxation time (T_2) was observed after labeling as compared to unlabeled. IO-PLL complex was more effective than iron oxide alone in labeling and a detectable lower limit found to be hundred with optimized concentration. Significant increase in Oct-4 expression on day-3 after labeling was observed, whereas CD146 expression remains unchanged in real time RT-PCR. This optimized labeling method of MSCs may be very useful for cellular MRI and stem cells tracking studies.

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1. Introduction

Stem cells transplantation as a therapeutic approach has been emerged as a promising alternative to conventional treatments for a number of diseases. Mesenchymal stem cells (MSCs) are multipotent cells and they have the potential to produce variety of cell lineages [1]. The continuous and noninvasive monitoring of stem cell fate *in vivo* is a necessary step that facilitates post-transplantation. Magnetic resonance imaging (MRI) is an effective tool for imaging and *in vivo* tracking and homing of labeled stem cells used in infusion [2]. It is noninvasive, generates high-resolution 3D images, used for longitudinal studies and does not rely on the use of radioactive isotopes. Cellular MRI along with the ability of MRI contrasts agents providing dynamic assessment of cell migration into target tissues [3].

Superparamagnetic iron oxide (SPIO) nanoparticles with different surface modification were frequently used for labeling several cell types, like macrophages, cancer cells, dendritic cells and stem cells [4,5]. Ferumoxides/Feridex is a dextran coated SPIO (120–180 nm) and also FDA approved contrast agent, which already has been used for imaging of hepatic kupffer cells and reticuloendothelial cell. Senerem/Resovist is a carboxy-dextran coated SPIO (60 nm), used for clinical imaging. Monocrystalline iron oxide nanoparticles (MION) have also been used for lymph node imaging and are in phase-III clinical trials for blood pool agent [4,6,7]. The use of transfection agents like poly-L-lysine (PLL), protamine sulfate (PS), superfect and lipofectamine has shown to enhance cell labeling [8–10]. Mechanistically, PLL forms a complex with iron oxides through electrostatic interactions and effectively incorporated into the cells. Similarly the FDA approved Protamine sulfate, being low molecular weight polycationic peptide and forms Fe-Pro complex with Ferumoxides in serum free media and has been shown to transfect cells effectively.

A numbers of reports on stem cell labeling have been published

Abbreviations: ANOVA, Analysis of variance; DLS, Dynamic Light Scattering; FBS, Fetal Bovine Serum; FOV, Field of view; ISA, Imaging sequence analysis; IO-PLL, Iron oxide-Poly-L-lysine; MGE, Multi Gradient Echo; MRI, Magnetic resonance imaging; MSCs, Mesenchymal Stem Cells; mMSCs, mice MSCs; mRNA, Messenger RNA; MSME, Multi Slice Multi Echo; PCR, Polymerase chain reaction; PDI, Ploydispersity Index; PLL, Poly-L-lysine; ROI, Region of interest; RT-PCR, Real Time PCR; R_2, Relaxation rate; r_2, Relaxivity; SPIO, Superparamagnetic iron oxide; TEM, Transmission Electron Microscope; T_2, Relaxation time; TR, Repetition time; TE, Echo time; USPIO, Ultrasmall superparamagnetic iron oxide

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in previous years which suggest the optimum concentration of iron oxide to transfection agent to be used at optimun incubation time period. According to Jasmin et al., the efficient uptake of Feridex (SPIO) can be achieved using 25 μg/ml Fe and 0.75 μg/ml PLL at an incubation of 4 h [8]. Whereas, Hu et al. have compared four different concentration of Feridex (5.6, 11.2, 22.4 and 44.8 μg/ml) and showed that 44.8 μg/ml was toxic for hMSCs [11]. For the same context, Babic et al. have employed PLL modified iron oxide nanoparticles for successful labeling of MSCs [12]. Again, Kim et al. have compared the labeling efficiencies of three different SPIO contrast agents (Feridex, Resovist and MION) in hMSCs and optimized that 12.5 μg/ml iron oxide and 0.75 μg/ml PLL were efficient for stem cells labeling in terms of viability and toxicity [13]. Other reports have also demonstrated the optimization of stem cells labeling based on different IO-PLL ratio and viability-toxicity assay [14–16]. The relaxation properties being a dependable property for stem cells labeling has not been studied and reported extensively. To the best of our knowledge so far no one has optimized the labeling method of IO-PLL concentration in terms of relaxometry measurements and its effect on biological properties of MSCs.

The purpose of the study in particular was to determine the effective labeling method when using different ratio of USPIO to PLL with different incubation times. The serum proteins since form a large complex with iron oxide when present in cell culture pose a challenge and it reduce stem cell labeling efficiency. Hence, knowing the physical and biological properties of mesenchymal stem cells after labeling with iron oxides seems to be necessary. Therefore, we made an effort to evaluate the labeling efficiency by measuring proliferation, cytotoxicity and iron content in mesenchymal stem cells. The labeling was validated by phantom based relaxometry study of unlabeled and labeled MSCs and also using different cell numbers to determine the minimal detectable quantity of cells by 7T MRI. The combine effect of IO-PLL after cells labeling was observed on the expression of biological variables like differentiation and expression of stem cell markers.

2. Materials and methods

2.1. Synthesis of contrast agent

The magnetic USPIO nanoparticles were synthesized by modifying alkaline co-precipitation method reported earlier [17, 18]. Briefly, Fe^{2+} and Fe^{3+} salts of chlorides (1:1.75) were mixed in 2 M HCl and precipitated with 0.5 M NH_4OH under continuous N_2 protection. The resulting nanoparticles were oxidized with sodium hypochlorite, followed by surface adsorption with dextran (10 KDa). Subsequently formed Dextran-Fe_3O_4 was purified by dialysis and ultrafiltration. The relaxation time (T_2/T_2^*) of nanoparticles was measured in phantom study by using 7 T Bruker Biospec magnetic resonance imaging (MRI) system. Phantom was prepared in microcentrifuge tube containing deionised H_2O to generate a localizable suspension containing different concentrations of magnetic nanoparticles at each spots.

2.2. Isolation and culture of mMSCs

The study was commenced after obtaining approval from the Institute ethics committee (IAEA, INMAS, Delhi) and every procedure was done as per the manual of use and care of laboratory animals for experimental use. Bone marrow MSCs were isolated from the tibias and femurs of Balb/C mice (6–8 weeks) after cervical dislocation as reported earlier [19,20]. The epiphyses were removed and the bones were individually flushed with low glucose Dulbecco's modified Eagle's medium (DMEM-LG; Sigma-aldrich, St. Louis, MO) inserting 1 ml (26 Gz) insulin syringe. The media containing cells was filtered in 70 μm nylon cell strainer (BD falcon, USA) to separate cell debris. The media was centrifuged at 300*g* for 6 min and the pellets suspended in media. Subsequently, cells were plated in 60 mm cell culture plastic Petridishes at a density of 25×10^6 cells in DMEM-LG media, supplemented with 15% fetal bovine serum (FBS; Gibco, USA), 2 mM/L-glutamine (Sigma-aldrich, St. Louis, MO) and 1% Streptomycin/Penicillin/amphoterecin (Sigma-Aldrich, St. Louis, MO). Culture was maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. The isolation of MSCs was carried out on the basis of the frequent medium change in primary culture and diminishing the trypsinization time [21]. Medium was changed twice during initial 72 h to remove non-adherent RBCs and macrophages, thereafter once every 3–4 days. When primary cultures became nearly confluent (70–80%), passaging was carried out by treated with 1 ml of 0.025% Trypsin containing 0.02% EDTA for 2 min at room temperature. All furthers experiments were performed after the third passage of cells.

2.3. Protein adsorption assay

The concentration of serum added while labeling of stem cells labeling was determined by protein adsorption assay [12]. In this experiment, 500 μl of iron oxide (100 μg/ml) colloid solution with PLL (3 μg/ml) was mixed in 500 μl of DMEM containing different concentration of FBS to form a final concentration of 50 μg/ml iron oxide, 1.5 μg/ml PLL and different serum concentrations (0%, 2%, 5% and 10%). The mixture was mixed properly by vortexing for 1 h and the supernatant was separated from the pellet by centrifugation at 14,000 rpm for 1 h. The concentration of protein in supernatant was determined by UV-spectrophotometer at 280 nm. An aqueous solution of DMEM served as a control.

2.4. USPIO labeling method

mMSCs were grown in 24-well plate in MDEM supplemented with 15% FBS until the cells of growth reaches 80% confluence. The media was aspirated from the well and cells were washed in 1X PBS without disrupting the monolayer. The cells were labeled by incubating with different concentrations of USPIO (2.5 mg/ml stock) and PLL (0.01% stock) for different time periods (2, 4, 6 and 24 h). Different combination of iron oxide (0, 25, 50 and100 μg/ml) and PLL (0, 0.5, 1.0 and 1.5 μg/ml) were first mixed properly in separate tube and incubated for 15 min. The IO-PLL complex was formed due to electrostatic interaction. The complex after adding into the well mixed and incubated for 30 min. Subsequently, 50 μl of serum was added to each well to achieve a final concentration of 10% and incubated at different duration of time as shown at 37 °C and 5% CO_2. After the incubation, wells containing cells were washed three times with PBS and the cells present in the well were ready for experimental work.

2.5. Flow cytometric analysis

The cells were detached from culture dish with Trypsin/EDTA and counted. About 2×10^5 cells were resuspended in l ml PBS and pelleted by centrifugation for 5 minutes at 300 g. The cells in pallet were stained with Fluorescent isothiocyanate (FITC)-conjugated rat anti-mouse CD34, CD45, CD11b, Sca-1 and CD90.2 (Thy1.2) (BD Biosciences, San Diego, CA) at a dilution of 1:200 in PBS at 4 °C for 60 min. The cells stained with FITC-labeled rat anti-mouse IgG served as control. The cells were washed twice with PBS and fixed with 2% paraformaldehyde in PBS. Cells were examined by FACS cytometry (Becton Dickinson, San Jose, CA) and analyzed using cell quest software.

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