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Size, charge and concentration dependent uptake of iron oxide particles by non-phagocytic cells

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ABSTRACT

A promising new direction for contrast-enhanced magnetic resonance (MR) imaging involves tracking the migration and biodistribution of superparamagnetic iron oxide (SPIO)-labeled cells in vivo. Despite the large number of cell labeling studies that have been performed with SPIO particles of differing size and surface charge, it remains unclear which SPIO configuration provides optimal contrast in non-phagocytic cells. This is largely because contradictory findings have stemmed from the variability and imprecise control over surface charge, the general need and complexity of transfection and/or targeting agents, and the limited number of particle configurations examined in any given study. In the present study, we systematically evaluated the cellular uptake of SPIO in non-phagocytic T cells over a continuum of particle sizes ranging from 33 nm to nearly 1.5 μm, with precisely controlled surface properties, and without the need for transfection agents. SPIO labeling of T cells was analyzed by flow cytometry and contrast enhancement was determined by relaxometry. SPIO uptake was dose-dependent and exhibited sigmoidal charge dependence, which was shown to saturate at different levels of functionalization. Efficient labeling of cells was observed for particles up to 300 nm, however, micron-sized particle uptake was limited. Our results show that an unconventional highly cationic particle configuration at 107 nm maximized MR contrast of T cells, outperforming the widely utilized USPIO (<50 nm).

1. Introduction

Continuing advancements in cell-based therapies have recently led to the emergence of cellular imaging as a strategy to track the migration and biodistribution of target cells in living organisms. Pre-clinical studies have already shown that cellular imaging can be used to evaluate stem cell distribution and homing in cell-based regenerative therapies [1,2]. Recently, cellular imaging has also allowed for improved assessment of functional efficacy and applicability of immunotherapeutic treatments in disease models for cancer [3–5] and AIDS [6].

In addition to evaluating cell-based therapies, cellular imaging also promises to provide a great deal of insight into diverse physio- and pathological phenomena. Interesting applications include the observation of monocyte recruitment to atherosclerotic lesions for the mapping of disease development and therapeutic intervention [7], imaging embryonic stem cell movement during embryonic [8] and organ development [9] and monitoring the dynamics of metastatic cellular extravasation and tissue invasion [10,11].

Tracking of labeled cells has been accomplished with a variety of imaging modalities including optical methods, positron emission tomography (PET), single photon emission computed tomography (SPECT), and magnetic resonance (MR) imaging [12–14]. MR imaging presents a particularly promising approach because of its high spatial resolution in three dimensions and exquisite soft tissue contrast, which can be acquired concomitantly with the contrast-enhanced cellular distribution. MR detection of cells in vivo is often accomplished following labeling with superparamagnetic iron oxide (SPIO) particles. SPIO are negative contrast agents that are typically composed of an iron oxide crystal core surrounded by a polymer or polysaccharide shell [15]. A variety of manifestations of SPIO have been used to track cells, which can be broadly categorized as (1) ultrasmall SPIO (USPIO) with an overall diameter of 30–50 nm [16], (2) standard SPIO (SSPIO) with a diameter of 50–150 nm and (3) micron-sized paramagnetic iron oxide (MPIO) having a diameter approaching or greater than 1 μm [17].

To date, USPIO has perhaps been the most widely utilized SPIO configuration for cell labeling. Although they provide less contrast enhancement per particle compared with SSPIO and MPIO, large numbers of particles can be loaded into each cell [18,19]. As cationic surfaces have been shown to facilitate cellular internalization [20,21], USPIO is often modified with polycationic cell permeating peptides (CPPs) such as HIV transactivator (TAT) [22] or protamine [23]. Other transfection techniques, sometimes in concert with CPPs, are also used [24,25].

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An exciting new direction for cell tracking involves labeling cells with MPIO [26]. The large iron oxide cores present in these particles provide enough contrast for single cells to be imaged by MR. However, work with such large particles generally confines application of iron oxide labeling to phenotypes such as macrophages [18], dendritic cells [27] or hepatocytes that actively internalize foreign material. MPIO uptake in non-phagocytic cells has been accomplished, but is limited by the additional conjugation work and cost of using an antibody-mediated approach [28], which must be species specific and may induce adverse cellular events.

Recently, several studies have attempted to define an optimized particle configuration for iron oxide labeling of both phagocytic and non-phagocytic cell types. Although MPIO was excluded from all of these studies, it was found that phagocytic monocytes are more effectively labeled with SSPIO (150 nm) compared with USPIO (30 nm) [18,29]. Further, it was found that ionic carboxydextran-coated SSPIO (i.e. ferucarbotran) performed better than non-ionic dextran-coated SSPIO (i.e. ferumoxide) [18]. It remains unclear how MPIO compares with these agents; however, single cell detection has been achieved in phagocytic cells with both SPIO configurations [30,31].

The optimal SPIO configuration for labeling non-phagocytic cells has been much more elusive and findings have been contradictory. For example, in one study it was found that the delivery of carboxydextran USPIO and dextran-labeled SSPIO into non-phagocytic cancer cells and leukocytes (with the assistance of lipofection agents) was similar in terms of iron uptake [21]. Both particles led to higher iron uptake than USPIO. This indirectly suggests that larger particles with ionic coatings are superior to non-ionic USPIO. However, in a different study it was found that, in the presence of poly-L-lysine, ionic (aminated) USPIO exhibited significantly higher iron uptake in non-phagocytic cells compared with SSPIO. These data suggest that smaller ionic particles are internalized into non-phagocytic cells more efficiently [32]. These contradictory findings likely stem from the variability and imprecise control over surface charge and the limited number of particle configurations examined, particularly with respect to diameter (ranging only from ~17 nm to 150 nm).

In the present study we systematically evaluated the cellular uptake of SPIO in non-phagocytic T cells over a continuum of particle sizes ranging from 33 nm to nearly 1.5 μm and with precisely controlled surface properties. T cells were selected as a model non-phagocytic phenotype since visualization of their distribution is expected to be of importance for adoptive T cell therapy for cancer and T cell homing in autoimmune diseases. Extremely fine control was exerted on the surface properties of SPIO by direct chemical modification of particle surfaces rather than attempting to modulate the density of supplemental transfection agents. Concentration effects and incubation times were also tested in the interest of isolating the role particle size exerts on individual cell uptake and overall contrast enhancement. Our work shows that in a space between USPIO and MPIO exist configurations of relatively small particles (~100 nm) that efficiently label non-adherent, non-phagocytic T cells and generate higher relaxivity (per cell) relative to particles of other sizes.

2. Materials and methods

2.1. Nanoparticle synthesis

Three different formulations of dextran-coated superparamagnetic iron oxide nanoparticles were prepared using the co-precipitation method [33]. All three formulations were prepared following the same procedure, as described below, with the only difference being the amount of $FeCl_2$ and $FeCl_3$ added. Specifically, 25 g of dextran T10 (GE Healthcare, Piscataway, NJ) was dissolved in 50 mL of dH_2O and heated to 80 °C for 1 h. The solution was allowed to return to room temperature and continued to mix overnight. Subsequently, the dextran was cooled to 4 °C on ice and degassed with N_2 for 1 h. $FeCl_2$ (0.7313 g, 1.5 g, or 2.2 g) and $FeCl_3$ (1.97 g, 4 g, or 6 g, respectively) were each rapidly dissolved in 12.5 mL of degassed dH_2O and kept on ice for approximately 10 min. The iron solutions were added to the dextran simultaneously and allowed to mix for 30 min. Keeping this mixing solution at 4 °C, 15 mL of ammonium hydroxide was added. The resulting black viscous solution was then heated to 90 °C for 1 h then cooled overnight, followed by ultracentrifugation at 20 k rcf for 30 min. Pellets were discarded and the supernatant was continually diafiltrated using a 100-kDa MWCO cartridge (GE Healthcare) on a peristaltic pump (E323, Watson Marlow Bredel, Wilmington, MA). The particles were exchanged into 0.02 M citrate, 0.15 M sodium chloride buffer until all unreacted products had been removed. Aminated silica-coated iron oxide micro-particles were purchased from Bioclone Inc. (San Diego, CA). Amine functionalized styrene copolymer-coated iron oxide particles (Adembeads) were purchased from Ademtech SA (Pessac, France).

2.2. Amination of particles

Amination and crosslinking of the coating on the dextran–SPIO were accomplished through reaction of the SPIO with 25% 10 M NaOH and 33% epichlorohydrin [34]. After mixing for 24 h, additional ammonium hydroxide was added to the solution, bringing the volume fraction to 25% ammonium hydroxide, and the reaction was allowed to proceed for another 24 h. The particles were then exhaustively purified via diafiltration. The resulting particles were amine functionalized crosslinked iron oxide.

2.3. FITC labeling and amine-blocking of particles

All SPIO particles were labeled with FITC at a FITC-to-iron molar ratio of 19.2:1. FITC was reacted with particles for 4 h followed by two rounds of gel purification, once on a NAP-5 column and then on a PD10 column (GE Healthcare), both equilibrated with PBS. The FITC-labeled SPIO was subsequently reacted with various volumes of glycidol (0.01–50%) to produce populations of particles with different amine content. The particles were cleaned of excess glycidol through repeated precipitation in isopropanol and resuspension in PBS. Amine-blocking was also attempted with particles of 200 nm and greater, but this modification impelled immediate particle insolubility.

2.4. Measurement of particle size

The hydrodynamic diameter of the dextran-coated and commercial iron oxide particles was measured using a Zetasizer Nano-z (Malvern Instruments, Malvern, UK) through dynamic light scattering (DLS). The dextran-coated SPIO particles were diluted in PBS to a concentration of approximately 0.5 mg/mL and read in triplicate. The commercial particle diameters were read in the same manner, but only after undergoing three washes by precipitation in the presence of a strong magnet and resuspension in PBS. The values reported for all samples are the intensity peak values.

2.5. Measurement of particle cores

Transmission electron micrographs of all iron oxide particles were taken using a JEOL 2010 at 200 kV. Samples were prepared for imaging by evaporating the particles onto a carbon-coated copper grid (Holey carbon – mesh 200, Structure Probe Inc., West Chester, PA). Salt was removed from all of the samples prior to evaporation by exchanging the particles into dH_2O. Images of particle cores were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). Since many of the particles were found to be composed of a cluster of multiple iron oxide cores, the average diameter of each core and the average number of cores per particle were determined. Assuming each core to be spherical, the amount of iron per particle type was determined from the aggregate core volume.

2.6. Measurement of particle relaxivity (R_1 and R_2)

The longitudinal (R_1) and transverse (R_2) relaxivity of each particle was calculated as the slope of the curves $1/T_1$ and $1/T_2$ against iron concentration, respectively. T_1 and T_2 relaxation times were determined using a Bruker mq60 MR relaxometer operating at 1.41 T (60 MHz). T_1 measurements were performed by collecting 12 data points from 5.0 ms to 1000 ms with a total measurement duration of 1.49 min. T_2 measurements were made using $\tau = 1.5$ ms and two dummy echoes, and fitted assuming monoexponential decay.

2.7. Measurement of number of amines per particle

The number of amines per particle was determined following the general procedure described by Zhao et al. [35]. Briefly, iron oxide particles at a concentration of 2 mg/mL Fe were reacted with excess *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Calbiochem, San Diego, CA) for 4 h. SPIO was washed of excess SPDP through repeated precipitation in isopropanol and resuspension in PBS. The particles were then run through a 50-kDa MWCO centrifugal filter (YM-50, Millipore, Billerica, MA) either with or without the addition of disulfide cleavage agent TCEP. The difference of the absorbance of these two samples at 343 nm was used to determine the