REACTION: Release Activation of Iron Oxide Nanoparticles: A novel environmentally sensitive MRI paradigm

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INTRODUCTION: Magnetic cell labeling for the purpose of MRI based cell tracking has proved successful for over fifteen years, recently culminating in the detection of single cells in vivo in intact animals (1). A major focus moving forward is to use these non-invasive MRI methodologies to not only detect the position and movement of cells, but also gene expression. This will be particularly important for monitoring stem cell transplants and detecting their differentiation into appropriate cell types. MRI reporter gene strategies employing ferritin (2) or poly-lysine rich peptides (3) are promising, but currently achieve very small signal changes, hampering their use. Here we report on a new enzyme/contrast agent paradigm which to date has achieved enzymatically responsive changes in cellular relaxation times of ~ 35%. Cells are labeled with particles coated with a biopolymer which is cleavable by a specific enzyme. This coat restricts the approach of water to the particle, preventing the magnetic core from relaxing protons. The reactive enzyme potentially can be genetically engineered as a reporter protein, whose expression can be regulated either by environmental stimulus or during cellular differentiation. The enzyme cleaves the particle coating, revealing the magnetic center, increasing the relaxivity of the agent. We demonstrate the principal of enzyme mediated changes in nanoparticle relaxivity in cell free assays, *in cellulo*, and finally *in vivo* in animals.

MATERIALS AND METHODS: *Cell free system:* Dextranase is an enzyme which cleaves dextran at a pH of ~5-6, supplemented with Ca^{2+} as a cofactor. Feridex is a 150 nm dextran coated iron oxide nanoparticle. Here we use dextranase to cleave the coating from Feridex, releasing the magnetic core from the particle. Cleavage of Feridex nanoparticles (1mM iron) by 25nM dextranase was established in acetate and citrate buffers, pH 6.0 and 5.5 respectively, supplemented with Ca^{2+}. These buffers were chosen to approximate the chemical environment of low pH compartments in the cells, namely endosomes and lysosomes. Reaction (dextran cleavage) occurred within minutes as evidenced by precipitation of brown magnetic cores. Reaction mixtures were incubated for 3 days at 37°C on a turning wheel to achieve complete reaction. T_2 relaxation times were mapped at 4.0T for samples diluted 1:10 in 1% agar (0.1mM iron) using multi-echo spin echo experiments.
In cellulo: Feridex (1mM iron) was pre-incubated with poly-l-lysine (1nM) at 37°C for 1 hour and added to cells in suspension (STO fibroblasts or MCF7 cells, $1*10^6$ cells/ ml) for 2 hours of labeling, with occasional mixing (4). For enzymatic cleavage of the particles directly in cells, dextranase (25nM) was added to the labeling mixture. Samples were prepared immediately after labeling ($5*10^6$ cells/ ml, in 1% agar) and T_2 relaxation times were mapped. Confocal fluorescence microscopy of cells that were double labeled with a prepared green fluorescent Feridex and Texas Red labeled dextranase was performed to confirm double labeling.
In vivo: Two sets of MCF7 cells were prepared. One set was labeled only with Feridex, the other set labeled with both Feridex and dextranase as above. Following treatment with the anti-mitotic agent mitomycin C (10µg/ml), cells ($2.5*10^6$ cells, mixture of 80% unlabeled and 20% labeled cells) were injected into the hind limb muscles of mice (n=3). 3D T_2* gradient echo (TE 4 ms, TR 100 ms) and multi-slice T_2 weighted spin echo (TE 15 ms, TR 5 s) images of the mouse legs were obtained at 1 day and 4 days after injection.

RESULTS: *Cell free system:* The T_2 value of Feridex samples constituted at 0.1 mM iron in acetate and citrate buffer in agar was 115 ± 8 ms and 109 ±12 ms, respectively. Following incubation with dextranase, these T_2 values dropped to 67 ± 4 ms and 77 ± 18 ms. These changes following enzymatic cleavage of the Feridex were 48 ms and 34 ms or 42% (P=0.05) and 28%, respectively for acetate and citrate (P=0.17).
In cellulo (Figure 1): The T_2 value of cells alone was 153 ± 22 ms. T_2 dropped to 77 ± 9 ms after labeling with Feridex. When cells were co-labeled with Feridex and dextranase and incubated 2 hours, the T_2 further dropped to 50 ± 4 ms, a change in T_2 of 27 ms or 35%, significant with $p<0.05$. Confocal fluorescence microscopy indeed confirmed the presence of dextranase and Feridex in the same cells.
In vivo (Figure 2): MR images of hindlimb injections at 1 day and 4 days post injection demonstrate that the susceptibility effect generated from the labeled cells, that is detected in the gradient echo images, is enhanced in the side injected with cells co-labeled with Feridex and dextranase compared to the leg injected with Feridex labeled cells only. Spin echo images, however, revealed similar sizes of the two injected areas.

DISCUSSION: Increase in effective nanoparticle relaxivity by way of aggregation of nanoparticles to report on molecular events has been well demonstrated (5). Here we introduce a different approach to increasing nanoparticle relaxivity, potentially reporting on molecular events. We demonstrate *in vitro* and *in vivo* modulation in T_2 relaxation times of Feridex-labeled cells in the presence of dextranase, due to enzymatic digestion of the coat. Removal of the thick polymer coat reduces the distance of approach for water molecules to the magnetic core, thus allowing water to experience higher magnetic field inhomogeneities. This reveals itself as shorter T_2 relaxation times and larger susceptibility weighted signal voids in the in vivo gradient echo images. Indeed, further increase in the susceptibility induced signal void in the gradient echo images between days 1 and 4 reflects continuing cleavage of Feridex beads by the enzyme during this time. Changes in particle relaxivity requires colocalization of enzyme and particles in low pH environment, enabling optimal enzymatic activity. Fortunately, confocal microscopy revealed that upon endocytosis by cells, both magnetic particles and enzymes are shuttled in to low pH intracellular compartments, such as endosomes and lysosomes. These experiments form the backbone for new classes of potential in vivo MRI reporter genes/agents that can report on cellular changes at the molecular level, such as stem cell differentiation.

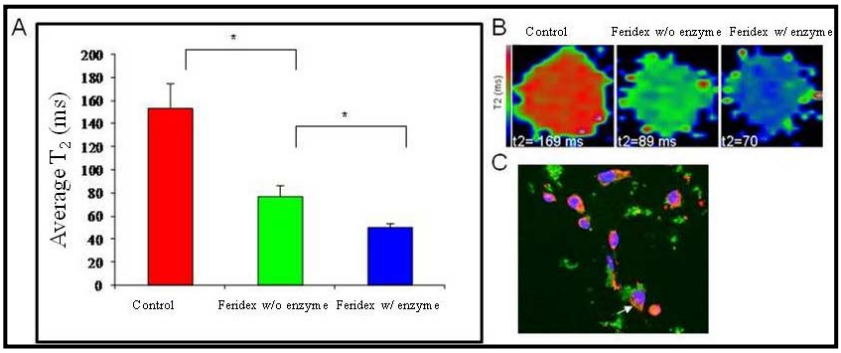

Figure 1: **In cellulo reduction of T_2 following enzymatic cleavage of Feridex coating.** A) Graphical presentation of T_2 values of cell samples. B) T_2 maps from one individual experiment at 24 hours incubation. C) Confocal fluorescence microscopy of cells co-labeled with green fluorescent feridex and red fluorescent dextranase.

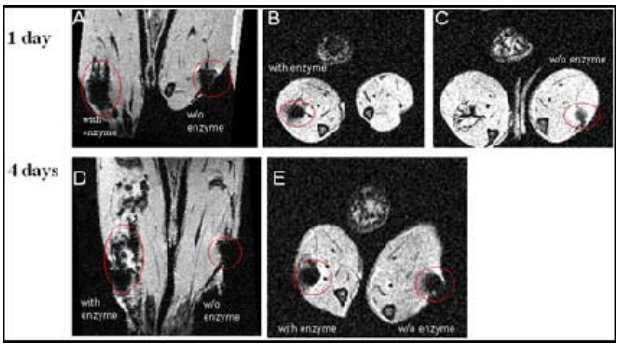

Figure 2: **In vivo demonstration of increase in magnetic susceptibility of Feridex loaded cells co-labeled with dextranase.** A, D) 3D GRE images from the same animal showing both sites of labeled cell injections (circled) at 1 day (A) and 4 days (D) post injection. Left side is cells co-labeled with Feridex and dextranase; right side is Feridex labeled cells only. B,C, E) SE images from the same animal taken 1 day (B,C) and 4 days (E) post injection, showing injected areas (circled).

References: 1) Shapiro, EM, et al, MRM (55) 2006; 2) Cohen, B, et al, Neoplasia (7) 2005; 3) Gilad, AA, et al, Nat Biotech (25) 2007; 4) Arbab AS, et al, Transplantation (76) 2003; 5) Bogdanov, A, et al, Mol Imaging (1) 2002.