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Pulsed magneto-motive ultrasound imaging to detect intracellular trafficking of magnetic nanoparticles

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Abstract

As applications of nanoparticles in medical imaging and biomedicine rapidly expand, the interactions of nanoparticles with living cells have become an area of active interest. For example, intracellular trafficking of nanoparticles – an important part of cell-nanoparticle interaction, has been well studied using plasmonic nanoparticles and optical or optics-based techniques due to the change in optical properties of the nanoparticle aggregates. However, magnetic nanoparticles, despite their wide range of clinical applications, do not exhibit plasmonic-resonant properties and therefore their intracellular aggregation cannot be detected by optics-based imaging techniques. In this study, we investigated the feasibility of a novel imaging technique – pulsed magneto-motive ultrasound (pMMUS), to identify intracellular trafficking of endocytosed magnetic nanoparticles. In pulsed magneto-motive ultrasound imaging a focused, high intensity, pulsed magnetic field is used to excite the cells labeled with magnetic nanoparticles, and ultrasound imaging is then used to monitor the mechanical response of the tissue. We demonstrated previously that clusters of magnetic nanoparticles amplify the pMMUS signal in comparison to signal from individual nanoparticles. Here we further demonstrate that pMMUS imaging can identify interaction between magnetic nanoparticles and living cells, i.e. intracellular aggregation of nanoparticles within the cells. The results of our study suggest that pMMUS imaging can not only detect the presence of magnetic nanoparticles but also provides information about their intracellular trafficking non-invasively and in real-time.

Keywords

Pulsed magneto-motive ultrasound imaging; superparamagnetic iron-oxide nanoparticles; macrophage; endocytosis; intracellular trafficking

1. Introduction

Nanoparticles have been introduced as a promising tool to expand the scope of many imaging modalities to the cellular and molecular levels. The small size of nanoparticles makes them a suitable tool to help identify and track the cells or molecules of interest, create contrast and provide information about events on small scale levels [1]. During the past decade, metal-based nanoparticles such as gold and iron-oxide have been used as suitable

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molecular imaging probes due to the fact that they significantly accumulate in the site of interest and provide sufficient imaging contrast [2]. Besides their applications for molecular imaging, nanoparticles have been utilized for other biomedical applications such as nanoparticle-assisted selective delivery of drugs and other therapeutic agents to reduce the undesirable side effects associated with these therapeutic procedures [3-5].

In many rapidly growing applications of nanoparticles in medical imaging and biomedicine, interactions between nanoparticles and living cells play a critical role. Intracellular trafficking of nanoparticles involves important cell-nanoparticles interaction which has been studied extensively during the past several years, especially in area of targeted drug delivery [6-14]. Among commonly used metal nanoparticles, intracellular aggregation of plasmonic nanoparticles is detectable through the significant plasmon red-shift and broadening in their absorption spectra [15, 16]. However, magnetic nanoparticles, despite their biocompatibility and thus availability for various clinical applications, do not exhibit plasmonic-resonant properties and therefore their intracellular aggregation cannot be detected through change in their optical properties.

Pulsed magneto-motive ultrasound imaging (pMMUS) has been introduced as an ultrasound-based molecular imaging technique capable of detecting the presence and distribution of magnetic nanostructures through their mechanical responses to an applied magnetic field [17, 18]. The pMMUS signal (i.e. magnetically induced and ultrasonically measured micro-displacement of the tissue) depends on several parameters including the distribution of contrast agents. We have recently demonstrated that magnetic contrast agents with larger geometries (i.e. nanoclusters) can enhance pMMUS signal [19]. These findings and the fact that endocytosed nanoparticles form large aggregates within the cells have been our motivation to explore the capability of pMMUS imaging to monitor intracellular trafficking of ultra-small magnetic nanoparticles.

Internalization of nanoparticles into cells takes place via different pathways such as fluid phase endocytosis, receptor-mediated endocytosis or phagocytosis [20]. Macrophage-like scavenger cells are highly active in the fluid-phase endocytosis of nanoparticles [21, 22]. The nanoparticles taken up by endocytosis will accumulate in the vesicles called endosomes [23]. When internalization occurs via this mechanism, the cell membrane is invaginated to form a pocket, which then pinches off into the cell to form a vesicle (0.5–5 μm in diameter) filled with a large volume of extracellular fluid and nanoparticles within it. The filling of the pocket occurs in a non-specific manner. The vesicle then travels into the cytosol and fuses with other vesicles such as endosomes and lysosomes [24]. In the case of magnetic nanoparticles (NPs) taken up by cells, aggregation of nanoparticles within nascent vesicles significantly changes the distribution of magnetic nano-agents and thus is expected to have an effect on pMMUS signal. In the study presented here we investigated the pMMUS imaging of intracellular aggregation of dextran-coated superparamagnetic iron-oxide (SPIO) nanoparticles (5 nm core diameters) in mouse macrophages (J774A. 1 cell line).

2. Materials and Methods

The monodispersed dextran coated iron oxide nanoparticles were synthesized by using a method described elsewhere [25]. Briefly, 15 mL of dextran (MW 10,000 kDa) aqueous solution (15% w/w) was titrated with 4 mL NH_4OH (>25% w/w) to pH 11.7 at room temperature. Five milliliters of freshly prepared $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (0.75 g) and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.32 g) aqueous solution was gradually injected into the alkali-treated dextran solution after passing through a 0.2 μm pore size filter. After 30 minutes, the black colloidal suspension was centrifuged at 10,000 rpm for 20 minutes to remove the aggregates. The supernatant was dialyzed in a dialysis bag with 25 kDa molecular weight cut off (Spectra/Pro 7,

Spectrum Laboratories Inc.) against deionized water for 36 hours to remove ammonia in order to reach a pH value of 7.0. A centrifugal filter device (Ultracel YM-30, Millipore Co.) was used with a relative centrifugal force of $1500 \times g$ to further purify and concentrate the dextran coated iron oxide dispersion. The size of the individual iron oxide nanoparticle cores measured by high resolution transmission electron microscopy (HRTEM) was 5.2 ± 0.8 nm (Fig. 1a) giving an overall hydrodynamic diameter of about 20 nm (Fig. 1b) measured by dynamic light scattering (DLS). The induced saturation magnetization of SPIO nanoparticles was measured as 54 emu/gr Fe at 300 K (Fig. 1c) using a super-conducting quantum interference device (SQUID – Quantum design MPMS).

Mouse macrophage cells (J774A.1 cell line) were selected for this study due to their high rate of non-specific uptake. To load the cells with dextran-coated magnetic nanoparticles, cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% Fetal bovine serum (FBS) at 37°C in 5% CO₂ and then were incubated with the suspension of nanoparticles at the concentration of 0.1 mg/mL Fe (i.e. 4×10^{14} NPs /mL suspension) for 24 hours. The average number of internalized dextran-coated SPIO nanoparticles was measured using inductively coupled plasma mass spectrometry (ICP-MS). For this purpose the culture was washed with $1 \times$ PBS six times to make certain that all non-internalized nanoparticles were removed. Then the labeled cells were removed from the culture, counted using a hemocytometer and dissolved in 35% trace metal-grade Nitric acid (HNO₃) and then kept in an oven at 60°C for 12 hours. After baking, the sample was diluted to 1-2% Nitric acid and mass spectroscopy measurements were performed. The results indicated that the cell uptake in culture was $(3.2 \pm 0.09) \times 10^4$ particles per cell and were consistent across multiple measurements.

After incubation with NPs, cells were divided for TEM and pMMUS studies. For TEM imaging, cells were fixed in a mixture of 3% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer at pH 7.4 for 30 minutes. Following three buffer rinses, the cells were post-fixed for 30 minutes in reduced osmium, a mixture of 2% osmium tetroxide and 2% potassium ferrocyanide in the cacodylate buffer. The fixed cells were then placed in 2% uranyl acetate for 30 minutes before dehydration in an ethanol series (50-70-95-100%). Dehydration was followed with two changes of absolute acetone after which the cells were infiltrated with a 1:1 mixture of Spurr and EMBED 812 epoxy resins (Electron Microscopy Sciences, Hatfield, PA) which was subsequently polymerized for 2 days at 60°C. Sections with thickness of 60-70 nm were cut from the epoxy blocks and picked up on copper grids for imaging at 80 kV in a Tecnai Spirit BioTwin transmission electron microscope without further staining. As a control, cells not incubated with NPs were prepared with the same protocol.

To demonstrate the ability of pMMUS imaging to detect the intracellular trafficking of SPIO nanoparticles, tissue mimicking phantoms were made out of 6% polyvinyl alcohol (PVA) by weight to mimic the mechanical and magnetic properties of soft tissue. For ultrasound imaging, 0.2% of 15 μ m silica particles were added to create acoustic backscattering. Two cylindrical-shape compartments with the diameter of 2.5 mm were created within the phantom. Both compartments were filled with 10% gelatin gel containing either (1) macrophages incubated with dextran-coated SPIO nanoparticles overnight and fixed in 10% formalin solution for 30 minutes, or (2) fixed macrophages mixed with the SPIO nanoparticles. In each inclusion, 5×10^6 macrophages were used, i.e., the number of cells was the same in each inclusion. Furthermore, the concentration of iron was the same in each inclusion – this was achieved by measuring the amount of iron (or number of SPIO nanoparticles) internalized by cells used in the first inclusion and then adjusting the number of nanoparticles in the second inclusion to match the concentration of iron. Therefore, the inclusions containing an equal number of cells and nanoparticles simulated the two separate

states of cell/nanoparticles before and after intracellular trafficking. Similar to the PVA background, 0.5% of 15 μm silica particles were added to the inclusions to act as ultrasound scatters. Once the inclusions solidified, the phantom was placed in a water cuvette for pMMUS imaging.

The diagram of the custom-built US/pMMUS imaging system is presented in Figure 2a. The 10 ms long magnetic excitation pulses were generated by a high-power voltage-controlled current amplifier driving the current to a solenoid magnetic coil. A cone shaped iron core made of ferritic stainless steel was embedded into the center of the coil to increase the magnetic flux density and also to focus it to the desired imaging region. The magnetic flux density of pulses was measured to be about 8000 G at 5 mm above the coil. An active cooling system was used to remove the heat generated within the coil due to the large amount of current passing through it. The ultrasound RF signals before, during and after application of the magnetic pulse were acquired at a high pulse repetition frequency of 1 kHz using a focused single-element ultrasound transducer operating at 25 MHz (focal depth = 25.4 mm, f # 4) interfaced with an ultrasound pulser/receiver. A block-matching motion-tracker algorithm was applied to calculate the magnetically induced displacement.[17, 19, 26] The cross-section of the phantom with two inclusions was imaged by mechanically moving the water cuvette with the phantom while the ultrasound transducer and the magnetic coil were kept fixed and stationary. The maximum displacement at each position within the imaging plane was then calculated (Fig 2b), normalized to the magnetic pulse strength and displayed in the magneto-motive ultrasound image[17-19] – an image that combines both the B-scan ultrasound image and spatially co-registered pulsed magneto-motive displacement image.

3. Results and Discussion

The general scheme of cellular uptake of magnetic NPs is shown in Figure 3a and Figure 3b. After diffusion of nanoparticles into extracellular environment, the cell membrane begins to sink in and to envelop the nanoparticles (Fig. 3a). As a result, the nanoparticles are captured inside of a capsule of cell membrane. When endocytosis is complete, the nanoparticles aggregate inside the endosomes (Fig. 3b). While the dark field microscopy of intact macrophages and labeled cells clearly indicated the uptake of SPIO nanoparticles by the cells (Fig. 3c and Fig. 3d), TEM images revealed the intracellular aggregation of nanoparticles within the endosomes (Fig. 3e and Fig. 3f). Different sizes of aggregates were observed within the cells ranging from several hundred nanometers up to a few micrometers. These large aggregates of nanoparticles were expected to enhance the pMMUS signal at the same concentration of iron. Aggregation of SPIO nanoparticles can also take place within the nanoparticles that are not taken up by cells. However, the sizes of those aggregates were much smaller than what we observed within endosomes.

Ultrasound and pMMUS images of both inclusions within the tissue phantom are presented in Fig. 4 where only the regions of interest with the inclusion in the center are shown. Both magnetic inclusions can be identified in B-scan ultrasound images (Fig. 4a and Fig. 4b) but the contrast in these images is due to the difference between the concentration of ultrasound scatterers in inclusions and background. Magnetically induced displacements were calculated within these relatively large regions of interest and the resulting combined US and pMMUS images (Fig 4c and Fig. 4d) were co-registered with respect to ultrasound images. The pMMUS images of both inclusions can clearly identify the presence of the magnetic inhomogeneities within the non-magnetic background. However, large aggregates of SPIO nanoparticles within the endosomes resulted in significantly larger mechanical response and, therefore, pMMUS signal. The average magnetically induced displacement measured within the inclusion containing labeled cells was about 42 μm while the second

inclusion where nanoparticles were mixed with cells exhibited only 25 μm motion although the concentrations of magnetic nano-agents were identical in both inclusions.

The temporal behavior of magnetically induced displacements, calculated within the small region in the center of each inclusion, is shown in Figure 5. The increase in pMMUS signal is caused by aggregation of endocytosed nanoparticles. The sizes of the aggregates, typically larger than a few hundred nanometers, can vary based on the size of the vesicles and also the uptake rate of the cells (Fig 3). A TEM image of an endosome filled with nanoparticles is shown at higher magnification in Figure 5 and was measured to be more than 300 nm which is much larger than the possible uncontrolled aggregation of SPIO nanoparticles in solution or extracellular space, e.g., cells mixed with SPIO nanoparticles (Fig. 5).

While we studied the endocytosis of SPIO nanoparticles that were passively taken up by macrophages, there are various mechanisms of nanoparticle internalization by the cell. Several recent studies have shown that capping specific molecules assembled on the surface of nanoparticles makes them aggregate in desired cells under specific conditions [27, 28].

Our results suggest that pMMUS imaging is capable of imaging and monitoring the intracellular trafficking of nanoparticles. For example, pMMUS imaging can be used to assess the targeting efficiency of nanoparticles used as drug carriers. Monitoring the pMMUS signal in time can provide spatial and temporal information about delivery of nanoparticles and interactions between nanoparticles and cells.

4. Conclusion

In summary, we have demonstrated the ability of pMMUS imaging to detect the presence of magnetic nanoparticles and their intracellular trafficking. Once fully developed, pMMUS imaging can provide this information in real time, in-vivo, non-invasively and at reasonable imaging depth. As such, pMMUS can be used in many applications where monitoring of intracellular trafficking of such nanoparticles is crucial: molecular imaging, functional cellular imaging, image guidance of molecularly targeted drug delivery, controlled drug release, enhanced thermal and other therapies using magnetic or hybrid (e.g. magneto-plasmonic) particles, etc. Overall, the combination of magnetic nanoparticles and pMMUS imaging is a clinically viable approach because both nanoparticles and the imaging modality are non-toxic, safe and can be used in in-vivo applications.

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Figure 1.

(a) High-resolution TEM image of the dextran-coated SPIO nanoparticles. (b) Dynamic light scattering (DLS) measurement of hydrodynamic diameter of dextran-coated SPIO nanoparticles. (c) Magnetization of dextran-coated SPIO nanoparticles at different applied magnetic field.

Figure 2.

(a) Block diagram of a pMMUS imaging system used to image a tissue mimicking phantom with two magnetic inclusions. Both inclusions have approximately identical concentration of iron and macrophages but in one of the inclusions the SPIO nanoparticles are aggregated within the cells while the second inclusion is simply the mixture of nanoparticles and cells. (b) Displacement of a normal tissue (dotted orange line) and magnetic inhomogeneity such as tissue labeled with magnetic nanoparticles (solid green line) in response to an externally applied pulsed magnetic field. The pMMUS signal represents the maximum induced displacement measured by analysis of ultrasound frames obtained before, during and after the magnetic excitation.

Figure 3.

(a,b) Diagrams describing the intracellular uptake of magnetic nanoparticles by macrophage-type cells. The size of the endosomes can be as large as 5 μm . (c-d): Dark-field microscopy images of murine macrophage cells (J774 A1 cell line): (c) control (non-labeled) cells, (d) cells incubated with 5 nm iron-oxide magnetic nanoparticles for 24 hours. The images were obtained using Xenon illumination and a 20 $\times$ darkfield objective (0.5 NA) with Leica 6000 DM upright microscope. The aggregation of nanoparticles within the cells causes a significant increase in reflectance, therefore loaded cells are more bright in dark-field image. (e-f) TEM images of fixed and sectioned (70 nm thicknesses) of (e) control (non-labeled) cells and (f) cells labeled with 5 nm iron-oxide nanoparticles. Red arrows indicate the endocytosed aggregates of SPIO nanoparticles within endosomes.

Figure 4.

(a,b) B-Scan ultrasound and (c,d) combined US and pMMUS images of PVA phantoms with gelatin inclusions containing: (a,c) 5×10^6 macrophages mixed with 16×10^{12} magnetic nanoparticles, (b,d) 5×10^6 macrophages incubated with SPIO nanoparticles for 24 hours. Concentration of nanoparticles was approximately the same in both inclusions. Each image covers the field of view measuring 6.04 mm laterally and 4.68 mm axially. The ultrasound images identify the location and extend of the inclusions within the imaging plane while the pMMUS images clearly show a signal enhancement due to intracellular trafficking of SPIO nanoparticles.

Figure 5.

Temporal behavior of magnetically induced displacement in an inclusion containing macrophages mixed with dextran coated SPIO nanoparticles (dashed blue line) and in an inclusion containing magnetically labeled macrophages with large nanoparticles aggregates inside endosomes (solid red line). Each measurement is accompanied by a diagram outlining the interaction of nanoparticles with individual cells and a corresponding TEM image. The average normalized pMMUS signal in each inclusion is shown in corresponding graphs where error bar represents plus/minus one standard deviation.