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Tracking Injectable Microspheres in Dynamic Tissues With Encapsulated Superparamagnetic Iron Oxide Nanoparticles^a

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Abstract

Trackable spheres of similar size to those typically used for sustained protein delivery are prepared by incorporating superparamagnetic iron oxide (SPIO) nanoparticles into the core of poly(lactide-*co*-glycolide) microspheres. The visibility of injections in static and temporally in dynamic tissue systems is demonstrated. This method improves upon other, less sensitive imaging modalities in their ability to track injectable delivery systems. The results obtained confirm the localization of microspheres to the injected target area and highlight the novelty of tracking delivery vehicles for other applications.

^aSupporting Information for this article is available from the Wiley Online Library or from the author.

Keywords

biomaterials; bioresorbable; controlled release; drug delivery; magnetic resonance imaging

1. Introduction

Poly[(D,L-lactide)-*co*-glycolide] (PLG) microspheres, are a well-characterized, synthetic drug delivery vehicle, able to temporally control drug release in a local environment.^[1] PLG microspheres are attractive in drug delivery applications, as they are injectable, biodegradable, and capable of encapsulating and releasing various types of biological molecules (e.g., small molecules, proteins, plasmid DNA) with some retention of biological activity. Local delivery studies typically involve measuring putative downstream effects of the released drug, and the effects are often compared with local bolus injection.^[2] However, drug release can be detrimental to healing and can cause unwanted adverse effects if the delivery vehicle transports outside the wound-healing region. Despite this, local retention of drug-releasing microspheres after injection has not been extensively studied,^[3] due in part to a lack of robust imaging techniques for microsphere tracking. The ability to visualize a delivery vehicle at the injection site and track its local retention over time could lead to valuable insights about the intended and unintended *in vivo* effects of current therapies. Because of the widespread use of PLG microspheres to deliver proteins and other drugs, a simple, non-invasive tracking mechanism for these microspheres could have widespread utility, particularly in dynamic, highly vascularized tissues in which local microsphere retention is likely to be a particular challenge. Ultrasound can potentially be used to track PLG microspheres, owing to the contrast provided by the microsphere/tissue interface. However, the acoustic enhancement produced with standard PLG microspheres is not high enough to be clinically significant,^[4] and ultrasound contrast requires a high level of PLG microsphere porosity, which is known to influence degradation and drug release rates.^[5] Thus, there is a need for microsphere tracking approaches that do not significantly impact the bulk properties of the microsphere and do not use types of radiation (e.g., ultrasound)^[5] that can influence hydrolytic degradation.

Superparamagnetic iron oxide (SPIO) nanoparticles have been widely used as effective contrast agents for conventional magnetic resonance imaging (MRI),^[6] as magnetic domains

of the iron oxide core can align and create MRI contrast in the presence of an external magnetic field. SPIO nanoparticles have also been used as a drug delivery system, which can enable simultaneous visualization and localized therapy with guidance from an externally applied magnetic field.^[7] We hypothesized that SPIO nanoparticles could be incorporated into bioresorbable, PLG microspheres using standard processing techniques, and that the resulting SPIO-encapsulated microspheres would be detectable in vitro and in vivo using MRI. We further hypothesized that SPIO nanoparticles would remain encapsulated within a standard PLG microsphere formulation for multiple weeks, allowing for microsphere tracking over time in vivo. A particular goal of our approach was to provide MRI contrast after microsphere injection in vivo, and to thereby enable microsphere tracking over relatively short (≈ 30 d) timescales associated with common drug release kinetics in dynamic, highly vascularized tissues.

2. Experimental Section

2.1. SPIO-Encapsulated Microsphere Fabrication

PLG 85:15 Sigma-Aldrich (St. Louis, MO) was fabricated into microspheres as described previously via water-in-oil-in-water (W/O/W) double emulsion technique with some modifications.^[8] The organic phase consisted of 5% w/v PLG in ethyl acetate, while the aqueous phase consisted of $1 \text{ mg} \cdot \text{mL}^{-1}$ of SPIO nanoparticle solution ($10 \text{ mg} \cdot \text{mL}^{-1}$, FeREX, Biopal, Worcester, MA). The aqueous and organic phases were mixed and sonicated briefly. The resulting first emulsion was added immediately into a 1% poly(vinyl alcohol) in ethyl acetate and vortexed to form the second emulsion. The resulting solution was added to a hardening solution containing 3% poly(vinyl alcohol) and 0.7% ethyl acetate stirred for 4 h to allow for organic solvent evaporation. The resulting SPIO-encapsulated microspheres were collected, washed, resuspended, and purified via coarse filtration. The efficiency of SPIO nanoparticle encapsulation was measured with a colorimetric Ferrozine assay that measures total iron.^[9] Specifically, microspheres encapsulated with SPIO nanoparticles and those that were fabricated in a similar fashion but were blank in their core were dissolved in a solution of 0.5 N NaOH (Fisher) for 15 min, until all of the particles were gone. 1 N HCl (Fisher) was added dropwise to the dissolved microsphere solution until it reached pH = 7. Aliquots of dissolved SPIO particles were measured for their total iron concentration above that of the blank microsphere control.

2.2. In vitro Imaging in an Agarose Phantom

To determine if there was a concentration dependence of SPIO particles on MRI signal, we compared the MRI signal generated from SPIO-encapsulated microspheres to that of blank microspheres in an agarose gel phantom. To accomplish this, agarose powder was dissolved into double distilled H₂O with stirring and heat for 30 min. At the critical point of saturation, the 2% agarose solution was poured into a plastic frame to set. 200 μL aliquots of the same 2% agarose solution were pipetted inside of five Eppendorf tubes to provide a base for the microsphere suspension. The Eppendorf tubes were then placed inside the congealing agarose for imaging SPIO-encapsulated microspheres of varying masses (0.5, 1, 2, 3 mg) and blank microspheres (1 mg spheres, 0 mg SPIO) were measured, suspended in a 1000 μL phosphate-buffered saline (PBS) solution and added to the 2% agarose gel foundation (Figure 2A and B). Centrifugation at 9.8×1000 rpm for 3 min pelleted the microspheres on the agarose platform within the Eppendorf tube, concentrating the microspheres at an equal height. The phantom was imaged on a 1.5 T magnetic resonance imaging (MRI) system (Signa HDxt, GE Healthcare, Milwaukee, WI), using an 8-channel receive-only cardiac coil.

2.3. In vitro Imaging in Static Muscle Tissues

In a similar experiment, SPIO-encapsulated microspheres (3 mg total mass per 200 μL of PBS) or blank PLG microspheres of an equal concentration were injected into explanted skeletal muscle (cow flank, butchered locally) 1.5 cm thick and visualized on MRI. The muscle specimen was subsequently immersed in water and imaged with the same modality as mentioned above. An explanted pig heart was secured from a local slaughterhouse and used within 24–48 h of receipt. Prior to injection, sites were chosen and marked along the surface of the heart tissue in order to correlate the location of injection with image slices seen on MRI. SPIO-encapsulated microspheres in 3, 6.67, and 16.67 $\text{mg} \cdot \text{mL}^{-1}$ concentration, free SPIO particles and 5 $\text{mg} \cdot \text{mL}^{-1}$ of PLG microspheres (non-SPIO-encapsulated) were injected into the pericardial tissue in the pig heart. The explanted heart muscle was imaged with a fast 3D spoiled gradient echo (3D FSPGR) sequence in the coronal plane. Imaging parameters were: repetition time (TR) 6.2 ms, echo time (TE) 4.2 ms, number of exposures (NEX) 1, bandwidth (BW) ± 62.5 kHz, matrix 256×256 , no. of slices 32, field of view (FOV) 36 cm, slice thickness 2 mm.

To further characterize SPIO-encapsulated microspheres in heart tissue, we next injected 1 mg/60 μL of SPIO-encapsulated micro-spheres into a pig heart in vivo using a trans endocardial catheter (Cardio3 Biosciences, Belgium). The injection was administered 3 d after a 90 min balloon occlusion in the LAD to cause a myocardial infarction (MI). Post injection, the heart was imaged with standard ECG-gated, balanced SSFP (bSSFP) imaging sequences, which were used to define axial, ≈ 2 -chamber, and left ventricular (LV) short-axis sections of the heart. To visualize the SPIO-encapsulated microspheres, an ECG-gated, 2D gradient-echo imaging sequence in the LV short-axis orientation was used with the infarcted heart. Imaging parameters were: TR 7.5 ms, TE 4.0 ms, NEX 2, BW ± 15.625 kHz, matrix 256×160 , no. of slices 20, FOV 35 cm, slice thickness 5 mm, delay after trigger = 500 ms.

2.4. In vivo Imaging of SPIO-Encapsulated Microspheres in Dynamic Tissues

Three male Wistar rats (Harlan Laboratories) born on the same date and 242–278 g at time of injection were injected with 0.5 mg/50 μL of SPIO-encapsulated microspheres. The left leg of each rat received three injections, each in a different location: the Achilles tendon, the medial collateral ligament (MCL), and the gastrocnemius muscle. The right leg received a matched injection containing 0.5 $\text{mg} \cdot \mu\text{L}^{-1}$ of blank PLG microspheres (without SPIO). Prior to injection, the microspheres were incubated for 30 min in a sterilization cocktail, which contained 2.5 $\mu\text{g} \cdot \text{mL}^{-1}$ of amphotericin B (Fisher Scientific), 10 $\mu\text{g} \cdot \text{mL}^{-1}$ ciprofloxacin hydrochloride (TCI America), 100 U mL^{-1} /100 $\mu\text{g} \cdot \text{mL}^{-1}$ penicillin/streptomycin (Hyclone) and 50 $\mu\text{g} \cdot \text{mL}^{-1}$ gentamycin sulfate (MP Biomedicals). Microspheres were then pelleted via centrifugation, washed $3\times$ with deionized water, and suspended in PBS for subsequent injection.

Animals were sedated briefly with isoflurane (0.5–3.0%) with an induction chamber and then a facemask, while the hindlimbs were shaved and prepared for injection. After injection the animals were allowed to recover at least 1 h, before being sedated again with isoflurane for initial “ $T=0$ ” imaging. An Agilent 4.7 T magnet gradient echo sequence (gems) T_2 -weighted with TR = 5–5 ms, TE = 8, flip angle = 20, NT (averages) = 2, sagittal view, 45×45 FOV 1 mm slice thickness, 40 slices was used to track microspheres in vivo. Subsequent images were taken weekly until sacrifice (days 5, 14, 21, 28), and rats were able to move freely throughout the study.

2.5. MRI Analysis

MRI images were analyzed with ImageJ software. Images were enlarged and a black and white threshold was applied to determine the region of interest created by the site of the initial injection. Once the area of MRI contrast was determined and region of interest compared to the contra lateral leg, the total pixel area of detectable MRI signal was calculated. Areas were measured 3–5 times and the region of best approximation of the pixel area recorded. If a region could not be approximated, the last three values were averaged. The result was normalized to the day 0 pixel area to determine temporal changes. Student *t*-tests were used to determine statistically significant differences.

3. Results and Discussion

SPIO nanoparticles were efficiently encapsulated into the core of PLG microspheres, and the resulting microspheres had similar size and morphology when compared to PBS-loaded “control” microspheres (Figure 1A). Scanning electron microscopy demonstrated that SPIO-encapsulated microspheres and control microspheres each had a spherical morphology (Figure 1B), which is consistent with previous studies using the water-in-oil-in-water emulsion technique. The size distribution of the SPIO-encapsulated microspheres closely mirrored that of the control microspheres, with the majority of microspheres $<10\ \mu\text{m}$ in diameter. The average diameters of the SPIO-encapsulated microspheres ($6.1 \pm 0.1\ \mu\text{m}$) and control microspheres ($5.4 \pm 0.2\ \mu\text{m}$) were not significantly different ($p > 0.05$) (Figure 1C). Taken together, these results indicate that SPIO encapsulation does not significantly influence the size or morphology of PLG microspheres.

SPIO-encapsulated microspheres included into an agarose gel phantom produced a signal loss on T_2 -weighted MRI in a concentration dependent manner (Figure 2A). Previous results have shown that dispersed iron oxide particles injected into a phantom or tissue results in low T_2 relaxivity^[10] and small amounts of signal. Similarly, when SPIO-encapsulated microspheres were dispersed in solution, we were unable to detect a signal on MRI. However, when SPIO-encapsulated microspheres were injected into an agarose phantom in a concentrated solution, MRI pixel area was directly dependent on the microsphere mass (Figure 2B, $R^2 = 0.9694$). Therefore, SPIO-encapsulated microspheres could produce MRI contrast in an MRI phantom above a critical concentrated mass of $\approx 0.5\ \text{mg}$. To determine whether SPIO-encapsulated microspheres would be likely to lose MRI contrast over time due to hydrolytic microsphere erosion, we also measured release of the encapsulated SPIO nanoparticles in vitro. $26.40 \pm 3.18\%$ of the encapsulated iron was released from the microspheres during a 45-d incubation in a simulated body fluid (Figure 2C, Figure S1 in Supporting Information), indicating that the majority of SPIO particles are not lost in the short term. These data were supported by daily iron release measurements (Figure S1), which demonstrate that a significant amount of iron was released during each of the first 3 d of incubation ($8.42 \pm 0.85\%$ of cumulative iron release), but the amount of iron released during each subsequent day of release was below the detectable range of the assay (previously shown to be $0.2\ \text{nmol}$).^[9b] Taken together, these data indicate that minimal iron was released during the 45 d incubation in simulated body fluid, and significant iron release only occurred during the first 72 h. These data suggest that the majority of SPIO nanoparticles remained encapsulated within the delivery vehicle over multiple weeks.

In order to characterize the utility of SPIO-encapsulated microspheres for localized, in vivo tracking, we next injected varying concentrations of microspheres into two static muscle tissue models. Injections of SPIO-encapsulated microspheres and free SPIO particles were each detectable on MRI from the base to the apex of a static (non-beating) in vivo pig heart – post MI – just prior to animal sacrifice, while control PLG microspheres were not visible (Figure 2D–F). Thus, SPIO-encapsulated microspheres of at least $3\ \text{mg} \cdot \text{mL}^{-1}$ per injection

were detectable in static pig cardiac muscle. SPIO-encapsulated microspheres were also detectable in explanted skeletal muscle at concentrations of $15 \text{ mg} \cdot \text{mL}^{-1}$ (Supplementary Figure 2) and $16.67 \text{ mg} \cdot \text{mL}^{-1}$ in a pig heart (Figure S2, S3, Supporting Information). This successful detection of injected SPIO-encapsulated microspheres in static tissues motivated further studies to track retention and distribution of microspheres in dynamic musculoskeletal tissues.

Interestingly, imaging of SPIO-encapsulated micro-spheres in dynamic tissue environments indicated that a substantial portion of injected PLG microspheres were localized to the site of injection for at least 28 d. We analyzed and compared images of SPIO-encapsulated microspheres injected into the rat lower limb, specifically into rat gastrocnemius muscle, MCL or Achilles tendon. These are highly vascularized tissues that undergo routine motion, and were therefore an attractive test-bed for microsphere tracking. SPIO-encapsulated microspheres were detectable in these tissues at dosage ranges similar to those described above for static explanted muscle models. In vivo injections of $10 \text{ mg} \cdot \text{mL}^{-1}$ SPIO-encapsulated microspheres into the rat gastrocnemius muscle, MCL or Achilles tendon resulted in a detectable MRI signal in each of the injected areas, while there were no detectable signals present in contra lateral control tissues injected with the same mass of “blank” PLG microspheres (Figure 3).

Quantification of MRI pixel area from each of the three tissue types over time demonstrated a decrease of MRI signal between day 0 and day 5 (Figure 3B), suggesting that individual microspheres are not entirely retained in dynamic, vascularized tissue. This initial decrease in microsphere pixel area is likely due to low level SPIO nanoparticle release (see Figure 2G, Figure S1) or due to transport of microspheres away from the injection site rather than microsphere erosion, as erosion of this PLG composition has been shown to take place over weeks to months in vivo,^[11] and would not be significant during a 5 d period. Interestingly, there was no significant decrease in the MRI pixel area between day 5 and 28 in any of the injection sites tested (p -values = 0.36, 0.60, 0.47 for the gastrocnemius, MCL, and Achilles, respectively), and >45% of the MRI pixel area was retained at 28 d for each of the tissue types tested. These data indicate that SPIO-encapsulated microspheres that were retained during the first 5 d after injection tended to remain at the initial injection site long term, even in highly vascularized tissues undergoing motion.

It is noteworthy that the changes in the average pixel density when comparing time points may also be partially attributable to the subtle variations in the imaging protocol at each time point. Subtle differences in the orientation angle or thickness of the MRI slices taken through the rat limbs, and a difference in relaxivity of the background tissue between bone and surrounding tissue may have some impact on the detectable pixel area. However, normalizing all experimental regions to the day 0 injection for each animal and using the contra lateral leg as a negative control likely allowed for consistent measurements across days in this study.

Our results are consistent with some portions of previous studies, which have incorporated iron oxide particles within PLG delivery vehicles to remotely control their location using an external magnetic field. Specifically, our results in Figure 2 are consistent with the previous findings of Patel et al.^[12] that SPIO-loaded microspheres are visible in T_2 images on MRI in static tissue. These previous studies indicate that SPIO-encapsulated microspheres may be useful not only to track microspheres, but also to induce microsphere movement in vivo.

4. Conclusion

Collectively, these results indicate that SPIO nanoparticles can be efficiently encapsulated into PLG microspheres, and the resulting microspheres can be detected and tracked at sites

of in vivo injection, including explanted muscle tissue and highly vascularized musculoskeletal tissues that are commonly in motion. Although this study did not focus on measuring long-term effects of these contrast agents in vivo, SPIO nanoparticles have been reported to have a lower toxicity than other MRI contrast agents and may therefore be particularly suitable as a long-term tracking agent for injectable microspheres in drug delivery applications. Further, trace amounts of the released iron can be removed by red blood cells, potentially decreasing the concern of toxicity from the contrast agent.^[6b,13] The results motivate the continued use of SPIO tracking in polymeric drug delivery systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A): SPIO encapsulated microsphere fabrication modeled with a schematic; (B) size and spherical morphology is maintained with FeREX encapsulation, visualized on scanning electron microscopy, scale bar = 3 μm ; (C) PLG size distribution with and without FeREX incorporated is not statistically different and follows a similar trend of number of spheres per size

Figure 2.

Analysis of SPIO encapsulated microspheres in static environments: (A) microspheres pelleted on an agarose layer demonstrate different relaxivity with varying concentrations; (B) quantification of pixel area demonstrates a concentration dependence on relaxivity; (C) SPIO encapsulated, PLG microspheres, and bolus FeREX were injected into an explanted pig heart; (D–F): MRI slices through heart tissue from the base to the apex; (G) total iron released from SPIO encapsulated microspheres over time.

Figure 3.

In vivo analysis of SPIO encapsulated microspheres injected into a dynamic tissue environment (A): SPIO encapsulated micro-spheres injected into three tissue types (muscle, ligament and tendon) and tracked over 4 weeks on MRI. Contra lateral control leg [C] was injected with PLG microspheres of a similar mass is pictured at day 0 (B): Quantification of average pixel area from MRI of three rats in three hind limb tissues suggests a decrease in pixel area between day 0 and day 5, but no significant difference after day 5.