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Polystyrene Microsphere–Ferritin Conjugates: A Robust Phantom for Correlation of Relaxivity and Size Distribution

Preeti A. Sukerkar¹, Uzma G. Rezvi¹, Keith W. MacRenaris¹, Pinal C. Patel¹, John C. Wood², and Thomas J. Meade^{1,*}

¹Departments of Chemistry, Molecular Biosciences, Neurobiology and Physiology, Radiology, Northwestern University, Evanston, Illinois, USA

²Division of Cardiology, Department of Radiology, Children's Hospital of Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles, California, USA

Abstract

In vivo iron load must be monitored to prevent complications from iron overload diseases such as hemochromatosis or transfusion-dependent anemias. While liver biopsy is the gold standard for determining in vivo iron load, MRI offers a noninvasive approach. MR phantoms have been reported that estimate iron concentration in the liver and mimic relaxation characteristics of in vivo deposits of hemosiderin. None of these phantoms take into account the size distribution of hemosiderin, which varies from patient to patient based on iron load. We synthesized stable and reproducible microsphereferritin conjugates (ferribeads) of different sizes that are easily characterized for several parameters that are necessary for modeling such as iron content and bead fraction. T_1 s and T_2 s were measured on a 1.41-T low-resolution NMR spectrometer and followed a size-dependent trend. Ferribeads imaged at 4.7 and 14.1 T showed that signal intensities are dependent on the distribution of ferritin around the bead rather than the iron concentration alone. These particles can be used to study the effects of particle size, ferritin distribution, and bead fraction on proton relaxation and may be of use in mimicking hemosiderin in a phantom for estimating iron concentration.

Keywords

ferritin; transverse relaxation; MRI

Iron overload is a serious health issue that results from transfusion-dependent anemias in thalassemia (1), hereditary disorders of iron metabolism such as hemochromatosis (2,3), cirrhosis (4), and viral hepatitis (5). Excess iron deposits in tissues as hemosiderin (aggregates of partially digested ferritin within lysosomes) that is associated with iron-catalyzed oxidative damage to proteins, lipids, and nucleic acids (6–10). Increased iron levels in the brain, heart, liver, lungs, endocrine organs, or other tissues eventually cause

organ failure. This may lead to diabetes, cirrhosis, cardiac dysfunction, or even death (6–10).

To prevent organ toxicity, iron must be efficiently regulated. Thus, treatment for hemochromatosis requires weekly phlebotomy until normal iron levels are obtained, followed by phlebotomy several times a year to maintain stasis (11), whereas transfusion-dependent iron overload requires iron chelation therapy to remove excess iron (12,13).

Laboratory tests such as serum ferritin and serum transferrin saturation are often used to assess iron levels in the body. These serum tests are inexpensive and widely available, but isolated measurements cannot reliably diagnose iron overload (where serum ferritin levels have been shown to be only weakly correlated with iron load) and can be influenced by various factors such as inflammation and transfusion rate. Liver biopsy is currently the gold standard test because it is highly sensitive for liver iron levels, is quantitatively accurate, and provides a good estimate of iron burden in the body (11). However, this technique has several disadvantages. In addition to the pain associated with this invasive procedure, there is a significant risk of hemorrhage, infection, and damage to surrounding organs (14,15). Further, biopsies are prone to sampling error from uneven iron distribution. Finally, liver iron concentration may not correlate with iron levels in the rest of the body and is poorly correlated with cardiac iron deposition (16).

A noninvasive method to quantify in vivo iron load would prove to be a significant clinical tool as it would avoid the complications associated with biopsies and sampling errors (17). One noninvasive technique to measure iron load is magnetic resonance imaging (MRI). MR scanners are widely available and able to determine pathological state of organs and iron content in areas besides the liver (18). Therefore, MR imaging is a promising alternative for noninvasive quantification of iron content. Numerous studies have been conducted to determine iron–relaxivity relationships in iron overload diseases. Transverse relaxation time (T_2), effective transverse relaxation time (T_2^*) (17,19–21), and liver signal intensity ratios have been correlated to iron content in the liver (22–25). While MR imaging is a promising solution, there are inconsistent results from various centers, an inability to determine higher iron loads, and methodological inaccuracies, leading to inaccurate liver quantification (26–28).

To overcome these barriers, an external reference MRI phantom would provide quantitative calibration between different exams, imaging sequences, patients, and scanners. However, due to the heterogeneous and complex nature of the liver and other biological tissues, phantoms possessing characteristics of an iron overloaded liver have been difficult to develop. Ideally, the MRI phantom should have background relaxation times similar to human tissue with homogeneous relaxation times throughout, be easy to handle, and possess long-term shelf life with regard to chemical and physical stabilities. Previously published phantoms include manganese-doped agarose (10) and ferritin-coated liposomes embedded in agarose (17). However, none of these studies take into account the size variability of the hemosiderin deposits and their effect on the interaction between the iron and water protons within the liver (28,29). Further, while the liposomes can be produced in various sizes, the ferritin is not covalently attached, leading to relatively unstable structures (they cannot

withstand heat or high salt concentrations due to the electrostatic interaction between the ferritin and the liposome surface).

Here, we describe the synthesis and relaxation properties of ferritin-coated latex microspheres that possess many similarities to hemosiderin including insolubility in aqueous media and 25- to 30-fold increase in relaxivity (r_2) compared with ferritin in solution. Additionally, latex microspheres are available in a wide range of monodisperse sizes and can be functionalized for stable covalent protein attachment. This produces a chemically stable material whose synthesis is highly reproducible and is an excellent choice for studying the effects of ferritin size distribution on relaxation rate changes.

MATERIALS AND METHODS

Preparation of Ferribeads (and Apoferribeads)

1.0 mL each of the 1.0 μm and 400 nm aldehyde-/sulfate modified polystyrene microspheres (IDC microspheres, Invitrogen, Carlsbad, CA) were centrifuged at 1500g for 15 min and the supernatant was removed. The microspheres were washed with acetate buffer (pH 4.5) containing 15 mM sodium cyanoborohydride [$\text{Na}(\text{CN})\text{BH}_3$] followed by centrifugation at 1500g for 15 min. This cycle was repeated three times. Microspheres of 20 nm were too small to be centrifuged and were cleaned instead by dialysis in [$\text{Na}(\text{CN})\text{BH}_3$]/acetate buffer over 3 days. Microspheres were suspended in 1.0 mL [$\text{Na}(\text{CN})\text{BH}_3$]/acetate buffer and stored at 4.0°C prior to use.

Different concentrations of ferritin (or apoferritin) from horse spleen (in buffered saline, 93 mg/mL ferritin, 1460 Fe atoms per ferritin protein based on ICP-OES, Sigma Chemical, St. Louis, MO) were added to [$\text{Na}(\text{CN})\text{BH}_3$]/acetate buffer (pH 4.5). 50 μL of the microsphere suspension was added to the ferritin solution and gently mixed on a rotator at room temperature for 24 h. The proteincoated microspheres (called ferribeads or apoferribeads) were centrifuged at 1500g for 15 min and the supernatant removed. The pellets were resuspended in [$\text{Na}(\text{CN})\text{BH}_3$]/acetate buffer and centrifuged at 1500g for 15 min, which was repeated three times to remove unbound protein. Following washes, ferribeads were resuspended in 1.0 mL acetate buffer (pH 4.5) and stored at 4.0°C prior to use.

Preparation of Ferribead/Agarose Phantoms

Low-gelling temperature agarose ($25 \pm 5.0^\circ\text{C}$, Fisher Scientific, Pittsburgh, PA) was added to ultrapure water and boiled via microwave heating for 30–60 sec to produce a 1.0% (w/v) agarose stock solution. Ferribeads were serially diluted in acetate buffer (pH = 4.5) to attain five ferribead concentrations. These solutions were added to 7.5 mm (outer diameter) NMR sample tubes followed by addition of an equal volume of agarose. The solution was mixed to ensure homogeneity followed by gelation at 4.0°C (placed in ice) prior to relaxation time measurements. Air bubbles were removed prior to gelation by gently tapping the NMR tube until the bubbles moved to the top of the sample.

Relaxation Time Measurements at 1.41 T (60 MHz) and 37°C

T_1 and T_2 relaxation times were measured on a Bruker mq60 NMR analyzer equipped with Minispec V2.51 Rev.00/NT software (Billerica, MA) operating at 1.41 T (60 MHz) and 37°C. T_1 relaxation times were measured using an inversion recovery pulse sequence (t1_ir_mb) using the following parameters: four scans per point, 10 data points for fitting, monoexponential curve fitting, phase cycling, 10 ms first pulse separation, and a recycle delay and final pulse separation $5T_1$. T_2 relaxation times were measured using a simple spin echo (SE, t2_Co_mb) or Carr–Purcell–Meiboom–Gill (CPMG, t2_Cp_mb) pulse sequence. The following parameters were used for the SE pulse sequence: four scans per point, 10 data points for fitting, monoexponential curve fitting, no phase cycling, 5 ms first pulse separation, 500 ms final pulse separation, and a 15 sec recycle delay. The following parameters were used for the CPMG pulse sequence: four scans per point, 100–5000 data points for fitting (longer T_2 = more data points for fitting to produce a good monoexponential curve fit), monoexponential curve fitting, phase cycling, 1 ms pulse separation (unless otherwise specified), and a 15 sec recycle delay. T_2^* measurements were attempted using a fid_fit application (courtesy of Dr. Supriyo Ghosh, Bruker Optics, The Woodlands, TX), which is a monoexponential fit of the free induction decay signal, using the following parameters: 5 ms duration of data acquisition, four scans per point, 100 data points for fitting, monoexponential curve fitting, and no phase cycling. The free induction decay line width (~0.26 ms) of the Bruker mq60 minispec did not allow for accurate T_2^* measurements as any sample (including water and nonferritin containing iron oxide nanoparticles) placed in the minispec returned a T_2^* value of 0.25–0.26 ms or approximately the free induction decay line width.

Magnetic Resonance Imaging

MR images of the ferribead/agarose phantoms were acquired on a General Electric/Bruker Omega 600WB (wide bore, 89 mm) 14.1 T (600 MHz) imaging spectrometer fitted with Accustar shielded gradients at ambient temperature (25°C) and 4.7 T images were acquired on a 4.7 T Bruker Biospec MR Imager at ambient temperature (25°C) courtesy of the Center for Basic Magnetic Resonance Research at Northshore University Health Systems in Evanston, IL. Spin lattice relaxation times (T_1) and spin–spin relaxation times (T_2) were measured using a simple SE pulse sequence. Specifically (at 14.1 T), a multislice multiecho variable repetition time sequence with eight echo times (TEs) (7.6, 15.2, 22.8, 30.4, 38, 45.6, 53.2, and 60.8 ms) and 10 repetition times (TRs) (200, 400, 650, 1000, 2000, 4000, 8000, and 15,000 ms) was used for each set of samples. Imaging parameters were as follows: field of view = $20 \times 20 \text{ mm}^2$, matrix size = 128×128 , number of axial slices = 5, slice thickness = 1.0 mm, interslice distance = 1.5 mm, and averages = 4 (or eight depending on imaging time allotted). Specifically (at 4.7 T), a multislice multiecho variable repetition time sequence with 16 TEs (40, 80, 120, 160, 200, 240, 280, 320, 360, 400, 440, 480, 520, 560, 600, and 640 ms) and eight TRs (150, 600, 1150, 1800, 2650, 3850, 5875, and 15,000 ms) was used for each set of samples. Imaging parameters were as follows: field of view = $40 \times 40 \text{ mm}^2$, matrix size = 256×256 , number of axial slices = 5, slice thickness = 1.0 mm, interslice distance = 1.5 mm, and averages = 2. T_1 and T_2 analyses (for both magnet systems) were carried out using the image sequence analysis tool in Paravision 3.0.2

software (Bruker BioSpin, Billerica, MA) with monoexponential curve fitting of image intensities of selected regions of interest for each axial slice.

ICP Sample Preparation

Iron concentration was measured using inductively coupled plasma optical emission spectroscopy (ICP-OES). Concentrated nitric acid (500 μ L) was added to each NMR sample tube and heated at 65°C for 4.0 h. After cooling to room temperature, samples diluted with nanopure H₂O and indium internal standard to produce a final solution of 3.0% nitric acid (v/v) and 1.0 μ g/mL indium up to a total sample volume of 10 mL. Samples were syringe filtered through 0.2- μ m nitrocellulose filters into fresh 15-mL conical tubes. ICP-OES was performed on a computer-controlled (running ICP-Expert software) Varian Vista-MPX CCD Simultaneous ICP-OES (Varian, Palo Alto, CA) equipped with an autosampler at the Integrated Molecular Structure Education and Research Center at Northwestern University.

Scanning Electron Microscopy

All materials were purchased from Ted Pella Inc. (Redding, CA). A drop of diluted ferribead sample was placed on a silicon wafer and dried overnight. Samples were sputter coated on a Denton Desk III TSC sputter coater (Denton Vacuum, Moorestown, NJ) using a Au/Pd target with a coat thickness of 4.0 nm. Images were acquired on a LEO Gemini 1525 field emission gun scanning electron microscope (Carl Zeiss SMT, Inc., Peabody, MA) at the Electron Probe Instrumentation Center facility of the NUANCE Center at Northwestern University. Specifically, images were acquired at 3.00 keV accelerating voltage at a working distance of 3.0 mm using the InLens detector.

Bead Counting via Flow Cytometry

The fluorescent excitation of aldehyde/latex fluospheres was 505 nm with an emission at 515 nm. Fluospheres (before and after ferritin attachment) were counted via a Guava EasyCyte mini personal cell analyzer (microcapillary flow cytometer) using the Guava Express software module (Guava Technologies, Hayward, CA). Five thousand trials of three separate dilutions of beads were run to assess machine reproducibility and pipetting accuracy (1000, 100, and 10 beads per microliter).

RESULTS

Synthesis and Characterization

The schematic in Fig. 1 depicts the reaction conditions for the covalent attachment of ferritin to the surface of aldehyde/sulfate latex microspheres (ferribeads). After ferribead synthesis, the particles began to sediment and were therefore suspended in 0.5% agarose to mimic the tissue environment and provide a homogeneous sample for NMR relaxometry and MR image acquisition. To ensure maximum particle coating and homogeneity, various pH levels and ferritin incubation concentrations were tested for their effects on T_2 , T_1 , iron content, and appearance. These conditions were optimized for 20, 400, and 1000 nm aldehyde/latex beads to determine the effect that particle size has on T_1 , T_2 , and, consequently, contrast enhancement in MR images. These bead sizes were chosen based on data obtained from

Wood et al. and Brissot and coworkers (28) where siderosome radii of iron overload patients was between 0.1 and 1.4 μm with the maximum $\sim 0.4 \mu\text{m}$ for the highest iron loads.

Initial experiments were conducted to determine optimum ferritin loading based on the chemistry of ferritin and the aldehyde/latex microspheres. The pH for ferritin coupling that resulted in the highest R_1 (Supporting Information Fig. S1), transverse relaxation rate (R_2) (Fig. 2), and iron concentration (Fig. 2, inset) was 4.53 for all bead diameters. The optimal ferritin loading, however, was different for all three bead sizes (presumably as a result of bead surface area and the number of accessible aldehyde functional groups on the bead surface). The optimal ferritin loading that resulted in the highest R_1 (Supporting Information Fig. S2), R_2 (Fig. 3a), and iron concentration (Fig. 3a, inset) for the 1000 nm beads was determined to be 18.6 mg ferritin per milliliter reaction mixture, resulting in 146 mg iron per liter gel. The most favorable loading concentrations for 400 and 20 nm beads were 46.5 and 37.2 mg ferritin per milliliter reaction mixture, respectively, resulting in 897 and 357 mg iron per liter gel for the 400 and 20 nm beads, respectively. Ferritin loading beyond the optimal level reduced iron concentration of the final sample. This was possibly due to intermolecular interactions between the ferritin proteins at higher concentrations, which decreased their availability to bind to the microspheres. Apoferrinbeads were synthesized as controls using the same apoferritin loading concentrations as the optimal ferritin loading concentrations to ensure the reaction conditions did not influence changes in T_2 , T_1 , or MR image intensity.

Scanning electron micrographs of the 1000 nm beads were taken as a qualitative measure of ferritin coating to ensure protein binding to the surface of the microspheres. Images of the 1000 nm beads are shown in Fig. 3b (1000 nm beads were chosen due to their larger diameter, which gives a better depiction of ferritin coating on the bead surface). The uncoated beads show a smooth surface and have the expected diameter of 1000 nm (left). Beads coated using a nonoptimal ferritin concentration show amorphous, nonuniform coating of the protein (right). Optimally coated beads show a rough surface demonstrating the presence of a protein coating. However, these beads still retain their spherical shape, indicating a relatively homogeneous coating of ferritin (middle).

An important parameter for analysis of clustered particles is bead fraction. The use of fluorescent microspheres allowed for accurate and reproducible bead counting that was not possible for our previous liposomal/ ferritin preparations. Dynamic light scattering could not be attempted due to the insoluble nature of the particles after ferritin attachment. Therefore, 1000 nm fluorescent beads were counted via flow cytometry. Smaller beads could be counted using a hemacytometer and fluorescence microscope. Fluorescent ferribeads of 1000 nm were counted before and after initial cleaning and after ferritin coupling. The overall bead count stayed approximately the same (within 5.0%) throughout the procedure, indicating minimal loss of beads during the cleaning and coupling process. After determining the iron content of the ferritin and ferribeads as well as the bead count, we determined that each 1000 nm ferribead contained 3.8×10^{-11} mg of iron per bead that translates into 2.7×10^{-9} mg ferritin per bead or $\sim 3.7 \times 10^6$ ferritin proteins per bead. Combining this data with the parameters given in the certificate of analysis for the aldehyde/

latex microspheres (5.4×10^6 aldehydes per bead), the total bead coverage is 68.5% of the available aldehyde groups.

Relaxation Time Measurements

Relaxivity (r_1 and r_2) measurements of ferribeads suspended in 0.5% agarose were obtained at 1.41 T (60 MHz) and 37°C and the data are summarized in Table 1. Longitudinal relaxivity (r_1) did not differ between the three ferribead sizes but was approximately three to four times higher than ferritin. Transverse relaxivity using a SE pulse sequence [$r_{2(SE)}$] was highest for the 20 nm ferribeads, while the 400 and 1000 nm ferribeads SE relaxivities were not significantly different from each other. Overall $r_{2(SE)}$ was ~20 to 35 times greater for ferribeads compared with ferritin. Transverse relaxivity using a CPMG pulse sequence [$r_{2(CPMG)}$], however, showed no significant differences between the three different size ferribeads (using a 1 ms interecho spacing). Uncoated beads and apoferribeads showed little or no change in T_1 or T_2 in response to changes in bead concentration.

To determine the effect of interecho spacing (τ) on the transverse relaxation of different size ferribeads, T_2 s were measured (using a CPMG pulse sequence) for five different ferribead concentrations at six different τ values from 0.1 to 5 ms (Fig. 4). The R_2 s for all ferribead sizes varied significantly with interecho spacing. R_2 increases steeply with τ from 0.1 to 0.5 ms and less sharply from 0.5 to 5 ms; the strength of this interaction increases with iron concentration but does not vary for the different ferribead sizes.

We measured T_2^* (using a fid_fit application) but the values were not different between the three ferribead sizes (Supporting Information Fig. S3). On closer evaluation, T_2^* did not change with different ferribead concentrations (where T_2^* should be linear with iron concentration), and agarose alone produced a very short T_2^* .

MR Imaging of Ferribeads

To determine the contrast enhancement of ferribead/agarose phantoms, T_1 - and T_2 -weighted MR images were acquired of different concentrations of ferribeads at 14.1 T (600 MHz; Fig. 5a). As expected, as iron concentration increases, the signal intensity drops due to shorter T_2 for all beads sizes. R_2 increases with iron concentration in a linear fashion until the T_2 of the sample approaches the TE (7.6 ms) of the SE imaging pulse sequence (~0.75 mM Fe concentration; Fig. 5b).

Different sized ferribeads at the same iron concentration (~0.50 mM Fe) were compared at 4.7 and 14.1 T (Fig. 6). Despite identical iron levels for each sample, the T_2 -weighted image intensities (and consequently T_2 s) differ with the biggest differences observed at 14.1 T. The slopes for the three bead sizes differ slightly where smaller ferribead diameters correlate to smaller R_2 (14.1 T): R_2 (4.7 T) ratios (Fig. 6c and Table 2). As expected, the r_2 is greater at the higher field strength for all samples.

DISCUSSION

We have reported the preparation of ferritin-coated liposomes (17). However, ferritin was not covalently attached to the liposome surface, which led to low reproducibility and instability of the structures. These characteristics along with the inability to assess bead fraction prevented further use of liposomal/ferritin preparations as reliable long-term phantoms. Polystyrene latex beads, however, are available in many monodisperse diameters with many different fluorophores and surface functionalities that allow for accurate bead counting and visualization as well as robust covalent protein attachment, providing a more characterizable and stable long-term phantom. Our assessment of phantom stability is based on relaxation time measurements of ferribead/agarose phantoms conducted over the course of 2 weeks to 2 months. In between experiments, samples were stored covered at 4°C. Prior to the continuation of relaxation time measurements, samples that were measured previously were measured again and compared with the original values. All values were less than 5% different from the original relaxation time measurements.

We coupled ferritin to commercially available polystyrene latex beads with three different surface functionalities: carboxylic acids, amines, and aldehydes. Superactive aldehyde-functionalized beads provided the highest degree of covalent ferritin attachment with the least amount of nonspecific binding. The low amount of ferritin on the carboxylic acid- and amine-functionalized beads is likely due to the inefficiency of peptide coupling in aqueous conditions. Multiple coupling conditions and coupling reagents were used to optimize and increase the overall amount of ferritin attachment but no conditions used produced ferribeads approaching the coverage of the superactive aldehyde beads (15 and 5% coverage for carboxyl- and amine- functionalized beads, respectively, vs. 60–80% coverage for superactive aldehyde beads—% coverage based on the number of available functional groups on the bead surface).

To optimize coupling of ferritin to aldehyde/latex beads, the coupling process was tested at pHs ranging from 3 to 9. Above pH 6, very little protein attached to the bead surface whereas acetate buffer at pH 4.5 was optimal. Under these conditions, lysines of ferritin react with aldehydes on the particle surface to form imine bonds. The presence of multiple imine bonds between a single ferritin protein and the aldehydes on the bead surface leads to an overall thermodynamically stable attachment of the protein to the surface of the bead. These results correlate well with the mechanism of imine formation, which is known to be acid catalyzed and proceeds most rapidly at a pH of 4.5.

Initially, bead coating was inhomogeneous and significant amounts of ferritin would detach from the bead surface over time, followed by aggregation. This was due to the labile imine bond cleaving at even slightly acidic pH. The addition of the reducing agent $\text{Na}(\text{CN})\text{BH}_3$ to the buffer reduces the imines to stable amines that are less labile in acidic conditions and do not cleave easily. Furthermore, the synthesis was significantly more reproducible after amine bond formation between three different batches of ferribeads (Table 1). Thus, by optimizing these ferritin attachment conditions, we have produced a stable and reproducible synthetic ferritin phantom compared with previous liposomal/ferritin preparations that varied significantly over time and at different temperatures (4, 25, and 40°C).

After optimizing reaction conditions for the synthetic ferribeads, we determined the effect that ferritin attachment to the bead surface had on phantom properties such as solubility and relaxivity. Immediately after ferritin attachment, the ferribeads became insoluble in aqueous media, which is a characteristic of hemosiderin as well as our previous liposomal/ferritin preparations (17). The transverse relaxivity (SE and CPMG) of the ferribead/ agarose phantoms at 1.5 T was 15–30 times higher than ferritin in solution, as seen in vivo where iron-containing tissues have at least a 3-fold higher relaxation rate than ferritin in solution (30). The increased relaxivity in our system is likely not due to the agarose because ferritin relaxivity in solution and in agarose are the same (31). Instead, it is hypothesized that agglomerated ferritin and/or hemosiderin causes an increase in R_2 (based on outer sphere theory) via water molecule diffusion within the local magnetic field inhomogeneities caused by the superparamagnetic ferrihydrite ($5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$) crystal in ferritin (31–33). The more drastic increase in relaxivity (15- to 30-fold vs. 3-fold) seen with ferribeads (or previously with our liposomal/ferritin preparations) could be due to the homogeneous distribution of ferritin in our phantoms as opposed to the inhomogeneous distribution seen in hemosiderin. In addition, our ferribeads are hollow spheres composed of a hydrophilic latex core decorated with an evenly distributed amount of homogeneous ferritin (previously termed “paramagnetic shells”) while hemosiderin is a dense aggregate of ferritin and an inhomogeneous distribution of its lysosomal breakdown products (previously termed “paramagnetic spheres”) (17). These structural or “morphological” differences could alter the access of water to the ferrihydrite crystal as well as the diffusion of water relative to the particle radius and the interparticle spacing, all of which are currently under investigation.

Multiple models have been described to understand the behavior of ferritin in solution and iron in tissue including: outer sphere theory, the proton exchange dephasing model, and the static dephasing regime model (34–36). The two major components of these models is their predictions of the relationship between $1/T_2$ and field dependence and between $1/T_2$ and interecho spacing of ferritin or iron-laden tissue. It is difficult to fully assess field strength dependence on $1/T_2$ without the use of multiple NMR spectrometers working at different magnetic fields, yet we were able to identify interesting characteristics using 4.7 and 14.1 T MR imaging spectrometers. Although a majority of the literature concerning iron quantitation via MRI (or NMR) uses spectroscopy, we wanted to acquire MR images to show differences in image intensity based on ferritin distribution to determine their feasibility as external calibration phantoms (using a basic imaging SE pulse sequence).

The images and accompanying data presented in Figs. 5 and 6 show there is visible contrast enhancement between ferribeads of different sizes in samples with the same iron concentration (Fig. 6). At high iron concentrations, the R_2 begins to taper off, likely due to the T_2 approaching TE. In addition, while at 1.5 T, the 20 nm ferribeads appear to have the highest relaxivity [$r_{2(\text{SE})}$] and the 400 and 1000 nm are similar, this trend reverses at 4.7 and 14.1 T. At these high field strengths, 20 nm ferribeads appear brighter than the other samples. However, the ratio of $R_{2\text{S}}$ at 14.1 T:4.7 T increases with ferribead size (Fig. 6c). This may be due to a significant increase in bead fraction for 20 nm ferribeads as the amount of ferritin per bead is significantly lower for 20 nm ferribeads compared with 400 and 1000 nm ferribeads. The smaller bead diameter and higher bead fraction result in shorter

interparticle distances. This could decrease the diffusion length relative to the interparticle spacing for 20 nm ferribeads. Additionally, measurements at 1.5 T were performed at 37°C, while those at 4.7 and 14.1 T were performed at 25°C. Thus, a combination of temperature difference and field strength seems to be responsible for this trend reversal. Further studies on temperature and field dependence will be carried out using nuclear magnetic resonance dispersion. Nevertheless, there is a dependence of R_2 on ferribead size at a given iron concentration at all field strengths and temperatures tested.

Although we were unable to gain access to multiple NMR spectrometers, we were able to study the dependence of $1/T_2$ on T. Ferritin in solution is described by the proton exchange dephasing model where there is no effect on $1/T_2$ with respect to T while our ferribead phantoms (and our previous liposomal/ferritin preparations) show significant effects of T on $1/T_2$ (Fig. 4). This is consistent with either outer sphere theory or the static dephasing regime model but without accurate field dependence measurements, it is not possible to fit our ferribeads into a specific model (outer sphere would predict a quadratic dependence of $1/T_2$ with field strength and static dephasing regime a linear dependence). Work is ongoing to determine the effect of field strength on $1/T_2$. In addition, we are studying larger particles (~10 to 20 μm) to mimic the spleen in which ferritin accumulates inside macrophages and is more accurately described by the static dephasing regime model (35).

Another observation we made was the nonmonoexponential behavior (based on the discrepancy in the monoexponential curve fit with the data points) of our ferribead phantoms, especially at higher iron loads. Ideally, transverse relaxation should be fitted by the sum of exponential functions (35,37). The decision was made, however, to use monoexponential curve fitting as the nonmonoexponential behavior was subtle and seen mostly at higher iron loads. Although there have been multiple reports describing models for CPMG- τ data (including chemical exchange, nonexponential, and biexponential), there is no consensus on what model reflects the true behavior of iron in tissue. Each report suggests either using combinations of models or specific models based on tissue type and iron load (34,35). In the future, multiple models will be used to describe the R_2 - τ behavior as CPMG acquisitions at multiple interecho spacings provides a means to probe the interactions between diffusing water protons and iron centers (35).

Finally, by comparing the ferribeads to uncoated beads and apoferribeads, we determined that the presence of the bead alone, or protein-coated beads without iron, has no effect on relaxation times. Normalization of data to bead number showed that relaxation rate is independent of bead number. As expected, longitudinal relaxivity was not significantly affected by bead size or iron concentration.

In conclusion, we have synthesized and characterized ferritin-coated polystyrene beads as long-term synthetic ferritin/hemosiderin mimics. These ferribeads are stable and are easily and reproducibly synthesized. In addition, they embody key relaxometric and physical properties portrayed by hemosiderin deposits in iron overloaded livers. Finally, we determined that spatial distribution of iron determines relaxation rates, indicating that different ferribead sizes can be used to help understand the effects that particle size, ferritin distribution, and bead fraction have on proton relaxation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Schematic representation of the covalent attachment of ferritin to aldehyde/sulfate latex microspheres (ferribeads). Attachment is accomplished via covalent bond formation between aldehydes on the bead surface (left inset) and the lysine amines of the ferritin protein-creating ferribeads. $[\text{Na}(\text{CN})\text{BH}_3]$ is added to reduce the imine to a more stable amine (right inset). Sulfate groups are required for charge stabilization and distribution on the bead surface and were omitted for clarity.

Fig. 2.

Effect of pH on ferritin loading (all microspheres were incubated with 30 mg/mL ferritin) for 400 nm ferribeads suspended in 0.5% agarose gel. (Main graph) R_2 (SE and CPMG) as a function of pH (using acetate buffer) where the highest R_2 corresponds to a pH of 4.53. (Inset) Iron concentration (determined via ICP-OES of acid digested samples) as a function of pH (same samples as main graph) where the highest iron loading corresponds to a pH of 4.53. All measurements were carried out at 1.41 T (60 MHz) and 37°C. Error bars represent $\pm$ one standard deviation (SD) of the mean of two separate samples (from one batch of ferribeads).

one batch of ferribeads). **b:** Scanning electron micrographs of 1000 nm ferribeads incubated with (or without = control) 18.6 or 55.8 mg/mL ferritin. Scale bars represent 500 nm.

Fig. 4.

R_2 as a function of interecho spacing for 20, 400, and 1000 nm ferribeads suspended in 0.5% agarose gel. Data were acquired at 1.41 T (60 MHz) and 37°C. Error bars represent ± 1 SD of the mean of two separate samples (from one batch of ferribeads).

Fig. 5.

MR images and image-based r_2 s (SE) of different concentrations of 20, 400, and 1000 nm ferribeads suspended in 0.5% agarose gel. MR images (a) and R_2 analysis (b) of multiple concentrations of 20, 400, and 1000 nm ferribead/agarose phantoms at 14.1 T and 25°C. **a:** Images were acquired using a SE pulse sequence with TE = 7.6 ms and TR = 1000 ms using the following parameters: field of view = $20 \times 20 \text{ mm}^2$, matrix size = 128×128 , number of axial slices = 5, slice thickness = 1.0 mm, interslice distance = 1.5 mm, and averages = 4. Scale bar = 400 μm . **b:** R_2 s of MR phantoms from a, error is the width of the data point and

equals ± 1 SD of the mean of five separate axial slices taken of the same samples. R_2 plateaus as the T_2 of the samples approaches TE (~ 0.75 mM Fe).

Fig. 6.

Magnetic field dependence of R_2 for 20, 400, and 1000 nm ferritebeads suspended in 0.5% agarose gel at 14.1 and 4.7 T (25°C). MR images (a), R_2 s (b), and R_2 ratios (c) of 0.50 mM Fe samples from Fig. 5 (1000 nm ferritebeads = 0.46 mM Fe) of 20, 400, and 1000 nm ferritebeads. **a:** 14.1 T Images were acquired using the same parameters as Fig. 5a, and 4.7 T images were acquired using a SE pulse sequence with TE = 20 ms and TR = 1000 ms using the following parameters: field of view = $40 \times 40 \text{ mm}^2$, matrix size = 256×256 , number of axial slices = 5, slice thickness = 1.0 mm, interslice distance = 1.5 mm, and averages = 2.

Scale bar = 400 mm. **b:** R_2s at 14.1 T versus R_2s at 4.7 T of MR phantoms from **a**, error bars represent 6 1 SD of the mean of five separate axial slices taken of the same samples (if error bars are not visible, they are the width of the data point). **c:** The ratio of R_2s at 14.1 T versus R_2s at 4.7 T.

Table 1
Relaxivities (r_1 and r_2 s) of 20, 400, and 1000 nm Ferribeads Suspended in 0.5% Agarose Gel

	Relaxivity (mM Fe ⁻¹ s ⁻¹)			r_2/r_1 ratio		
	r_1	r_2 (SE)	r_2 (CPMG)	r_2 (SE)/ r_1	r_2 (CPMG)/ r_1	
20 nm Ferribeads	0.067 ± 0.017 ^a	4.92 ± 1.18	2.12 ± 0.184	73.5 ± 0.988	29.1 ± 0.863	
400 nm Ferribeads	0.054 ± 0.011	2.92 ± 0.190	2.42 ± 0.594	54.8 ± 7.96	41.4 ± 6.20	
1000 nm Ferribeads	0.069 ± 0.016	3.08 ± 0.106	2.22 ± 0.332	45.9 ± 11.9	34.8 ± 0.968	
Ferritin ^b	0.017	0.14	0.17			

All data was obtained at 1.41 T (60 MHz) and 37°C.
SE, spin echo pulse sequence; CPMG, Carr-Purcell-Meiboom-Gill pulse sequence.

^aError bars represent ± one standard deviation of the mean of three separate batches of synthetic ferribeads.

^bStock ferritin solution suspended in 0.5% agarose. Measured once.

Table 2

MR Image–Based Relaxivities (r_1 and r_2) of 20, 400, and 1000 nm Ferribeads Suspended in 0.5% Agarose Gel at 14.1 and 4.7 T (25°C)

	4.7 T (25°C)		14.1 T (25°C)	
	r_1 (mM Fe ⁻¹ s ⁻¹)	r_2 (mM Fe ⁻¹ s ⁻¹)	r_1 (mM Fe ⁻¹ s ⁻¹)	r_2 (mM Fe ⁻¹ s ⁻¹)
20 nm Ferribeads	0.0709 ± 0.005	29.88 ± 3.14	0.0737 ± 0.006	74.59 ± 10.4
400 nm Ferribeads	0.0773 ± 0.006	37.26 ± 3.23	0.0657 ± 0.005	113.2 ± 11.6
1000 nm Ferribeads	0.0695 ± 0.005	28.42 ± 2.96	0.0657 ± 0.006	100.4 ± 11.1

r_2 s were calculated based on the plot of the R_2 (SE) of an apoferritebead/agarose phantom (no iron control) and the 0.50 mM Fe ferribead/agarose phantom from Fig. 6. r_1 s were calculated based on the plot of the R_1 (saturation recovery) of an apoferritebead/agarose phantom (no iron control) and the 1.25 mM Fe ferribead/agarose phantom from Fig. 6.