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# Synthesis of Iron Nanoparticles for Enhanced MRI Contrast Agents

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# Abstract

Iron nanoparticles (metal and oxides) are being heavily researched for biomedical applications including hyperthermia therapies and MRI contrast agents. Synthesizing metal iron nanoparticles would take advantage of its high room temperature saturation magnetization, shorter relaxation time, and large magnetic moment to improve MRI technologies by increasing resolution and providing a substitute to patients unable to use current Gd contrast agents. Preserving the edge iron metal nanoparticles have over their iron oxide counterparts is particularly difficult as iron metal is extremely susceptible to oxidation. Iron metal nanoparticles have been previously synthesized, but the methods used to prevent oxidation rendered the nanoparticles nonbiocompatible. The goal of this work was to produce a metal iron nanoparticle to be used as an MRI contrast agent.

Iron metal nanoparticles and superparamagnetic iron oxide nanoparticles were successfully synthesized. The samples are a result of three batches created using a reduction method with various coatings and techniques. These results were confirmed by Mössbauer and TEM. Cell toxicity was evaluated to ensure the biocompatibility required for medical applications. The iron metal sample was evaluated three years post synthesis to determine the rate of degradation. It was found to have mostly oxidized with some remnants of iron metal. This result is significant in that it did not completely oxidize; this verifies the stability of the coated nanoparticles in air.

**Keywords:** Iron nanoparticles, Mössbauer Spectroscopy, MRI contrast agent, Reduction synthesis, Magnetic nanoparticles, Biomedical applications.

# Introduction

Nanoparticles containing iron, including metallic iron (Fe), maghemite ( $\gamma$ -Fe<sub>3</sub>O<sub>4</sub>) and magnetite (Fe<sub>3</sub>O<sub>4</sub>), are being heavily

researched for their applications in medicine, including magnetic hyperthermia and Magnetic Resonance Imaging (MRI) [1]. Iron nanoparticle contrast agents offer improvement to current MRI technologies in use and can be used in combination with hyperthermia therapies to precisely treat tumors. Image-guided hyperthermia treatments have shown to be an efficient diagnostic and therapeutic tool in recent oncology clinical trials [2].

MRI contrast agents are fast-relaxing superparamagnets which shorten the relaxation time of surrounding protons. They typically reduce T2 and T2\*-weighted relaxation times resulting in a negative contrast. This spin-spin relaxation is due to induced local field inhomogeneities and is described by relaxivity R2 (1/T2). The relaxivity of the particles is associated with their magnetic susceptibility as a function of particle size and composition [3]. Metallic iron has the highest room-temperature magnetic susceptibility of any element, giving it the strongest response to an applied field [4]. Along with a low magnetocrystalline anisotropy, metallic iron has more magnetism per unit volume than nickel, cobalt or its oxide counterpart. It also exhibits a greater magnetic moment than any other metal [4].

Induced hyperthermia for cancer treatment is another significant contribution magnetic nanoparticles can make to medicine. They are introduced directly at the tumor or through systemic injection. The high magnetic saturation of the nanoparticles would allow them to absorb energy from an alternating magnetic field and convert it to heat through hysteresis losses or Néel relaxation [5]. The hypoxic environment of tumor cells cause them to be more sensitive to heat than normal cells [6]. This allows the tumor to be eliminated with minimal collateral damage to surrounding tissue [5].

Since nanophase toxicity is widely disputed, it is extremely important to test the iron nanoparticles thoroughly before putting them into use [7]. The nanopartricle medium must be

biocompatible to serve as a viable hyperthermia or MRI contrast agent. Producing metallic iron nanoparticles in a form that can be used in biomedical applications is challenging due to the risk of oxidation in biocompatible solvents [8].

Previous researchers have successfully made metallic iron nanoparticles, but they were embedded in layers, such as graphene oxide sheets [9] and polymer layers [10] or onto surfaces [11]. For clinical use it is necessary to make free, metallic iron particles which can be made into a suspension for injection into a patient's blood stream.

To achieve MRI contrast, materials must have high saturation magnetization [12]. Metallic iron has a much greater saturation magnetization ( $\mu_0 M_s = 2.16$  T) than iron oxide (0.54 T) and would allow for less contrast medium to be used while achieving better resolution [13]. Hence iron is a highly attractive option of nanoparticles for medical applications and is the subject of this paper.

Another important characteristic is size as size affects magnetic moment and the response magnetic nanoparticles have to external magnetic fields. A decreased magnetic nanoparticle (MNP) size will cause a faster relaxation time, which will increase their heating rate. This increased heating rate will make smaller sized nanoparticles more efficient as an MRI contrast agent and hyperthermia therapy [8].

# Methods

#### Participants

Iron nanoparticles were synthesized and subsequently characterized using Transmission Electron Microscopy at the Center for Nanophase Materials at Oak Ridge National Laboratory, Oak Ridge, TN. Cytotoxicity Studies were performed at Vanderbilt University, Nashville, TN. Mössbauer Spectroscopy was performed at The University of Tennessee Space Institute, Tullahoma, TN.

#### Materials

Iron (II) chloride (Sigma Aldrich), ethanol (Decon Labs), sodium borohydride (Sigma Aldrich), Brij 30 (Sigma Aldrich), and polyethylene glycol 600 diacid (Fluka Analytical) were used in the synthesis of iron nanoparticles. Phosphate buffered saline, trypsin, minimum essential media with l-glutamine, trypan blue, and luciferin were used in cytotoxicity studies.

#### Procedure

**Synthesis:** Samples were synthesized by reacting 2.4 g iron (II) chloride and 30 mL pure ethanol with 1.2 g sodium borohydride and 30 mL deionized water. The two mixtures were collected into 30 mL syringes and placed in a dual syringe pump; the pump was set to a rate of 5 mL per minute. The nanoparticles were formed when the borohydride solution interacted with the chloride solution by reducing it. The reduction reaction continued down the separatory funnel and into a mixture of 10 mL Brij 30 and 50 mL ethanol as seen in Figure 1.

Sample A was synthesized without a separatory funnel; the reduction reaction was performed in a 250 mL three neck round flask before falling into the same coating mixture. Sample B was produced using the separatory funnel but with polyethylene glycol 600 diacid directly replacing the Brij 30 as a coating. The particles were stirred at 250 rpm for 30 minutes to break up the agglomerations and sufficiently coat the particles.



Figure 1: Sample C Synthesis

**Washing:** The coated particles were centrifuged at 9000 rpm for 10 minutes and washed with ethanol twice then transferred to a sample jar and sonicated for 45 minutes to break up agglomerates.

# Characterization

**Transmission electron microscopy:** Transmission electron microscopy (TEM) was performed using a Zeiss Libra 120. Three samples were prepared by diluting the nanoparticles with ethanol until the solution was barely tinted. A copper TEM grid was dipped into the solution and dried in air. Sample A, B, C and D were characterized using this technique.

**Cytotoxicity:** Cytotoxicity studies were performed using varying concentrations of Sample C. Sample C was selected for this experiment because it appeared the most promising with respect to size from TEM results (Figure 2); ideal diametersare 3-7 nm. Mössbauer had not been completed at this time but Sample C was later determined to possess metallic iron (Figure 7).

Luc-231 cells, human breast cancer MDA-MB-231 cells that are stably transfected to constitutively expressed firefly luciferase, were cultured in DMEM (Gibco, Carlsbad, CA) with 10% FBS (Gibco, Carlsbad, CA0 and 50  $\mu$ g/mL gentamicin (Gibco, Carlsbad, CA). Cells were isolated by incubation for 5 minutes in 0.05% trypsin. Isolated cells were centrifuged at 1000 rpm for 5 minutes in conical tubes to separate the cells from the media. The supernatant was discarded and cells were re-suspended in DMEM. The concentration and viability of cells were assessed using trypan blue and measured in a hematocytometer. Cells



Figure 2: TEM of iron nanoparticles Samples C, A and B respectively



Figure 3: Cytotoxicity studies at 17 hours, 24 hours and 48 hours

were cultured in 96 well plates with an initial seeding density of 50,000 cells per  $cm^2$ .

Iron nanoparticles were added to the cells at various dilutions and untreated wells served as controls. Nanoparticle concentrations were estimated by volume. Nanoparticles were in contact with cells for 17, 24 or 48 hours prior to the replacement of cell culture medium with DMEM containing 1:200 luciferin. Plates were imaged using a Xenogen IVIS 200 imaging system and the luminescence was measured in radiance (photons). Cell growth suppressed by iron nanoparticles was detected by a reduced luminescence intensity relative to untreated controls in direct proportion to cell number and metabolism.

**Mössbauer Spectroscopy:** The Mössbauer Spectroscopy experiments were performed at 6 and 9 Kelvin (K) to avoid potential relaxation effects. Nanoparticles below a critical size are superparamagnetic at room temperature and can yield a non-Zeeman split spectrum. Sample A, suspended in ethanol, was dried in air and run at 9 K and again at room temperature (25° C). Sample C, also in ethanol, was dried in air and run at 9 K and again at 6 K three years post synthesis. Sample B in ethanol was dried in air and run at room temperature.

An iron calibration spectrum was obtained from the Mössbauer Spectrometer to compare with our synthesized samples. A <sup>57</sup>Co source was used for the calibration and sample runs. The chemical isomer shift of metallic iron is 0 mm/s. The hyperfine field is shown by the distance between the first and last lines.

Hyperfine fields are the measure of the internal field in the nucleus of a particular iron atom. A metallic iron atom has a hyperfine field of 330 kG, which will Zeeman split and span the spectrum between  $\pm 5.1$  mm/s. However, the oxides have a much

larger field (anywhere from 490 kG upwards), which will span the spectrum over a range  $\pm 8$  mm/s.

# Results

# Characterization

**Transmission electron microscopy:** TEM results show that the diameter of the iron core for Sample A is 8 nm, 3 nm for Sample C, and 20 nm for Sample B (see Figure 2). TEM also indicates all the nanoparticles are coated and agglomerated. From these results, we concluded that the longer reaction time created by using the separatory funnel produced a smaller iron core. The Brij 30 appeared to be a superior coating over the polyethylene glycol 600 diacid because coated Sample B was 50 nm in diameter - which is too large for our desired product.

**Cytotoxicity:** Cytotoxicity studies of Sample C were carried out and analyzed at Vanderbilt University. The two cell plates used were imaged using an *in vivo* optical imaging system. It measured the luminescence of the wells in radiance (photons). The higher radiance in the wells represents more surviving cells. As seen in Figure 3, the brightly colored cells (red, yellow, and green) had excellent survival rates and the dark wells (dark blue and black) represent cell death. The process was repeated for the 24 hour and 48 hour time points for both plates. A summary of these results can be seen in Figure 4 with the luminescence of untreated control samples normalized to 100.

Cell proliferation and viability was comparable to untreated and ethanol controls for the 1:640,000 iron nanoparticle dilution and all incubation durations, suggesting the lack of acute or chronic toxicity at this dose. Toxicity was broadly proportional to iron nanoparticle dose with significant cell death at elevated concentrations of iron nanoparticles, which was especially



# Cytotox Summary

Figure 4: Summary of cytotoxicity results

Dilution

evident for dilutions less than approximately 1:10,000. Toxicity was not consistently different as a function of incubation duration, suggesting a rapid suppression of cell metabolism at high iron nanoparticle doses.

With a dilution rate of 1:640,000 by volume, the cells had a good survival rate compared to the ethanol control. At 24 hours, the average radiance of cells diluted to 1:640,000 was 90; by comparison, the ethanol control had an average radiance of 68 at the same time point. The 1:400,000 dilution rate represented a median amount of cell survival. These results indicate that the sample is not toxic to cells at a diluted rate and can intentionally kill cells (if desired) at higher concentrations.

Toxicity of concentrated iron nanoparticles could also serve as a treatment for inoperable brain tumors. In this treatment, uncoated iron nanoparticles would be directly injected into the tumor. Toxicity of the uncoated nanoparticles would cause cell death to the tumor and not to the surrounding healthy cells [6].

Mössbauer Spectroscopy: In a paramagnetic material, spins are oriented when an external magnetic field is applied. This is reversible, i.e. the spins are disoriented when the magnetic field is removed. Magnetically ordered materials (ferromagnets, ferrimagnets) are composed of domains consisting of interacting spins, which are oriented when an external magnetic field is applied. But when the magnetic field is removed the domains are not completely disoriented (Hysteresis). MNPs that are the size of a single magnetic domain respond to an external magnetic field but do not become permanently magnetized, i.e. they are superparamagnetic [14]. The domain magnetic moments fluctuate with a relaxation time. Magnetite, or Fe<sub>3</sub>O<sub>4</sub>, has a domain size of 15-80 nm [14].

The detection of Fe<sup>3+</sup>in Mössbauer would represent oxidation. Any particles smaller than 10 nm could be superparamagnetic and would therefore present as a broad line. This could split into 6 lines at low temperatures when the relaxation has slowed down.

Sample A dried in air and run at room temperature was depicted as a broad singlet (see Figure 5, and confirms the particles are small (around 10 nm in size). However, it is unlikely sample A contains nanoparticles less than 5 nm in diameter- the peak would appear more narrow. This is confirmed by TEM (Figure 2). Sample A at 9 K mainly presents as oxide, specifically  $\gamma$ -Fe<sub>2</sub>O<sub>2</sub> in the spectra seen in Figure 6. Sample B yielded mostly oxides



Figure 5: Mössbauer spectroscopy of iron nanoparticle Sample A at room temperature



Figure 6: Mössbauer spectroscopy of iron nanoparticle Sample A at 9 K

according to Mössbauer spectroscopy.

The spectra of Sample C, run at 9 K in the cryostat (Figure 7) closely matches the iron calibration spectra ( $\pm$ 5.2mm/sec or 330 kG) but with broader lines. Sample C was run again three years after synthesis, having been stored in a sealed vial and kept in air over this period, at 6 K, room temperature and with a 10 kG field. The results can be seen in Figure 8. Three years post-synthesis the sample had maintained 16% metallic iron. The remainder of the sample had mostly oxidized to  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> resembling Sample A. We believe the smaller iron metal nanoparticles fully oxidized to  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> and the larger iron metal nanoparticles maintained a metallic core with a  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> shell. The preservation of metallic iron could be attributed to Sample C's longer reaction time and/or the efficiency of the coating. The 10 kG spectra at room temperature confirms Sample C contains nanoparticles due to its large susceptibility.



Figure 7: Mössbauer spectroscopy of iron nanoparticle Sample C at 9 K



**Figure 8:** Mössbauer spectroscopy of iron nanoparticle Sample C ata) 9K after synthesis, **b**) 6 K three years post synthesis, **c**) room temperature three years post synthesis, and **d**) room temperature with a 10 kG field three years post synthesis. Red lines represent iron, green lines represent superparamagnetic  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub>, and blue lines represent intermediate size particles.

#### Conclusions

The characterization results show that small nanoparticles (3-20 nm) were successfully synthesized, most likely due to a longer reaction time. Synthesis utilizing the separatory funnel resulted in smaller particles than those synthesized without the funnel, which could be due to a longer reaction time or faster synthesis rate. The polyethylene glycol 600 diacid coating led to more cross linkage and larger particles.

We attribute the agglomeration of samples to the nature of the synthesis – the particles form as agglomerates during the reaction due to the synthesis rate (in both uncontrolled and controlled by a syringe pump). We also believe the coatings utilized to ensure biocompatibility and prevent oxidation bind the agglomerates together. When the agglomerates are broken up via sonication, the coating is disrupted and the particles are left vulnerable to oxidation.

Cytotoxicity results show that cells are able to survive when introduced to the iron nanoparticles at low concentrations. At higher, intense concentrations, the cells did not survive; this is advantageous if the nanoparticles are intended for direct injection into diseased cells.

Mössbauer Spectroscopy shows that Sample A, syntheized with a short reaction time, contains superparamagnetic  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> and metallic iron was produced in Sample C with a longer reaction time. The most promising results are the discovery of metallic iron nanoparticles and the presence of metallic iron after three years of storage in air.

#### **Future Work**

Future work should include the exploration of different coatings, sonication before coating, or an entirely different synthesis procedure to prevent any agglomeration. Solving the agglomeration issue is necessary for biomedical applications as the agglomerated clumps of iron act as one large particle; this affects the magnetic properties of the product and clearance from the body. Both the magnetic properties and size distribution of the nanoparticles are important for use as MRI contrast agents; the nanoparticles should be small (less than 10 nm) and monodispersed. We will also study the reasons for different oxidation rates over time between the samples. Once these issues are addressed, we will optimize the iron nanoparticles for hyperthermia and MRI and test them in a synthetic cadaver brain.

#### **Author Note**

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