Magnetic Characterization of Ferritin

Honors Thesis

By

Kevin Zeng

Undergraduate in Biomedical Engineering

The Ohio State University

Thesis Committee:

Dr. Gunjan Agarwal, Advisor

Dr. Mark Ruegsegger

Table of Contents

Acknowledgements

I would like to thank Tanya Nocera for guiding me throughout the entire process and teaching me the necessary lab techniques. Tanya has been a great source of knowledge and was always there when I needed help. Her enjoyment of research really helped motivate me through the entire project.

I would also like to thank Dr. Gunjan Agarwal for giving me the opportunity to work in her lab on this project. I could always depend on her if I ever needed any guidance or encouragement. I learned a lot about experimentation and scientific writing under her tutelage. Not only that, but she also created a very enjoyable research environment that bolstered my interest in research.

Finally, I want to thank both Tanya Nocera and Dr. Gunjan Agarwal again for giving me their time and an amazing research experience.

List of Figures

List of Tables

Abstract

Many diseases including hemochromatosis, chronic anemia, and cirrhosis result in abnormal levels of iron stores. Due to its correspondence with different physiological conditions, iron content is often used as a disease marker. 30%-50% of iron storage within the body is performed by the protein ferritin. Currently, there are indirect methods of measuring iron content using immuno-detection of iron binding proteins. The objective of this project is to develop a highly sensitive technique that can directly measure iron content based upon the magnetic character of iron; the first step is to understand the magnetic characterization of ferritin. Magnetic force microscopy (MFM), a mode of the atomic force microscope (AFM), has been shown by other research groups and our own group to be a promising method of characterizing magnetic particles. Our research uses this method to characterize ferritin in its iron bound and unbound states (termed apoferritin). MFM uses a magnetized probe to record topographical and magnetic information from samples. We compare the widely used medium moment probe to the less widely used high moment probe for our project; high moment probes were found to detect ferritin proteins with greater sensitivity than medium moment probes. Our work also reveals that MFM can characterize and differentiate between ferritin and apoferritin. MFM phase data is used to measure the iron content of our ferritin samples. The high resolution and sensitivity of MFM makes it a promising method for measuring iron content in blood samples.

Introduction

Abnormal levels of iron storage have been linked to many disorders. Low iron levels can indicate anemia, hypothyroidism, and other deficiencies, while excess iron levels may be caused by hemochromatosis, adult-onset Still's disease, and cirrhosis [1-3]. Because of body iron content's correlation to many diseases, it is often used as a marker for various physiological conditions. Up to fifty percent of the body's iron stores lie in ferritin, a globular intracellular protein that regulates body iron content by controlled loading and release. Ferritin stores up to 4500 iron atoms in its hollow core as shown in Figure 1.a [4]. The iron is stored in the Fe(III) oxidation state and is released when it is changed to the Fe(II) oxidation state. When the ferritin protein is in its unbound state, it is termed apoferritin (Figure 1.b [5]).

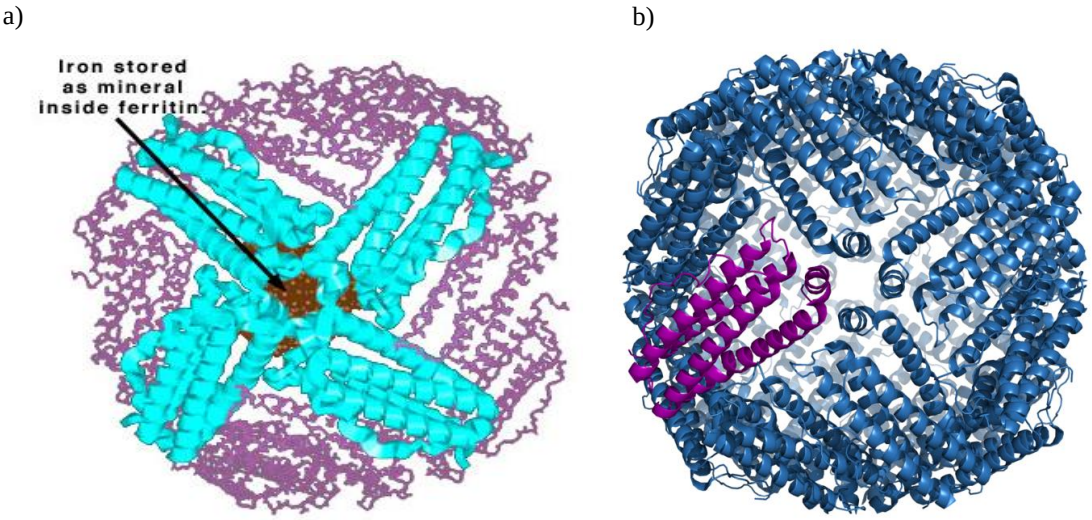

Figure 1. a) Ferritin protein with iron stored in its hollow core [4]. b) Apoferritin protein, ferritin without bound iron [5].

The amount of ferritin present in body is usually proportional to the amount of stored iron. There are a few currently available tests that exploit this proportionality through immuno-

detection of ferritin. Serum ferritin test is one of the most commonly used iron content assays, but it has limitations. Serum ferritin tests can be unreliable as an iron content indicator because serum ferritin levels are affected by inflammation, infection, and ascorbate deficiency [6-8]. Under these conditions, ferritin may be upregulated in the apoferritin(ironless) state, or with lower or higher than normal iron atoms bound to its core. Serum ferritin test measures the amount of ferritin in a sample (both apoferritin and iron bound ferritin), but cannot distinguish between the two. In these cases, the amount of ferritin (apo and iron-bound) measured by the serum ferritin test will not be proportional to the amount of iron in the body.

Our goal is to develop a procedure that will directly measure the iron content in blood based upon the magnetic character of iron rather than immuno-detection. Current iron magnetism-based techniques include MRI and biosusceptometry, but these two methods are not sensitive enough as they can only detect large quantities on the order of milligrams, while body iron content is on the order of micrograms. Superconducting quantum interference device (SQUID) magnetometry is another method of measuring Fe content, but due to its complexity and cost, its usage is limited. From previous studies, it was found that magnetic force microscopy (MFM), an atomic force microscopy technique, can detect and quantify magnetic moments of single and aggregate superparamagnetic nanoparticles (SPNs) [9-11]. In this study, we employed a similar MFM technique to characterize ferritin and apoferritin at the single particle level.

MFM is a specialized atomic force microscopy (AFM)-based technique that maps the magnetic force gradient above a sample surface without making direct contact with the sample. This is performed using a two pass technique termed LiftMode. In LiftMode, a probe makes two passes over one area of a sample. The first pass collects topographical (height) data, while the

second pass uses this data to maintain the probe tip at a constant specified height (lift height) above the sample surface for long-range magnetic force data collection (Figure. 2a). The magnetic field gradient above the sample induces changes in the cantilever's resonance frequency or phase, which is recorded as an amplitude or a phase shift measurement (Figure 2b [12]). These measurements are then compiled by the AFM program and rendered as an image. We can use the phase shift measurements to analyze magnetic moment strength and iron content of the sample.

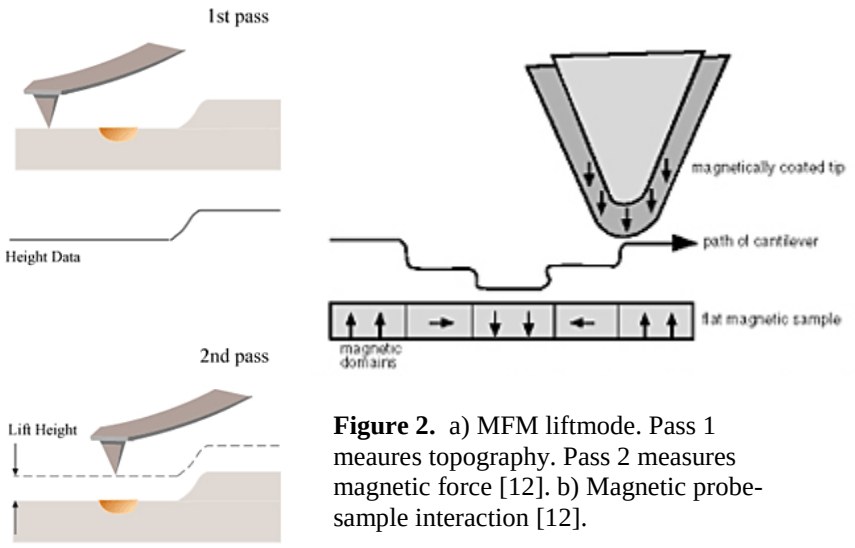

Figure 2. a) MFM liftmode. Pass 1 meaures topography. Pass 2 measures magnetic force [12]. b) Magnetic probe-sample interaction [12].

The magnetic force data is collected by a probe coated with a ferromagnetic thin film. Multiple types of magnetic probes can be utilized for MFM imaging. Most current research employs medium moment (1e-13 emu) probes. Less widely used are high moment (3e-13 emu), probes, which are have a stronger magnetic moment and therefore should be more sensitive to interaction with magnetic samples [9]. This study will utilize both probe types and compares

their relative abilities to collect magnetic data from ferritin. We then use MFM to detect and characterize the magnetic moments and iron content of ferritin, apoferritin and mixed samples.

Objective 1: *Compare the relative abilities of high magnetic moment probes and medium magnetic moment probes to image ferritin using magnetic force microscopy*

Objective 2: *Use MFM to distinguish ferritin from apoferritin, and characterize the proteins' magnetic properties.*

Methods

Sample Preparation

Ferritin, apoferritin, and mixed samples were synthesized using a series of dilutions. The ferritin samples were produced from a stock solution of 85 mg/mL horse spleen ferritin (SIGMA). 11.76 uL of the base solution was diluted with 1 mL DI water and then vortex mixed (Vortex Genie Scientific Instruments) for 10 seconds. 0.5 uL of the new solution is diluted with 1 mL distilled water and then votex mixed for another 10 seconds. The vortex mixing is crucial to sample preparation because it separates conglomerates of ferritin into individual proteins. This procedure creates a final ferritin concentration of 0.5 ug/mL. The apoferritin samples are produced from a stock solution of 47.4 mg/mL horse spleen apoferritin (SIGMA). There are two dilutions in which the stock solution of apoferritin is first diluted to 0.55 mg/mL and then to 0.22 ug/mL. 10 seconds of vortex mixing was performed after each dilution. The mixed apoferritin/ferritin sample was created by mixing equal amounts of the apoferritin and ferritin samples; the combination was then vortex mixed for 10 seconds. The final concentrations were chosen to minimize formation of large conglomerates so that individual proteins can be characterized. 30 uL of each liquid sample was immediately aliquoted onto freshly cleaved mica

substrate (ruby muscovite, S&J Trading) and was allowed to dry overnight under ambient conditions.

Magnetic Force Microscopy

MFM imaging was conducted using a Multimode AFM in conjuction with a Nanoscope IIIa controller and Quadrex Extender (Digital Instruments). The proteins were imaged with an applied magnetic field of ~2000 G, emitting from the magnet at the base of the Multimode AFM [9]. We used NSC18/CoCr (Micro Masch) medium moment probes and CoCr ASYHMFM (Asylum Research) high moment probes. To increase the magnetic sensitivity, each probe was pre-magnetized with a permanent magnet for ~1 minute. The medium moment probes were auto-tuned to a resonance frequency of approximately 70 kHz with a 5% offset for main controls and 0% offset for interleave controls. The high moment probes were auto-tuned to a resonance frequency of approximately 90 kHz with the same offset settings as the medium moment probes. Tapping mode was used at a scan rate of 2 Hz and with 512 lines/scan direction. Topographical height images were collected in the main scan while the phase images were collected in the interleave lift mode at various lift heights (10 nm – 100 nm). Images collected from the AFM were flattened and analyzed using the section analysis tools in the NanoScope Analysis software (Digital Instruments). Multiple independent experiments were conducted per sample type and n = 50 particles from each sample type were analyzed.

Results

MFM of Ferritin

Magnetic force microscopy was performed on ferritin to test if it was a viable technique for quantifying iron levels in blood. The average diameter (d) measured by medium moment

probes was 95.9 nm, while the average diameter measured by high moment probes was 105.6 nm. The diameters are inflated because in tapping mode, the lateral dimension is distorted due to the tip-sample convolution effect. The true diameter (2r) of our ferritin protein was found using the geometric de-convolution equation [9].

$$d \approx 4\sqrt{Rr} \quad (1)$$

The average radius (R) of our high moment MFM probe was ~110 nm, as denoted by the manufacturer. We found that the true diameter of the ferritin particle was ~7.2 nm. Data for ferritin was collected with both medium moment and high moment probes examining whether high moment probes were significantly more sensitive than their medium moment counterparts. The images in Figure 3 compares phase and height images collected at different lift heights with medium moment probes to those collected with high moment probes. To quantify the difference in sensitivities of the two probes, we measured the phase shift of individual ferritin particles and plotted the average values against the lift height in Figure 4.

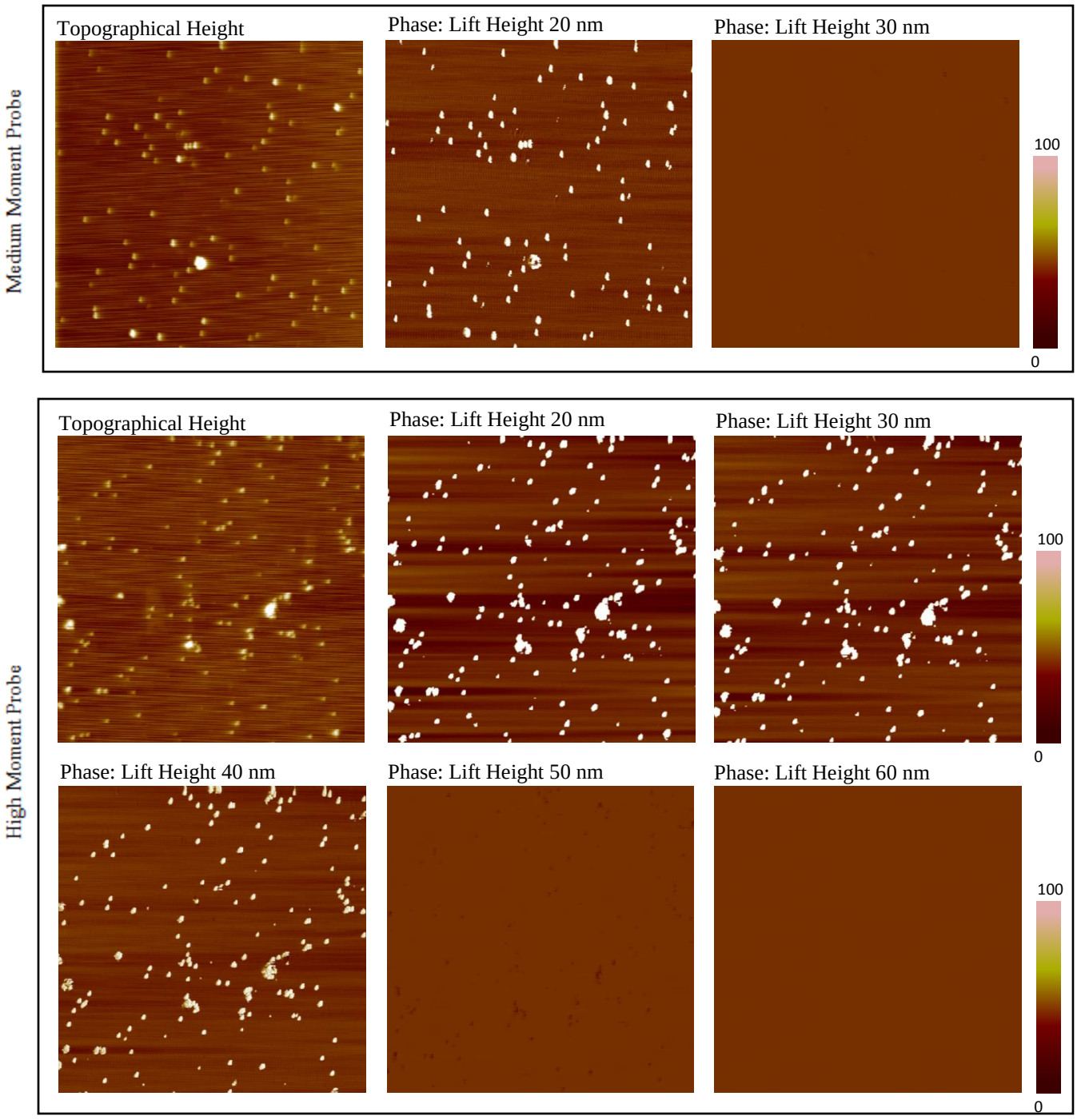

Figure 3. a) Height and phase shift images of ferritin captured at different lift heights using medium moment probe. b) Height and phase shift images of ferritin captured at different lift heights using high moment probe. These images indicate that the MFM technique is a viable means of detecting the magnetic moment of ferritin. Both samples indicate that the phase shift signal decreases as lift height increases, but the signal of the medium moment probe decreases at a much higher rate. It is possible to collect phase images up to 30 nm lift height using the medium moment probe, while high moment probes can capture images at up to 60 nm lift height. Height image qualities are similar.

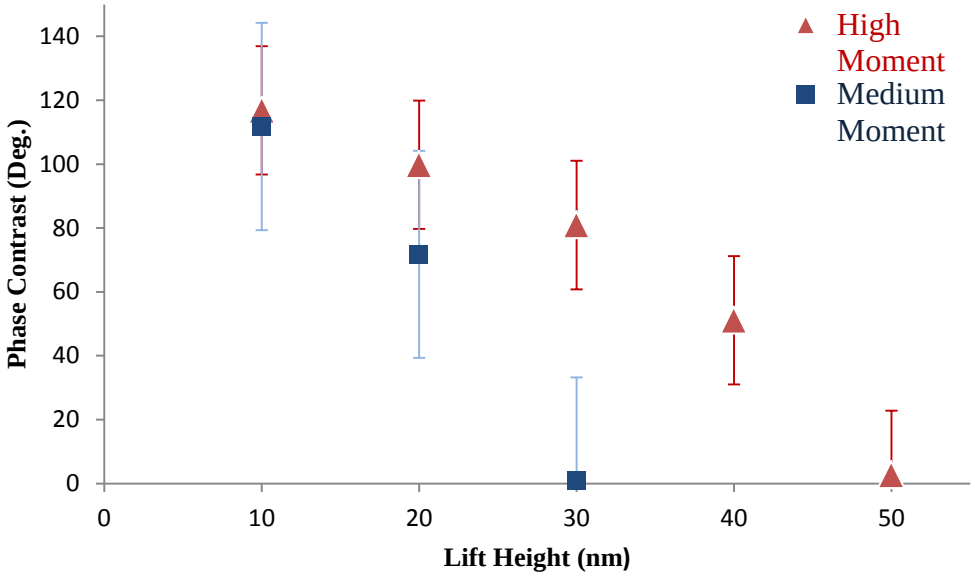

Figure 4. Plot of phase shift data as a function of lift height collected using high moment and medium moment probes. Similar to the graphical data, the numerical data shows a decreasing relationship between lift height and phase shift. While both medium moment and high moment probes start at similar phase values, medium moment probes lose their ability to image at 30 nm while high moment probes loses its ability at 60 nm.

As shown by the quantitative and graphical data, although the phase shift of both medium and high moment probes decreases as lift height increases, the maximum lift height at which signals could be measured is much greater for high moment probes (50 nm) than medium moment probes (30 nm). Because of the significant difference in signal detection, the rest of our experiments were conducted using the high moment probe.

MFM of Apoferritin

To understand the differences between ferritin in its bound state and unbound state, we also analyzed apoferritin with the MFM technique. The height image and the phase images are shown in Figure 5.

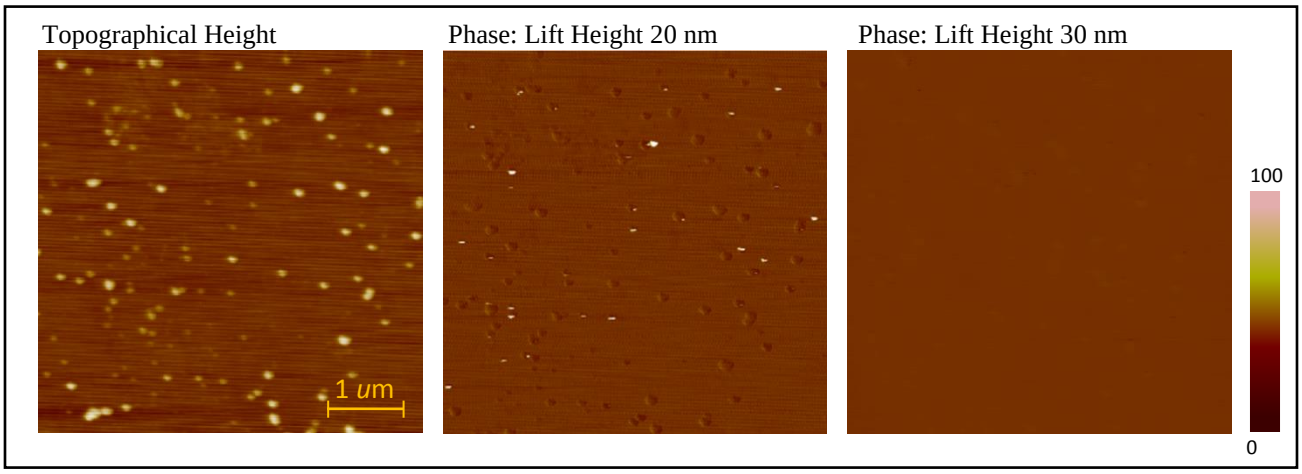

Figure 5 Height and phase shift images of apoferritin at various lift heights collected with a high moment probe. Apoferritin phase shift decreases much faster than ferritin.

The average diameter found for apoferritin was similar to that of ferritin at 7.4 nm. Figure 6 compares the phase shifts of ferritin and apoferritin as a function lift height.

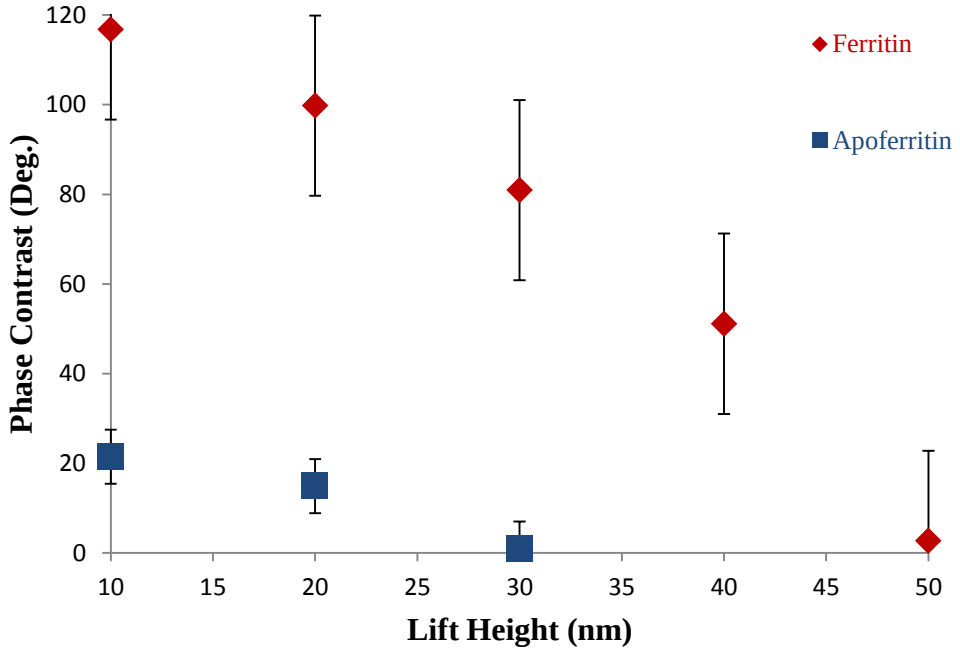

Figure 6. Phase shift plot over lift height for ferritin and apoferritin. As the graphical data suggests, the magnetic moment of apoferritin is much lower than that of ferritin.

MFM of Mixed Apoferritin and Ferritin

A sample where ferritin and apoferritin was mixed was imaged using the MFM technique because in humans, apoferritin may exist in the bound and unbound states simultaneously. Figure 8 compares the height and phase images of the same sample at 20 nm lift height, where ferritin and apoferritin can be easily distinguished. Every ferritin particle bound or unbound shows up as a bright dot on the height image, but only iron loaded ferritin will be bright on the phase image. In Figure 7, yellow circles denote ferritin in their respective height and phase images while red circles denote apoferritin. The average true diameter of the ferritin and apoferritin were 7.3 nm and 7.4 nm respectively.

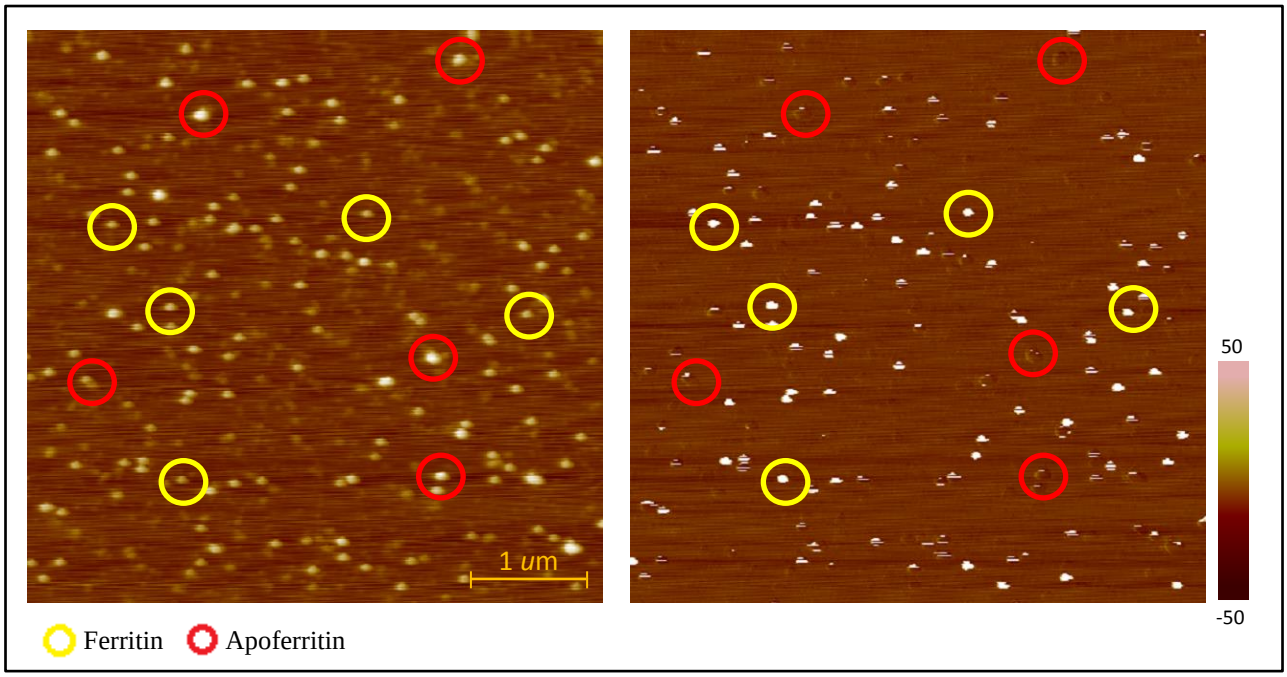

Figure 7. Height topographical image and phase shift image of mixed solution at 20 nm lift height. The difference between ferritin and apoferritin in the mixed sample can be easily seen. Red circles indicate corresponding apoferritin particles, while yellow circles indicate corresponding ferritin particles.

Magnetic Property Characterization of Ferritin

A series of equations were used to convert the magnetic phase shift data into magnetic moment. The relationship between lift height, phase shift, and sample magnetic moment can be described by the equation [11]:

$$\delta\varphi = \frac{\mu_0}{4\pi}\frac{12\pi Q}{k(\frac{d}{2}+R+c+s)^5} m_p m_s \frac{180}{\pi} \qquad (2)$$

Where μ_0 is the permeability constant of a vacuum, k (high moment: 2 N/m, medium moment: 2.8 N/m) is the spring constant of the MFM cantilever, Q (high moment: 270, medium moment: 380) is quality factor, d is the diameter of the ferritin protein, R is the outer shell radius of the MFM probe (high moment: 110 nm, medium moment: 90 nm), c is the thickness of the Cr layer preventing oxidation of the magnetic coating (high moment: 30 nm, medium moment: 20 nm), s is the lift height, m_s is the particle magnetic moment, and m_p is the probe magnetic moment, given as [10]:

$$m_p = \frac{4}{3}\pi(R^3 - R_1^3)M_p \quad (3)$$

where M_p (1.4e6 A m^{-1})[10] is the saturation magnetization of Co coating on the MFM cantilever and R_1 is the inner shell radius of the MFM probe (~10nm).

Using equation 2 and data collected with the high moment probe, we found that the average magnetic moment of an individual ferritin protein, m_s, was 3.77e-17 A m^2 (range: 2.71e-17 A m^2 – 3.33e-17 A m^2). The moment of an individual apoferrtin protein was 8.08e-18 A m^2

(range: 1.95e-18 A m^2 – 2.59e-17 A m^2). In the mixed sample, the average magnetic moment of ferritin and apoferritin at 20 nm lift height were 2.31e-17 Am^2 and 4.96e-18 Am^2. A summary of our measurements is shown in Tables 1 and 2.

Table 1. Summary of data for Ferritin and Apoferritin trials.

	Ferritin	**Apoferritin**
Diameter (nm)	7.2	7.3
Rng. Magnetic Moment (Am^2)	2.71e-17 - 3.33e-17	1.95e-18 – 2.59e-17
Ave. Magnetic Moment (Am^2)	3.77e-17	8.08e-18

Table 2. Summary of data for mixed solution trials.

	Ferritin	**Apoferritin**
Ave. Magnetic Moment (Am^2)	2.31e-17	4.96e-18

Discussion

Prior studies have confirmed that the MFM technique can be used to detect superparamagnetic nanoparticles [9]. In this work, we used magnetic force microscopy to characterize single ferritin and apoferritin proteins as part of a greater goal of developing a technique for measuring the iron content of ferritin in the blood. Our research revealed that the MFM technique can detect single ferritin proteins across a wide range of lift heights. In a comparison of medium moment and high moment probes, we found that high moment probes can detect magnetic signals at much greater lift heights (60 nm) than medium moment probes (30

nm). The difference in magnetic moment sensitivity will be important when developing a technique for characterizing magnetic properties of liquid samples, such as blood. This is usually difficult because when an AFM probe is placed inside a liquid, probe movement is dampened and measurement accuracy is low. If we are able to characterize a liquid sample from a distance, then dampening will not be an issue. Because of the difference in sensitivity, the rest of the experiment was conducted using high moment probes.

Our MFM studies characterized ferritin proteins as spherical shells with lateral diameters of 7.4 nm, which is smaller than 13 nm found by previous studies [13]. This might be due to differences in calibration of the atomic force microscope or differences in sample preparation. For our procedure, distilled water was used to dilute the base ferritin solution, but ferritin's shell is hydrophobic. The hydrophobic forces might have then compressed the ferritin shell, reducing its diameter and height.

Using equation 2 and our phase shift data, we calculated that the average magnetic moment of individual ferritin proteins was 3.77e-17A m^2. This value is similar to the 3.7e-17 A m^2 that was found by an MFM study of similarly sized superparamagnetic nanoparticles [9]. However, SQUID analysis found a slightly different magnetic moment values. Using a magnetization curve shown in Figure 8 [], we find that the magnetization of ferritin using a 2000G magnetic field is ~0.05 emu/g, which translates into 1.68e-20 A m^2. These values indicate consistency among MFM characterizations of ferritin, but differences between MFM measurements and SQUID measurements. Further optimization of the MFM technique is necessary to bridge this discrepancy.

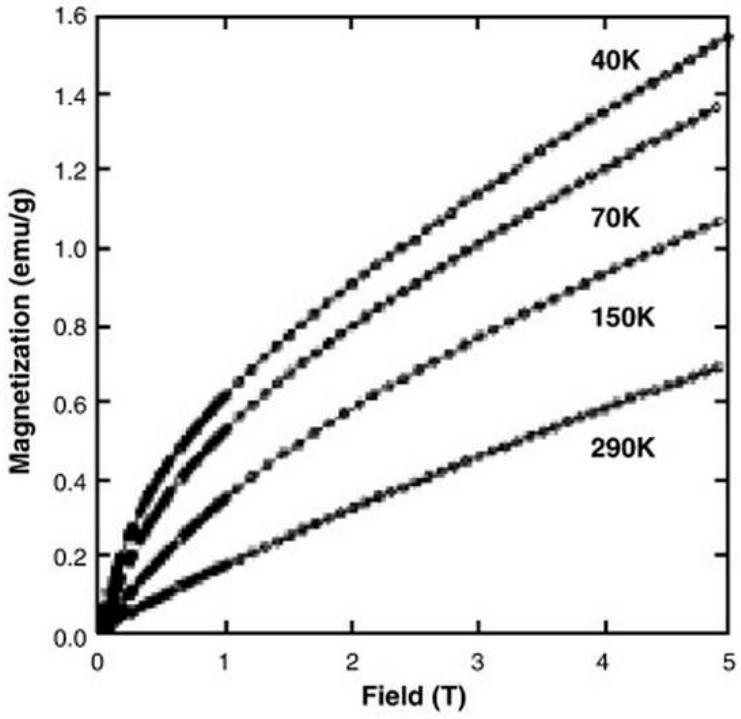

Figure 8. Magnetization graph for ferritin. []

MFM studies on apoferritin recorded lateral diameters of 7.41 nm, which is similar to our ferritin measurements. As expected, the magnetic moment of apoferritin (8.08e-18 A m^2) is much lower than the values measured for ferritin at an average value of 3.77e-17 A m^2, but it was not at 0 A m^2. MFM Appoferritin's magnetic moment is probably not caused by the presence of iron particles, but rather it was due to van der waals and other short range interactions. To obtain the true magnetic moment of ferritin, we subtracted the 8.08e-18 A m^2 caused by unrelated short range forces from both ferritin and apoferritin to achieve final results of 3.69e-17 Am^2 and 0 Am^2. Results from the apoferritin study confirm that the MFM technique can accurately characterize apoferritin and differentiate separate samples of ferritin and apoferritin.

Because blood carries both apoferritin and ferritin, we conducted MFM on mixed apoferritin ferritin solutions. Figure 9 shows that the MFM technique is sensitive enough to

differentiate between ferritin and apoferritin. Significant differences between ferritin and apoferritin magnetic moment can be found (2.31-17 A m^2 vs. 4.96e-18 A m^2). The magnetic moment of ferritin was lower than expected, which might indicate that some of the iron was picked up from the ferritin by the apoferritin during the preparation process. Overall, the MFM technique shows promise as an accurate method of ferritin characterization.

Conclusion

In summary, we demonstrated here that MFM can accurately identify and characterize both ferritin and apoferritin. Magnetic phase shift measurements can be collected at greater lift heights when using high moment probes rather than the more widely used medium moment probes. If we are to image ferritin in blood, high moment probes will be necessary for iron detection. The MFM technique has enough sensitivity to quantitatively differentiate between ferritin and apoferritin in mixed solutions. Magnetic moment measurements will allow direct measurements of iron content for disease diagnosis. We conclude that MFM is a viable method of ferritin characterization and iron quantification; the ability to capture magnetic data without direct contact to the sample makes magnetic force microscopy a promising technique for biological samples like blood in the future.

References

[1] K. V. Kowdley, "Iron, hemochromatosis, and hepatocellular carcinoma," *Gastroenterology*, vol 127, no. 5, pp. S79-S86, 2004.

[2] B. Mehta and P. Efthimiou, "Ferritin in adult-onset still's disease: just a useful innocent bystander?" *International Journal of Inflammation*, vol. 2012. 2012.

[3] H. Chandarana, R. K. G. Do, T. C. Mussi, J. H. Jensen, C. H. Hajdu, J. S. Babb, and B. Taouli, "*The Effect of Liver Iron Deposition on Hepatic Apparent Diffusion Coefficient Values in Cirrhosis*," American Journal of Roentgenology, vol. 199, no. 4, pp. 803-808, 2012.

[4] R. Casiday and R. Frey. "Ferritin, the iron storage protein. http://www.chemistry.wustl. edu/~edudev/LabTtorials/Ferritin/Ferritin.html. Nov 2009. Viewed 2/17/2013

[5] T. Grainer, D'E Langlois, B. Gallois, J.M. Chevalier, G. Precigoux, P. Santambrogio, P. Arosio. *Structural description of the active sites of mouse L-chain ferritin at 1.2 A resolution* J. Biol. Inorg. Chem. v8 pp.105-111, 2003.

[6] V. A. Pilon, P. J. Howanitz, J. H. Howanitz, and N. Domres, "Day-to-Day Variation in Serum Ferritin Concentration in Healthy-Subjects," *Clinical Chemistry*, vol. 27, no. 1, pp. 78-82, 1981.

[7] M. J. Cooper and S. H. Zlotkin, "Day-to-day variation of transferrin receptor and ferritin in healthy men and women," *American Journal of Clinical Nutrition*, vol. 64, no. 5, pp. 738-742, 1996.

[8] J. C. Dale, M. F. Burritt, and A. R. Zinsmeister, "Diurnal variation of serum iron, iron-binding capacity, transferrin saturation, and ferritin levels," *American Journal of Clinical Pathology*, vol. 117, no. 5, pp. 802-808, 2002.

[9] T. Nocera, J. Chen, C. Murray, and G. Agarwal, "Magnetic anisotropy considerations in magnetic foce microscopy studies of single superparamagnetic nanoparticles," *Nanotechnology*, vol. 23, 2012.

[10] T. Nocera, J. Chen, C. Murray, and G. Agarwal, "Imaging Superparamagnetic Nanoparticles in Cells," *Proceedings of AACR special conference: Nano in Cancer*, 2011.

[11] S. Schreiber et al., "Magnetic force microscopy of superparamagnetic nanoparticles," *Small*, vol. 4, no.2, pp. 270-278, 2008.

[12] "Atomic force microscope-home," http://ipc.iisc.ernet.in/~ipcafm/about%20us.html, Indian Institute of Science, Visited 3/17/13.

[13] C. W. Hsieh, B. Zheng, and S. Hsieh, “Ferritin protein imaging and detection by magnetic force microscopy,” Chemical Communications, vol. 46, no. 10, pp. 1655-1657, 2010.

[14] G. C. Papaefthymiou, “The Mossbauer and magnetic properties of ferritin cores,” Biochimica et Biophysica Acta (BBA) – General Subjects, vol. 1800, no. 8, pp. 886-897.