Zinc Oxide Nanoparticle Synthesis in Apoferritin

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ABSTRACT

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Zinc oxide is a semiconductor with a wide direct band gap and optical properties advantageous for three-photon absorption. Apoferritin is a hollow protein previously used to achieve uniform growth of nanoparticles of other materials. I demonstrate that zinc oxide nanoparticles can be synthesized within ferritin using a Slow Chemical Reaction (SCRY) scheme and that native impurities can be utilized to exhibit strong visible luminescence during the three-photon absorption process. My results were analyzed and verified through TEM imaging, spectrophotometry, ICP-MS, and various other methods.

Keywords: Zinc Oxide, Ferritin, Semiconductor, Nanoparticle, Three-Photon Absorption, Apoferritin, SCRY, Cancer Screening

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Chapter 1

Background

1.1 Significance

The material presented in this thesis is part of a larger work. The goal of this project is to create a reliable, non-invasive procedure to conduct cancer screenings, utilizing the ability of ferritin to attach to a cell-specific targeting peptide without compromising the non-native core within [1] and its subsequent ability to work as a molecular probe [2]. I seek to achieve this through creative use of the optical properties of semiconducting nanoparticles and the potential intrinsic defects found within the zinc oxide nanoparticles I create. Other experiments have been conducted in which nanoparticles were created inside of apoferritin [3] and defects were introduced to zinc oxide nanoparticles for three-photon absorption [2]; I seek to combine their research and create zinc oxide nanoparticles inside apoferritin that have the proper defects needed for three-photon absorption.

1.2 Zinc Oxide

I chose to focus on the optical properties of zinc oxide, as it is a semiconductor with a direct band gap in the ultraviolet range (3.37 eV); this is of particular importance because a wide band gap will

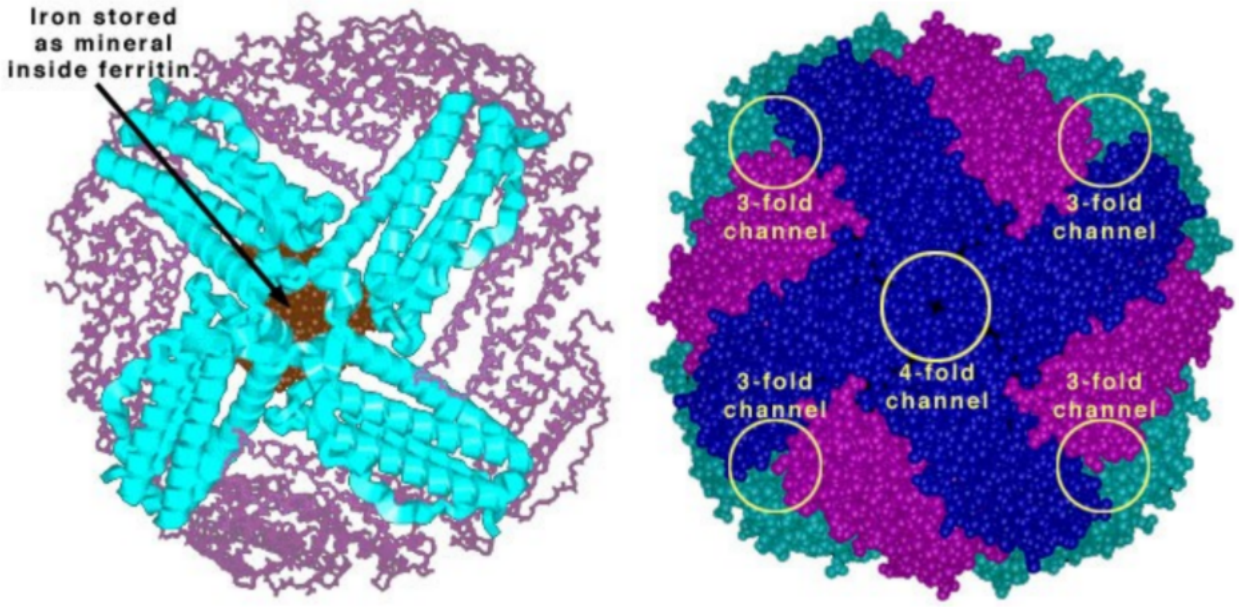

Figure 1.1 (Left) The structure of ferritin with an iron core. (Right) The ion channels of ferritin are represented. Acquired from Washington University in St. Louis. Used with permission. [4]

allow for more freedom when introducing the defects. Materials are classified as semiconductors if they have a band gap, or distance between the valence and the conducting band, that can be bridged by adding energy to an electron in the ground state to force it into an excited state. This energy can be introduced in various ways and the electron, when returning from the excited state back to the ground state, will release a photon corresponding to the size of the band gap of the material.

1.3 Ferritin

To contain the zinc oxide nanoparticles, I utilize a structure called ferritin. Ferritin is a protein that is universally used to store iron. Ferritin is a protein with a spherical shell, around 12 nm in size, surrounding an iron core. It is thermally [5] and chemically stable [6], and only starts to break down above a temperature of 75°C [5]. It is stable within a pH range of 3.5 - 11. One of the primary

reasons for ferritin's stability lies in its subdivisions of amino acids, called peptides. Ferritin has two types of peptides: H-chain and L-chain, referring to heavy and light peptides respectively [7]. These peptides fold together to create channels within the structure with either threefold or fourfold symmetry [8] (see Fig. 1.1), thereby changing its electrostatic properties: the threefold channel is negative, while the fourfold is positive. This difference in charge reflects how molecules enter the channel. Positive iron ions will enter the negative threefold channel and exit through the positive fourfold channel [8]. As iron ions enter the ferritin, some attach to nucleation sites on the inside of the ferritin. The iron will continue to consolidate within the center of the protein until it forms a spherical shell [9].

1.4 Apoferritin

The native ferrihydrite material making up the core of the ferritin can be removed from the ferritin; the resulting protein structure is called apoferritin. A non-native core can then be introduced into the material using the threefold or fourfold channels under a process known as cation-anion synthesis. This method was discovered by Douglas *et al.* and utilizes the electrostatic gradients alongside the ion channels to pull cations into the center of the hollow protein [10]. Once the cationic portion of the desired core has been injected into the buffer containing the solution, it travels into the cavity using the threefold channels. Immediately following that process anions are added to the solution, which are drawn in through the fourfold channel and react strongly with the cations to form a crystalline core [11–13], bounded by the inner diameter of the ferritin itself. This allows for other materials to be housed within the ferritin, creating a biological packet that can be utilized for other processes.

1.5 Three-photon Absorption

I intend to artificially create defects in the material in order to facilitate a process known as three-photon absorption. Three-photon absorption involves utilizing the defects found in a material to create virtual states between the ground state and the lowest excited state of the material. These virtual states are extremely short-lived quantum states that allow for movement between the ground state and first excited state. It is helpful to think of this process as an intermediate state existing between the ground state and the lowest excited state. Quantum tunneling can result in energy from multiple photons combining to bring an atom from the ground state to an excited state. Similarly to the three-photon absorption process, defects found in a material can be used to used to change the resulting photons so the nanoparticles emit in visible light. For zinc oxide, however, the band gap is close enough to the visible range that they can emit in the visible spectrum even when excited with near infrared light.

1.6 Cancer Screening

This capability is of particular importance for use in cancer screenings. Generally, cancer screenings are completed by obtaining and testing samples taken from affected organs in the body. This method, in contrast, would be more like a CAT scan - significantly less invasive. However, unlike a CAT scan, this method could be completed in an operating room while surgery is ongoing. By connecting these apoferritin packets to a peptide that seeks out cancerous cells and attaches to them, medical professionals could inject a patient with these packets. Once the peptides have attached to the cancerous cells, the optical properties of the zinc oxide core can be exploited. Infrared light can pass through tissue, is non-harmful, and will provide enough energy to raise the zinc oxide nanoparticles into a virtual state. Once multiple photons have struck the nanoparticles, the zinc oxide will then be in an excited state, and in dropping back down to the ground state, will luminesce. This light can

easily be seen by the medical professional and additional tests to ensure the presence of cancer, or lack thereof, can be run.

I know this is viable due to the efforts of Raghavendra *et al.* [14]. This group utilized zinc oxide nanoparticles, created through a lengthy process delineated in the paper and suspended in de-ionized water, that had defects induced in them through a polyol method. Following the creation of the nanoparticles and the subsequent introduction of defects for three-photon absorption, the nanoparticles were functionalized with peptides for selective uptake and evaluated using breast cancer MCF-7 cells. The uptake was measured using ICP-MS, and the biocompatibility was evaluated through various cell viability assays. They determined that three-photon absorption imaging was possible for these cells with low background noise under these circumstances.

By creating the zinc oxide nanoparticles inside of ferritin, I seek to decrease the work necessary to make the zinc oxide nanoparticles biocompatible and see how that impacts the resultant imaging.

Chapter 2

Method

2.1 Apoferritin Preparation

To achieve the goal of a biological packet capable of non-invasive cancer screening, one must first prepare the protein for use in containing the nanoparticles. To effectively insert another core into ferritin, one must first remove the native iron core. I started this core removal process by immersing a solution of equine spleen ferritin purchased from Sigma Aldrich inside a bath made primarily of thioglycolic acid. Precise instructions for synthesis are included in appendix A.1. The acid reacts with the iron core while passing inertly through the outside protein structure, thereby breaking down the core while the outer horse spleen ferritin remains untouched. To avoid losing the sample within the significantly larger amount of thioglycolic acid, I injected the ferritin into a hydrated dialysis cassette.

This dialysis cassette has a semipermeable membrane and an allowance of 7000 g/mol. Because the horse spleen ferritin shell is much larger than 7000 g/mol, it remains inside the dialysis cassette while the iron core flows out, leaving me with only apoferritin within the dialysis cassette, indicated by a purple tinge added to the acid. After the successful breaking down of the core, further removal

is achieved through a bath primarily in sodium bicarbonate. The sodium bicarbonate serves to both neutralize any remaining thioglycolic acid and to ensure that the core has been fully removed. The apoferritin is then removed from the dialysis cassette, and can be evaluated using a modified Bradford method explained in the paper by Keyes *et al.* [11].

However, the resulting concentration of apoferritin from dialysis can differ, so this concentration must be measured before moving forward. Because the protein sequence of apoferritin has been studied extensively [7–9], it is known that some of the amino acids present absorb at 280 nm. The presence of iron can influence the absorbance, so most of the iron core must be removed prior to taking these measurements. Ferritin has an extinction coefficient, ε, of 471,600 M^{-1} cm^{-1}. To determine concentration, the Beer-Lambert law put forth in the equation

$$A = \varepsilon BC$$

is utilized. Here, A is the log of the absorbance, ε is the extinction coefficient, B is the pathlength (1 cm for our spectrophotometer), and C is the concentration in M. Using this law, I can calculate the concentration of the apoferritin through a relatively simply process. First, I added around 1 mL of buffer to a UV transparent cuvette (found in drawer G-7) and blanked the spectrophotometer. That precise amount is not necessary, but care must be taken to fill the cuvette sufficiently so that a blank spectra can be taken. Next I added a small amount of apoferritin to the buffer. Again, the precise amount is not important, but it needs to be enough that the apoferritin is present in the solution and the spectrophotometer can take a reading. 10-50 µL of apoferritin is sufficient. I then recorded the absorbance. This displayed the concentration of the resulting apoferritin solution to be around 30.7 ± 0.5 mg/mL. The uncertainty stems from variations in recordings from the spectrophotometer, as well as the possibility that the iron core was not fully removed and might interfere at 280 nm. However, this value is close to the previous concentration of the ferritin so I believe it to be accurate. Performing this calculation allows me to accurately create zinc oxide in the following section.

2.2 ZnO Core Synthesis

Following the removal of the native core, a reaction is necessary to form a structure of zinc oxide within the now-hollow protein structure; merely inserting ZnO into the same solution as the apoferritin will not suffice. The exact method is delineated in Appendix A.2. I used a buffer of 3-(Cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) to guide this process and prevent large changes in the pH of the final sample. I inserted a small amount of the apoferritin solution into the buffer, then added ammonia, then added zinc nitrate to form the reaction. This disjointed reaction using the Slow Chemical Reaction System (SCRY) results in the formation of zinc oxide on the inside of the ferritin shell due to the electrostatic forces at play [8], as described in the next paragraph.

Ammonia serves as a reactive deterrent, so once the zinc oxide is added it can only react in areas where the ammonia is not present [3]. Because ammonia is cationic, zinc oxide is anionic, and the center of the protein structure is similarly cationic [4], the ammonia cannot enter the protein until after the zinc oxide already has. This causes the chemical reaction to only take place within the apoferritin [10]. A strong reaction occurs between the zinc nitrate and the oxygen piece of the CAPSO, resulting in the formation of a non-native zinc oxide core within the ferritin at the nucleation sites. This reaction is confined to the space in which occurs, so by regulating the size of the apoferritin using gel column filtration, also called gel filtration chromatography, I can obtain uniformly sized nanoparticles inside of the ferritin packets [11, 12].

2.3 Gel Column Filtration

Gel filtration chromatography can be performed in order to separate apoferritin into various sizes of protein; the larger protein structures will emerge first, while the smaller ones will emerge later. This can be achieved by using S-300 Sephadex spheres and a sodium chloride solution, however, I did

not reach a stage in which I believed it was necessary to pursue to improve the results, so a detailed procedure will not be provided. An explanation of the process is related in the paper by Suzumoto et al. [3].

The resulting mixture from the synthesize of ferritin with a non-native zinc oxide core is also evaluated through the use of a size-limiting gel column. The form of gel column filtration that I use utilizes gravity to split the sample based on the size of the particles found within, and is captured in ten equal-sized fractions. These fractions, typically 1 mL apiece, separate the size of the molecules from largest to smallest. The gel in the column traps smaller molecules while larger molecules can pass through uninterrupted [7], resulting in large molecules like ferritin passing through in the fourth or fifth fraction. Because ferritin is very nearly uniformly sized, each instance of the protein will escape from the gel column at a similar time in a similar fraction, thereby ensuring that this gel filtration is a viable method to separate the ferritin from the buffer. This was achieved by using both a PD-10 column and a gel column created using S-100 Sephadex beads and flushing the sample through with the buffer. A detailed explanation is included in Appendix A.3.

2.4 Fraction Analysis

The resulting fraction of apoferritin was analyzed through several different methods. One such method is a process known as spectrophotometry, in which light rays shine through a sample and a program records the resulting absorption spectrum caused by the material. This process works best when utilized alongside methods such as the Bradford protein assay, which allows for the determination of the protein concentration within each fraction [15]. This concentration is determined by testing samples with known quantities of protein within them and comparing those results to the fractions filtered. Because the concentration of the apoferritin solution was 30.7$\pm$0.5 mg/mL, 100 µL was taken from each fraction to obtain enough of the protein to be easily

measurable. I then added 1 mL of the Bradford reagent to each fraction. This amount was chosen because the standards were created with 1 mL of Bradford reagant as well; this number can be changed accordingly as long as the standards and samples agree with each other. After five minutes, the samples have sufficiently changed to allow for the testing of the sample. The brown Bradford reagent reacts to become blue in the presence of protein, which allows for visual confirmation regarding the success of this piece of the experiment. The color also represents whether the sample is anionic, neutral, or cationic [15]. These pieces of the fractions, combined with the 1 mL Bradford reagent, have a higher absorbance at 595 nm than any of the other fractions, resulting in a definitive path to determining the presence of the ferritin. This process allowed me to see which fraction the ferritin appeared in, as there was a resulting peak shown in the graphs and data taken (see Fig. 2.1).

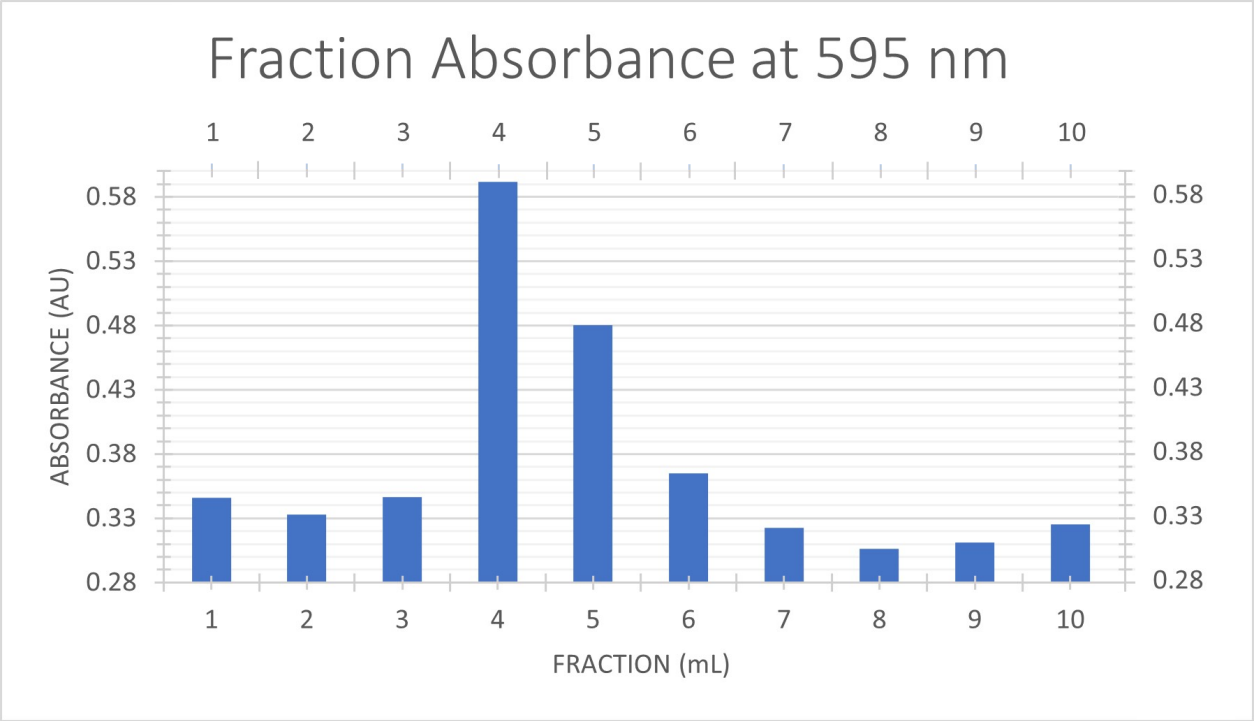

Figure 2.1 Recorded absorption of different fractions of the sample with Bradford reagent added after it passed through the gel column. The different levels correspond to resulting amounts of the protein (ferritin) within the sample.

However, to determine the presence of the zinc oxide core within the ferritin, techniques other

than the Bradford method are required. One such technique is known as inductively coupled plasma mass spectrometry, or ICP-MS. Detailed instructions for ICP-MS sample preparation can be found in Appendix A.4. ICP-MS ionizes a sample, then atomizes it to create small ions which can be detected, which is then compared to an existing template of metal samples. This results in a printout of the different elements present within the sample and their respective concentrations, which is useful for indicating whether or not a metal that would normally not be present, such as zinc, appears within the fraction where the highest protein concentration lies. If there is zinc inside of that fraction, then there are two possibilities for the result: either the zinc oxide core formed as intended, or zinc oxide bonded on the outside of the ferritin and did not create a core. As such, more tests must be run.

2.5 Transmission Electron Microscope (TEM)

One of the best ways to examine the samples is through the use of a transmission electron microscope (TEM). Specifically, I used a Tecnai-F20. To prepare the sample, a drop of the sample solution is added to a negatively charged copper grid, which is allowed to set before it is rinsed off and the excess liquid is removed. Then the copper grid and sample are stained with uranyl acetate, ensuring better pictures and more clear results. Detailed instructions on sample prep are included in Appendix A.5. The TEM is used in STEM mode, which results in dark-field images of the sample. I then check the ferritin to see if there is zinc oxide present outside of the sample or if it is encased inside. In the event that the TEM data is inconclusive, testing the sample using ICP-MS or during the TEM session using energy dispersive X-ray analysis (EDX) may prove to be necessary. ICP-MS has previously been discussed, and EDX images the sample by bombarding the sample with an electron beam while recording the X-rays that occur from this process. A program evaluates how the X-rays interact with the sample and can determine the chemical composition found within the

sample based on elemental standards within the program itself.

Chapter 3

Results/Conclusions

3.1 ZnO Synthesis

I believe that zinc oxide nanoparticle synthesis was successfully synthesized, as indicated by the ICP-MS data indicated later in the paper. To synthesize these nanoparticles, the slow chemical reaction system (SCRY), designed to suppress all chemical reactions outside of the ferritin [3] was utilized; the detailed method can be found in Appendix A.2. This system relies on the ionic concentration of the substance; first, 7 µL of Apoferritin is added to 0.778 mL CAPSO buffer, then 150 µL of ammonia is added to stabilize the Zn^{2+} ions. 65 µL of $Zn(NO_3)_2$ is then added to the mixture, resulting in the zinc ions being drawn into the apoferritin, where there are many negatively charged amino acids. These form nucleation sites for the nanoparticles to be grown, a lattice of ZnO is formed within the apoferritin. The aforementioned numbers were chosen for several reasons. I wanted the resulting sample to be 1 mL with an apoferritin concentration of 0.2 mg/mL, so I calculated the amount of apoferritin necessary to add based on that result and the concentration of our apoferritin we found previously. The amount of ammonia was chosen to sufficiently suppress the reaction outside the ferritin, the amount of zinc nitrate was chosen to create a good chance for

the reaction to take place, and the CAPSO included was utilized to bring the resulting solution up to the desired 1 mL amount.

A higher pH level results in more OH^-, resulting in a more optimal reaction by providing more molecules with whcih the zinc nitrate can react [3]. However, having a pH level that is too high will denature the apoferritin [16]. Both apoferritin and ferritin begin to denature at a pH of about 11, so I chose to conduct my experiments with the CAPSO buffer held at a pH of 9.8. This provides an adequate environment in which to perform the experiment, while still ensuring that the apoferritin can retain its shape.

Bradford Protein Assay of Gel Column Sample Fractions

Fraction	Abs <595 nm> ($\pm$0.0005)	Concentration ($\pm$0.005)
1	0.3460	0.171
2	0.3331	0.164
3	0.3467	0.171
4	0.5918	0.292
5	0.4802	0.237
6	0.3652	0.180
7	0.3227	0.159
8	0.3061	0.151
9	0.3110	0.153
10	0.3255	0.161
CAPSO (blank)	0.3242	0.161

Table 3.1 Recorded absorption of different 1 mL fractions of a sample after it passed through the gel column (column two). Note that this is the same data found in Fig 2.1. The different concentrations correspond to resulting amounts of apoferritin (column three).

Analysis through use of the Bradford protein assay and a spectrophotometer showed the highest concentration of apoferritin was concentrated within the fourth fraction, while the fifth fraction contained some as well. The concentration was calibrated using standards made with apoferritin in quantities of 0 μL, 10 μL, 20 μL, 30 μL, 40 μL, and 50 μL held in a CAPSO buffer (see Table 3.1).

Because the CAPSO buffer is the basis against which I test the changes, I can ascertain that a significant change in the concentration or absorbance stems from other materials present in the fraction. The other variation occurs primarily due to defects within the vials I used to test the samples, as well as slight variations within the instrument itself. At a glance, however, it is clear that the fourth and fifth fractions exceed the variations present in the other columns, and must stem from ferritin coming out in those fractions.

After determining that the apoferritin came out in both the fourth and fifth fractions, I created more samples. I chose to look at the fourth fraction under a transmission electron microscope as it had the highest concentration of ferritin in it. I believed this would allow me to see whether or not the zinc oxide was truly present within the fraction (see Fig. 3.1).

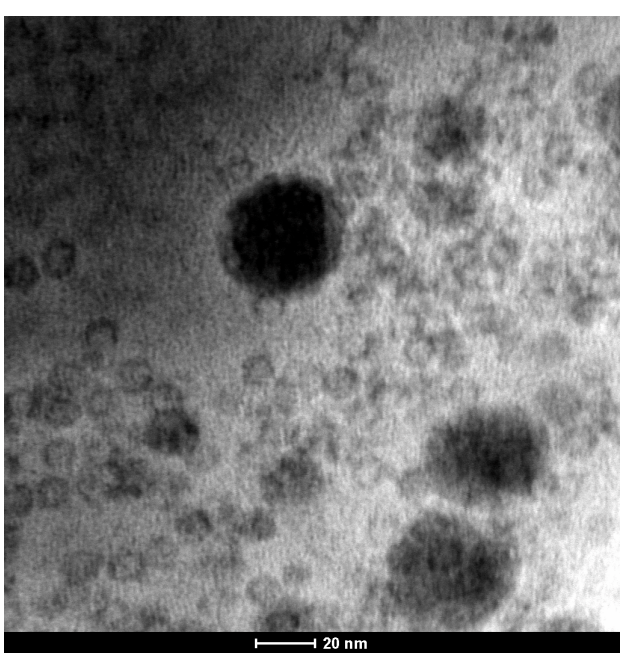

Figure 3.1 Several ferritin molecules. Note the lack of contrast between the ferritin H and the zinc oxide core as zinc is a light metal.

However, because zinc is a light metal I was unable to visually confirm that zinc made up the core of the ferritin, even after staining with uranyl acetate. As such, testing the sample using ICP-MS or EDX proves to be necessary.

ICP-MS was completed on these fractions to test for the presence of zinc. When running the gel column, we ascertained that that the protein appeared in the fourth and fifth fractions, and the protein is significantly larger than the zinc oxide created from this reaction. If zinc appears within this fraction, then we can be comfortably certain that it was formed inside of the apoferritin. Standards of 0, 50, 100, 150, 200, and 1000 ppb of zinc were created to evaluate the samples, and the process for creating the standards and samples is delineated in Appendix A.4.

Each of the four samples tested was above the value at which our standard with 0 ppb of zinc was found, and after plotting on the graph made from the standards I found the resulting concentrations to range from 1.84 ppb to 123.11 ppb, as shown in Fig. 3.2.

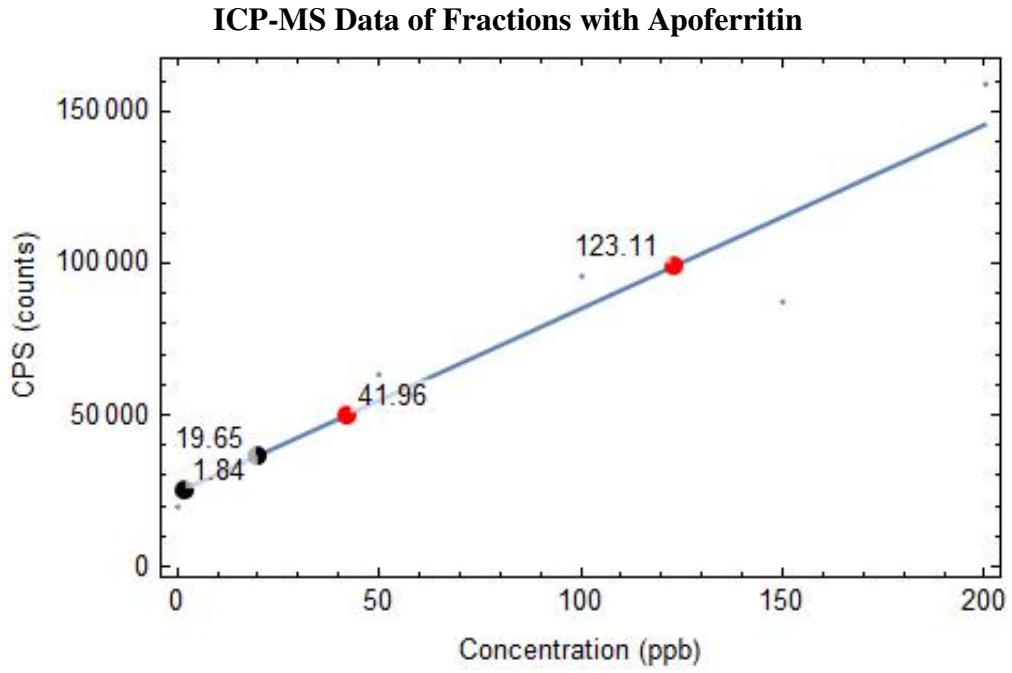

Figure 3.2 The data obtained from performing ICP-MS. The blue line is the fit from the standards, fitting the blue dots. The red dots are from one sample measured with quantities of 20 µL and 30 µL included, and the black dots are from a separate sample measured with quantities of 20 µL and 30 µL.

As both samples were created in the same manner and using the same process, it is readily apparent that there was a large amount of uncertainty in the process of sample creation and the reaction. Those sources of uncertainty are to be explored further in the future work section 3.2. Regardless of the uncertainty, a non-negligible amount of zinc is present in each of these samples, thus indicating the presence of zinc oxide nanocrystals inside of the apoferritin. Once this process has been optimized, we can then utilize the concentration of the ferritin along with the ICP-MS results to determine the amount of zinc atoms per ferritin.

I also utilized EDX to confirm the results of the ICP-MS and test for the presence of zinc oxide. Deposition upon our sample quickly occurred, leading to the pictures taken appearing washed-out.

EDX Taken on this Portion of Sample

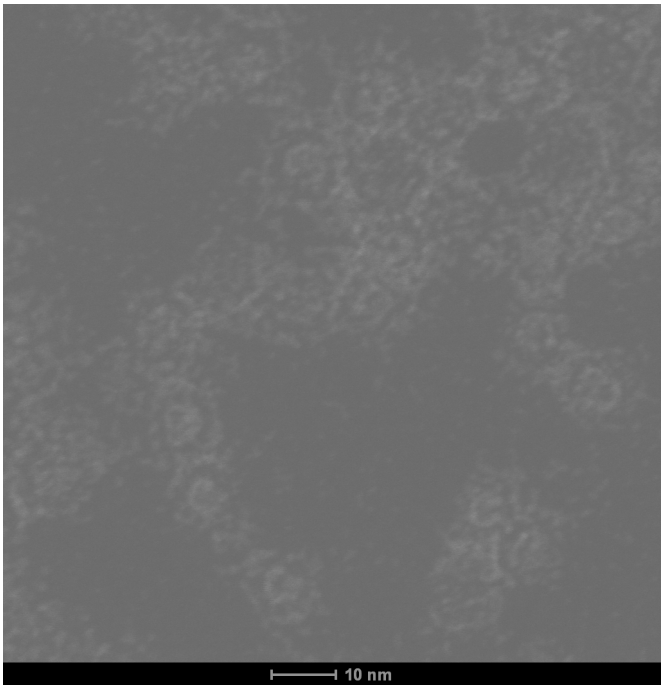

Figure 3.3 Image of Ferritin. Note the deposition on sample causing it to lighten significantly.

EDX Data from Ferritin in CAPSO

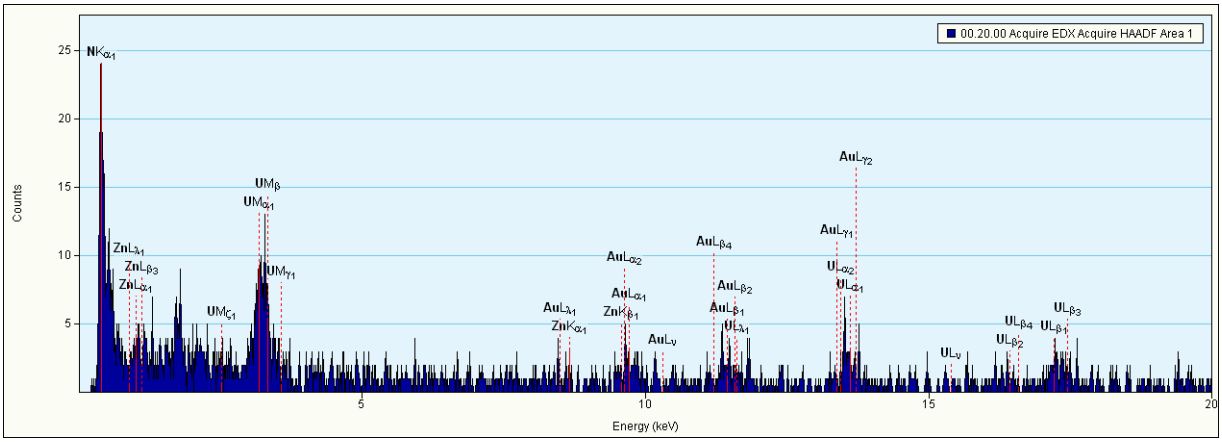

Figure 3.4 EDX data from our sample.

From the graph above, the highlighted elements are uranium, zinc, nitrogen, and gold. The uranium stems from the uranyl acetate stain I utilized, and the zinc comes from the zinc oxide inside

the ferritin. The presence of nitrogen is not significant, but the presence of gold is perplexing. After discussing it with the TEM supervisor, I believe that the grid upon which I deposited the sample was coating with a thin layer of gold, which helped contribute to the intense carbon deposition upon the grid that obscured the pictures. Furthermore, the amount of zinc present in the sample was not enough to conclusively determine that zinc oxide is present in our apoferritin - but there is too much to reliably claim that it is not present in the sample, either. Further testing must be undergone in order to conclusively determine the presence of zinc oxide in the apoferritin through EDX. Due to the results of the ICP-MS, however, I am certain that zinc oxide is present in our samples, and a retest of the sample using EDX is likely to succeed.

3.2 Future Work

As the goal of the project revolves around introducing impurities for three-photon absorption, one must first reliably recreate the nanoparticles to avoid as much uncertainty as possible when introducing new impurities. As such, it is recommended that any who continue this project seek to compare the different parameters included in the synthesis of the nanoparticles and how they impact the creation of the zinc oxide nanocrystals. Once the nanoparticles can be reliably created and as much uncertainty as possible has been removed, defects can be introduced to the non-native apoferritin core and three-photon absorption can be tested using a wavelength of light in the infrared. Infrared light should be used because it can pass through tissue, then observed to determine whether or not it will luminesce in the visible spectrum.

Appendix A

Procedures

A.1 Apoferritin

Apoferritin preparation through dialysis consists of six different 8-hour baths. The first three involve a solution of thioglycolic acid, while the remaining three involve a bath with sodium bicarbonate. Nothing magical occurs at the eight hour mark in steps 6 and 9 below – it can be removed up to an hour early and neither will the ferritin will not be harmed by increased time spent in the bath.

All preparation was done in Dr. Watt's north lab.

1. Take a 7000 molecular weight cut off 0.5-3.0 mL dialysis cassette and hydrate it by soaking it in a bath of dH_2O.

2. Using a small syringe (around 5 mL), inject 3 mL of ferritin into one of the holes in the corner of the cassette. Mark the hole used. Be careful not to puncture the membrane.

3. Attach a foam buoy to the dialysis cassette, ensuring that the hole punctured is at the top and will not be submerged in the bath.

4. Fill a beaker to 1 L with dH_2O, 7.54 mL thioglycolic acid, and 34.02 g sodium acetate. The

order they are added is inconsequential. Sodium acetate can be found in powdered form in the glass cabinets. Thioglycolic acid is stored in the large glass fridges, the vial is in a metal cylinder.

5. Insert stir bar and stir the mixture. A strong smell from this chemical reaction should emanate from the beaker, indicating successful synthesis. Cover with tin foil to confine the smell.

6. Place dialysis cassette in bath and place on magnetic stir plate in refrigerator. Allow to stir for eight hours. The cassette should be able to spin. The first time, the solution should turn purple from the displaced iron. If that is not the case, then something is wrong.

7. Remove cassette and dispose of solution by pouring into container labeled "aqueous waste" (this solution is acidic, so if a distinction is made, use the acidic aqueous waste container). Return to step 4. and repeat the process at least two more times. More can be done if you believe the iron core has not been sufficiently broken down, but this should be sufficient.

8. Fill beaker to 1 L with dH_2O and 5 g of sodium bicarbonate (found below the glass cabinet where powders are stored.

9. Insert cassette and allow to stir for eight hours.

10. Remove cassette and dispose of solution down normal drain. Return to step 8. and repeat the process for two more cycles.

11. OPTIONAL: Fill beaker to 1 L with CAPSO at pH 9.8. Place cassette in bath for eight hours in refrigerator, then remove. This step is considered optional because small amounts of the sodium bicarbonate solution could be present in the dialysis cassette when removing the sample, and a bath in CAPSO would remove it. However, it may also not be present, and may not impact the solution significantly regardless.

12. Using a 5 mL syringe, extract the apoferritin from the cassette and store in refrigerator.

A.2 Nanoparticle Synthesis

Nanoparticle synthesis is completed as a slow reaction, so the order of additions is important. It is possible that mixing the zinc and ammonia together prior to combination with ferritin and CAPSO would work as well, but otherwise the order is necessary.

Note: The ultimate desired apoferritin concentration is 0.2 mg/mL with a 1 mL sample, so if the apoferritin concentration differs from what is written here the experiment should be adjusted accordingly to allow for it. Using the following equation, this experiment can be tweaked.

$$(DAC)(DSA) = (AA)(AC)$$

Here DAC is the desired apoferritin concentration, DSA is the desired sample amount, AA is the amount of apoferritin added, and AC is the concentration of the added apoferritin. The CAPSO buffer will be increased or decreased to keep the final sample amount the same.

1. This procedure for the reaction uses 50 mM CAPSO buffer at pH 9.8, 30 mg/mL apoferritin, 100 mM $Zn(NO_3)_2$, and 1.0 M stock of ammonia. Gather or prepare these. Zinc nitrate hydroxide is a powder by the window. Ammonia can be created with 0.69 mL of concentrated ammonium hydroxide in 10 mL of water, or found in the lab. CAPSO can be prepared using the CAPSO powder. Apoferritin preparation is found in Appendix A.1.

2. Add together 0.778 mL CAPSO and 7 µL of apoferritin. Shake/stir lightly - apoferritin is a durable protein, but prolonged vortexing could still result in denaturing.

3. Add in 150 µL ammonia and lightly mix.

4. Add 65 µL $Zn(NO_3)_2$ and shake sample lightly.

A.3 Gel Filtration Chromatography

There are two different ways to create a gel column. In Dr. Watt's lab there are premade PD-10 columns that are significantly faster than running a column with Sephadex G-100. As such, the PD-10 columns are preferred. If you don't have access to one of those columns and wish to make and run a column in the meantime, follow the steps below.

Note: The columns must be stored with buffer in them. If you store them without buffer, the gel will crack and liquid will pass through the crack rather than the gel, rendering the column filtration less effective.

A.3.1 Column Creation

1. Mix 1.1 g Sephadex G-100 with 30 mL. You can use different bead sizes depending on what you are doing, for ferritin anything above about ten works well.

2. Place beaker in fridge and let sit for at least 30 minutes. The gel should sink to the bottom of the beaker, with a visible boundary between the water and the gel.

3. Insert a mesh circle into the bottom of the empty column. The end should be sealed with either a plastic cap or parafilm to prevent leakage.

4. Pour water layer into sink, then siphon gel into the 20 mL column. Seal top with additional plastic cap or parafilm. The gel must resettle in the column. Do not run a sample for at least 2 hours.

5. Remove top covering and insert another mesh circle between the gel and water. Pushing it down with a metal spoon or tweezers is generally effective. Using too much force will displace the gel, which is not preferable.

6. Remove bottom covering and allow ~45 mL of buffer (three times the size of the column) to pass through. You can reuse the buffer that falls to conserve it if necessary.

A.3.2 Running a Column

When running a column it is important to start taking fractions when the sample is first added to the top of the column.

1. Run some of your desired buffer through the column. This will flush out any previous samples used in the column. If extreme accuracy is needed, do three times the column length of buffer. Otherwise, a small amount should be fine.

2. Remove any remaining buffer above the mesh circle divided and place column above the first 1 mL or 1.5 mL vial.

3. Insert 0.5-2.5 mL of sample above the top mesh circle. Do not continue until sample has run past mesh circle. Change fractions underneath as necessary.

4. Insert 1 mL of buffer and let it run past mesh grid. Change fractions underneath as necessary.

5. Top off column with desired amount of buffer. If taking ten 1 mL fractions, then combined sample and buffer ought to be just above 10 mL.

6. After taking each fraction, close the bottom with parafilm or plastic cap, insert buffer to somewhere above the mesh circle, and close top using parafilm or plastic cap. Store.

A.4 ICP-MS Procedures

A.4.1 Standard Preparation

Dr. Watt and the ICP-MS lab have standards prepared. The standards from the ICP-MS lab have a chance of contamination, depending on the students who used them, but Dr. Watt's lab is generally pretty good. Recommended to ask before creating your own.

1. Six different standards of 0, 50, 100, 150, 200, and 1000 parts per billion (ppb) must be created of whichever metal you wish to measure.

2. Fill six test tubes with 5 mL of HNO_3. The ICP-MS operator may want you to increase the amount of 0 ppb solution to 10 mL. Defer to their wisdom in that case.

3. Dissolve the metal you are using into each of these with its respective concentration. Because 1 ppb = 10^{-6} g/L, you are working with tiny amounts of the metal that are difficult to accurately measure. It is recommended that you create a 10 mM solution, then dilute it to make a 0.1 mM solution and pipette the proper amount.

4. Shake by hand and let sit overnight.

A.4.2 Sample Preparation

Figure A.1 Examples of syringe, PES filter, and tubing necessary to create samples. Often found in drawer H-19. 15 mL vials can be found in drawer E-10.

1. You need to prepare 2 ICP samples for each sample you want to know the metal atom concentration of. Start by obtaining 5 mL HNO_3.

2. Inject 20 µL and 30 µL of your gel filtered sample into the vials containing 5 mL of HNO_3.

3. Shake by hand and let sit overnight.

4. Centrifuge at 4000 rpm for five minutes.

5. Push a segment of plastic tubing over a 5 mL syringe tip. Syringes, tubing, and filters can be found in drawer H-19. If a precipitate was formed, draw in the supernatant (liquid over the precipitate); otherwise, draw in the entire sample.

6. Remove the plastic tubing and screw a 2 µm PES filter onto the syringe.

7. Expel the liquid from the syringe into a new 15 mL vial.

8. Contact ICP-MS operator. They will ask several questions, including the metal you wish to measure, in whose lab you are working, and the date to drop off the sample or receive results.

9. Drop off the samples where they inform you (generally C295).

A.5 TEM Procedures

Recommended that you read the entire procedure before beginning.

The Electrode-Vacuum System is located in a closet beside the door to Dr. Watt's lab. His key will open the door. It is on a giant black cart. The TEM grids are located in drawer H-13, labeled "TEM." It is found on the north side of his lab, across from two refrigerators.

1. Remove a TEM grid from blue box using tweezers. Tweezers should have smooth edges so as to not mess up the grid.

2. Place grid on glass slide and insert into glass chamber of electrode-vacuum system underneath electrode. Screw on air-tight cap.

3. Turn on vacuum using the black switch on the cord and let it pump down. After two or three minutes, close vacuum valve while leaving vacuum running.

4. Switch on electrode using switch. A visible arc will appear. Wait ~30 seconds before turning off.

5. Turn off vacuum and remove cap to chamber slowly. Remove glass slide without touching the grid.

6. Mount the grid on tweezers that default to being closed. Remember to use ones with a smooth edge. Have the dark carbon mesh of the grid facing up.

7. Place a 3.5 µL droplet of your sample onto the grid. Let sit for 1 minute.

8. Rinse the grid in a dH_2O droplet for a second.

9. Before five seconds have elapsed, gently touch a folded Kimwipe to the edge of the grid. This will attract all excess liquid from the entirety of the grid, so only one touch is necessary.

10. OPTIONAL: Stain grid with uranyl acetate (kept in top right of glass refrigerator). Place a 3.5 µL droplet of uranyl acetate onto the grid. Let sit for 1 minute. Repeat steps 8 and 9.

11. Let sample sit overnight.

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