Synthesis of Fluorophore Encapsulated Silica Nanoparticles for the Evaluation of the Biological Fate and Toxicity of Food Relevant Nanoparticles

DISSERTATION

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Abstract

This dissertation focuses on three interconnected projects: the characterization of commercial food-relevant nanoparticles during a simulated digestion, and their internalization and toxicity to intestinal epithelial cells; the study of nucleation and growth of quantum dots in a microwave synthesis; and the encapsulation of fluorophores within silica shells for \textit{in vitro} and \textit{in vivo} studies to elucidate their fate after ingestion.

We show that commercially available TiO$_2$, SiO$_2$, and ZnO nanoparticles are all internalized by C2BBe1 intestinal epithelial cells, but do not appear to be toxic, even after long term repeat-exposures. When particles were exposed to a simulated digestion protocol mimicking the stomach and intestinal environment, TiO$_2$ particles did show mild toxicity by MTT assay, indicating a decrease in metabolic activity. IR spectra of these particles indicate presence of material from the digestion media, and these absorbed species may be responsible for the effects noted. Though the three particles were not significantly toxic, we note internalization by the intestinal epithelial cells, opening a possibility for absorption into circulation where they may localize in organs throughout the body. This will be observed by functionalizing the particles with fluorophores, after which they can be measured via fluorescence.
To optimize the quantum yield efficiency, and thus the brightness, of one such fluorophore, we seek to improve a microwave synthesis of CdSe/CdS/ZnS quantum dots our lab has previously reported. By coupling the microwave reactor to a fluorescence spectrometer via fiber optic cables, we were able to monitor the development of the particles throughout the microwave heating. Time-dependent fluorescence shows the development of an early fluorescence peak at 502 nm attributed to CdSe cores. We then note two isosbestic points which we attribute to the development of CdS layer around CdSe cores, and eventually the formation of outer ZnS shell. We utilize this in situ monitoring along with a study of various nucleation temperatures ranging from 0 to 100 °C, and pre-and-post microwave heating UV exposure treatments to obtain optimized CdSe/CdS/ZnS particles with a QY of 40%. This is an improvement over our previous particles’ 13% QY, and the highest yet reported for an aqueous synthesis of CdSe/ZnS type particles.

Finally, we incorporate these QDs as well as two organic fluorophores, rhodamine 6G and rhodamine 800, into silica shells for direct monitoring in intestinal epithelial cells and tissues of exposed mice. We show that, for small nanoparticles, a typical Stober-type ammonia driven synthesis does not yield stable fluorescence. This has been observed in literature and is attributed to incompletely hydrolyzed silica precursor causing partial dissolution of the silica shell. We remedy this by applying an arginine driven silica shell synthesis, which is known to produce a denser and more stable product at smaller particle sizes. We show that all three fluorophores can be coated in a simple generalized procedure, and the resulting particles all show stable fluorescence with no evidence of
dye leakage. Using these particles, we demonstrate that silica nanoparticles can be observed internalizing into C2BBe1 intestinal epithelial cells, and in the tissues of mice that were fed the particles by gavage. We find direct evidence that the particles are absorbed into circulation and subsequently localize in organs throughout the body. Future efforts will attempt to better quantify this accumulation, as well as generalize the procedure to other food relevant nanoparticles such as TiO$_2$. 
Dedication

This document is dedicated to my family.
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I would first like to thank my advisor, Dr. Prabir Dutta. He has served as an excellent example to me of what a scientist should be. I have admired how hard he has always worked for his students, and the tremendous dedication he has to his art. Dr. Dutta has given so much helpful advice, and shown me considerable patience while keeping me on track through the last several years, and I am grateful looking back to see the effects his mentorship has had on me as a scientist and a person.

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Chapter 1. Introduction

This dissertation will focus on three interconnected projects: the characterization of commercial food-relevant nanoparticles during a simulated digestion, and their internalization and toxicity to intestinal epithelial cells; the study of nucleation and growth of quantum dots in a microwave synthesis; and the encapsulation of fluorophores within silica shells for in vitro and in vivo studies to elucidate their fate after ingestion.

1.1. Nanoparticles and Exposure via Food

Nanomaterials, generally considered to be a material with at least one dimension smaller than 100 nanometers, have generated great interest due to the discovery that they can have vastly different properties than bulk materials. These changes can be due to an increase in surface area by mass: as particle sizes decrease, less material is contained within particles, and a greater portion of the atoms are present on the surface and exposed to potential reactants. For example, from a simple surface area calculation, 1 gram of silicon dioxide particles with diameters of 10, 100, and 1000 nm would have surface areas of 227, 22.7, and 2.27 m², respectively. A decrease in particle size can also lead to a
change in material properties, such as the band gap of semiconducting materials, which will be discussed in further detail later.

The food industry has identified many potential applications for nanotechnology. Examples include nanoencapsulation to increase bioavailability of nutrients and provide controlled release, flavor enhancers, sensors using nanotechnology in food packaging and nanoparticles with antimicrobial properties.\textsuperscript{1–3} Many of these applications are still being researched and may eventually find their way to the food products on the market. The Project on Emerging Nanotechnologies has compiled and periodically updates an inventory of consumer products containing nanoscale components, including products in the “Food and Beverage” category.\textsuperscript{4} This inventory lists 41 products containing nanoscale silicon dioxide at the time of writing. Most of these products are categorized as cleaning products and coatings which would pose potential exposure after particles entered the environment. Seven of the products are categorized as supplements, personal care, and cosmetics, which clearly have a more direct route of exposure. Tables and Figures

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investigate the safety of foods incorporating nanoparticles, but as of now, many of the inorganic nanoparticles are generally regarded as safe for use in foods.\textsuperscript{5}

**Current Dietary Exposure to Nanoparticles:**

In foods, silica (SiO\textsubscript{2}) is used as an anti-caking agent and to clarify liquids, while titania (TiO\textsubscript{2}) is commonly used as a whitening agent. The average size of food-grade TiO\textsubscript{2} (E171) and SiO\textsubscript{2} (E551) particles is a few hundred nanometers (nm), but both powders have broad size distributions. In an analysis of 89 consumer food products for TiO\textsubscript{2} content, 36\% of E171 particles were found to have one dimension less than 100 nm.\textsuperscript{6} Table 1.2 summarizes the products from this study that contained the highest concentration of titanium. One can see that these products are mostly candies and foods that are marketed to children, making them the group most likely to be exposed to more titania nanoparticles. Based on the TiO\textsubscript{2} detected in the tested food products, Weir et al.\textsuperscript{6} estimated dietary exposure to TiO\textsubscript{2} for the US population to be 0.2-0.7 mg TiO\textsubscript{2}/kg body weight/day for consumers over the age of 10 and 1-2 mg TiO\textsubscript{2}/kg body weight/day for children under age 10 due to greater consumption of candies and sweets that tend to contain TiO\textsubscript{2}. Food-grade SiO\textsubscript{2} was found to contain up to 33\% silica with a size between 10-200 nm,\textsuperscript{7} some of which would be considered nanoparticles according to the conventional definition of a nanoparticle as less than 100 nm. Table 1.3 summarizes the concentrations of silica nanoparticles found in 10 commercial food products from this study. All of the products highest in nano-silica were powdered pre-made mixes of
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**Potential Bioaccumulation and Toxicity:**

As orally ingested nanoparticles traverse the digestive tract through the mouth, stomach, and intestines, they will be exposed to a number of digestive enzymes. The epithelial cells lining the small intestine absorb most nutrients obtained from food by endocytosis or diffusion, and transport them across the epithelium where they can enter the bloodstream. As nanoparticles move through the small intestine, they will come into contact with intestinal epithelial cells, which may be able to internalize nanoparticles as they take up nutrients.

Previous *in vitro* and *in vivo* research has shown that nanoparticles do cross the intestinal epithelium, apparently by transcytosis, i.e. transport through the enterocytes.⁸⁻¹⁰ Desai, et al.⁸ found that biodegradable polylactic polyglycolic acid co-polymer particles of 100 nm were internalized by intestinal tissue 15-250 times more than larger size (1-10 µm) particles. Koeneman et al.⁹ exposed an epithelial-lining intestinal model to 40 nm titania particles and found that the particles crossed this lining via transcytosis, with no effects on the cells from 10 µg/mL to 100 µg/mL. Jani et al.¹⁰ fed Sprague Dawley rats
1.25 mg/kg doses of 50 nm to 3 µm polystyrene particles for 10 days. These particles were found to have accumulated in the rats: 34% of the 50 nm particles remained, while 26% of the 100 nm particles remained. The percent uptake in liver, spleen, blood, bone marrow, and kidney combined was found to increase exponentially with decreasing particle diameter.

Several studies have begun to investigate the toxicity of inorganic nanoparticles to intestinal epithelial cells both in vitro and in vivo. Koeneman’s study, mentioned above, found that titania particles above a concentration of 100 µg/mL began to disrupt the integrity of the epithelial layer of an intestinal model. Wang et al. exposed adult mice to 5 g/kg body weight 25 and 80 nm titania and found evidence of damage to liver, kidney, and myocardial tissue, though no signs of acute toxicity were noted. Zhang et al. found that the same concentration (5 g TiO2/kg body weight) of 50 and 120 nm titania particles in conjunction with exposure to lead acetate (500 mg/kg body weight) resulted in damage to the liver. It is of note that the particle loadings in these two studies are significantly higher than the estimated level of exposure on the order of 1-10 mg/kg body weight discussed above. Pasupuleti et al. exposed Sprague Dawley rats to varying concentrations of 86 nm ZnO particles for 14 days, and found that the lowest doses (5 mg/kg body weight) resulted in the highest incidence of microscopic lesions of the liver, pancreas, heart, and stomach, with the effect diminishing as concentration increased. Onishchenko et al. found that titania nanoparticles of rutile and anatase phases, as well as food grade titania, injected directly into the ileac loop of rats showed no toxicity to these cells, but did accumulate in the liver. Awaad et al. determined that fluorescent
silica particles were internalized by Peyer’s patches in the intestinal epithelium more as particle size decreased from 1050 nm to 95 nm.

Recently, nanoparticle characteristics other than size, including composition, solubility, crystal structure, surface charge, surface modifications, and shape have been recognized to substantially influence nanoparticle toxicity. Warheit et al.\textsuperscript{16} showed that different forms of titania affected rats differently upon pulmonary exposure: an 80/20 anatase/rutile mixture cause more lung inflammation, cytotoxicity, and lung parenchymal cell proliferation than three different rutile samples. Our group has previously studied how properties of various nanoparticles affect their interactions and toxicity with cells. It was shown that carbon nanoparticles containing iron are significantly more biologically active than plain carbon nanoparticles, based on their chemical reactivity toward decomposition of hydrogen peroxide to form hydroxyl radicals.\textsuperscript{17} We have also reported on differing internalization behavior of quantum dots depending on positive or negative charge, finding that negatively charged CdSe/CdS/ZnS quantum dots associated with cell surface scavenger receptors, while positively charged QDs did not, and were subsequently internalized more rapidly.\textsuperscript{18} Lastly, our group has reported that silver nanoparticles embedded into zeolite membranes show antibacterial effects, but that this is due to silver ions released from the membrane and not from direct interaction with silver nanoparticles themselves.\textsuperscript{19} Based on the toxicity studies that have been conducted in different systems, it is apparent that toxicity depends upon many different properties of nanoparticles.
Toxicity to Intestinal Epithelial Cells

There is conflicting data in the literature on toxicity of silica to intestinal epithelial cells. Lack of toxicity of SiO$_2$ in Caco-2 cells was observed despite particle internalization by cells and even nuclear localization of particles.$^{20}$ Mitochondrial activity (WST assay) in Caco-2 cells treated with SiO$_2$ nanoparticles (NP) showed no significant changes as compared to controls, even at dosage levels of 200 µg/mL.$^{20}$ In another study, SiO$_2$ NP were found to reduce cell viability only in undifferentiated Caco-2 cells, even at loadings of 5 µg/cm$^2$.$^{21}$

With nanoparticles of TiO$_2$, there appears to be agreement of a lack of toxicity towards Caco-2 cells.$^{22}$ Commercial TiO$_2$ used in sunscreens, made up of a rutile core surrounded by Al(OH)$_3$ shell and fibrous-type morphology with lengths of 50 ±10 nm and widths of 7 ± 2 nm were not internalized, and no toxic effects were noted in response to loadings of up to 100 µg/mL.$^{23}$ TiO$_2$ at concentrations of 1-20 µg/cm$^2$ had no effect on Caco-2 cell viability in the presence or absence of serum.$^{24}$ TiO$_2$ at loadings of 0.1-100 mg/L did not influence Caco-2 cell viability, as measured by MTT assay.$^{22}$ TiO$_2$ at loading levels of 1-10 µg/mL did not cause cell death, either after acute or chronic exposure in Caco-2 cells.$^{9}$ Anatase/rutile mixtures, similar to the NP examined in the present study, did not induce toxicity (by LDH assay) to Caco-2 cells at loading levels of 20 µg/cm$^2$, but toxicity was observed at 80 µg/cm$^2$. The WST-1 assay indicated reduction in metabolic activity only at loadings of 80 µg/cm$^2$.$^{25}$ Using the MTT assay, different
mammalian cell types have been shown to respond differently to TiO$_2$ NP, with only some showing a toxic response.$^{26}$

There is agreement in the literature that ZnO induces some cytotoxicity in Caco-2 cells.$^{22}$ ZnO have been reported to be cytotoxic to both undifferentiated and differentiated Caco-2 cells at doses $> 5$ µg/cm$^2$.\textsuperscript{21}

Only one of these studies (Gerloff\textsuperscript{21}) included a simulation of the digestive system, which the particles will pass through before reaching the intestinal epithelial cells. In Gerloff’s work, the digestion simulation involved acidifying the solution to match stomach acid (pH=2.7) and then neutralized with a carbonate/bicarbonate buffer. As we have described above, the surface properties of the particles can clearly have an impact on their behavior and toxicity. There are many chemical species along the digestive tract that the particles could interact with, so it is important to mimic the digestion as closely as possible to determine if any of these bind to and affect the properties of the particles.

\textit{Contributions of This Work:}

In Chapter 2 of this dissertation, we investigate the properties of three commercially available nanoparticles; SiO$_2$, TiO$_2$, and ZnO, during a simulated digestion, and examine their toxicity on C2BBe1 intestinal epithelial cells using several assays. To simulate the \textit{in vivo} environment before interaction with the intestinal epithelium, nanoparticles were incubated with representative gastric and small intestinal
digestive enzyme solutions before cell exposure. Long-term exposure during which cells were repeatedly treated with nanoparticles was also conducted. Data generated by these studies, the first to include long-term exposure in an *in vitro* intestinal epithelial cell model, suggest that SiO$_2$ and TiO$_2$ exposed to culture media do not cause significant toxicity *in vitro* in their interactions with intestinal epithelial cells, whereas ZnO exhibits mild toxicity. However, TiO$_2$ treated with simulated digestion media do exhibit mild toxicity.

In order to further explore these particles’ interactions with the digestive system and potential accumulation should they be absorbed into the bloodstream, we will utilize fluorescent molecules and quantum dots which can be encapsulated within metal oxide shells, allowing for their visual observation.

1.2. Semiconducting Quantum Dots

Quantum dots (QDs) are nanosize semiconductors that fluoresce, the origin of fluorescence arising from quantum confinement effects. In semi-conducting materials, an excited-state consists of an electron excited to the conduction band, and a hole left behind in the valence band. This is known as an exciton. The electron and the valence-level hole have a natural spatial separation, called the Bohr exciton radius. If a particle is made smaller than the Bohr exciton radius, the confinement of the exciton to this space leads to an increase in the band gap energy, the quantum confinement effect. In a quantum dot, the particle size is smaller than the Bohr exciton radius, typically on the order of 10 nm,
and thus the quantum confinement manifests itself as an increase in the band gap energy. As the exciton is further constrained, the band gap energy continues to increase. This increase in energy results in a decrease of fluorescence wavelength. By controlling the size of the quantum dot, a precise fluorescence wavelength can be chosen. Using this strategy with various semiconducting materials, nanometer-sized quantum dots have been made which fluoresce across the entire visible spectrum and in the infrared. Figure 1.1 shows an image published by Shuming Nie’s group of a size series of CdSe/ZnS quantum dots with fluorescence wavelengths spanning the visible spectrum, from 443 to 655 nm.

QDs are useful in a variety of fields because they are less prone to photo-bleaching and chemical degradation, have discrete emission wavelengths based on size, and their wide absorption profile makes them very suitable for biological imaging. Materials scientists are exploring QDs for solar cells, sensors, and improved lighting. A Nature report published in 2009 details a potential booming market for quantum dots. Early commercial uses for quantum dots were for bio-imaging, with Evident being a major supplier to biologists. More recently, quantum dots are being used for LED lights and display panels. In 2013, Amazon.com released the first consumer tablet device with a quantum dot display, produced by Nanosys and 3M. ASUS is subsequently releasing a laptop using the same quantum dot display in 2014. A market research report by Allied Market Research published in 2014 predicts the world QD market will grow from $316 million in 2013 to $5.04 billion by 2020. The report expects this growth to be driven by increased biological imaging, optoelectronics, and renewable energy applications. These
increases depend on the further maturation of QD production, allowing more for economical production. With the potential growth of this market due to existing demand, advances in the efficiency of QD production have potential to be highly impactful.

*Synthetic Methods*

Control of nucleation and growth of QDs is essential to optimize size and optical properties. Conventional organic-based hot-injection synthesis involves rapid nucleation by introducing precursor solutions to a hot (>300 °C) solvent. For the cadmium chalcogenides, these typically involve injection of dimethyl cadmium or cadmium oxide with other organometallic reagents into high temperature coordinating solvents such as trioctylphosphine (TOP) and trioctylphosphine oxide (TOPO).\textsuperscript{39} Controlled growth then occurs more slowly at a lower temperature and the final size of the particles is determined by the temperature during the growth phase and the organic capping agent which sterically inhibits growth.\textsuperscript{39} Such methods of quantum dot production are well developed and produce high quality quantum dots with quantum yields (QY) between 50-80 %.\textsuperscript{40}

The QY typically decreases with surface defects that create trap states resulting in nonradiative decay and a broad red-shifted trap-state emission.\textsuperscript{41} To enhance the fluorescence and stability of the core particle, protective shells with higher band gap are often grown around them.\textsuperscript{40,41} Figure 1.2 shows a simplified energy diagram of an example CdSe/ZnS core/shell quantum dot. Ideally, all electron/hole recombination would be from the conduction band to valence band of the CdSe. Trap states of various
energies may be present, however, and recombination from these states results in a wide band of lower energy red-shifted emission. Addition of a shell layer can passivate these trap states, resulting in a greater portion of the emission coming from the CdSe bandgap. The shell material chosen must have a larger energy bandgap than the core material to prevent electron/hole migration into the shell.

Quantum dots prepared in organic solvents do not disperse in water and require ligand exchange, which can significantly alter the optical properties of the particles. By careful optimization of post-treatment conditions, Pong et al.\textsuperscript{42} were able to ligand exchange TOPO capped CdSe/ZnS QDs with mercaptopropionic acid (MPA) and retain the particles’ original QY of 40\%. This was achieved by removal of MPA’s thiolic hydrogen with strong base, and optimization of MPA added. The amount of MPA added proved to be of importance: adding 70 µL led to 42\% QYs while adding 50 µL resulted in a reduction of QY from 40\% to 24\%. Due to the added difficulty of ligand exchanging QDs for water-solubility, it is advantageous to synthesize the QDs directly in aqueous solution.

Aqueous based methods of quantum dot synthesis have been developed which yield water-soluble quantum dots suitable for use in biological research. In these methods, cadmium chalcogenides (Se, Te) are nucleated by reacting cadmium salts (CdCl\textsubscript{2}) with NaH(Se, Te) in the presence of a passivating ligand, often utilizing thiol groups for attachment.\textsuperscript{43,44} These reaction methods have the benefit of being safer, less expensive, and yielding water-soluble particles without the need for ligand exchange; however, their quantum yield is typically lower compared to organic methods. While the
well-developed organic hot-injection method routinely yields QYs in excess of 50% for CdSe/ZnS, the highest QY reported for CdSe/ZnS in an aqueous synthesis is 38%.

The advantages of microwave heating for aqueous synthesis of QD have been enumerated. Li et al. have shown this of CdTe QDs, which had previously had QYs in the range of 50-80% for organic-phase synthesis, but 3-10% for aqueous-phase synthesis. In addition, the aqueous synthesis required several hours to days to prepare. By utilizing a microwave synthesis, aqueous CdTe with QYs of 40-60% were prepared within 1-2 hours. Similarly, Qian et al. reported improvements in the synthesis of ZnSe QDs. Organic-based methods typically resulted in 20-50% QYs, and initial attempts at aqueous synthesis gave particles with a 1-10% QY and took 1-2 days to produce. Using a microwave synthesis, particles with a 17% QY were produced in 55 minutes. Microwave synthesis has also been used to improve organic-based methods, as reported by Gerbec et al. By utilizing a microwave synthesis in an ionic-liquid, TOPO capped QDs with 74% QY were produced in as little as 30 seconds, and did not require high-temperature injection as required in typical hydrothermal routes. Microwave heating is generally understood to increase reaction rates mostly due to uniform dielectric heating. In a hydrothermal reaction route, heat is transferred from a heat source to the reaction vessel, and then to the reaction solution from the outside and propagating inward. In a microwave synthesis, microwave energy penetrates the microwave-transparent reaction vessel (glass, Teflon) and directly heats any polar molecules in the reaction solution. This heat is then transferred to the rest of the solution, resulting in an overall increase in temperature. This heating process can be very rapid, and additionally can result in
localized temperatures of microwave-absorbing reactants being much higher than the overall solution. Besides uniform dielectric heating of the reaction volume, influence on the collisions between reactants, as well as entropic effects due to the rotation of the dipoles are considered important in explaining kinetics and reaction yields.

Contributions of This Work

In Chapter 3, we investigate the microwave synthesis of CdSe/CdS/ZnS quantum dots, with the end goal of producing particles with a higher quantum yield and greater potential for use in subsequent biological studies. We have previously reported a fast, “one-pot” synthesis that produces stable, aqueous CdSe/CdS/ZnS core/shell quantum dots. While they are relatively easy to produce and show promising stability during biological experiments, these particles have a low quantum yield of 13%. By coupling the microwave reactor to a fluorescence spectrometer via fiber optics, the growth of the QD is monitored during synthesis, and this real-time data has provided insight into the growth mechanism. We also examine the effect of temperature during both the initial nucleation phase and the microwave-assisted crystal growth, and found a way to form QDs with varying emission wavelengths. When combined with UV-visible light exposure during nucleation and after microwave-based QD growth, CdSe/CdS/ZnSe QDs with quantum yields of 40% were obtained.
Having obtained a significantly brighter QD, we next explore the silica coating of these particles along with two organic dyes, rhodamine 6G and rhodamine 800, and their observation within cells and tissue samples.

1.3. Fluorescence Functionalized Silica for Biological Studies

As discussed previously in Section 1.1, silica is an important and commonly used additive in food products. While there is increasing interest in using nano-scale silica particles, evidence shows that currently used food grade silica already contains up to 33% of particles within 10-200 nm. Nanoparticle interactions with biological systems are dependent on size as well as surface charge and material properties. One method commonly used to study these interactions is the imaging of nanoparticles functionalized with a fluorescent component. Because particle interactions depend on surface material and charge, it is important that the dye remains within the particle and is not present in the solution or bound to the particle surface, where it may affect their properties.

Silica Synthesis

Two common methods of silica nanoparticle synthesis are a sol-gel based Stober synthesis, and a modification of this which takes place in a reverse microemulsion water-
in-oil droplet. Both of these involve an ammonia catalyzed hydrolysis of an alkyl silicate followed by condensation, shown here for TEOS:

\[
\text{Hydrolysis: } \quad \text{Si(OC}_2\text{H}_5\text{)}_4 + \text{H}_2\text{O} \rightarrow \text{Si(OC}_2\text{H}_5\text{)}_3\text{OH} + \text{C}_2\text{H}_5\text{OH} \\
\text{Condensation: } \quad \text{Si-O-H + H-O-Si} \rightarrow \text{Si-O-Si} + \text{H}_2\text{O} \\
\text{Si-OC}_2\text{H}_5 + \text{H-O-Si} \rightarrow \text{Si-O-Si} + \text{C}_2\text{H}_5\text{OH}
\]

The Stober silica synthesis is an adaptation of a typical sol-gel synthesis in which tetra alkyl silicates undergo hydrolysis and condensation in the presence of ammonia.\textsuperscript{51,52} Under optimal basic conditions, a gel phase is not formed, and a solution of easily purified silica nanoparticles is obtained. This reaction has been utilized to add a silica coating to various materials, though typically only with polar ligands attached to the surface.\textsuperscript{53}

Silica synthesis involving a reverse-microemulsion was developed to further control over Stober type methods.\textsuperscript{54} In essence, a Stober synthesis is performed within water-in-oil droplets. Figure 1.3 shows an example water-in-oil droplet, or micelle. Water droplets are stabilized in an organic solvent by surfactant molecules. The hydrophilic head-groups surround the water droplet, and the hydrophobic tail-groups lend stability in the non-polar solvent. Water-soluble alkyl silicates and ammonia localize within the surfactant-stabilized water droplet, which serves as a border for their growth. This method has led to small, monodisperse nanoparticle formation. Creating the water-
in-oil emulsion requires a large amount of surfactant, so particles of this type require more extensive post-synthesis washing than those formed in a typical Stober synthesis.

_Dye Encapsulation_

There are a variety of choices of fluorophores to use with silica coating for biological studies. The variables desired are good fluorescence stability/lack of photobleaching, stability in biological media, bright fluorescence/high quantum yield, and a longer emission wavelength, particularly for in-vivo imaging where the emitted light must pass through tissues. Fluorescence in the near-IR and IR region benefits from minimal scattering and absorption in biological tissues.\(^{55,56}\) In this research, we focus on CdSe/CdS/ZnS quantum dots (QDs), rhodamine 6G, and rhodamine 800. CdSe/CdS/ZnS QD synthesis has been previously optimized in our lab to inexpensively and quickly produce particles with a 40% quantum yield and \(\lambda_{\text{em}}\) of 574 nm.\(^{57}\) Quantum dots in general have been popular for biological studies due to their bright fluorescence and resistance to photobleaching. As opposed to photobleaching, UV light in some cases actually increases the fluorescence intensity of QDs.\(^{58-62}\) In CdSe quantum dots, this is due to the dissolution of surface trap states, freeing atoms to subsequently rejoin the surface crystal structure in a more stable conformation. Rhodamine 6G is a commonly available and inexpensive organic dye, with very strong emission (90% quantum yield in water) at 558 nm.\(^{63}\) Rhodamine 800 is a dye in the rhodamine family with a 25% QY emission maximum at 700 nm.\(^{64}\) While this dye has the lowest quantum yield of the three
we have chosen, it is easily adapted to syntheses involving rhodamine 6G, and has a near-IR fluorescence emission, which is desirable for in-vivo imaging. Figure 1.4 shows the structure of each fluorophore discussed here. Rhodamine 6G and rhodamine 800 are both positively charged at neutral pH.

Potential for Dye Leakage

An important issue that has arisen in the encapsulation of dyes in Stober-silica shells is the instability of the silica to dissolution. Recent publications show evidence of silica NP dissolution, including from the inside, leaving hollow spheres. Wang and Chen\(^65\) show that silica coated gold NPs approximately 110 nm in diameter etch differently depending on synthetic conditions; dissolving completely when reacted for 4 hours at room temperature, leaving a shell when reacted for 14 hours at room temperature, and remaining stable when reacted at room temperature for 4 hours and 60\(^\circ\)C for 10 hours. The nature of these shells must be porous if material is dissolving and leaving empty spheres. This poses an obvious problem for dye-encapsulation: the dye may leave the particles. This dissolution is found to be size-dependent and more relevant to smaller nanoparticles. Park reports that silica nanoparticles of 21 nm initially develop internal pores and eventually form an interconnected matrix, particles of 49 nm formed hollow spheres, and particles of 123 nm remained stable.\(^66\) The dissolution is also found to be particularly strong in biological media.\(^67,68\) A more recent development to the Stober synthesis of silica particles has been the use of weakly basic amino acids in place
of ammonia. This includes the coating of core particles, where higher temperatures and lower amino acid concentrations result in more stable particles. Using this strategy, dense stable coatings of dye-incorporated silica have been synthesized which minimize dye leakage.

Contributions of This Work

In Chapter 4 we investigate the functionalization of these three fluorophores with a silica shell, with the goal of observing the particle fluorescence in an in vivo study to determine if food-relevant silica nanoparticles escape the digestive system and accumulate within other organs. For each fluorophore, we investigate two synthetic methods of silica coating: driven by ammonia and driven by more weakly basic arginine.

Rhodamine 6G/rhodamine 800 dye coating: First, a more common Stober method will be used to coat the organic rhodamine 6G and rhodamine 800 dyes electrostatically bound to commercial silica nanoparticles. This method is advantageous in its simplicity; no covalent linkage is required, instead relying on attraction of the positively charged dye to the negatively charged silica core’s surface, and no surfactants are required which necessitate extensive washing of the synthesized particles. We base our synthesis on a literature report of Stober-silica coated rhodamine 6G dye, and we expect this should be adaptable to rhodamine 800 due to their similar structure and charge (Figure 1.4). While this method was determined to be stable for silica particles above 150 nm in diameter, it is known that smaller silica nanoparticles can undergo dissolution, and this will need to
be monitored to determine if the smaller particles are stable against dye leakage. Next, to counter this potential dissolution, the more recent arginine-driven method discussed previously will be generalized to these two molecules.67

**QD/silica coating:** There are many reports on coating quantum dots with silica for increased biocompatibility72,73 and biodetection.74 These particles are of varying quality and stability depending on the specific core/shell structure and passivating ligands present.75 For the coating of quantum dots, microemulsion synthesis has been found to be robust against many reaction conditions and result in monodisperse particles.53 We will apply this method to our lab’s previously developed MPA capped CdSe/CdS/ZnS quantum dots. The arginine-driven method reported by Dawson will also be generalized to the QD particles to determine if its more dense silica coating is beneficial.67

**Biological Studies:** After the optimal particle of each type is determined, they will each be observed in a murine alveolar macrophage cell line to demonstrate their utility in the study of biological systems. These cells provide a good starting point for a feasibility study, as macrophages typically show strong uptake of foreign materials. We will then expose an intestinal epithelial cell line, C2BBe1, to the particles to investigate their propensity to internalize within cells more relevant to the digestive system. Finally, the particles will be administered orally to mice, followed by confocal microscopy of tissue samples to determine if particles escape the digestive system, and where they may localize.
1.4. References


Table 1.1. Number of products currently sold containing nanoparticles of selected inorganic materials

<table>
<thead>
<tr>
<th>Particle</th>
<th>All Products</th>
<th>Foods, Beverage, Supplements</th>
<th>Cosmetic Products, Personal Care</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver</td>
<td>434</td>
<td>19</td>
<td>141</td>
</tr>
<tr>
<td>Silica</td>
<td>41</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Titania</td>
<td>91</td>
<td>0</td>
<td>43</td>
</tr>
<tr>
<td>Zinc Oxide</td>
<td>38</td>
<td>1</td>
<td>29</td>
</tr>
</tbody>
</table>
Table 1.2. List of food products containing the most titanium by weight. Adapted from Weir.⁶

<table>
<thead>
<tr>
<th>Product</th>
<th>Titanium Content (µg Ti/mg Food)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dickinson’s Coconut Curd</td>
<td>3.59</td>
</tr>
<tr>
<td>Mentos Freshmint Gum</td>
<td>2.64</td>
</tr>
<tr>
<td>Hostess Powdered Donette</td>
<td>2.42</td>
</tr>
<tr>
<td>Good and Plenty Candy</td>
<td>2.08</td>
</tr>
<tr>
<td>Kool Aid Blue Raspberry</td>
<td>1.69</td>
</tr>
<tr>
<td>Eclipse Spearmint Gum</td>
<td>1.64</td>
</tr>
<tr>
<td>M&amp;M Chocolate Candy</td>
<td>1.25</td>
</tr>
<tr>
<td>Albertsons Vanilla Pudding</td>
<td>1.00</td>
</tr>
<tr>
<td>Betty Crocker Whipped Cream Frosting</td>
<td>0.922</td>
</tr>
<tr>
<td>M&amp;M Chocolate with Peanuts</td>
<td>0.839</td>
</tr>
</tbody>
</table>
Table 1.3. 10 food products highest in nanosilica concentration. Adapted from Dekkers.7

<table>
<thead>
<tr>
<th>Product</th>
<th>Nano-silica Concentration (mg nano-silica/g Food)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coffee Creamer</td>
<td>1.0</td>
</tr>
<tr>
<td>Roasted Vegetable Rub</td>
<td>0.6</td>
</tr>
<tr>
<td>Sea Food Rub</td>
<td>0.5</td>
</tr>
<tr>
<td>Sweets Sticky Rub</td>
<td>0.4</td>
</tr>
<tr>
<td>Lasagna Sauce Mix</td>
<td>0.3</td>
</tr>
<tr>
<td>Burrito Seasoning Mix</td>
<td>0.3</td>
</tr>
<tr>
<td>Minced Meat Seasoning Mix</td>
<td>0.2</td>
</tr>
<tr>
<td>Steak House Rub</td>
<td>0.2</td>
</tr>
<tr>
<td>Instant Asparagus Soup</td>
<td>0.2</td>
</tr>
<tr>
<td>Instant Noodles</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>
Figure 1.1. Image from Shuming Nie's group\textsuperscript{32} showing CdSe/ZnS QDs ranging from 443 to 655 nm.
Figure 1.2. Simplified energy diagram of CdSe/ZnS core/shell quantum dot.
Figure 1.3. Example of a water-in-oil micelle. Polar hydrophilic head-groups surround a droplet of water, while non-polar hydrophobic tails make the droplet soluble in a non-polar organic solvent.
Figure 1.4 CdSe/CdS/ZnS QD, Rhodamine 6G, and Rhodamine 800 structures. Charged sites at neutral pH are indicated on Rhodamine 6G and Rhodamine 800.
Chapter 2. Minimal intestinal epithelial cell toxicity in response to short- and long-term food-relevant inorganic nanoparticle exposure

2.1. Introduction

The food industry has identified many potential applications for nanotechnology. Examples include nanoencapsulation to increase bioavailability of nutrients and provide controlled release, flavor enhancers, sensors using nanotechnology in food packaging and nanoparticles with antimicrobial properties.\textsuperscript{1-3} Many of these applications are still being researched and may eventually find their way to the food products on the market. The Project on Emerging Nanotechnologies has compiled and periodically updates an inventory of consumer products containing nanoscale components, including products in the “Food and Beverage” category.\textsuperscript{4} Despite the increased interest in using nanoparticles in food, the FDA currently has no specific regulations related to nanoparticles. A 2012 draft guidance suggested that food manufacturers investigate the safety of foods incorporating nanoparticles, but as of now, many of the inorganic nanoparticles are generally regarded as safe for use in foods.\textsuperscript{5}
Silica (SiO\textsubscript{2}) is used as an anti-caking agent and to clarify liquids, while titania (TiO\textsubscript{2}) is commonly used as a whitening agent. The average size of food-grade TiO\textsubscript{2} (E171) and SiO\textsubscript{2} (E551) particles is a few hundred nanometers (nm), but both powders have broad size distributions. In an analysis of 89 consumer food products for TiO\textsubscript{2} content, 36% of E171 particles were found to have one dimension less than 100 nm.\textsuperscript{6} Based on the TiO\textsubscript{2} detected in the tested food products, Weir et al.\textsuperscript{6} estimated dietary exposure to TiO\textsubscript{2} for the US population to be 0.2-0.7 mg TiO\textsubscript{2}/kg body weight/day for consumers over the age of 10 and 1-2 mg TiO\textsubscript{2}/kg body weight/day for children under age 10 due to greater consumption of candies and sweets that tend to contain TiO\textsubscript{2}. Food-grade SiO\textsubscript{2} was found to contain up to 33% silica with a size between 10-200 nm,\textsuperscript{7} some of which would be considered nanoparticles according to the conventional definition of a nanoparticle as less than 100 nm. A study assessing the silica content of various foods estimated a “worst-case” intake of 1.8 mg/kg body weight/day nanosilica for the average adult (9.4 mg/kg body weight/day total silica).\textsuperscript{7} These studies reveal that consumers are currently being exposed to nano-sized fractions of both food-grade TiO\textsubscript{2} and SiO\textsubscript{2}. However, individual exposure will vary greatly with diet.

As orally ingested nanoparticles traverse the digestive tract through the mouth, stomach, and intestines, they will be exposed to a number of digestive enzymes. The epithelial cells lining the small intestine absorb most nutrients obtained from food by endocytosis or diffusion and transport them across the epithelium where they can enter the bloodstream. As nanoparticles move through the small intestine, they will come into
contact with intestinal epithelial cells, which may be able to internalize nanoparticles as they take up nutrients.

Previous \textit{in vitro} and \textit{in vivo} research has shown that nanoparticles do cross the intestinal epithelium, apparently by transcytosis, i.e. transport through the enterocytes.\textsuperscript{8-10} Several studies have begun to investigate the toxicity of inorganic nanoparticles to intestinal epithelial cells both \textit{in vitro}\textsuperscript{9} and \textit{in vivo}.\textsuperscript{11-14} Based on the toxicity studies that have been conducted in different systems, it is apparent that toxicity depends upon many different properties of nanoparticles. Particle size influences toxicity, with nanoparticles showing greater toxicity than their larger counterparts.\textsuperscript{15} Smaller particles have also been shown to be more readily internalized by intestinal epithelial cells.\textsuperscript{8,10,15} Recently, nanoparticle characteristics other than size, including composition, solubility, crystal structure, surface charge, surface modifications, and shape have been recognized to substantially influence nanoparticle toxicity.\textsuperscript{16-19}

In this chapter, we investigated the internalization of commercially available nanoparticulate SiO\textsubscript{2}, TiO\textsubscript{2}, and ZnO, and examined their toxicity on C2BBe1 intestinal epithelial cells using several assays. To simulate the \textit{in vivo} environment before interaction with the intestinal epithelium, nanoparticles were incubated with representative gastric and small intestinal digestive enzyme solutions before cell exposure. Long-term exposure during which cells were repeatedly treated with nanoparticles was also conducted. Data generated by these studies, the first to include long-term exposure in an \textit{in vitro} intestinal epithelial cell model, suggest that SiO\textsubscript{2} and TiO\textsubscript{2} exposed to culture media do not cause significant toxicity \textit{in vitro} in their
interactions with intestinal epithelial cells, whereas ZnO exhibits mild toxicity. However, TiO₂ treated with simulated digestion media do exhibit mild toxicity.

2.2. Experimental Procedures

The cell cultures, exposure of cells to particles, cell growth studies, staining, and toxicity assays described in this section were performed by Christie McCracken in Dr. James Waldman’s laboratory.

Nanoparticles

Zinc oxide, titania, and silica nanoparticles were purchased from Sigma-Aldrich (St. Louis, MO). The specifications of the ZnO particles were size ≤ 100 nm, with a specific surface area of 15-25 m²/g. For TiO₂, the particle size was specified as 21 nm, with surface area of 35-65 m²/g, and purity of ≥ 99.5%, with a trade name of Aeroxide P25. Silica particle specification included size of 12 nm, with surface area of 175-225 m²/g, and purity of 99.8%.

Particle Size and Surface Charge

A Zetasizer Nano ZS (Malvern, Westborough, MA) was used to determine the size and zeta potential of the particles. The Nano ZS uses a 633 nm laser as its light
source. For size measurements, a 173° backscatter angle was used for collecting scattered light. The instrument was set to automatically determine the number of runs, the run duration, and the optimal focal point for each sample. The analysis model was set to general purpose, with default size limits of 0.4-10000 nm in diameter. Three replicate measurements were taken for all samples and averaged. All particle sizes are given as radii. For zeta potential measurements, a forward angle of 12° was used for collecting light. The default Smoluchowski model in the software program was used. Each measurement included 20 runs and monomodal analysis provided by the vendor was used for analysis. Three replicate measurements were taken for all samples and averaged. Samples were titrated versus pH using an attached MPT-2 Autotitrator. The titrator was supplied with 0.1 M HCl and 1.0 M HCl. Three replicate measurements were taken at each pH with a two minute pause between all measurements.

*TEM Images of Commercial Nanoparticles*

Images were taken using a Tecnai F20 Transmission Electron Microscope. Ten mg/mL solutions of each particle in ethanol were prepared and sonicated for 30 minutes. The solutions were dropped on to lacy carbon copper TEM grids (Ted Pella, Inc., Redding, CA) and allowed to dry for several hours.
Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) was performed on particles which had undergone simulated digestion treatment. After the final digestion step, the particles were washed twice by centrifugation and replacement of supernatant with water. The digested particles were isolated by centrifugation and frozen with liquid nitrogen, then placed in a Millrock Bench Top Manifold Freeze Dryer (Millrock, Inc.) to preserve any potential protein coating. The digestion protocol was performed on another set of particles using water in place of digestive enzymes to use as reference. DRIFTS analysis was performed with a Spectrum 400 FTIR Imaging System (Perkin Elmer). Five hundred runs were performed in % Reflectance mode using an air background (mirror in the diffuse reflectance cell) for both the water and digested samples (50 mg); the samples were packed into the infrared cell without any dilution. Kubelka Munk analysis was performed on these samples using an in-house developed program using the air reference. The Kubelka Munk spectra of each water-exposed particle was subtracted from the spectra of each digested particle using Origin software, leaving only peaks related to the digestion enzymes and media (normalized relative to the 1260 cm\(^{-1}\) band observed in both TiO\(_2\) and ZnO, and for SiO\(_2\), the subtraction was done to maximize/symmetrize the band at 1300 cm\(^{-1}\)). An example of this for TiO\(_2\) is shown in Figure 2.1. The top spectrum shows TiO\(_2\) exposed to water, followed below by the spectrum of TiO\(_2\) exposed to the digestion treatment. In both of these, a band is seen at
1260 cm\(^{-1}\). After the subtraction this band is gone, and only peaks associated with the digestive solutions remain, seen in the bottom spectrum of Figure 2.1.

**X-Ray Diffraction**

X-Ray diffraction (XRD) analysis of the commercial particles was performed using a Rigaku Geigerflex diffractometer. Particles were packed onto glass slides with no further preparation. The diffraction angle was scanned from 0 to 100° (2θ) with a 0.2 step size and 10 second dwell time.

**Atomic Absorption Spectroscopy**

Atomic absorption spectroscopy was performed to test for the presence of dissolved Zn ions after exposure of ZnO particles to the acidic pepsin enzyme solution. A Buck Scientific Accusys 211 spectrophotometer (East Norwalk, CT) was used with a Zn hollow cathode lamp (Heraeus). Standard solutions were diluted from 1000 ppm standard (Buck Scientific). The digestion protocol was performed on ZnO particles and stopped after exposure to pepsin solution. Following this, the solution was centrifuged at 209,000g for 30 minutes. The supernatant was removed and analyzed for Zn ion concentration.
Simulated gastrointestinal nanoparticle digestion

Pepsin, pancreatin, and bile salts were used to simulate the gastric and small intestinal digestive environments \textit{in vitro}. The concentrations used were based on \textit{in vitro} digestion methods used in previously published studies. The stomach enzyme pepsin (146 U/mL, Sigma-Aldrich) was dissolved in water (pH 2, adjusted with 1N HCl). The small intestinal enzyme mixture pancreatin (2mg/mL, Sigma-Aldrich) was dissolved in water (adjusted to pH 7 with 1M NaHCO\textsubscript{3} and HCl). A solution of intestinal bile extract (porcine, Sigma-Aldrich) was made at a concentration of 0.024mg/mL in water (adjusted to pH 7 with 1M NaHCO\textsubscript{3} and HCl).

To simulate the digestive process, nanoparticles (50 mg/L) were incubated first in the pepsin solution for one hour (37°C). The nanoparticles were pelleted by centrifugation (200,000 x g for 30 minutes) and resuspended in the pancreatin solution. After a one-hour digestion (37°C), the particles were pelleted and resuspended in bile extract (one hour, 37°C). Nanoparticles were centrifuged and resuspended in phosphate-buffered saline (PBS) and used for the assays.

\textit{Cell Culture}

C2BBe1 cells were obtained from the American Type Culture Collection (Manassas, VA) at passage 47 (as specified by the vendor). For short-term exposure assays, cells were used between passages 55-70, consistent with previous studies.\textsuperscript{24-26}
Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Serum Source International, Inc., Charlotte, NC), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.3% penicillin/streptomycin, 0.3 ug/mL Amphotericin B (fungizone), and 10 µg/mL transferrin (all from Life Technologies). Cells were incubated in 10% CO₂/90% room air at 37°C. Cells were passaged every 5-7 days and plated on flasks or plates pre-coated with collagen I (0.05 mg/mL, rat tail, Life Technologies). Cells were incubated at least 24 hours prior to treatment with nanoparticles.

Treatment of cells with nanoparticles

The nanoparticles were weighed and placed in a glass vial prior to steam-sterilization. Sterile nanoparticles were suspended in PBS to make a 1 mg/mL solution. Immediately prior to treating cells, nanoparticle solutions were sonicated using a VC130 Sonics Vibra-Cell sonicator (Sonic Materials, Inc. Norwalk, CT) pulsing for one second on, one second off for approximately 15 seconds in order to break up nanoparticle agglomerates before exposing them to cells. Nanoparticles were added to cells at a dose of 10ug/cm² (100µL of the 1 mg/mL suspension per well of a 6-well plate in 2 mL total volume per well, or 20µL per well in a 24-well plate in 0.5 mL total volume per well). For assays described below, cells were seeded in 24-well tissue culture plates at a density of 2×10⁴-1×10⁵ cells/well and grown to 75-100% confluency. To promote contact of nanoparticles with cells, plates were centrifuged at 300×g for 15 minutes. Cells were
incubated with nanoparticles for 24 hours at 37°C at 10% CO₂/90% air, and then washed twice with PBS. Toxicity assays were performed immediately after washing. For long-term nanoparticle exposure studies, culture media was added and cells were incubated at 37°C at 10% CO₂/90% air.

*Long-term nanoparticle exposure studies*

For long-term exposure studies, one exposure cycle consisted of plating cells in a 6-well tissue culture plate (Corning-Costar, Tewksbury, MA) pre-coated with collagen. After two days to allow the cells to firmly attach, cells were treated with 10 µg/cm² nanoparticles and centrifuged to promote nanoparticle-cell contact. After a 24-hour incubation at 37°C, cells were washed with PBS and media was replaced. Cells were cultured 4-6 days to reach confluency. Cells were then passed 1:10 into new 6-well plates and the nanoparticle treatment was repeated after each passage. This was continued for 29 nanoparticle exposure cycles. To perform toxicity assays, a portion of these long-term cultured cells were plated into 24-well tissue culture plates (Corning-Costar). Cells were treated with nanoparticles (10 µg/cm²) for 24 hours prior to each assay, as in single exposure studies. Long-term exposure studies were only conducted with commercially-obtained nanoparticles (i.e., no enzymatic digestion).
**Cell growth**

To assess the effects of long-term nanoparticle exposure on treated cells, we assessed their proliferation over a 10 day period. The cells were seeded at a density such that they reach confluence after 10 days. Cells were plated in triplicate into 6-well tissue culture plates at a density of $1 \times 10^5$ or $5 \times 10^4$ cells/well. The time at which cells were plated was considered time zero. Cells were counted daily using a Z2 Beckman Coulter Counter (Indianapolis, IN) and mean counts of triplicate wells were plotted versus time.

**Sytox Red staining**

To evaluate necrotic cell death (cellular membrane damage) in nanoparticle-treated cells, a flow cytometric analysis was performed using Sytox Red Dead Cell Stain (Life Technologies). Cells were treated with nanoparticles and briefly centrifuged. After a 24-hour incubation at 37°C, cells were washed twice with PBS and detached from the culture plate with trypsin. Each cell sample was suspended in 1 mL calcium- and magnesium-free Hanks Balanced Salt Solution (Life Technologies) and stained with 1 µL of the Sytox Red Dead Cell Stain. Samples were incubated for at least 15 minutes at room temperature before analyzing for fluorescence using a FACScalibur flow cytometer (BD Biosciences) at an excitation wavelength of 635 nm. All experimental conditions were performed in triplicate. As a positive control for cell death, a subset of wells were treated with 10 or 20 mM hydrogen peroxide ($\text{H}_2\text{O}_2$) in DMEM for one hour. Culture
medium was replaced and cells were incubated for 23 hours prior to staining and flow cytometric analysis.

**Annexin V-FITC staining**

To examine whether cells treated with nanoparticles underwent apoptosis, cells were stained with FITC-conjugated Annexin V (BD Biosciences, San Jose, CA). Cells were plated, treated with nanoparticles, and harvested with trypsin as described above. Cells were resuspended in 100 µL of Annexin binding buffer (Life Technologies). Five µL of FITC Annexin V was added to each tube and the samples were incubated for 15 minutes in the dark at room temperature before adding an additional 400 µL of Annexin binding buffer. Fluorescence of bound FITC Annexin V was then detected by flow cytometry, excitation wavelength of 488 nm. All experimental conditions were performed in triplicate. As a positive control for apoptosis, a subset of wells were treated with 10 or 20 mM H₂O₂ in DMEM for one hour. Culture medium was replaced and cells were incubated for 23 hours prior to flow cytometric staining and analysis.

**LDH Assay**

To further assess cell death by detecting cellular membrane damage, release of the cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium was examined by colorimetric assay. Cells were plated and treated with nanoparticles as described
above. After a 24-hour incubation, 50 µL of culture media was collected from each well and placed in a flat-bottom 96-well plate (BD Falcon). LDH activity was assessed using a commercially available kit (LDH Assay Kit, Sigma-Aldrich) according to manufacturer’s instructions. Briefly, LDH Assay Substrate Solution, LDH Assay Cofactor Preparation, and LDH Assay Lysis Solution were added to samples and incubated at room temperature for 15-30 minutes. The absorbance was read on a microplate reader at 490 nm and 690 nm. Sample absorbance values were corrected by subtracting the background reading at 690 nm from the 490 nm reading. As a positive control for LDH release, cells were treated with a 1% solution of Triton X-100 (Sigma-Aldrich) in DMEM for 5 minutes to lyse all cells before collecting the supernatants. Sample absorbance values were normalized to the Triton positive control (considered as 0% cell viability) and untreated negative control (considered as 100% cell viability). All experimental conditions were performed in quadruplicate.

MTT Assay

To measure the metabolic activity of nanoparticle-treated cells, we used a commercially available MTT assay kit (Cayman Chemical, Ann Arbor, MI). In this assay, the tetrazolium dye, MTT, is reduced by mitochondrial NAD(P)H-dependent oxidoreductase enzymes to an insoluble purple crystal, formazan. Cells were plated and treated with nanoparticles as described above and incubated for 24 hours at 37°C prior to the assay. As a positive control for complete cell death, cells were treated with a 1%
solution of Triton X-100 in DMEM for five minutes immediately before the assay was performed. After the incubation, all but 150 µL of the cell media was removed from each culture well before the addition of 15 µL of the MTT reagent. The cells were incubated for 3-4 hours at 37°C. The culture media/MTT reagent was removed and formazan crystals were dissolved with the provided MTT solvent. The solution was transferred to a flat-bottom 96-well plate and absorbance values were read using a microplate reader at 570 nm and 690 nm. Absorbance values were corrected by subtracting the background absorbance reading at 690 nm from the absorbance at 570 nm. To analyze and compare the data, absorbance values were normalized to the untreated control cells such that the untreated control represented 100% mitochondrial activity and an absorbance of 0 represented the absence of mitochondrial activity (i.e., 0%). All experimental conditions were performed in quadruplicate.

TEM of nanoparticle-treated cells

To verify that treated cells internalized nanoparticles, TEM was performed. Cells were grown to near confluence in a 6-well tissue culture plate. Cells were then treated with nanoparticles and briefly centrifuged. After a 24-hour incubation at 37°C, the treated cells were washed twice with PBS before detachment with trypsin. Detached cells were washed with PBS and resuspended in 3% glutaraldehyde in PBS. Incubation steps were carried out at room temperature on a Lab Line orbital shaker (Barnstead/Thermolyne, Melrose Park, IL) operating at 700 rpm. The cell suspension was centrifuged at 1000×g
for 5 minutes between each processing step. Fixed cells were washed twice with sodium cacodylate buffer (pH 7.4, 10 minutes each), then post-fixed in 1% osmium tetroxide in sym-collidine buffer (pH 7.6) for 1 hour at room temperature. Following two washes with s-collidine buffer (10 minutes each) the cells were en-bloc stained with a saturated aqueous uranyl acetate solution (pH 3.3) for 1 hour. Cells were dehydrated in a graded ethanol series up to absolute (10 minutes each). Acetone was used as the transitional solvent for two 10-minute washes. Cells were infiltrated overnight with a 1:1 mixture of acetone and Spurr’s epoxy resin (Electron Microscopy Sciences, Fort Washington, PA). Finally, the cells were centrifuged and the pellet was placed into BEEM™ embedding capsules containing 100% Spurr’s resin. Polymerization of epoxy blocks was carried out at 70° C overnight. Polymerized blocks were sectioned with a Leica Ultracut UCT ultramicrotome (Leica Microsystem GmbH, Wein, Austria). Ultrathin (80 nm) sections were collected on 200 mesh copper grids (Electron Microscopy Sciences) and post-stained with lead citrate (3 minutes). Electron micrographs were generated on with a JEOL JEM-1400 TEM (JEOL Ltd. Tokyo, Japan) equipped with a Veleta digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).
2.3. Results

Nanoparticle characteristics

The toxicity experiments were conducted with readily available commercial TiO₂, SiO₂, and ZnO nanoparticles. Particle specifications provided by the vendor appear above (Experimental Procedures, Nanoparticles) and results of characterization studies conducted in our laboratory are as follows. Figure 2.2 A - C shows the TEM pictures of the three particles; the primary particles of silica and titania are almost spherical with radii of ~ 10-15 nm for SiO₂, ~ 20 - 25 nm for TiO₂. For ZnO, there is a polydispersity in size and morphology with both spherical (~10-20 nm) and rod-like (5-10×50-200 nm) particles. There is clearly some aggregation of these primary particles, and we discuss below the size based on dynamic light scattering which is used in this study rather than the primary size. Figure 2.3 shows the X-ray powder diffraction patterns, indicating that the SiO₂ is amorphous, TiO₂ is a mixture of anatase and rutile (marked on the figure, as is expected for P25), and ZnO is primarily wurtzite.

To simulate the digestion process that the particles would undergo in the gastrointestinal tract, particles were sequentially treated with pepsin at pH 2 to mimic the stomach, and then pancreatin and bile salts at pH 7 to mimic the small intestine. Particle characterization by light scattering and zeta potential measurements at each of these stages was measured and is summarized in Table 1. In the culture medium DMEM, all nanoparticles were negatively charged, with zeta potentials of -11 millivolt (mV) for
TiO$_2$ and SiO$_2$, -19 mV for ZnO. The radii varied with ~96 nm for silica, ~112 nm for ZnO, and ~115 nm for titania. Titania particles remained stable in pepsin solution at approximately ~119 nm radii, but with a positive zeta potential of 14 mV. At the acidic pH (~ 2) of the pepsin solution, the surface of TiO$_2$ is protonated, consistent with the isoelectric point (IEP) of TiO$_2$ being pH ~ 6. Silica particles had surface charge close to zero in the pepsin solution and aggregated to ~3000 nm particle radii, consistent with their IEP reported as pH 2, and with our pH titration studies.

ZnO particles dissolved during incubation in pepsin at pH 2. This is consistent with reports in literature and was confirmed by measuring the dissolved Zn in the supernatant by atomic absorption spectroscopy, and the dissolved zinc correlated with the amount of nanoparticles introduced in the medium. Figure 2.4A is a plot of the size and zeta potential as a function of pH for ZnO in water and in the presence of media. In water, the ZnO particles are not tracked by light scattering below pH 6.5, indicating dissolution, whereas in the media there is a protective effect on the ZnO, and particle size only begins to decrease below pH 4. Dissolution studies were not done on the SiO$_2$ and TiO$_2$ particles, since these particles could be isolated after the various treatments and were used for the in vitro studies. Figure 2.4B shows the difference infrared spectrum between ZnO that was exposed to media and that which was exposed to water (both samples lyophilized prior to infrared measurements). Weak bands at 1661, 1534, 1444, 1395 cm$^{-1}$ are characteristic of adsorbed protein, with the Amide 1 band at 1661 cm$^{-1}$ suggesting the presence of β-sheet structures. Similar bands were also noted for SiO$_2$. 

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and TiO₂ exposed to media (shown in Figure 2.5), indicating that protein adsorption is occurring on all particles exposed to media.

Upon treatment with pancreatin and bile salts, the zeta potentials of the TiO₂ and SiO₂ reversed and became strongly negative, in the range of -30 to -40 mV. TiO₂ particles retained their radii of ~104 nm, whereas SiO₂ deagglomerated and had radii of ~109 nm. Figure 2.6 shows the difference infrared spectra between the SiO₂ and TiO₂ that were exposed to pepsin, pancreatin and bile salts and those just exposed to water. The bands observed at 1682, 1527 and 1415 cm⁻¹ are different from that observed with the media and stronger in intensity by about two orders of magnitude, indicating that the pepsin treatment alters the surface and promotes adsorption of material from the pancreatin/bile salt mix. Just based on the spectra, it is difficult to assign the exact types of species adsorbed on these particles. Infrared spectra reported in the literature on bile salts show presence of a band at 1680 cm⁻¹, and IR spectrum of just the bile salts used in this study show bands at 1625 and 1670 cm⁻¹ (Figure 2.7). These bands can be assigned to carboxylate groups of the bile salts, indicating that such species are adsorbed on the nanoparticle surface after treatment with the digestion medium.

Nanoparticle interaction with C2BBel cells

The intestinal epithelial cell line C2BBel was established as a sub-clone from Caco-2 cells, originally isolated from a human colon cancer, to be a more homogeneous population of brush border-expressing cells. These cells have been shown to form
polarized monolayers of cells with microvilli maintained by cytoskeletal proteins, as is observed for intestinal epithelium *in vivo*.\textsuperscript{35,36} For these studies, we used actively proliferating cells rather than differentiated cells since proliferating cells are generally more sensitive to toxic agents as has previously been observed.\textsuperscript{37} This level of sensitivity likely more accurately simulates the sensitivity of proliferating intestinal stem cells *in vivo*. As shown in Figure 2.8, morphology of C2BBe1 cells following exposure to TiO\textsubscript{2} (Figure 2.8B) is indistinguishable from that of untreated cells (Figure 2.8A).

In order to observe whether these nanoparticles are internalized by C2BBe1 cells, TEM was performed on cells that were treated with nanoparticles for 24 hours. Cells were treated with 10 \(\mu\)g/cm\(^2\) of SiO\(_2\), TiO\(_2\), or ZnO and centrifuged. After 24 hours, cells were washed and processed for TEM. Each type of nanoparticle was able to be visualized within C2BBe1 cells, as shown in Figure 2.8, although there seemed to be differences in the amount of nanoparticle uptake as a function of the particle type. For all of the treated cells, nanoparticles were only found in a fraction of the cells. TiO\(_2\) seemed to be taken up most readily by cells. All of the nanoparticles inside cells seemed to be clustered together, probably in vesicles. No particles were observed within the nucleus, only in the cell cytoplasm.

*Toxicity*

Cells were treated with nanoparticles at a dose of 10 \(\mu\)g/cm\(^2\), centrifuged to promote nanoparticle-cell interaction, and incubated for 24 hours. Toxicity assays were
then performed on the treated cells. Four different toxicity assays were performed. These short-term treatment experiments were conducted over a range of 5-12 cell passages (varying by assay).

Sytox Red is a nuclear stain that will bind DNA. However, it will only enter cells with damaged membranes and is thus a marker for cell necrosis or late apoptosis. Cells were stained with Sytox Red and analyzed by flow cytometry to measure the percent of stained cells (Figure 2.10A). Results are shown as percent viability based on normalization with unstained cells. The nanoparticle-treated cells showed comparable viabilities to the untreated control cells, approximately 100% viability, whereas H₂O₂ (used as a positive control) reduced viability of cells to 25%. Thus, SiO₂, TiO₂, and ZnO do not seem to cause necrotic cell death in C2BBe1 cells.

Flow cytometric analysis was also performed on cells stained with Annexin V to label apoptotic cells. Annexin V will bind to phosphatidylserine that is exposed on the outer surface of cells, which occurs in early apoptosis when phosphatidylserine is flipped to the outer layer of the cell membrane. C2BBe1 cells were stained with Annexin V and analyzed by flow cytometry (Figure 2.10B). Nanoparticle-treated cells revealed approximately 100% cell viability, which was similar to the untreated control cells. Inducing apoptosis with H₂O₂ as a positive control reduced cell viability to 10%. These results indicate that SiO₂, TiO₂, and ZnO do not induce apoptotic cell death in C2BBe1 cells.

The LDH assay indirectly measures cell death based on the amount of lactate dehydrogenase released by cells with damaged membranes. LDH release was measured
in supernatants from C2BBe1 cells treated with nanoparticles for 24 hours and data were converted to percent viability of cells based on LDH release from positive control cells (treated with Triton X-100) and untreated control cells (Figure 2.10C). Both TiO\textsubscript{2} and SiO\textsubscript{2} showed comparable viability to the untreated control (near 100% viability) which supports the results of the flow cytometric assays. However, cells treated with ZnO displayed an approximately 20% (\( p < 0.01 \)) decrease in viability from the control cells, indicating that ZnO is causing toxicity.

We also used the MTT assay to evaluate mitochondrial activity of these cells. The MTT assay measures the ability of cells to reduce a tetrazolium dye to the insoluble formazan, which can be measured colorimetrically. SiO\textsubscript{2} and TiO\textsubscript{2} particles induced no change in the mitochondrial activity, but ZnO treatment reduced mitochondrial activity to approximately 70% of untreated controls (\( p < 0.0001 \)) (Figure 2.10D).

Toxicity of nanoparticles treated with digestive enzymes

C2BBe1 cells were also treated with SiO\textsubscript{2} and TiO\textsubscript{2} nanoparticles that had undergone simulated \textit{in vitro} digestion. Since the ZnO dissolved in pepsin at pH 2, we could not treat cells with digested ZnO. Cells were treated with the digested nanoparticles, centrifuged, incubated for 24 hours, and toxicity assays were performed as described above. Flow cytometric analysis after Sytox Red staining revealed no decrease in cell viability below that of the untreated control cells, suggesting no necrotic cell death induced by the digested nanoparticles (Figure 2.11A). Annexin V staining also revealed
comparable cell viability between cells treated with digested nanoparticles and untreated control cells, suggesting that the digested nanoparticles do not induce cell apoptosis (Figure 2.11B). The digestion solutions appeared to interfere with the LDH assay (increase in apparent cell viability was observed after treatment of cells directly with only the digestive solutions; data not shown). Thus, LDH release could not be used as an indicator of digested nanoparticle toxicity. In the MTT assay, only the digested TiO$_2$ induced a slight decrease (10%) in mitochondrial activity (Figure 2.11C, p < 0.02), indicating that treatment with the digestion media caused minor nanoparticle toxicity.

*Long-term C2BBe1 exposure to nanoparticles*

Consumption of nanoparticles in foods can realistically be expected to result in repeated exposure of the intestinal epithelium to these particles over a protracted period. To examine the effects of long-term nanoparticle exposure on intestinal epithelial cells, C2BBe1 cells were treated with nanoparticles for 24 hours after each cell passage. Cells were plated and allowed to adhere and acclimate for 2 days before being treated with nanoparticles. After 24-hour treatment, free nanoparticles were removed and cells were grown to confluence. Cells were then passed into new plates and the particle exposure process was repeated. To examine the effects of repeated nanoparticle exposure on cells, some cells were also plated in 24-well plates for toxicity assays after certain cell passages. These cells were treated with nanoparticles for 24 hours before performing the toxicity assays described above.
Toxicity of nanoparticles on the long-term exposure cells (26 nanoparticle exposures) was determined using the same toxicity assays used for the acute exposure studies (Figure 2.12). Staining with Sytox Red and flow cytometric analysis revealed no changes in cell viability from the untreated control. Flow cytometric analysis of cells stained with Annexin V likewise showed no decrease in viability of the cells. This suggests that the nanoparticles do not induce apoptosis in C2BBe1 cells even after repeated exposure. No decrease in viability was observed for cells treated with TiO$_2$ or SiO$_2$ by LDH assay. Measurement of LDH release from cells revealed a 30% decrease in viability in cells treated with ZnO ($p<0.01$). A similar pattern was observed with ZnO treatment after all exposure cycles evaluated. This demonstrates that ZnO again may be inducing modest toxicity in cells exposed to ZnO long-term. No change in mitochondrial activity was observed after treatment with TiO$_2$ or SiO$_2$, however the MTT assay revealed a significant decrease of 55% ($p < 0.0001$) in mitochondrial activity in cells treated with ZnO.

Because long-term exposure to nanoparticles may induce effects other than cell death, growth curves were performed on long-term-exposed cells to measure changes in cell proliferation. Growth curves were conducted with cells repeatedly exposed to nanoparticles subsequent to the most recent nanoparticle treatment. Cell proliferation was measured by counting cells daily for 10 days, and growth curves were performed after 7, 11, 16, and 29 nanoparticle exposures. Figure 2.13 shows the growth curves obtained after 11 cycles. The lack of differences among these growth curves suggests that long-term nanoparticle exposure did not affect cell proliferation. In Figure 2.14, we show two
replicate growth curves performed after 29 nanoparticle exposures. Although individual curves indicated slight differences between differentially treated cell populations, no reproducible trends were observed between the curves performed at any time points, and thus no further analysis was performed.

2.4. Discussion

Particle Characteristics

From the X-ray diffraction patterns shown in Figure 2.3, silica is amorphous, ZnO has the wurtzite-type structure, and TiO₂ is a mixture of anatase and rutile. Table 1 shows that the size and charge of the particles undergo significant changes in different environments. In serum-containing media, the particles appear aggregated as compared to the primary particle size (Figure 2.2), indicating that the as-obtained particles were aggregated, consistent with previous reports. Titania becomes positively charged and the silica particles are almost neutral in the “stomach” fluid (pepsin, pH 2.0). In the case of silica, the neutrality of the particles results in formation of large aggregates (~3 µm radii). The charges revert back to negative in the neutral intestinal fluid and in the case of silica, the particles deagglomerate.

ZnO dissolved completely in the pepsin solution (pH 2.0). There are conflicting reports in the literature, with most studies suggesting that treatment at pH ~ 2 (simulating gastric conditions) will increase the solubility of ZnO, but at least one study suggests
otherwise.\textsuperscript{37} Light scattering studies as a function of pH shown in Figure 2.4A indicate that ZnO is indeed stabilized by the serum, and dissolution occurs at pH<4, as compared to ZnO in water, where rapid dissolution occurs at pH < 6.5. Infrared spectrum (Figure 2.4B) shows that upon suspension in serum, characteristic protein bands\textsuperscript{31} are observed on the ZnO. It has been proposed that the presence of serum proteins will inhibit dissolution of ZnO,\textsuperscript{40} however it is unlikely that serum can stabilize against dissolution in the highly acidic pepsin solution. Protein coronas are also observed on the TiO\textsubscript{2} and SiO\textsubscript{2} particles (Figure 2.6).

\textit{Particle Uptake by Cells}

Our choice of a sub-clone (C2BBe1) of the Caco-2 cell line is based on their ability to mimic human intestinal cells. The Caco-2 cell line has been extensively characterized, and is recommended for use as an \textit{in vitro} model of the GI tract by an international toxicity screening workgroup.\textsuperscript{42} Figure 2.8 demonstrates that these cells form a confluent layer with intercellular tight junctions, the morphology of which is unaffected by nanoparticle exposure. The TEM data in Figure 2.9 indicate that particles are being internalized. Previous studies have also noted that silica with primary particle size of 32 and 83 nm were taken up by Caco-2 cells, formed larger agglomerates (200 - 300 nm), and even showed up in the nucleus after 72 hour exposure with the smaller particles.\textsuperscript{43} Silica nanoparticle (NP) uptake by Caco-2 cells by elemental analysis of the cells has been reported, although surface binding versus internalization is not readily
distinguished by this method.\textsuperscript{37} Internalization of TiO\textsubscript{2} NP in Caco-2 cells has also been reported.\textsuperscript{9}

\textit{Toxicity: Undigested Particles}

Engineered nanoparticles have been reported to interfere with optical assays due to light absorption/scattering effects.\textsuperscript{44-46} In particular, for the MTT and LDH assays, it was suggested that loading levels be kept below 50 µg/cm\textsuperscript{2}. With ZnO at a loading level of ~ 10 µg/cm\textsuperscript{2}, slight effects were even observed with the LDH assay, but not with the MTT assay.\textsuperscript{44} TiO\textsubscript{2} beyond loading levels of 100 µg/mL were found to adsorb on LDH.\textsuperscript{45} SiO\textsubscript{2} has been reported to interfere with the MTT assay in HeLa cells by promoting exocytosis of the formazan crystals, even at loading levels of 10 µg/mL.\textsuperscript{46} Because of these literature reports, the experiments in the present study were conducted at a dose of 10 µg/cm\textsuperscript{2}.

Silica/titania dispersed in cell media did not exhibit any toxicity by any of the assays, including the long-term study with repeated exposures (~ 29 cycles) (Figure 2.10, Figure 2.12). There is conflicting data in the literature on toxicity of silica. Lack of toxicity of SiO\textsubscript{2} in Caco-2 cells was observed despite particle internalization by cells and even nuclear localization of particles.\textsuperscript{43} Mitochondrial activity (WST assay) in Caco-2 cells treated with SiO\textsubscript{2} NP showed no significant changes as compared to controls, even at dosage levels of 200 µg/mL.\textsuperscript{43} In another study, SiO\textsubscript{2} NP were found to reduce cell viability only in undifferentiated Caco-2 cells, even at loadings of 5 µg/cm\textsuperscript{2}.\textsuperscript{37}
With nanoparticles of TiO\textsubscript{2}, there appears to be agreement of a lack of toxicity towards Caco-2 cells\textsuperscript{41}. Commercial TiO\textsubscript{2} used in sunscreens, made up of a rutile core surrounded by Al(OH)\textsubscript{3} shell and fibrous-type morphology with lengths of 50 ±10 nm and widths of 7 ± 2 nm were not internalized, and no toxic effects were noted in response to loadings of up to 100 µg/mL\textsuperscript{47}. TiO\textsubscript{2} at concentrations of 1-20 µg/cm\textsuperscript{2} had no effect on Caco-2 cell viability in the presence or absence of serum\textsuperscript{40}. TiO\textsubscript{2} at loadings of 0.1-100 mg/L did not influence Caco-2 cell viability, as measured by MTT assay\textsuperscript{41}. TiO\textsubscript{2} at loading levels of 1-10 µg/mL did not cause cell death, either after acute or chronic exposure in Caco-2 cells\textsuperscript{9}. Anatase/rutile mixtures, similar to the NP examined in the present study, did not induce toxicity (by LDH assay) to Caco-2 cells at loading levels of 20 µg/cm\textsuperscript{2}, but toxicity was observed at 80 µg/cm\textsuperscript{2}. The WST-1 assay indicated reduction in metabolic activity only at loadings of 80 µg/cm\textsuperscript{2}\textsuperscript{48}. Using the MTT assay, different mammalian cell types have been shown to respond differently to TiO\textsubscript{2} NP, with only some showing a toxic response\textsuperscript{49}.

**Zinc Oxide**

In response to ZnO exposure, we found evidence of low-level cell toxicity as determined by the LDH assay, but this was not corroborated by assays of necrosis or apoptosis which demonstrated no toxicity (Figure 2.10C). Mitochondrial activity was also diminished by ZnO treatment (Figure 2.10D). Both these effects of ZnO were also observed in response to long-term treatment (Figure 2.12C,D). There is agreement in the
literature that ZnO induces some cytotoxicity in Caco-2 cells.\(^{41}\) ZnO have been reported to be cytotoxic to both undifferentiated and differentiated Caco-2 cells at doses > 5 \(\mu\)g/cm\(^2\).\(^{37}\)

Considering that TEM demonstrated that ZnO is internalized by cells, one hypothesis is that if the internalized ZnO NP are taken up in endosomes, the particles can dissolve and provide a burst of Zn\(^{2+}\) within the cell. The kinetic profile of pH change in Caco-2 endosomes upon internalization of particles indicates a drop in pH from 7.4 to ~ 5.\(^{50}\) The intracellular dissolution hypothesis could explain the decreased mitochondrial activity observed in ZnO-treated cells. Zinc ions have been shown to be pro-apoptotic under certain circumstances\(^{51}\) and the solubilized ZnO released from the endosomes could be causing toxicity by inhibiting energy metabolism.\(^{51-53}\)

However, as Figure 2.13 shows, none of the three particles had any influence on the rate of proliferation of the cells.

**Toxicity: Digested Particles**

The MTT experiments suggest a slight decrease in metabolic activity induced by titania nanoparticles only after exposure to the simulated digestion solutions (Figure 2.11C). IR data shown in Figure 2.6 indicates that in both silica and titania, there is strong adsorption of material from the simulated digestive media. Bile salts/proteins appear to be on the surface of these particles, with the acidic pepsin treatment promoting the amount of adsorbed material (since particles exposed to media have much less material).
Micelles of lecithin and the bile salt sodium deoxycholate at concentrations > 0.2 mM have also been reported to decrease cell viability of Caco-2 cells. Thus, the nanoparticles could be transporting toxic material from the digestion media into the cells. It is unclear at this point why silica exposed to the simulated digestion media is not showing the same type of toxicity as the titania. It is important to note that the toxicity manifested by titania, as measured by the MTT assay, is mild and possibly can be related to the different levels of adsorbed bile salts being transported by the two particles.

**Implications of this Study**

The implications of this *in vitro* study of nanoparticles of SiO₂, TiO₂ and ZnO in media towards C2BBe1 cells are as follows. The cells internalized all three particles. ZnO were the only nanoparticles that induced mild toxicity by the LDH and MTT assay. Repeated exposure of cells to SiO₂, TiO₂ or ZnO did not alter their growth patterns or render them any more susceptible to toxicity. With the SiO₂ and TiO₂ treated with the simulated digestion medium, there was no indication of necrosis or apoptosis, but diminished mitochondrial activity was noted with titania, possibly due to transport of bile salts/proteins into the cell. The importance of the corona formed on nanoparticles is being recognized as important in toxicity, since not only is the response of the living cell to the particle influenced by the composition of the corona layer, but it is also a mechanism to get material into the cells.
Even though we did not find SiO$_2$ and TiO$_2$ to be toxic, it is clear that they are internalized by the epithelial cells as nanoparticles and may subsequently enter the circulation and migrate to other parts of the body. The surface charge of SiO$_2$ and TiO$_2$ particles becomes neutral/positive within the “stomach” solution and can therefore interact with the negative mucus proteins and influence their transport. With ZnO, toxicity is not relevant in its nanoparticulate form, unless stabilized against dissolution in the stomach. This is consistent with the observation that when ZnO was administered to rats by oral gavage, Zn$^{2+}$ were found in tissues, but not as ZnO particles.$^{38}$ Thus, at least for silica and titania, it becomes essential to monitor transport of these nanoparticles in vivo to determine whether they do indeed enter the circulation and if so, to map their route(s) of distribution and potential sites of accumulation. Several studies have examined the fate of ingested nanoparticles. TiO$_2$ injected into rat ileum did not disrupt the structure of the epithelial layer, though the particles were taken up by the cells and eventually reached the liver.$^{14}$ In vivo studies with mice fed with SiO$_2$ exhibited liver toxicity.$^{56}$ Dynamics of the motion of nanoparticles into systemic circulation, localization in tissues and clearance from the body still needs to be mapped out.

The functionalization of particles with fluorophores presents an opportunity for further study, allowing for these particles to be visualized in cells and tissues. In Chapter 3, we will explore the synthesis and optimization of one such fluorophore, CdSe/CdS/ZnS quantum dots, and in Chapter 4, we will discuss the coating of these quantum dots along with two organic dyes with a silica shell for use in biological studies to determine if the particles are escaping the digestive system.
References


Table 2.1. Summary of dynamic light scattering data showing hydrodynamic radius and zeta potential of commercial nanoparticles during each step of a simulated digestion process.

<table>
<thead>
<tr>
<th></th>
<th>SiO$_2$</th>
<th>TiO$_2$</th>
<th>ZnO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Size (r, nm)</td>
<td>Zeta Potential (mV)</td>
<td>Size (r, nm)</td>
</tr>
<tr>
<td>DMEM/FBS</td>
<td>96 ± 4</td>
<td>-11 ± 0</td>
<td>115 ± 2</td>
</tr>
<tr>
<td>Pepsin</td>
<td>3039 ± 321</td>
<td>+1 ± 2</td>
<td>119 ± 2</td>
</tr>
<tr>
<td>Pancreatin</td>
<td>109 ± 1</td>
<td>-38 ± 2</td>
<td>110 ± 0</td>
</tr>
<tr>
<td>Bile Salts</td>
<td>109 ± 4</td>
<td>-29 ± 2</td>
<td>104 ± 2</td>
</tr>
</tbody>
</table>
Figure 2.1. Example of DRIFTS subtraction process: TiO$_2$ exposed to water and freeze dried; TiO$_2$ treated with digestive solutions and freeze dried; and the subtraction of the water exposed spectra from the digested sample, leaving peaks related to the digestive enzymes.
Figure 2.2. Representative TEM images of commercial nanoparticles. A) SiO₂, B) TiO₂, and C) ZnO. Long strands seen in the images are from the carbon support films, and indicated by arrows.
Figure 2.3. X-Ray Diffraction Patterns of commercial nanoparticles. A) SiO$_2$, B) TiO$_2$, (anatase and rutile peaks are marked by “A” and “R,” respectively), and C) ZnO.
Figure 2.4. A) Particle size and zeta potential of ZnO during pH titration in media (diamond markers) and water (squares) (symbols used are on top left of figure). The water samples are circled for clarity. B) Difference infrared spectrum in diffuse reflectance mode between ZnO particles exposed to DMEM/FBS media and water (y-axis in Kubelka-Munk arbitrary units). Labeled peaks are 1661, 1534, 1444, and 1395 cm⁻¹.
Figure 2.5. Difference infrared spectrum in diffuse reflectance mode between TiO$_2$ and SiO$_2$ particles exposed to DMEM/FBS media and water (y-axis in Kubelka-Munk arbitrary units). Labeled peaks are 1661, 1534, 1444, and 1395 cm$^{-1}$. 
Figure 2.6. Difference infrared spectrum in diffuse reflectance mode between TiO\textsubscript{2} and SiO\textsubscript{2} nanoparticles treated with simulated digestion media and water (y-axis in Kubelka-Munk arbitrary units). Labeled peaks are 1682, 1527, and 1415 cm\textsuperscript{-1}. 

[Diagram showing the difference infrared spectrum with labeled peaks at 1682, 1527, and 1415 cm\textsuperscript{-1}.]

Figure 2.6. Difference infrared spectrum in diffuse reflectance mode between TiO\textsubscript{2} and SiO\textsubscript{2} nanoparticles treated with simulated digestion media and water (y-axis in Kubelka-Munk arbitrary units). Labeled peaks are 1682, 1527, and 1415 cm\textsuperscript{-1}.
Figure 2.7. DRIFTS spectra of freeze-dried digestive enzyme solutions. Three major peaks from the digested TiO$_2$ and SiO$_2$ spectra in Figure 2.6 are marked for comparison.
Figure 2.8. Cell morphology after nanoparticle treatment. A) Untreated control cells were compared to B) cells treated with TiO$_2$. Images were taken after TiO$_2$ was washed off cells.
Figure 2.9. Representative TEM images of C2BBel cells which were treated with nanoparticles for 24 hours before being harvested, fixed in glutaraldehyde, and further processed for TEM. A) SiO$_2$-treated cell, B) TiO$_2$-treated cell, C) ZnO-treated cell.
Figure 2.10. Results of cytotoxicity assays for C2BBel cells treated with nanoparticles in media for 24 hours. Hydrogen peroxide was used as a positive control to cause cell death in flow cytometric assays (A and B) while Triton X-100 served as a positive control for cell death in the colorimetric assays (C and D). A) Cells were stained with Sytox Red and analyzed by flow cytometry to monitor necrosis. B) Cells were stained with Annexin V and analyzed by flow cytometry to monitor apoptosis. C) LDH release into the supernatant was measured for membrane leakage. D) Reduction of MTT to formazan by mitochondrial enzymes was measured as an indicator of mitochondrial activity. Significance as compared to untreated controls in the LDH and MTT assays was measured by Student’s $t$-test (* indicates $p<0.01$; ** indicates $p<0.0001$). Data are a compilation of five experiments, each containing three replicates.
Figure 2.11. Cytotoxicity of C2BBe1 cells after treatment with simulated digestion media-treated SiO2 and TiO2 nanoparticles. Hydrogen peroxide was used as a positive control for cell death in flow cytometric assays (A and B) while Triton X-100 served as a positive control for cell death in the MTT assay (C). A) Cells were stained with Sytox Red and analyzed by flow cytometry to measure necrosis. Flow cytometry assay data are a compilation of three experiments, each containing three replicates. B) Cells were stained with Annexin V and analyzed by flow cytometry to measure apoptosis. C) Reduction of MTT to insoluble formazan was measured to indicate mitochondrial activity of these cells. Significance as compared to untreated controls in the MTT assay was measured by Student’s t-test (* indicates p<0.02). MTT data are a compilation of four experiments, each containing three replicates.
Figure 2.12. Toxicity assays were performed on C2BBe1 cells that had been repeatedly exposed to nanoparticles, approximately weekly. The assays were performed 24 hours after the most recent nanoparticle treatment. Representative data shown were obtained after 26 nanoparticle exposures. A) Cells were stained with Sytox Red and analyzed by flow cytometry to measure necrosis. B) Cells were stained with Annexin V and analyzed by flow cytometry to measure apoptosis. Flow cytometry assay experiment data (A and B) consists of 3 replicates for each treatment. C) LDH release by cells due to membrane damage was measured. D) Mitochondrial activity was assayed by reduction of MTT to formazan. LDH and MTT data consist of 4 replicates for each treatment. Significant differences in viability or mitochondrial activity as compared to untreated control was measured by Student’s t-test (* indicates p<0.01; ** indicates p<0.0001) in LDH and MTT assays.
Figure 2.13. Growth curves after 11 nanoparticle exposure cycles. Growth was measured using cells that had undergone 11 cycles of repeated exposure to nanoparticles of SiO$_2$, TiO$_2$, or ZnO, along with untreated controls. Cells were plated at a starting concentration of $5 \times 10^4$ cells in 6-well tissue culture plates at time zero such that three replicate wells for each treatment could be counted daily for 10 days. This data is representative of growth curves performed after 7, 11, 16, and 29 nanoparticle exposures.
Figure 2.14. The growth curves shown above represent two replicate experiments conducted concurrently with cells that had undergone 29 nanoparticle exposures to SiO$_2$, TiO$_2$, or ZnO, as well as untreated controls cultured identically. Cells were plated at a concentration of $5 \times 10^4$ cells/well at time zero in 6-well tissue culture plates. This starting cell density allowed the three replicate wells for each treatment to be counted daily for 10 days without plateauing at confluence.
3.1. Introduction

Quantum dots (QDs) are nanosize semiconductors that fluoresce, the origin of fluorescence arising from quantum confinement effects. In a quantum dot, the particle size is smaller than the Bohr exciton radius, and this process manifests itself as an increase in the band gap energy.\(^1\) Using this strategy with various semiconducting materials, nanometer-sized quantum dots have been made which fluoresce across the entire visible spectrum\(^2\)–\(^4\) and in the infrared.\(^5\)

QDs are useful in a variety of fields because they are less prone to photo-bleaching and chemical degradation, have discrete emission wavelengths based on size, and their wide absorption profile makes them very suitable for biological imaging.\(^6\) Materials scientists are exploring QDs for solar cells, sensors, and improved lighting.\(^7\)–\(^9\)
Control of nucleation and growth of QDs is essential to optimize size and optical properties. Conventional organic-based hot-injection synthesis involves rapid nucleation by introducing precursor solutions to a hot (>300 °C) solvent. For the cadmium chalcogenides, these typically involve injection of dimethyl cadmium or cadmium oxide with other organometallic reagents into high temperature coordinating solvents such as trioctylphosphine (TOP) and trioctylphosphine oxide (TOPO). Controlled growth then occurs more slowly at a lower temperature and the final size of the particles is determined by the temperature during the growth phase and the organic capping agent which sterically inhibits growth. Such methods of quantum dot production are well developed and produce high quality quantum dots with quantum yields (QY) between 50-80 %. The QY typically decreases with surface defects that create trap states resulting in nonradiative decay and a broad red-shifted trap-state emission. To enhance the fluorescence and stability of the core particle, protective shells with higher band gap are often grown around them.

Organically prepared quantum dots do not disperse in water and require ligand exchange, which can significantly alter the optical properties of the particles. Aqueous based methods of quantum dot synthesis have been developed which yield water-soluble quantum dots suitable for use in biological research. In these methods, cadmium chalcogenides (Se, Te) are nucleated by reacting cadmium salts (CdCl₂) with NaH(Se, Te) in the presence of a passivating ligand. These reaction methods have the benefit of being safer, less expensive, and yielding water-soluble particles without the need for
ligand exchange, however, their quantum yield is typically lower compared to organic methods.

Microwave irradiation has been explored to decrease the time of aqueous quantum dot synthesis, and synthesis of CdSe(S), ZnSe(S), and CdSe/CdS/ZnS have been reported. Microwave heating of the precursors that are typically used in conventional convective heating has also been reported to produce QDs at shorter reaction times.

We have previously reported a fast, “one-pot” synthesis that produces stable, aqueous CdSe/CdS/ZnS core/shell quantum dots. While they are relatively easy to produce and show promising stability during biological experiments, these particles have a low quantum yield of 13%. In this research, we examine strategies for improving the quality of the microwave-produced QDs. By coupling the microwave reactor to a fluorescence spectrometer via fiber optics, the growth of the QD is monitored during synthesis, and this real-time data has provided insight into the growth mechanism. We also examine the effect of temperature during both the initial nucleation phase and the microwave-assisted crystal growth, and found a way to form QDs with varying emission wavelengths. When combined with UV-visible light exposure during nucleation and after microwave-based QD growth, CdSe/CdS/ZnSe QDs with quantum yields of 40% was obtained. We demonstrate with an intestinal epithelial cell line, C2BBBe1, that these particles are internalized and can be observed at lower concentrations than we have achieved with our previous 13% QY particles.
Materials: Cadmium chloride hemipentahydrate (CdCl$_2$·2.5 H$_2$O, >98 %) and sodium borohydride (NaBH$_4$, 99 %) were obtained from Aldrich (Milwaukee, WI, USA). Zinc chloride (ZnCl$_2$, 99.99 %), 3-mercaptopropionic acid (MPA), and selenium powder (Se, 99.5+ %, 200 mesh) were obtained from Acros (Geel, Belgium). Sodium hydroxide (NaOH) and ammonium hydroxide (NH$_4$OH, 28 – 30 %) were obtained from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). All chemicals were used without further purification. The H$_2$O used in this study was purified by a Barnstead NANOpure Infinity ultrapure water system (Dubuque, IA, USA).

Precursor Solutions:

Cadmium/MPA: A 250 mL solution of 1.05 mM Cd and 5.26 mM MPA was prepared by adding 60 mg of cadmium chloride hemipentahydrate and 114.5 μL of MPA to approximately 225 mL water. The pH was adjusted to 9.5 with 1 M NaOH, and the volume was adjusted to 250 mL in a volumetric flask. The final solution was stored in a plastic bottle and wrapped in aluminum foil.

Zn(NH$_3$)$_4^{2+}$: A 25 mL solution of 26.67 mM Zn(NH$_3$)$_4^{2+}$ was prepared using anhydrous zinc chloride and ammonium hydroxide. The hygroscopic zinc chloride was dried in a vacuum oven for one hour at 200 °C and capped prior to weighing. To minimize error in weighing due to absorption of water from the air, approximately 90.9
mg of zinc chloride was quickly added to a previously weighed beaker containing 20 mL of water. Ammonium hydroxide was then added drop wise. A white precipitate formed and then disappeared as more ammonium hydroxide was added. Once the solution was fully clear, the volume of the solution was adjusted to 25 mL in a volumetric flask. The final solution was stored in a plastic bottle, wrapped in aluminum foil to protect from light, and stored at 4 °C.

**NaHSe:** 152 mg of sodium borohydride was added to a glass test tube and chilled to 0 °C in an ethylene glycol/water bath. In quick succession, 2 mL of chilled water was pipetted into the test tube, followed by 158 mg of selenium powder. The test tube was capped with a rubber stopper with two needles sticking through; one of the needles attached to a nitrogen gas tank, and the other left open to vent. The test tube was quickly placed back into the cold bath. The reaction showed intense bubbling from the evolution of hydrogen gas, which lasted for approximately 30 minutes. By this time, the black selenium was reduced into the solution and a white Na₂B₄O₇ precipitate formed. After completion, the vial was moved to a nitrogen filled glove bag. 0.5 mL of the supernatant was pipetted into a 50 mL 3-neck round bottom flask containing 24.5 mL of nitrogen saturated water. This yielded a 25 mL solution of 20 mM NaHSe. The flask was kept capped and nitrogen was constantly bubbled though the solution. Within 12 to 18 hours, the presence of a red tint indicated that the selenium had oxidized, and the solution was no longer useful.

**CdSe Nucleation:** 19 mL of the Cd/MPA solution was added to a 25 mL Erlenmeyer flask and vigorously stirred on a stir plate. 0.25 mL of the NaHSe solution
was quickly added, immediately resulting in a yellow color due to formation of CdSe nuclei. The solution was stirred for 1 hour. After nucleation, 0.75 mL of the Zn(NH$_3$)$_4^{2+}$ solution was added. The final 20 mL solution was 1 mM Cd, 1mM Zn, 0.25 mM Se, and 5 mM MPA. To nucleate the CdSe cores at various temperatures, the 19 mL Cd/MPA solution was added to a 25 mL round-bottom flask and heated in an oil bath under reflux. After the appropriate temperature was reached, the reflux was temporarily removed while the NaHSe solution was added via pipet under rapid stirring (1200 rpm). The solution was stirred for 1 hour at the set temperature and allowed to cool to room temperature before addition of Zn(NH$_3$)$_4^{2+}$ and microwaving. Additionally, for lower temperatures, cores were nucleated at 0 °C by submerging the reaction flask into an ice bath.

**Microwave-Assisted Growth:** The solution was placed in a Discover SP (CEM Corp.) microwave system and heated for various times and temperatures, with maximum power set to 200 watts. After completion, the solution was cooled to 40 °C in the microwave with a stream of room temperature air, and then stored in a glass vial wrapped in aluminum foil at 4 °C until needed.

**UV-Visible Light Aging:** Samples were subjected to IR-filtered irradiation using a 150 W Xe lamp (model UXL151HXE, PTI) at room temperature under rapid stirring (1200 rpm). Samples were exposed to light for 2 hours after initial 1 hour nucleation, for 6 hours after microwave crystal/shell formation, or both.
Particle Characterization

**Optical Properties:** UV/Visible and fluorescence spectra were measured for QDs diluted 10:1 with water. Spectra were recorded with a Shimadzu UV-2501PC spectrophotometer. Fluorescence measurements were recorded with a Horiba Jobin-Yvon Fluorolog 3, using 2 nm slit widths for the emission and excitation monochromators, 0.3 second integration time, and a 375 nm excitation wavelength. The instrument was equipped with a photomultiplier tube detector.

Quantum yields were measured using a Quanta-Phi integrating sphere attachment for the Fluorolog 3. The detector was set to measure counts rather than the default counts per second. Slit widths for the emission and excitation monochromators were set to 5 nm, and the samples were excited with 480 nm light. Spectra were measured from 460 nm to 750 nm, to include both the excitation and emission wavelengths. An initial blank water sample was measured; adjusting the integration time to obtain an excitation peak height of 1 million counts to avoid oversaturation of the detector. The sample was then measured using the same settings for comparison. The areas under the excitation and emission curves in each spectrum were integrated, allowing for calculation of the quantum yield:

\[
QY = \frac{\text{emission}_{\text{sample}} - \text{emission}_{\text{blank}}}{\text{excitation}_{\text{blank}} - \text{excitation}_{\text{sample}}} \times 100\%
\]
Fluorescence lifetime was measured with the Fluorolog 3 time correlated single photon counting (TCSPC). Fluorescence was excited with a pulsed 455 nm LED (1.5 ns pulse duration). Repetition rate was set to 1 MHz.

**In situ Fluorescence:** In situ fluorescence measurements were taken during the microwave irradiation step of the synthesis. The Discover SP system is configured with a porthole for use with a camera attachment. We were able to couple the fluorometer to the microwave reactor via the camera porthole using a fiber optic cable attachment. Figure 3.3 shows a close-up of the fiber-optic inserted into the camera port inside of the microwave.

Batch runs were set up using the fluorometer software to take approximately one spectrum every minute. A significant fluorescence near 500 nm was present when the typical 375 nm excitation wavelength was used. This peak was observed when no sample was present, and is attributed to the lining of the microwave chamber. An excitation wavelength of 480 nm was chosen, which adequately stimulated quantum dot emission while avoiding the background fluorescence of the microwave chamber. A 6 nm bandwidth was used for both the excitation and emission monochromators. The emission wavelength was scanned from 500 to 750 nm with an integration time of 0.2 seconds.

**X-Ray Diffraction:** As-prepared QD solutions were washed twice with water by centrifugation (290,000 x g), replacement of supernatant, and re-dispersion. After the second wash, particles were dried overnight in a vacuum oven. The sample was loaded into a 0.5 mm capillary and XRD spectra were recorded using a Bruker D8-advance system with nickel-filtered Cu Kα radiation (1.5405 Å).
**Electron Microscopy:** High resolution transmission electron microscopy (HRTEM) images were obtained using a Tecnai-F20 system. Particles were washed twice by centrifugation and replacement of supernatant, resuspended in a dilute solution in ethanol, and deposited onto a lacey-carbon coated copper grid.

**Biological Imaging:**

Cell cultures, flow cytometry, and confocal microscopy described in this section were performed by Christie McCracken in Dr. James Waldman’s laboratory.

**Cell culture:** C2BBe1 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Serum Source International, Inc., Charlotte, NC), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.3% penicillin/streptomycin, 0.3 µg/mL Amphotericin B (fungizone), and 10 µg/mL transferrin (all from Life Technologies). Cells were incubated in 5% CO₂/95% room air at 37 °C. Cells were passaged every 5-7 days and plated on flasks or plates pre-coated with collagen I (0.05 mg/mL, rat tail, Life Technologies). Cells were plated at a density of 70,000 cells/well in 24-well tissue culture plates (Corning-Costar, Tewksbury, MA) for comparison of QD fluorescence intensity by flow cytometry. Cells were plated in 8-chamber slides (Thermo Scientific) at a density of 90,000-100,000 cells/chamber for analysis by confocal microscopy. Cells were incubated for at least 24 hours after plating before treatment with QDs. Directly prior to treatment on cells, QDs
were sonicated using a Sonics Vibra-Cell sonicator (Sonic Materials, Inc., Norwalk, CT) pulsing for one second on, one second off for approximately 15 seconds in order to minimize QD agglomeration. Experiments were generally performed at a dose of 157 nM which was achieved by adding 107 µL of the QDs (732 nM) per well in a 24-well plate with a 0.5 mL total volume per well with cell culture media and 64 µL of QDs per chamber in an 8-chamber slide with a total volume of 0.3 mL per chamber. After treating cells with the appropriate dose of QDs, cells were centrifuged to promote contact of QDs with cells. The 24-well plates were centrifuged at 300×g for 15 minutes. Due to greater fragility, 8-chamber slides were centrifuged at 75×g for 15 minutes. Cells were incubated with QDs for 24 hours before performing experiments.

**Flow Cytometry:** After 24-hour treatment of cells with QDs, cells were washed twice with phosphate-buffered saline (PBS) and detached from the culture plate with trypsin. Cells were suspended in PBS and analyzed for fluorescence using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA) at an excitation wavelength of 488 nm. All experimental treatments were performed in triplicate. Mean fluorescence intensity values were used to compare the fluorescence of QDs made by different syntheses in cells.

**Confocal Microscopy:** After 24-hour treatment of cells with QDs, the chambers were removed from 8-chamber slides for staining. All staining was performed at room temperature. Slides were washed twice with PBS and fixed in 4% paraformaldehyde for 45 minutes. Cells were washed again in PBS before permeabilization in a 0.2% Triton X-100 (Sigma-Aldrich) solution in PBS for 15 minutes. Cells were washed in PBS and
incubated in a 1% bovine serum albumin (BSA; Sigma-Aldrich) blocking solution for 1 hour. Cells were incubated with a 1:150 dilution of Alexa Fluor 647 mouse anti-E-cadherin (BD Biosciences) in 1% BSA in PBS for 90 minutes. Cells were washed with PBS and stained with a 0.25 µg/mL solution of 4’,6’-diamidino-2-phenylindole (DAPI, Life Technologies) in 1% BSA in PBS for 10 minutes. Cells were washed with PBS before coverslips were mounted using ProLong Gold Antifade Reagent (Life Technologies). Mounting media was allowed to cure overnight at room temperature before analysis using a Zeiss LSM 700 confocal fluorescence microscope (Jena, Germany).

3.3. Results

_Synthetic Strategies_

Figure 3.1 shows the synthetic pathways discussed in this paper. The overall composition Cd₄:Se₁:Zn₄:MPA₂₀ is maintained the same for all experiments. The effect of temperature during the microwave heating phase of the synthesis was examined between 125-160 °C in 5-degree increments. At temperatures below 125 °C and above 160 °C, very weakly fluorescent products were obtained. Only the reactions between 130 and 155 °C were studied further. The fluorescence of the quantum dots was observed _in situ_. To determine an approximate reaction time for each temperature, the time at which the fluorescence of the quantum dot stopped increasing was chosen based on the _in situ_
data. Approximate reaction times varied from 27 minutes at 155 °C to 4 hours and 20 minutes at 130 °C for a room temperature nucleated sample. Using these reaction times as a starting point, the optimal time for each temperature was determined by performing the synthesis at several times bracketing this time and measuring the quantum yields of all recovered samples. This process was repeated with nucleation of CdSe between 0-100 °C, followed by microwave irradiation (MWI) at temperatures between 130 and 155 °C for time periods ranging from 45 to 340 min. In addition, UV-visible light illumination was carried out during nucleation as well as after microwave treatment.

**Room Temperature Nucleation**

The nucleation of CdSe during the initial mixing of Cd$^{2+}$, NaHSe, and 3-MPA was examined over a range of temperatures. We focus first on the room temperature nucleated samples. To examine the initial steps during this nucleation event, the sulfur source 3-MPA was omitted from the reaction. Without 3-MPA, there was immediate formation of bulk CdSe particles upon mixing the cadmium and NaHSe solutions, indicating that the role of 3-MPA is crucial in complexing the Cd$^{2+}$, and gradually releasing it to react with the NaHSe. The second set of experiments excluded the Zn(NH$_3$)$_4^{2+}$ and heated in the microwave at 150 °C. The reactions were stopped at 10-minute intervals, solution cooled and the fluorescence spectrum was measured. Figure 3.4 shows that there is an initial broad emission peak at 502 nm, along with a trapped state band at 705 nm. Both these bands disappear, along with growth of a band at 537 nm,
which red shifts to 545 nm in 30 min and increases in intensity. With further heating, the 545 nm emission decreases in intensity and redshifts to 557 nm. At the conclusion of the experiment at 100 min, the solution had a dark red color and broad, strong emission at >700 nm from trap-states.

A similar experiment was repeated with all the reactants, but here the fluorescence spectra were recorded by removing the solution at 10 minute intervals, and diluting it by a factor of 10 before recording the spectra. As Figure 3.5 shows, there is a broad band at 517 nm at 10 min, which gradually redshifts and increases in intensity, reaching a maximum at 559 nm at 80 min. The trapped state emission is significantly quenched in the presence of zinc.

In order to evaluate the intermediate steps during the microwave treatment without disturbing the system, *in situ* fluorescence measurements were carried out. Figure 3.6a shows a three-dimensional plot of the *in situ* time-dependent fluorescence at the temperature of microwave operation, 150 °C (x and y axes are emission wavelength and time, respectively, and the z axis is the intensity). Within 20 min, a band at 502 nm is observed. This band disappears over the next 20 minutes, as the band around 550 nm develops, which gradually red-shifts to 574 nm and grows in intensity. After 60 minutes, the band at 574 nm decreases. This can be compared with Figure 3.5, where an identical reaction was carried out, but was cooled to room temperature and diluted before the spectra were recorded. When the spectra are recorded at room temperature instead *in situ* at 150 °C, the optimal fluorescence intensity is observed at 80 rather than 60 minutes, and $\lambda_{\text{max}}$ at 556 rather than 574 nm. This is because at higher temperatures, $\lambda_{\text{max}}$ red shifts and
the intensity decreases, due to change in the relative position of the conduction and valence bands from a temperature-dependent change in lattice dilatation and electron lattice interactions.\textsuperscript{22}

Figure 3.6b shows another view of the data in Figure 3.6a, starting from the lowest fluorescence intensity 5 minutes into the experiment, and ending with the maximum fluorescence intensity 60 minutes into the experiment. Simultaneous growth of two emissions, one at 502 nm and one starting near 540 nm, occurs in the first 20 minutes of the synthesis. The 502 nm emission begins to decrease in intensity and the 540 nm emission gradually red shifts to 574 nm, and increases gradually in intensity. An isosbestic point at 550 nm is observed between times of 40-60 min, indicating that within this time period, two species are interconverting.

The microwave synthesis was repeated as a function of temperature. Using the \textit{in situ} fluorescence to monitor the times around which the fluorescence reached its maximum, the appropriate time for each temperature was optimized for the range of 130 to 155 °C. At temperatures higher or lower that this range, the fluorescence spectra were significantly degraded, and not discussed in this study. Figure 3.7 shows all of the \textit{in situ} data as a function of time for each temperature. Table 1 shows the samples at each temperature with the optimal quantum yield. Similar quantum yields (QY, 17-19\%) were obtained at all microwaving temperatures between 130 and 155 °C, as long as the time for microwave treatment is optimized. Lower temperatures required longer times to reach the optimum QY.
Figure 3.8ab shows a plot of the peak fluorescence intensity with time at 135 and 155 °C. There is an increase in intensity before leveling off or decreasing during the final 10-15% of the monitored time. The derivative plots (rate of change in intensity with time, Figure 3.8cd provide further insight. In particular, the data at 135 °C shows four distinct regions. Region A between 10-30 min depicts an upward slope, Region B and C have downward slope (though still positive growth in fluorescence intensity). Region D beyond 120 min exhibits a decreasing rate of fluorescence intensity. As the temperature was raised to 155 °C, all stages merged into one another with a downward slope (all positive until the final 10% of the reaction).

Nucleation at Various Temperatures

The nucleation step was carried out between 0-100 °C, followed by microwave treatment at 150 °C for 80 minutes, and the fluorescence spectra and QY were measured. Figure 3.9 shows that the emission maximum is altered systematically with nucleation at different temperatures, with the 0 °C emitting at 546 nm and that at 100 °C at 574 nm. Table 2 shows the characteristic λ_{max} (nm) and QY of the samples obtained as a function of the various nucleation temperatures. Except for the sample nucleated at 0 °C, all other samples exhibited QY of 17-18%.

The in situ fluorescence experiments over the range of temperatures 130-155 °C were repeated with the sample nucleated at 100 °C, and these data are shown in Figure 3.10. At each temperature, 130 (part a), 145 (part b), and 155 °C (part c), the appearance
and disappearance of a band at 502 nm in the early stages of the reaction, as well as the development of QD peak followed by red-shifting, was identical to the room temperature nucleated samples.

Figure 3.11ab shows a plot of the peak fluorescence intensity with time at 130 and 155 °C for the 100 °C nucleated sample. As observed in the room temperature nucleated sample in Figure 3.8, there is an increase in intensity before leveling off and eventually decreasing before the reaction was halted. Figure 3.11cd show the derivative plots of Figure 3.11ab. As with the room temperature data in Figure 3.8, the data for the lower temperature 130 °C reaction shows four distinct regions, labeled A (the first ~50 minutes), B (~50-100 minutes), C (~100-200 minutes), and D (200+ minutes). For the higher temperature reaction (155 °C), the regions again merge together into one downward slope.

Effects of light illumination

The influence of UV-visible irradiation during nucleation was examined with two samples, one nucleated at room temperature and the second nucleated at 100 °C. Samples that were irradiated for two hours were then heated in the microwave at 150 °C for 80 min. Table 3 lists the QY of the various samples. These data show that light illumination during room temperature nucleation decreases the QY considerably (19 to 3%), while for the 100 °C nucleated sample, the QY remained unchanged (18 to 19%). Illumination after the microwave treatment was also carried out for a period of 6 hours. Table 3 shows that
for the room temperature nucleation, the QY almost recovered (15%), but for the sample nucleated at 100 °C, the QY reached 40%. Illumination only after the microwave treatment did improve the QY for both room temperature and 100 °C nucleated samples from 19 to 28% and 18 to 31%, respectively. Thus, light illumination during the nucleation period is having a negative effect on the room temperature nucleated sample, while a positive effect on the sample nucleated at 100 °C. With UV-visible light illumination both during the nucleation and after microwave treatment, there were marked improvements in the QY. For the room temperature nucleation, the QY increased from 3 to 15%, and for the sample nucleated at 100 °C, the QY increased from 19 to 40%. Several syntheses were repeated with the sample nucleated at 100 °C, with QY always between 40-41%.

Characterization of Final QDs

We characterized the QD sample with the 40% QY, and Figure 3.12 shows the optical spectra, TCSPC, XRD, and TEM of this sample. Absorption maximum is observed at 542 nm, with peak emission at 574 nm (Figure 3.12a). Figure 3.12b shows the high resolution TEM, twenty particles were selected in the TEM, and an average diameter of 4.6 ± 0.3 nm was calculated for the QD. The image in the insert shows lattice fringes of with a spacing of 0.33 nm, which extend out to the edges of the crystal indicative of a single crystal. Figure 3.12c shows the lifetime (TCSPC) data, and was fit best fit by a 3-exponential equation (lifetimes, 3.3, 16, 110 ns), with a majority
contribution from the 16 ns lifetime which falls in a range typically reported for QDs in literature.\textsuperscript{19,23,24} The 3.3 ns contribution is possibly arising from QDs with surface traps.\textsuperscript{19} We assign the longer lifetime component at 110 ns to an impurity, since its contribution decreases as we dialyze the QD samples. We could not get rid of it completely since with dialysis, the MPA capping ligand is also lost and the QDs become unstable. Figure 3.12d shows the XRD pattern with four broad peaks at 26.6, 43.9, 45.8, and 52.2 degrees (2θ). The figure also shows the expected patterns of the wurtzite and zinc blende structure of CdSe, CdS and ZnS. Because of the width of the bands, it is difficult to pinpoint the structure, but because the peak at 45.8° appears to match closely the (1\,0\,3) plane of wurtzite structure of CdSe, we assign the QD to have a wurtzite structure with all three chalcogenides of this form.\textsuperscript{2,25}

\textbf{Biological Imaging Studies}

We have reported previously imaging of microwave-based QDs with QY<20% with macrophages.\textsuperscript{18} With the present QDs with twice the QY, we examined the improvement possible for biological imaging. These experiments were performed in C2BBE1 cells,\textsuperscript{26} which are a human intestinal epithelial cell line, and relevant for uptake of food-relevant nanoparticles.\textsuperscript{27} Comparing mean fluorescence intensities (MFI) in a flow cytometry experiment revealed that fluorescence could be reliably detected only in cells treated with the QDs (40\% QY) at concentrations of 8-39 nM (Figure 3.13a), as compared to the QDs with 19\% QY which were not detected at similar concentrations of
11-57 nM. The flow cytometer will detect fluorescence in cells that have both internalized and membrane-bound QDs. Thus, to investigate internalization, fluorescence confocal microscopy was carried out and QDs with 40% QY were detected at concentrations as low as 0.8 nM, shown in Figure 3.13b. Cell nuclei appear blue, and clusters of QD particles are green. Particles appear to be internalized within cells.

3.4. Discussion

The advantages of microwave heating for synthesis of QD have been enumerated. Besides uniform dielectric heating of the reaction volume, influence on the collisions between reactants, as well as entropic effects due to the rotation of the dipoles are considered important in explaining kinetics and reaction yields. We have reported earlier, that with the composition Cd₄:Se₁:Zn₄:MPA₂₀, microwave synthesis led to QD of nominal structure with a CdSe core and an alloyed shell involving CdS and ZnS, and represented the QD as CdSe/CdS/ZnS. The QY of these QDs were <15%. In the present paper, we examine the same composition, with the goal to understand and optimize this synthesis, and evaluate the mechanistic steps involved in the synthesis. The use of fluorescence spectroscopy during the microwaving process is also being used for the first time, previous efforts have reported on the use of infrared spectroscopy.
We propose a reaction mechanism for the microwave synthesis of the QDs. A population of CdSe seed nanoparticles forms immediately upon mixing cadmium and selenium ions. These particles are protected from aggregation and further reactions by the thiol-linked protection of 3-MPA since as noted, the absence of 3-MPA leads to formation of bulk CdSe. With 3-MPA, but in the absence of \( \text{Zn(NH}_3\text{)}_4^{2+} \), an emission band at 502 nm is formed immediately in the microwave at 150 °C, and persists for about 20 minutes and replaced gradually with a band at 545 nm (Figure 3.4). The particles emitting at 545 nm are due to CdS deposition on the CdSe nuclei, the red-shift occurring from the higher band gap shell deposition. The CdS cap forms after 3-MPA decomposition and the resulting release of sulfur. The QD isolated from this reaction has poor optical properties, with large trap state emissions. QDs with a CdSe core and a predominantly CdS shell with a gradient alloy shell structure prepared in the microwave have been reported.\(^{16}\) Previous aqueous based thioalcohol-capped CdSe QD were found to have low QY (\(< 0.1\%\)).\(^{29}\)

In the presence of \( \text{Zn(NH}_3\text{)}_4^{2+} \), the growth process continues with the \( \text{Zn}^{2+} \) depositing as an outer shell ZnS on the CdSe/CdS particles. Figure 3.5 shows that deposition of ZnS results in a red shift of the emission. The isosbestic point in Figure 3.6b is assigned to the disappearance of the CdSe/CdS particle (at 545 nm) and formation of CdSe/CdS/ZnS QD (emitting at 575 nm). This would indicate that the ZnS deposition
occurs later than CdS because of the higher energy necessary to decompose the Zn(NH$_3$)$_4^{2+}$ complex.

The derivatives of the peak fluorescence intensity obtained during the \textit{in situ} experiments provide more insight. The four regions in Figure 3.8c for room temperature nucleated sample and heated in the microwave at 135°C (Figure 3.11 shows similar data for a sample nucleated at 100°C) can be correlated with the observations above as follows. Region A between 10 -30 min depicts an upward slope, and is related to the growth of the CdSe nuclei. Region B has a downward slope (though still positive growth in fluorescence intensity) and is proposed to arise from the deposition of the CdS shell. Region C is the incorporation of the ZnS shell. Region D continues with the incorporation of the ZnS shell, but as the quality of the QD improves, the self-quenching of the QDs becomes more pronounced, and leads to a decreasing rate of fluorescence intensity. If the microwaving temperature is increased to 155°C, these regions merge into one another (Figure 3.8d). Similar observations were also made with the sample nucleated at 100°C (Figure 3.10, Figure 3.11).

The reaction temperature during the microwave process does not impact the optical properties of the QDs, as long as the temperatures are within the range of 130 to 155°C (Table 1), and enough time is given to reach the optimized state. The unchanged $\lambda_{\text{max}}$ with microwave temperature (Table 1) indicates that the nuclei formed during the nucleation are responsible for the final optical properties of the QD. The microwave temperature is influencing the rates at which the various processes occur, including growth of nuclei by incorporation of the limiting nutrients, deposition of CdS, and then
ZnS. With continued microwave treatment beyond the optimum time at a fixed
temperature, the QY drops, and can be related to deposition of thicker ZnS layers.\textsuperscript{2,30} Also, at microwave temperatures exceeding 155 °C, the QY decreases, and could be due
to deposition of thicker ZnS layers, and extensive MPA decomposition, with loss of the
surface passivation of QD.\textsuperscript{3} Scheme 1.2 provides a mechanistic description of the
growth.

\textit{Controlling }\lambda_{\text{max}}\text{ of Emission}

As observed in Figure 3.9, the primary effect of the temperature at which CdSe
cores are formed prior to microwave irradiation is to alter the emission maximum, with
the peak emission wavelength red shifting from 549 to 574 nm. At the lower temperature
of nucleation, smaller size nuclei are formed, since the Cd\textsuperscript{2+} is coordinated with the
MPA. The influence of ligand detachment on growth of ZnSe QDs has been carefully
studied.\textsuperscript{31} As nucleation temperatures increase, more of the Cd\textsuperscript{2+} is released, forming
larger nuclei, assisted by ligand detachment from the surface of the nucleated state. Once
these nuclei are subjected to microwave, they grow into CdSe cores, with the lower
temperature nucleated state producing smaller CdSe cores and more of them as compared
to the higher temperature nucleation, since the nutrient pool is the same in all cases. So,
for a fixed composition, making fewer nuclei by manipulating the nucleation temperature
controls the core particle size.
There have been many reports on improvement in QY by light illumination after QD synthesis.\textsuperscript{32–36} The improvement arises from dissolution of surface defects and their passivation. We show that light illumination during nucleation is also a method to manipulate nuclei structure. Indeed, as Table 3 shows, light illumination during nucleation (2 hour) and after microwave treatment (6 hour) had a profound effect on the QY, especially for the nucleation carried out at 100 °C. The QY is improved considerably (18→40%), with unchanged $\lambda_{\text{max}}$ at 574 nm. For the nucleation at 22 °C, illumination during nucleation followed by microwaving led to significant decrease in QY (19% → 3%), which could be improved by illumination after microwave treatment (QY: 19% → 28%). We explain these results as arising from the fact that the nuclei formed at 22°C are less stable with irradiation. Nanocrystals of CdTe with a large defect structure have been reported to dissolve during photochemical etching.\textsuperscript{29} However, for the nuclei prepared at 100 °C, the nuclei are larger and more stable, and light illumination restructures the surface, vacancies can be satisfied with the 3-MPA, and there will be minimal change in size. This restructured surface is further improved with post-microwave illumination. Table 4 summarizes current literature on microwave synthesized aqueous CdSe/ZnS QDs. To date, the highest quality microwave synthesis of aqueous CdSe/ZnS particles were 38% quantum yield, and thus the method reported in this paper is an improvement.
The biological imaging studies shown in Figure 3.13 clearly demonstrate that with the higher QY (40%), both flow cytometry and confocal imaging can be done with more dilute concentrations of QDs: we were able to detect the newly optimized particles as low as 8 nM in cells via flow cytometry, while the previous particles were not observed at concentrations up to 57 nM. From a biological perspective, the uptake of QDs by the epithelial C2BBBe1 cells is of interest, since transport of nanoparticles in food that can then make it through the GI tract into circulation needs to go through these epithelial cells, and thus QDs can be used as a model for evaluating the rate of uptake of nanoparticles by these cells.

3.5. Conclusions

Several synthetic strategies for QD synthesis all starting with the composition Cd₄:Se₁:Zn₄: MPA₂₀ are being reported. In situ fluorescence spectroscopy during microwave synthesis of the QD provided insight into the growth process, including growth of the CdSe nuclei and deposition of the CdS and ZnS shell layers. The temperature during the microwave treatment (130 – 155 °C) led to QDs with the same QY and λₘₐₓ. By changing the nucleation temperature (0 – 100 °C), followed by microwave growth led to QD of varying size and with increasing λₘₐₓ (range 546 - 574 nm). UV-visible light illumination in the nucleation step and post microwave treatment
led to optimal QDs with QY of 40 - 41%. These QDs were examined as biological imaging agents. Uptake of QDs into intestinal epithelial cells could be readily observed at 0.8 to 8 nM QD concentrations by flow cytometry and confocal microscopy.

Figure 3.14 shows a summary scheme of this work. The microwave and fluorometer are coupled together and allow for fluorescence spectra to be recorded during the microwave reaction. This plot shows distinct regions corresponding to three stages of the reaction, labeled on the plot showing CdSe nuclei, CdSe/CdS, and finally CdSe/CdS/ZnS. TEM of the resulting QD is shown along with flow cytometry data of newly optimized 40% vs previous 19% QDs.

Having shown that these particles are brighter and are able to be detected at lower concentrations in biological samples, we next will investigate their encapsulation, along with rhodamine 6G and rhodamine 800 dyes, into silica shells in an effort to study their potential internalization through the digestive tract and localization within organs throughout the body.
3.6. References


3.7. Tables and Figures

Figure 3.1. Scheme of QD synthesis and optimization strategy
Figure 3.2. Scheme of Synthetic pathway of CdSe/CdS/ZnS microwave assisted synthesis
Figure 3.3. Fiber optic coupling of the fluorometer (Fluorolog 3) with the microwave oven (Discover SP) allowing for *in situ* fluorescence measurements during synthesis.
Figure 3.4. Fluorescence spectra of the composition Cd$_4$:Se$_1$:MPA$_{20}$ heated in the microwave at 150°C, with spectra recorded at room temperature while in the microwave after heat treatment every 10 minutes, $\lambda_{ex}$ 480 nm.
Figure 3.5. Fluorescence spectra for the composition Cd$_4$:Se$_1$:Zn$_4$:MPA$_{20}$ at 150 °C, stopped at 10 minute intervals, removed from the microwave, cooled to room temperature, diluted by a factor of 10 and spectrum recorded, $\lambda_{ex}$ 375 nm.
Figure 3.6. *In situ* fluorescence spectrum recorded during microwave heating at 150 °C. 
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b) Superposition of the spectra recorded during the first 60 minutes of a), $\lambda_{ex}$ 480 nm.
Figure 3.7. In situ fluorescence plots for room temperature nucleated QDs during microwave irradiation at a) 130, b) 135, c) 140, d) 145, e) 150, and f) 155 °C
Figure 3.8. Plot of the maximum intensity of the *in situ* fluorescence as a function of time for synthesis at a) 135 °C and b) 155 °C and the corresponding derivative plots as a function of time for c) 135 °C d) 155 °C.
Figure 3.9. QDs nucleated from 0 to 100 °C and reacted in microwave at 150 °C for 80 minutes, showing a red-shift of the $\lambda_{\text{max}}$ with increasing nucleation temperature, $\lambda_{\text{ex}}$ 375 nm.
Figure 3.10. In situ fluorescence plots for 100 °C nucleated QDs during microwave irradiation at a) 130, b) 145, and c) 155 °C
Figure 3.11. Plot of the maximum intensity of the in situ fluorescence as a function of time for 100 °C nucleated particles synthesized at a) 130 °C and b) 155 °C and the corresponding derivative plots as a function of time for c) 130 °C and d) 155 °C.
Figure 3.12. Characteristics of the QD with the 40% QY.  a) UV-Vis absorption and fluorescence spectra. Absorption maximum at 542nm, Emission maximum at 574 nm. b) Representative HRTEM image, average diameter $4.6 \pm 0.3$ nm calculated from 20 particles. Inset shows close-up of a 4.3 nm particle with clearly visible lattice fringes, measured to be $0.33 \pm 0.02$ nm. c) Fluorescence lifetime (TCSPC) decay curve, 455 nm LED light source with pulse width of 1.3 ns. Data was best fit to a 3 exponential curve with lifetimes of 3.3, 16.8, and 110 ns. d) Baseline corrected capillary XRD pattern. Peaks positions are $26.6^\circ$, $43.9^\circ$, $45.8^\circ$ and $52.2^\circ$ (expected peaks for wurtzite and zinc blende (ZB) structures of the three chalcogenides are also shown).
Figure 3.13. Biological imaging. a) Flow cytometry showing mean fluorescence intensity of cells treated for 24 hours with QDs nucleated at room temperature (19% QY, old) and optimized QDs nucleated at 100°C (40% QY, new). b) Confocal microscopy of cells treated with 16 nM (left) and 0.8 nM (right) QDs (40% QY) for 24 hours.
Figure 3.14. Summary of work. Microwave and fluorometer are coupled together and allow for fluorescence spectra to be recorded during reaction, showing distinct regions corresponding to three stages of the reaction. TEM of the resulting QD is shown along with flow cytometry data of newly optimized 40% vs previous 19% QDs.
Table 3.1. Quantum yields for a room temperature nucleated sample heated in the microwave at 130-155 °C for optimal times for best QY.

<table>
<thead>
<tr>
<th>Reaction Temperature (°C)</th>
<th>Optimal Time (min)</th>
<th>Quantum Yield (%)</th>
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<tbody>
<tr>
<td>130</td>
<td>340</td>
<td>17</td>
</tr>
<tr>
<td>135</td>
<td>170</td>
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</tr>
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<tr>
<td>155</td>
<td>45</td>
<td>19</td>
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Table 3.2. $\lambda_{\text{max}}$ and quantum yield values of QDs nucleated from 0 to 100 °C and then reacted in the microwave at 150 °C for 80 minutes

<table>
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<th>Nucleation Temperature (°C)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>Quantum Yield (%)</th>
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<tbody>
<tr>
<td>0</td>
<td>546</td>
<td>8</td>
</tr>
<tr>
<td>22</td>
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<td>17</td>
</tr>
<tr>
<td>40</td>
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<td>50</td>
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<td>100</td>
<td>574</td>
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Table 3.3. Summary of light illumination experiments both pre microwave (during nucleation) and post microwave (after synthesis)

<table>
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<tr>
<th>Nucleation Temperature</th>
<th>Light Treatment</th>
<th>$\lambda_{\text{max}}$</th>
<th>Quantum Yield</th>
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<tr>
<td>22 C</td>
<td>None</td>
<td>549 nm</td>
<td>19%</td>
</tr>
<tr>
<td></td>
<td>2 hours pre</td>
<td>537 nm</td>
<td>3%</td>
</tr>
<tr>
<td></td>
<td>6 hours post</td>
<td>548 nm</td>
<td>28%</td>
</tr>
<tr>
<td></td>
<td>2 hr pre/6 hr post</td>
<td>539 nm</td>
<td>15%</td>
</tr>
<tr>
<td>100 C</td>
<td>None</td>
<td>574 nm</td>
<td>18%</td>
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<td>2 hours pre</td>
<td>574 nm</td>
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<td>31%</td>
</tr>
<tr>
<td></td>
<td>2 hr pre/6 hr post</td>
<td>574 nm</td>
<td>40%</td>
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Table 3.4. Comparison of QYs of water-soluble microwave-based CdSe/ZnS QDs reported in literature

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<th>Reference</th>
<th>QY</th>
<th>Comments</th>
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<tr>
<td>Roy et al.(^{21})</td>
<td>28%</td>
<td>Organic-based core synth. in microwave, 150 °C for 2 minutes. Water soluble shell addition.</td>
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<tr>
<td>Han et al.(^{19})</td>
<td>38%</td>
<td>Citrate capped, core and shell separately synthesized, MWI 120 °C for 2 minutes each.</td>
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<tr>
<td>Schumacher et al.(^{18})</td>
<td>13%</td>
<td>MPA capped, room temperature nucleated, MWI 150 °C 90 minutes.</td>
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<tr>
<td>Zhang et al.(^{38})</td>
<td>26-33%</td>
<td>Organic-based synthesis, MPA ligand exchanged. QY increase to 33% after functionalization with denatured transferrin</td>
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<tr>
<td>This paper</td>
<td>40%</td>
<td>MPA capped, 100 °C nucleation with UV/Vis irradiation, MWI 150 °C 80 minutes, 6 hours UV/Vis irradiation post-treatment</td>
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Chapter 4. The Synthesis of Silica Coated Nanoparticles with Stable and Bright Fluorescence Emission

4.1. Introduction

Silicon dioxide is an important and commonly used material in consumer goods. Silica is used in foods as an anti-caking agent and to clarify liquids. While silica particles used in foods (E551) typically have an average size of a few hundred nanometers, they have a broad size distribution. Food-grade silica was found to contain up to 33% silica with a size between 10-200 nm, leading to an estimated 1.8 mg/kg of body weight/day intake of nanosize silica particles.\(^1\) In addition to this unintentional exposure, there are increasingly more products specifically using nanosize silica. The Project on Emerging Nanotechnologies Consumer Products Inventory attempts to catalog these products, which as of the time of writing lists 41 products using or claiming to use nano-silica.\(^2\) Many of these products are coatings, paints, and cleaning supplies. Seven of the listed products are cosmetics and dietary supplements. Despite the increased interest in using nanoparticles in food, the FDA currently has no specific regulations related to nanoparticles. A 2012 draft guidance suggested that food manufacturers investigate the safety of foods incorporating nanoparticles, but as of now, many of the inorganic nanoparticles are generally regarded as safe for use in foods.\(^3\)
Nanoparticle interactions with biological systems are dependent on size\textsuperscript{4–7} as well as surface charge and material properties.\textsuperscript{8–11} One method commonly used to study these interactions is the imaging of nanoparticles functionalized with a fluorescent component. Because particle interactions depend on surface material and charge, it is important that the dye remains within the particle and is not present in the solution or bound to the particle surface, where it may affect their properties.

Two common methods of silica nanoparticle synthesis are typically a sol-gel based Stober synthesis and a modification of this which takes place in a reverse microemulsion water-in-oil droplet. Both of these involve an ammonia catalyzed hydrolysis of an alkyl silicate followed by condensation, shown here for TEOS:

\[
\begin{align*}
\text{Hydrolysis:} & \quad \text{Si}(\text{OC}_2\text{H}_5)_4 + \text{H}_2\text{O} \rightarrow \text{Si}(\text{OC}_2\text{H}_5)_3\text{OH} + \text{C}_2\text{H}_5\text{OH} \\
\text{Condensation:} & \quad \text{Si-O-H} + \text{H-O-Si} \rightarrow \text{Si-O-Si} + \text{H}_2\text{O} \\
& \quad \text{Si-OC}_2\text{H}_5 + \text{H-O-Si} \rightarrow \text{Si-O-Si} + \text{H}_2\text{O}
\end{align*}
\]

\textit{Stober silica synthesis}

The Stober silica synthesis is an adaptation of a typical sol-gel synthesis in which tetra alkyl silicates undergo hydrolysis and condensation in the presence of ammonia.\textsuperscript{12,13} Under optimal conditions, a gel phase is not formed, and a solution of easily purified silica nanoparticles is obtained. This reaction has been utilized to add a silica coating to various materials, though typically only with polar ligands attached to the surface.\textsuperscript{14}
Silica synthesis involving a reverse-microemulsion was developed to further control over Stober type methods. In essence, a Stober synthesis is performed within water-in-oil droplets, as alkyl silicates and ammonia localize within the surfactant stabilized water droplet. This method has led to small, monodisperse nanoparticle formation. Creating the water-in-oil emulsion requires a large amount of surfactant, so particles of this type require more extensive post-synthesis washing than those formed in a typical Stober synthesis.

An important issue that has arisen in the encapsulation of dyes in Stober-silica shells is the instability of the silica to dissolution. Recent publications show evidence of silica NP dissolution, including from the inside, leaving hollow spheres. Wang and Chen show that silica coated gold NPs approximately 110 nm in diameter etch differently depending on synthetic conditions; dissolving completely when reacted for 4 hours at room temperature, leaving a shell when reacted for 14 hours at room temperature, and remaining stable when reacted at room temperature for 4 hours and 60 °C for 10 hours. The nature of these shells must be porous if material is dissolving and leaving empty spheres. This poses an obvious problem for dye-encapsulation: the dye may leave the particles. This dissolution is found to be size-dependent and more relevant to smaller nanoparticles. Park reports that silica nanoparticles of 21 nm initially develop internal pores and eventually form an interconnected matrix, particles of 49 nm formed hollow spheres, and particles of 123 nm remained stable. The dissolution is also found
to be particularly strong in biological media.^{18,19} A more recent development to the Stober synthesis of silica particles has been the use of weakly basic amino acids in place of ammonia.^{20} This includes the coating of core particles, where higher temperatures and lower amino acid concentrations result in more stable particles.^{21} Using this strategy, dense stable coatings of dye-incorporated silica have been synthesized which minimize dye leakage.^{18,19}

There are a variety of choices of fluorophores to use with silica coating for biological studies. The variables desired are good fluorescence stability/lack of photobleaching, stability in biological media, bright fluorescence/high quantum yield, and a longer emission wavelength, particularly for in-vivo imaging where the emitted light must pass through tissues. Fluorescence in the near-IR and IR region benefits from minimal scattering and absorption in biological tissues.^{22,23} In this research, we focus on CdSe/CdS/ZnS quantum dots (QDs), rhodamine 6G, and rhodamine 800. CdSe/CdS/ZnS QD synthesis has been previously optimized in our lab to inexpensively and quickly produce particles with a 40% quantum yield and λ_{em} of 574 nm (ref our paper). Quantum dots in general have been popular for biological studies due to their bright fluorescence and resistance to photobleaching. UV light in some cases increases the fluorescence intensity of QDs.^{24–28} Rhodamine 6G is a commonly available and inexpensive organic dye, with very strong emission (90% quantum yield in water) at 558 nm.^{29} Rhodamine 800 is a dye in the rhodamine family with a 25% QY emission maximum at 700 nm.^{30} While this dye has the lowest quantum yield of the three, it is easily adapted to syntheses involving rhodamine 6G, and has a near-IR fluorescence emission, which is desirable for
in-vivo imaging. Figure 4.1 shows the structure of each fluorophore discussed here. Rhodamine 6G and rhodamine 800 are both positively charged at neutral pH.

Using these three fluorophores, we adapt two different literature methods for each to explore their feasibility with our need for stable, bright fluorescence of silica nanoparticles.

**Rhodamine 6G/Rhodamine 800 dyes**

First, a more common Stober method will be used to coat the organic rhodamine 6G and rhodamine 800 dyes electrostatically bound to commercial silica nanoparticles. This method is advantageous in its simplicity; no covalent linkage is required, instead relying on attraction of the positively charged dye to the negatively charged silica core’s surface, and no surfactants are required which necessitate extensive washing of the synthesized particles. We base our synthesis on a literature report of Stober-silica coated rhodamine 6G dye,\(^{31}\) and we expect this should be adaptable to rhodamine 800 due to their similar structure and charge (Figure 4.1). While this method was determined to be stable for silica particles above 150 nm in diameter, it is known that smaller silica nanoparticles can undergo dissolution,\(^{17}\) and this will need to be monitored to determine if the smaller particles are stable against dye leakage. Next, to counter this potential dissolution, the more recent arginine-driven method discussed previously will be generalized to these two molecules.\(^{18}\)
**QD/silica coating**

There are many reports on coating quantum dots with silica for increased biocompatibility\textsuperscript{32,33} and biodetection.\textsuperscript{34} These particles are of varying quality and stability depending on the specific core/shell structure and passivating ligands present.\textsuperscript{35} For the coating of quantum dots, microemulsion synthesis has been found to be robust against many reaction conditions and result in monodisperse particles.\textsuperscript{14} We will apply this method to our lab’s previously developed MPA capped CdSe/CdS/ZnS quantum dots. The arginine-driven method reported by Dawson will also be generalized to the QD particles to determine if its more dense silica coating is beneficial.\textsuperscript{18}

**Biological Imaging**

After the optimal particle of each type is determined, they will each be observed in a murine alveolar macrophage cell line to demonstrate their utility in the study of biological systems. These cells provide a good starting point for a feasibility study, as macrophages typically show strong uptake of foreign materials. We will then expose an intestinal epithelial cell line, C2BBe1, to the particles to investigate their propensity to internalize within cells more relevant to the digestive system. Finally, the particles will be administered orally to mice, followed by confocal microscopy of tissue samples to determine if particles escape the digestive system, and where they may localize.
4.2. Experimental Section

Materials

Cadmium chloride hemipentahydrate (CdCl$_2$•2.5 H$_2$O, >98 %), sodium borohydride (NaBH$_4$, 99 %), Silica nanoparticles (included size of 12 nm, with surface area of 175-225 m$^2$/g, and purity of 99.8%), Tetraethyl orthosilicate (TEOS, 98%), L-arginine (>98%), rhodamine 6G (99% dye content), and rhodamine 800 were obtained from Sigma Aldrich (Milwaukee, WI, USA). Zinc chloride (ZnCl$_2$, 99.99 %), 3-mercaptopropionic acid (MPA), and selenium powder (Se, 99.5+ %, 200 mesh) were obtained from Acros (Geel, Belgium). Sodium hydroxide (NaOH) and ammonium hydroxide (NH$_4$OH, 28 – 30 %) were obtained from Mallinckrodt Chemicals (Phillipsburg, NJ, USA).

All chemicals were used without further purification. The H$_2$O used in this study was purified by a Barnstead NANOpure Infinity ultrapure water system (Dubuque, IA, USA).

Quantum Dot Synthesis

CdSe/CdS/ZnS quantum dots were first prepared using a previously published synthesis:
Precursor Solutions:

Cadmium/MPA: A 250 mL solution of 1.05 mM Cd and 5.26 mM MPA was prepared by adding 60 mg of cadmium chloride hemipentahydrate and 114.5 μL of MPA to approximately 225 mL water. The pH was adjusted to 9.5 with 1 M NaOH, and the volume was adjusted to 250 mL in a volumetric flask. The final solution was stored in a plastic bottle and wrapped in aluminum foil.

Zn(NH$_3$)$_4^{2+}$: A 25 mL solution of 26.67 mM Zn(NH$_3$)$_4^{2+}$ was prepared using anhydrous zinc chloride and ammonium hydroxide. The hygroscopic zinc chloride was dried in a vacuum oven for one hour at 200 °C and capped prior to weighing. To minimize error in weighing due to absorption of water from the air, approximately 90.9 mg of zinc chloride was quickly added to a previously weighed beaker containing 20 mL of water. Ammonium hydroxide was then added drop wise. A white precipitate formed and then disappeared as more ammonium hydroxide was added. Once the solution was fully clear, the volume of the solution was adjusted to 25 mL in a volumetric flask. The final solution was stored in a plastic bottle, wrapped in aluminum foil to protect from light, and stored at 4 °C.

NaHSe: 152 mg of sodium borohydride was added to a glass test tube and chilled to 0 °C in an ethylene glycol/water bath. In quick succession, 2 mL of chilled water was pipetted into the test tube, followed by 158 mg of selenium powder. The test tube was capped with a rubber stopper with two needles sticking through; one of the needles attached to a nitrogen gas tank, and the other left open to vent. The test tube was quickly placed back into the cold bath. The reaction showed intense bubbling from the evolution
of hydrogen gas, which lasted for approximately 30 minutes. By this time, the black selenium was reduced into the solution and a white Na₂B₄O₇ precipitate formed. After completion, the vial was moved to a nitrogen filled glove bag. 0.5 mL of the supernatant was pipetted into a 50 mL 3-neck round bottom flask containing 24.5 mL of nitrogen saturated water. This yielded a 25 mL solution of 20 mM NaHSe. The flask was kept capped and nitrogen was constantly bubbled though the solution. Within 12 to 18 hours, the presence of a red tint indicated that the selenium had oxidized, and the solution was no longer useful.

**CdSe Nucleation:** 19 mL of the Cd/MPA solution and a Teflon stir bar were added to a 25 mL round bottom flask and brought to 100 °C under reflux in a slowly stirring (100 rpm) oil bath. After removing the stir bar from the oil bath to avoid splashing of hot oil, the stirring speed was increased to 1200 rpm. Reflux was temporarily removed to quickly pipet 0.25 mL of the NaHSe precursor solution, immediately resulting in a yellow color due to formation of CdSe nuclei. The solution was stirred at temperature for 1 hour and then cooled to room temperature. This solution of seed nuclei was then subjected to IR-filtered irradiation for 2 hours using a 150 W Xe lamp (model UXL151HXE, PTI) at room temperature under rapid stirring (1200 rpm). After nucleation, 0.75 mL of the Zn(NH₃)₄²⁺ precursor solution was added. The final 20 mL solution was 1 mM Cd, 1 mM Zn, 0.25 mM Se, and 5 mM MPA.

**Microwave-Assisted Growth:** The solution was placed in a Discover SP (CEM Corp.) microwave system and heated for 70 minutes at 150 °C, with maximum power set to 200 watts. After completion, the solution was quickly cooled to 40 °C in the
microwave with a stream of room temperature air, and then allowed to cool to room temperature.

**UV-Visible Light Aging and Cleaning:** Samples were subjected to IR-filtered irradiation for 6 hours at room temperature under rapid stirring (1200 rpm). Particles were washed once by centrifugation at 290,000x g (Thermo Scientific Sorvall MX-150 Ultracentrifuge, S50-A rotor), removal of supernatant, and replacing with water. Extensive washing leads to loss of MPA capping ligand, and destabilized the particles. The final solution was covered with aluminum foil and stored at 4 °C until needed. Particles were stable for several months under these conditions.

**Fluorophore Entrapment by Ammonia-Driven Reaction (Stober Method)**

Rhodamine 6G, rhodamine 800, and previously prepared quantum dots were entrapped within silica shells by the following methods:

**Dye/Silica:** 1 g of commercial silica nanoparticles (Sigma Aldrich, ~20 nm) were added to 50 mL of dried ethanol and sonicated for 30 minutes (Branson 3510), 5 mg of rhodamine 6g or rhodamine 800 dye was added and the solution was stirred for 24 hours. 5 mL of 25% NH₃ was added to the solution, which was then brought to 50 °C in and water bath and stirred at 500 rpm. 250 µL TEOS was added and the solution was stirred for 4 hours at 50 °C, then at room temperature for 16 hours. Final reaction concentrations were 1.88x10⁻⁴ M rhodamine 6g, 1.82x10⁻⁴ M rhodamine 800, 1.21 M NH₃, and 2.03x10⁻⁵ M TEOS in ethanol. Particles were washed 4 times by centrifuging at 50,000 x g for one hour, removal of supernatant, and redispersion in ethanol. Following this, the particles
were dried in a vacuum oven at 70 °C for 14 hours, 100 °C for 2 hours, and 200 °C for 4 hours, then cooled to room temperature and dispersed in water for further analysis.

**QD/Silica:** Synthesized quantum dots were coated with silica according to a previously published reverse microemulsion method.\(^{14}\) 10 mL of cyclohexane, 1.3 mL of NP-5, 400 µL of as-synthesized quantum dot solution, and 80 µL of TEOS were added to a small flask and stirred at 1000 rpm for 30 minutes. 150 µL 25% NH\(_3\) was added and the solution was stirred for 24 hours. Final reaction concentrations were 0.168 M NH\(_3\), 3.00x10\(^{-5}\) M TEOS, and 25 nM QDs in cyclohexane. The resulting particles were washed in a sequence of 1-butanol, 1-propanol, ethanol, and water by centrifuging for 10 minutes at 3200 rpm (Clay Adams Compact II) removal of supernatant, and redispersion in new solvent.

**Fluorophore Entrapment by Arginine-Driven Reaction**

**Dye/Silica:** A 30 mL 10 ppm solution of dye was sonicated to ensure dissolution. 30 mg of commercial silica nanoparticles (Sigma Aldrich, ~20 nm) were added and the solution was placed in an ultrasonic bath for 30 minutes, resulting in silica particles electrostatically bound by the dye. The solution was then placed in a small polypropylene bottle with a Teflon stir bar. 15 mg L-arginine was added and the solution stirred for 15 minutes until fully dissolved. 600 µL TEOS was slowly added to the solution which resulted in two phases: TEOS on top and water/arginine/silica-dye below. Reaction concentrations were 6.82x10\(^{-4}\) M rhodamine 6g, 6.59x10\(^{-4}\) M rhodamine 800, 2.81x10\(^{-3}\) M
arginine, and 8.78x10^{-5} M TEOS in water. The solution was capped tightly and stirred slowly (100 rpm) in a 70 °C oil bath for 48 hours. After the reaction, the TEOS layer was no longer present and the solution appeared cloudy compared to the original clear silica-dye solution. The resulting particles were washed twice with ethanol then with water by centrifuging at 50,000 x g, removing supernatant, and replacing with new solvent. The solution was washed until fluorescence was not detected in the discarded supernatant, indicating any dye remaining was encapsulated, typically 3 times with water following the two ethanol cycles. The solution was covered with aluminum foil and stored at 4 °C until needed. If allowed to sit for several days, particles required sonication to re-suspend.

**QD/Silica:** A previously synthesized solution of quantum dots was diluted to 1 mg particles/mL (30 mL total) in a small polypropylene bottle. 15 mg L-arginine was added and the solution stirred for several minutes until dissolved. 600 µL TEOS was slowly added to the solution which resulted in two phases: TEOS on top and water/arginine/QDs below. Reaction concentrations were 2.81x10^{-3} M arginine, 8.78x10^{-5} M TEOS, and 478 nM QDs in water. The solution was capped tightly and stirred slowly (100 rpm) in a 70 °C oil bath for 48 hours. After reaction, TEOS layer was no longer present and the solution appeared cloudy compared to the original clear QD solution. The resulting particles were washed twice with ethanol then twice with water by centrifuging at 50,000 x g (Beckman-Coulter Allegra 64R), removing supernatant, and replacing with new solvent. The solution was covered with aluminum foil and stored at 4 °C until needed. If allowed to sit for several days, particles required sonication to re-suspend.
**Optical Properties:** UV/Visible absorption and fluorescence spectra were measured for particles diluted 10:1 with water. Spectra were recorded with a Shimadzu UV-2501PC spectrophotometer. Fluorescence measurements were recorded with a Horiba Jobin-Yvon Fluorolog 3, using 5 nm slit widths for the emission and excitation monochromators, 0.3 second integration time. Excitation wavelengths were 375 nm for QD/silica, 480 nm for rhodamine 6G/silica, and 635 nm for rhodamine 800/silica. A photomultiplier tube detector (Horiba Jobin-Yvon FL-1703) was used for QD/silica and rhodamine 6G/silica, while a CCD detector (Horiba Jobin-Yvon Synapse) was used for the near-IR rhodamine 800/silica.

Quantum yields were measured using a Quanta-Phi integrating sphere attachment (Horiba Jobin-Yvon) for the Fluorolog 3. The detector was set to measure counts rather than the default counts per second. Slit widths for the emission and excitation monochromators were set to 5 nm. The samples were excited with 480 nm light for QDs and rhodamine 6G, and 635 nm for rhodamine 800. Spectra were measured from 460 nm to 750 nm (620 nm to 800 nm for rhodamine 800), to include both the excitation and emission wavelengths. An initial blank water sample was measured; adjusting the integration time to obtain an excitation peak height of 1 million counts to avoid oversaturation of the detector. The sample was then measured using the same settings for comparison. The areas under the excitation and emission curves in each spectrum were integrated, allowing for calculation of the quantum yield:
\[ QY = \frac{\text{emission}_{\text{sample}} - \text{emission}_{\text{blank}}}{\text{excitation}_{\text{blank}} - \text{excitation}_{\text{sample}}} \times 100\% \]

Fluorescence lifetime was measured with the Fluorolog 3 time correlated single photon counting (TCSPC) attachment. Fluorescence was excited with a pulsed 455 nm LED (1.5 ns pulse duration). Repetition rate was set to 1 MHz.

**Zeta Potential and Isoelectric Point Measurement:** To determine surface charge properties of the coated particles, zeta potentials were measured (Malvern Zetasizer Nano ZS). For the QD/silica particles, the zeta potential was measured before and after encapsulation. For the dye/silica particles, the zeta potential of the initial silica cores, the silica cores coated with dye, and the final silica/dye/silica particles were measured. Commercial silica particles used in the dye/silica synthesis were titrated (Malvern MPT-1) to plot the pH vs. Zeta Potential. This was then repeated with the silica coated particles to compare with pure silica.

**Infrared Spectroscopy:** Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) measurements were taken using a Perkin Elmer Spectrum 400 spectrometer. Spectra were recorded from 600 to 4500 cm\(^{-1}\) with a 2 cm\(^{-1}\) resolution for 20 minutes. Samples were prepared by grinding approximately 1% by weight sample in KBr, and measured against pure KBr background. All samples were dried for two days in a Millrock Benchtop Manifold Freeze Drier, followed by vacuum drying for at least 12 hours at 110 °C.

**Surface Area:** Surface area was measured using a Quantachrome NOVA 2200e BET surface area analyzer. Samples were initially freeze dried for two days, then
degassed under vacuum at 110 °C for at least 12 hours. 11-point measurements were obtained for all samples.

**Electron Microscopy:** High resolution transmission electron microscopy (HRTEM) images were obtained using a Tecnai-F20 system. Particles were washed twice by centrifugation and replacement of supernatant, resuspended in a dilute solution in ethanol, and deposited onto a lacey-carbon coated copper grid.

**Fluorescence Stability**

**Dye Leaching:** To determine if significant dye-leaching was occurring, the Stober-silica and arginine-silica particles of both dyes were suspended in phosphate buffered saline (PBS) and checked periodically. The particles were centrifuged at 50,000 x g and a sample of the supernatant was checked for fluorescence. The particles were then re-suspended in fresh PBS and the fluorescence spectrum was recorded. This continued for up to one week.

**QD/Silica Quenching:** To observe any quenching of quantum dot fluorescence as a result of silica encapsulation, the fluorescence spectrum was recorded periodically for one week. The quantum yield was measured at the end of the experiment using the Quanta-Phi attachment.
Optimal reaction parameters were determined for arginine synthesis only, as the dye leaching and QD quenching studies revealed that the ammonia based synthesis did not yield particles with stable fluorescence.

**Dye Loading and TEOS:** Dye concentrations were optimized by repeating the arginine-silica coating with varying concentrations of both rhodamine 6G and rhodamine 800. 10, 20, and 40 ppm concentrations were used, corresponding to 6.82x10^-4, 1.36x10^-3, and 2.73x10^-3 M rhodamine 6G, and 6.59x10^-4, 1.32x10^-3, and 2.64x10^-3 M rhodamine 800. The best dye concentration was then used in a next set of reactions where TEOS was varied for a rhodamine 800 synthesis: 4.39x10^-6, 8.78x10^-6, 8.78x10^-5, and 4.39x10^-4 M TEOS.

**Arginine:** The arginine based QD/silica coating was repeated with the optimal 8.78x10^-5 M of TEOS and varying concentrations of arginine: 9.38x10^-4, 2.81x10^-3, 5.63x10^-3, and 1.13x10^-2 M arginine. The resulting particles were imaged by TEM to observe particle morphology and distribution of QDs within silica shells. In addition, a batch of particles was synthesized with 2.81x10^-3 M arginine and 8.78x10^-5 M TEOS, and aliquots were periodically removed to measure the quantum yield throughout the 48 hour reaction.
Cell culture: C2BBe1 and MH-S cells were obtained from the American Type Culture Collection (Manassas, VA). C2BBe1 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Serum Source International, Inc., Charlotte, NC), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.3% penicillin/streptomycin, 0.3 µg/mL Amphotericin B (fungizone), and 10 µg/mL transferrin (all from Life Technologies) and MH-S cells were cultured in RPMI Medium 1640 (Life Technologies) supplemented with 10% FBS (Serum Source International, Inc.), 0.3% penicillin/streptomycin, and 0.3 µg/ml Amphotericin B (fungizone; both from Life Technologies). Cells were incubated in 5% CO₂/95% room air at 37°C. C2BBe1 cells were passaged every 5-7 days and plated on flasks or plates pre-coated with collagen I (0.05 mg/mL, rat tail, Life Technologies). Cells were plated at a density of 100,000 cells/chamber in 8-chamber slides (Thermo Scientific) for analysis by confocal microscopy. MH-S cells were passaged every 3-5 days and cells were plated in 8-chamber slides (Thermo Scientific) at a density of 10,000-20,000 cells/chamber for confocal microscopy. Cells were incubated for at least 24 hours after plating before treatment with silica-R6G and silica-QDs. Directly prior to treatment on cells, silica-Rhodamine 6G and silica-QD solutions were sonicated using a Sonics Vibra-Cell sonicator (Sonic Materials, Inc., Norwalk, CT) pulsing for one second on, one second off for approximately 15 seconds in order to minimize particle agglomeration. Cells were treated with the appropriate dose of particles (generally 100 µg/cm²) and
immediately centrifuged at 75×g for 15 minutes to promote contact of QDs with cells. Cells were incubated with silica particles for 24 hours before fixing and staining for confocal microscopy. After 24-hour treatment of cells with silica-Rhodamine 6G and silica-QDs, the chambers were removed from 8-chamber slides for staining.

**Confocal microscopy:** All staining was performed at room temperature. Slides were washed twice with PBS and fixed in 4% paraformaldehyde for 45 minutes. Cells were washed again in PBS before permeabilization in a 0.2% Triton X-100 (Sigma-Aldrich) solution in PBS for 15 minutes. Cells were washed in PBS and incubated in a 1% bovine serum albumin (BSA; Sigma-Aldrich) blocking solution for 1 hour. Cells were incubated with a 1:150 dilution of Alexa Fluor 647 mouse anti-E-cadherin (BD Biosciences) in 1% BSA in PBS for 90 minutes. Cells were washed with PBS and stained with a 0.25 μg/mL solution of 4′,6′-diamidino-2-phenylindole (DAPI, Life Technologies) in 1% BSA in PBS for 10 minutes. Cells were washed with PBS before coverslips were mounted using ProLong Gold Antifade Reagent (Life Technologies). Mounting media was allowed to cure overnight at room temperature before analysis using a four-laser confocal LSM 700 microscope (Carl Zeiss Microscopy Ltd, Germany).

**Animal Model:** All animal experiments were approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC) on protocol 2012A00000020. Female mice of 8-12 weeks of age were purchased from Charles River Laboratories (Raleigh, NC). Once mice were received, they were given food and water **ad libitum.** Mice were fed Teklad Global Rodent Diet 2016 (Harlan Laboratories,
Indianapolis, IN) in order to minimize autofluorescence in the mice from their diet for 10
days prior to nanoparticle administration.

Silica-Rhodamine 6G and silica-QDs were administered by oral gavage to mice
once daily for four total administrations. In one experiment, silica-Rhodamine 6G and
silica-Rhodamine 800 were administered in 100 µl total volume at doses of 1 mg each. In
another experiment, silica-Rhodamine 6G and silica-QDs were administered in doses of
0.1 mg and 0.67 mg, respectively. Silica-Rhodamine 6G, silica-Rhodamine 800, and
silica-QDs were suspended in water and sonicated using a Sonics Vibra-Cell sonicator
(Sonic Materials, Inc.) pulsing for one second on, one second off for approximately 15
seconds in order to minimize particle agglomeration immediately prior to administration.
Mice were euthanized three hours after the final silica particle administration by carbon
dioxide asphyxiation. Organs were excised and frozen in Tissue-Tek O.C.T. Compound
embedding medium (Sakura Finetek, Torrance, CA). Organ blocks in OCT were
sectioned onto microscope slides using a Microm HM505E cryostat (Microm
International GbmH, Walldorf, Germany) (thickness) for fixation and staining for
confocal microscopy.
4.3. Results

I. Initial Synthesis

*Fluorophore Entrapment by Ammonia-Driven Reaction (Stober Method)*

Fluorescent silica particles were synthesized using ammonia hydrolysis of TEOS in the presence of organic dyes and QDs. Reaction scheme is shown in Figure 4.2. Due to the positive charge of the dyes, they electrostatically bind to the surface of the silica cores, which are then encapsulated within the silica framework as it develops around them via the hydrolysis and condensation of TEOS. For rhodamine 800 and rhodamine 6G, a standard aqueous Stober method was used: 1 g core silica nanoparticles (~20 nm) in ethanol with either $1.88 \times 10^{-4}$ M rhodamine 6g or $1.82 \times 10^{-4}$ M rhodamine 800 dye followed by $1.21$ M NH$_3$ and $2.03 \times 10^{-5}$ M TEOS at 50 °C 4 hours, then at room temperature for 16 hours. For QDs, a reverse microemulsion synthesis was used: $0.168$ M NH$_3$, $3.00 \times 10^{-5}$ M TEOS, and $25$ nM QDs in cyclohexane stirred at room temperature for 24 hours.

**Rhodamine 800:** Encapsulated rhodamine 800 was not strongly fluorescent enough to measure. These particles were not characterized further.

**Rhodamine 6G:** Figure 4.4 shows fluorescence spectrum of Stober coated rhodamine 6G, indicating significant fluorescence intensity immediately after sample preparation. To determine dye-leakage in biological conditions, the particles were
incubated in phosphate buffer saline (PBS, pH=7.4) for 4, 6, and 8 days. Figure 4.5a shows decreasing fluorescence intensity of these samples with time. Figure 4.5b shows strong fluorescence of the PBS supernatant, indicating significant dye leakage from the particles into the solution. Due to dye-leakage, these particles were considered not useful, and were not characterized further.

**Quantum Dots:** Figure 4.6 shows the fluorescence over time of quantum dots coated in a reverse microemulsion reaction. The emission maximum red-shifted in comparison to initial QDs to 591 nm from 574 nm. Fluorescence intensity decreased with time, until it was fully quenched within 3 days after silica coating. TEM image shown in Figure 4.7 shows QD entrapment within silica cores, with an average particle size of $37 \pm 9$ nm based on 51 measured particles.

*Fluorophore Entrapment by Arginine-Driven Reaction*

Fluorescent silica particles were synthesized in arginine-driven syntheses using organic dyes and QDs. Reaction scheme is shown in Figure 4.3. Similar to the scheme shown in Figure 4.2, the positively charged dyes are electrostatically bound to the negatively charged silica surface, and are subsequently entrapped during the shell formation. 1 mg/mL commercial silica particles ($\sim$20 nm) as substrate with $6.82 \times 10^{-4}$ M rhodamine 6G or $6.59 \times 10^{-4}$ M rhodamine 800, or 1 mg/mL (478nM) QDs with no silica cores. Solution adjusted to $2.81 \times 10^{-3}$ M arginine and $8.78 \times 10^{-5}$ M TEOS then stirred slowly (100 rpm) at 70 °C for 48 hours.
**Rhodamine 800:** In contrast to the ammonia driven (Stober) reaction, the arginine rhodamine 800/silica particles did exhibit measurable fluorescence. The particles just after washing are shown in Figure 4.8a. These particles were subjected to incubation in PBS media, but did not show loss of dye. Figure 4.8b shows the particles after 1 week in solution, along with the supernatant solution.

**Rhodamine 6G:** Silica/rhodamine 6G/silica arginine-driven particles just after washing are shown in Figure 4.9a. Similar to the rhodamine 800 sample, the rhodamine 6G samples were stable against dye leakage in PBS media. Figure 4.9b shows the particles after 1 week in solution, along with the supernatant solution.

**Quantum Dots:** The arginine-driven QD/silica sample did not exhibit any wavelength shift versus initial QDs (574 nm). The stability of these particles was observed by measuring fluorescence over time, shown in Figure 4.10. The sample had a quantum yield of 25% at both 0 days and 7 days. TEM show an average particle diameter of 29 ± 6 nm, and 0 to 5 QDs per particle are typically seen (Figure 4.18c).

**II. Synthesis Optimization - Arginine Driven Synthesis**

In all three cases, the arginine synthesis lead to stable fluorescence, while the Stober synthesis yielded particles which lost fluorescence within 3 to 7 days. The arginine based method was subsequently optimized.
The arginine dye/silica synthesis was further optimized by determining the amounts of dye and TEOS which resulted in the greatest fluorescence intensity. The quantum yields were measured in each step to determine the best quality particles, except in the case of rhodamine 800 particles, which did not have strong enough fluorescence to be measured in the Quanta-Phi attachment for the Fluorolog 3 fluorometer. For these particles, the fluorescence was measured directly and samples were compared by intensity.

Figure 4.11 shows the optimization for rhodamine 800/silica particles. Dye concentrations of 6.59x10^{-4}, 1.32x10^{-3}, and 2.64x10^{-3} M were observed. Increasing the dye concentration higher than 1.32x10^{-3} M resulted in decreased fluorescence intensity. We have noticed rhodamine 800 dye exhibits strong self-quenching at higher concentrations, and this is likely the cause of the behavior seen in Figure 4.11a. Figure 4.11b shows the variation of fluorescence intensity with 4.39x10^{-6}, 8.78x10^{-6}, 8.78x10^{-5}, and 4.39x10^{-4} M TEOS used during the reaction, indicating that 8.78x10^{-5} M is the optimal concentration.

The optimal TEOS concentration of 8.78x10^{-5} M from the rhodamine 800 particles was also used for rhodamine 6G. Particles synthesized with rhodamine 6G concentrations of 6.82x10^{-4}, 1.36x10^{-3}, and 2.73x10^{-3} M were observed and found to increase in quantum yield up to 1.36x10^{-3} M. Quantum yields were 7% for 6.82x10^{-4} M
and 20% for 1.36 × 10⁻³ M. At 2.73 × 10⁻³ M rhodamine 6G, the particles aggregated upon addition of arginine and were not suitable for coating.

**QD/Silica Synthesis: Influence of Arginine**

*Fluorescence During Silica Synthesis:* Figure 4.12 shows the change in quantum yield during the QD/silica arginine synthesis. The solution begins with the initial 40% quantum yield of the uncoated particles. The quantum yield decreases to a low of 34% after 1 hour. From this point the quantum yield increases, becoming higher than the initial particles and peaking at 56% after 10 hours. The quantum yield then decreases throughout the remainder of the reaction, ending at 36% after 48 hours. After washing, the final QY was 25%.

An additional synthesis was halted after 20 hours to test the stability of the increased quantum yield seen during the reaction. Within two days the quantum yield of this sample had decreased to 3%, indicating quenching similar to that seen in the Stober sample.

*Effect of Arginine Concentration:* Figure 4.13 shows TEM images of arginine QD/silica particles synthesized using 9.38 × 10⁻⁴, 2.81 × 10⁻³, 5.63 × 10⁻³, and 1.13 × 10⁻² M arginine. As the concentration of arginine increases, the particles become more aggregated, with more QDs per silica particle seen. At 5.63 × 10⁻³ and 1.13 × 10⁻² M, many large strands of coated QDs are seen rather than spherical particles, these samples had primary particle sizes of 29 ± 5 nm and 31 ± 8 nm, respectively. At 2.81 × 10⁻³ M,
spherical particles with an average diameter of $29 \pm 6$ nm and 0-4 QDs per particle are primarily seen, with fewer strand-like structures. The $9.38 \times 10^{-4}$ M arginine sample showed only spherical particles with an average diameter of $22 \pm 3$ nm and 0-4 QDs per particle.

Table 4.1 summarizes the quantum yields and average sizes of these particles. The $9.38 \times 10^{-4}$ M sample, which morphologically appears to be the ideal arginine concentration, has a quantum yield of 8%. The $2.81 \times 10^{-3}$ M sample had the highest quantum yield of 25%, while the $5.63 \times 10^{-3}$ and $1.13 \times 10^{-2}$ M samples, which showed significant aggregation, both had quantum yields of 7%.

III. Characterization

A summary of each optimized particles’ properties is listed in Table 4.2.

Surface Properties

**Zeta Potential:** The surface charge properties of the optimized particles were determined by analyzing their zeta potential across a range of pH values. The zeta potential of a particle at a given pH is determined by the surface and any species that are electrostatically or covalently bound to its outer surface. These titrations are shown in Figure 4.14, and illustrate the zeta potential as the pH of each solution was decreased from its initial value. All particles start at a zeta potential of approximately -30 mV at a
pH of 5, and approach a neutral zeta potential of 0 mV near a pH value of 1 to 2. The asymptotic approach of the zeta potential to 0 mV makes the isoelectric point (IEP) difficult to precisely determine; in fact the QD/silica particles did not appear to have an IEP. Both dye/silica particles had comparable IEP values to the commercial silica: pH 1.34 for rhodamine 6G/silica, pH 1.70 for rhodamine 800/silica, and pH 1.91 for commercial silica. These values are summarized in Table 4.2.

By comparing the synthesized dye/silica and QD/silica particles to identically treated commercial silica particles, we determine that the outer surface of the particles does behave similar to plain silica, and in the case of the dye/silica particles we see no evidence that dye is present on the surface.

**TEM:** TEM images of all three optimized particles were recorded and are shown in Figure 4.18, with particle sizes summarized in Table 4.2. Rhodamine 6G and rhodamine 800 particles appear spherical and similar in size: 28 ± 11 nm for rhodamine 6G and 32 ± 15 for rhodamine 800.

QD/silica particles are smaller, 29 ± 6 nm. The number of QDs in each silica particle varied from 0 to 4. EDX analysis confirmed presence of silicon and oxygen in a 1:2 ratio.

**Infrared Spectroscopy of QD/Silica:** To investigate potential differences between the microemulsion-silica coated QDs and arginine-silica coated QDs, infrared spectra were measured. All particles were prepared by washing, freeze drying, and heating at 110 °C overnight under vacuum. Figure 4.15 shows the DRIFTS spectrum of the final arginine QD/silica particles. Peaks at 800, 963, 1104, and 1206 cm⁻¹ closely match
literature values.\textsuperscript{36} 800 cm\textsuperscript{-1} is a symmetric stretching Si-O vibration within the Si-O-Si framework, 963 cm\textsuperscript{-1} is an in-plane stretching Si-O vibration attributed to Si-O-H groups, while 1104 and 1206 cm\textsuperscript{-1} are asymmetric stretching Si-O-Si vibrations. Several peaks are convoluted within the 3400 cm\textsuperscript{-1} O-H region and are attributed to Si-OH as well as hydrogen bonded water. The peak at 1635 cm\textsuperscript{-1} is due to residual water, and 1668 cm\textsuperscript{-1} is due to amide C=O from residual arginine. Figure 4.16 shows the DRIFTS spectrum of identically treated commercial silica nanoparticles. The same peaks are observed except for the absence of the 1668 cm\textsuperscript{-1} arginine C=O peak, and the appearance of a sharp 3747 cm\textsuperscript{-1} silanol peak. Figure 4.17 shows DRIFTS spectrum of identically prepared microemulsion QD/silica particles. Again, similar peaks are observed, minus 1668 cm\textsuperscript{-1} arginine C=O and minus the 3747 cm\textsuperscript{-1} silanol peak seen in the commercial particles. We note a difference in the intensity of the \(~960\) cm\textsuperscript{-1} silanol peak in comparison to the framework peaks. When we normalize these peaks to the 800 cm\textsuperscript{-1} Si-O stretch of similar intensity, we find that the \(~960\) cm\textsuperscript{-1} peak is 0.60 for commercial silica, 0.73 for arginine QD/silica, and 1.03 for microemulsion QD/silica, indicating a greater proportion of silanol compared to framework silica in the microemulsion QD/silica than either the commercial or arginine QD/silica.

**Surface Area of QD/Silica:** Continuing to investigate the differences between the microemulsion-silica coated QDs and the arginine-silica coated QDs, BET surface area measurements were taken for both. The microemulsion-silica particles had a surface area of 164 m\textsuperscript{2}/g while the arginine-silica particles had a surface area of 160 m\textsuperscript{2}/g.
Optical Properties

Figure 4.19 shows a comparison of the UV/Vis absorption and fluorescence spectra of rhodamine 6G, rhodamine 800, and QDs before and after silica coating. The UV/Vis absorption spectra (a, c, e) appear similar before and after coating, with more significant background after silica coating. This is due to the larger particles, which scatter light more intensely as wavelength decreases. Fluorescence spectra (b, d, f) show some shifting of the maximum emission wavelength. Rhodamine 6G (a, b) main absorption peak remained at 525 nm before and after coating, but the appearance of the H-dimer peak is noted in the coated sample. This peak overlaps the typical vibronic shoulder of monomeric rhodamine 6G seen in the free-dye spectrum.\textsuperscript{37} but has been reported previously depending on both concentration of dye, and, in the case of silica coating, on the density or porosity of the silica matrix. The fluorescence emission maximum blue-shifted from 552 nm to 543 nm. Rhodamine 800 (c, d) had two main absorption peaks at 686 and 627 nm for the uncoated dye. After silica coating, these peaks red-shifted to 697 and 635 nm, and the 635 nm peak became the dominant peak. This behavior has been reported in the literature and is again due to the formation of aggregate dimers.\textsuperscript{38} The fluorescence peak of rhodamine 800 broadened and red-shifted from 711 nm uncoated to 716 nm coated. QD (e, f) absorption shows more scattering noise after coating, but does not change otherwise. QD fluorescence did not significantly change after coating, with an emission maximum at 578 nm before and 576 nm after coating.
TCSPC fluorescence lifetimes were measured before and after silica coating for rhodamine 6G and QDs, and this data is shown in Figure 4.20. Rhodamine 800 was not sufficiently excited by the available LED light sources to be measured. Figure 4.20a shows rhodamine 6G decay curve, which fit well to a single exponential with a lifetime of 4.1 ns in agreement with literature values. Fluorescence lifetime decreased after adding the silica shell (Figure 4.20b). There were two lifetime components: 1.0 ns with 36% relative amplitude and 3.5 ns with 64% relative amplitude. QD samples also showed a decrease in fluorescence lifetime after silica coating. Figure 4.20c shows the pre coated QD data, which required a 3-exponential fit giving lifetimes of 3.3 ns (31.1% relative amplitude), 16 ns (53.5%), and 110 ns (15.4%). The 16 ns majority contribution is within the range of lifetimes typically reported in literature. The 3.3 ns contribution is possibly arising from QD surface traps. We assign the longer lifetime component at 110 ns to an impurity, since its contribution decreases as we dialyze the QD samples. We could not get rid of it completely since with dialysis, the MPA capping ligand is also lost and the QDs become unstable. Figure 4.20d shows QDs after silica coating. A 3 exponential fit was still required to obtain a quality fit, but all three lifetimes decreased in length. The main contributing time shifted from 16.1 to 12.3 ns, and its relative amplitude remained similar at 54.3%. The short-lifetime trap-state contribution decreased from 3.3 to 2.5 ns, and also decreased in relative amplitude from 31.1 to 18.6%. The impurity lifetime decreased significantly from 110 ns to 54.5 ns, and increased in relative amplitude from 15.4 to 27.1%.
IV. Biological Studies

**Macrophages:** The mouse macrophage cell line MH-S was used to readily observe internalization of QD/silica and rhodamine 6G/silica. These cells were chosen as an initial data-point due to their phagocytic nature to internalize foreign material. After 24-hour treatment, QD/silica particles were observed in almost all macrophage cells surrounding the nucleus but not within the nucleus (Figure 4.21a; nuclei are visible in blue due to DAPI staining, QD signal is green). Rhodamine 6G/silica was also internalized well by the macrophages and the gray differential interference contrast (DIC) image which is a contrast microscopy image that outlines cell features confirms that the silica particle signal is within the macrophages (Figure 4.21b).

**C2BBel Cells:** C2BBel cells are an intestinal epithelial cell line originally cloned from the human colon cancer cell line Caco-2. These cells are widely used as an *in vitro* model for normal intestinal epithelium. Since silica nanoparticles used in foods will traverse the GI tract and come into contact with intestinal epithelial cells, these cells are a good model to use to evaluate the interaction with fluorescent silica nanoparticles. Cells were treated for 24 hours with a 100 µg/cm² dose of rhodamine 6G/silica and then fixed and stained for confocal microscopy with DAPI (nuclei, blue) and for E-cadherin (junctions between cells, red). The silica-NP fluorescent signal is in green. For both QD/silica and rhodamine 6G/silica treated cells, silica particles were internalized by cells and are visible in the cytoplasm of cells surrounding the nucleus (Figure 4.22). The particles never appeared to localize within cell nuclei. While the same dose by weight
was used for the QD/silica and rhodamine 6G/silica particles, the rhodamine 6G fluorescent signal was much brighter in cells than the QD fluorescence.

**Mice GI Tract:** In order to evaluate the fate of silica nanoparticles that are consumed in foods, *in vivo* models provide more information that is possible with *in vitro* models. Thus, an experiment was conducted using an *in vivo* model where rhodamine 6G/silica and rhodamine 800/silica were administered orally to mice. Since rhodamine 800 signal cannot be detected by our confocal fluorescence microscopy instrument and was included to detect nanoparticle distribution using non-invasive imaging of live mice, it is assumed that all signal detected by confocal microscopy is due to the rhodamine 6G/silica and we focus on these particles. To observe nanoparticle distribution within the GI tract and throughout the body, nanoparticles were administered daily for four days with euthanasia three hours after the final administration. Tissue was frozen and then sectioned and fixed for confocal microscopy. Tissue sections were stained with DAPI to identify cell nuclei (blue) and for E-cadherin to indicate junctions between epithelial cells (red) in addition to analyzing the rhodamine 6G/silica (green). The stomach of the mouse administered particles over the four hours prior to euthanasia was full of rhodamine 6G/silica (Figure 4.23a). These particles appeared to be contained in the stomach contents but generally not internalized by the cells lining the stomach. Rhodamine 6G/silica was also present in an intestinal section of a mouse treated daily for four days and euthanized four hours after the final treatment (Figure 4.23b). Similarly, rhodamine 6G/silica was readily visible in cecum (Figure 4.23c) and colon (Figure 4.23d) sections in a mouse treated over four days with silica-Rhodamine 6G. The rhodamine 6G/silica particles
visible in these sections are often near the edge of the lumen of the GI tract, indicating that they may be trapped in the mucus layer that protects the epithelial cell lining.

**Mice Non-GI Tract:** It is expected to find orally-administered particles throughout the GI tract as they pass through the digestive system, but any particles outside of the GI tract must have traversed the epithelial cell lining to gain access to the rest of the body. When non-GI organs of mice administered rhodamine 6G/silica daily for four days were analyzed by confocal microscopy, rhodamine 6G signal was found in kidney, lung, brain, and spleen (Figure 4.24). These particles are within cellular regions of the tissues and may have been internalized by cells. When colocalization of nanoparticle signal with DAPI looks possible, it is clear that the majority of the silica nanoparticle signal is in a slightly different focal plane than the nuclei and thus there do not seem to be nanoparticles within the nuclei of cells. Signal from the silica-nanoparticles is much less common throughout these non-GI tissues than in the GI tract.

4.4. Discussion

*Dye Encapsulation*

**Ammonia Based Synthesis:** For both dyes, the Stober method resulted in particles with significant dye leaching when incubated in PBS. Figure 4.1 shows the structures of the rhodamine 6G and rhodamine 800 dyes; both are similar and we conclude they should be interchangeable in these reactions considering the particle-dye association is
electrostatic. Figure 4.2a shows the reaction schemes for dye encapsulation with the Stober method, in both cases the dyes electrostatically bind to commercial silica particles which are then encapsulated in a TEOS and ammonia Stober synthesis. We did not observe any fluorescence in the Stober rhodamine 800/silica particles. Figure 4.4 shows the initial fluorescence spectrum of the Stober rhodamine 6G/silica particles, indicating presence of dye, however, as demonstrated in Figure 4.5, the dye does not remain in these particles. We observe steady decrease in fluorescence intensity over the course of 8 days from near 200,000 counts to approximately 70,000 counts. Figure 4.5b shows that fluorescence intensity in the supernatant removed after a washing step was nearly 150,000 counts, in the same range as that seen in the particle solution.

As mentioned previously, the surface characteristics, including size surface charge, of particles determine their behavior in biological systems.4–10 Particles which leach dye are not suitable for biological studies. Fluorescence signal could be of free-dye rather than the particles intended to be studied. Additionally, dye particles could be associated with the particle surface, changing the surface charge. The literature particles on which we based this synthesis did not show any dye leakage within 10 days after synthesis.31 Particles in the literature synthesis were on the order of 200 nm, while the particles synthesized in this study were approximately 80 nm. The porosity and dissolution behavior of Stober and reverse microemulsion silica have been found to be dependent on size: in a recent literature report, particles of 21.4 nm initially developed internal pores and eventually interconnected into large silica networks, 48.7 nm particles developed pores and became hollow mesoporous particles, while 123.1 nm particles
showed some development of pores but no hollowing. The authors attributed this to the less dense and more porous nature of the smaller particles. Another report indicates that the overall concentration of silanol groups increases as nanoparticle size decreases (from 0.30 mmol OH/g silica for 130 nm particles to 3.56 mmol OH/g silica for 7 nm particles), giving smaller particles greater reactivity. With this in mind, we note that we did find evidence of greater proportion of silanol to framework silica in the DRIFTS data, which is discussed in more detail below. Based on these reports, we attribute the instability of the Stober silica coating in our smaller particles compared to those reported by Rezwan to the instability towards dissolution of the smaller particles.

**Arginine Based Synthesis:** The arginine method gave particles with much improved fluorescence properties. Figure 4.8 shows strong fluorescence of arginine rhodamine 6G/silica in the initially synthesized particles, as well as a lack of dye leaching after one week in PBS solution. Unlike the Stober method, fluorescence of the arginine rhodamine 800/silica particles was observable, and like the rhodamine 6G sample did not show evidence of dye leakage after one week in PBS solution, illustrated in Figure 4.9. In Dawson’s synthesis, on which our arginine method is based, this lack of dye leaching is attributed to a more dense and stable silica shell. Dawson noted a similar core dissolution of Stober synthesized silica as discussed above, but did not see this in similarly sized arginine synthesized silica particles. This was attributed to slower reaction kinetics allowing for complete hydrolysis of TEOS in silica monomers before addition to larger core particles. Our stability of dye within particles, seen in Figure 4.8b and Figure 4.9b, is in agreement with these findings. We find that, for rhodamine 6G and rhodamine
800, electrostatic binding of dye to core-silica is sufficient, avoiding the dye-silica conjugation utilized in Dawson’s covalently-bound synthesis.

Quantum Dot Encapsulation

**Reverse Microemulsion Synthesis:** Reverse microemulsion synthesis lead to coated 37 nm particles containing 0-4 QDs per particle, shown in the TEM image in Figure 4.7. These particles did not exhibit stable fluorescence. Figure 4.6 shows complete quenching of fluorescence within 3 days. It is likely that the silica shell grown by this method is porous and has allowed a quenching species access to the particle. As discussed for the Stober dye/silica particles, literature reports indicate that Stober and microemulsion synthesized silica particles less than 100 nm are less stable in solution, showing increased dissolution, including dissolution within the inner structure of the particles.\(^{17,18,42}\) This has been exploited in literature: microemulsion-silica shells were grown around QDs and used in the detection of hydrogen peroxide, as the porous shell allowed the hydrogen peroxide molecules to bind to the QD and quench fluorescence.\(^{34}\) Micropores in a similar QD/Silica microemulsion synthesis were determined to range from 0.58 nm to 0.91 nm and increased upon dialysis in aqueous solution.\(^{32}\)

This increased porosity has been suggested to be due to incomplete formation of silica framework, as reported by Galembeck.\(^{43}\) 15 and 33 nm silica particles were found to contain carbon (un-hydrolyzed TEOS) throughout their structure, whereas larger particles (115 nm) only had carbon associated with the particle surface. This is explained
in the context of silica nanoparticle growth, which is described as formation of seed nuclei followed by their aggregation into larger particles. Depending on the reaction kinetics, some portion of the seed nuclei may not be completely hydrolyzed and condensed. These incompletely formed sections within the core may give rise to more reactive sites and ultimately the dissolution of the particles, as described by Dove and De Yoreo. Silicon atoms within the silica framework can be of 4 different coordinations, depending on the number of bridging oxygen atoms they are bound to. These are known as Q1, Q2, Q3, and Q4, and are of increasing stability. Q2 sites (silica bound to two framework oxygens) will undergo hydrolysis until a surface rich in Q3 sites (silica bound to three framework oxygens) is formed:

\[
\text{SiO}_2 + 2\text{H}_2\text{O} \rightarrow \text{H}_4\text{SiO}_4
\]

An abundance of Q2 sites within the silica framework will therefore result in etching, and a more porous particle. Furthermore, the presence of alkalai cations was found in the same paper to dramatically increase the rate (50x) at which Q3 sites are lost from silica framework, resulting in the formation of unstable Q2 sites which are again etched away until a new Q3-rich surface is formed.

**Arginine Based Synthesis:** The arginine based synthesis resulted in 29 nm silica particles with 0-5 encapsulated QDs. TEM images (example shown in Figure 4.18c) indicate that QDs are fully coated with silica. Figure 4.10 shows the fluorescence for these particles after synthesis and after one week in PBS solution. No change in fluorescence is noted: the particles had a quantum yield of 25% at both time points. Due
to the stability of fluorescence, we conclude that the arginine silica synthesis resulted in more robust coating, as seen in the dye/silica particles.

To further investigate the differences between microemulsion and arginine synthesized QD/silica particles, the surface area and infrared spectra were observed: Microemulsion QD/silica particles had a surface area of 164 m²/g, while the arginine QD/silica particles had a surface area of 160 m²/g. A simple sphere volume and surface area calculation for these sizes of silica particles estimates that the surface areas should be 61 m²/g for the microemulsion method and 78 m²/g for arginine. Both particles have a higher surface area than predicted, indicating a porous or irregular surface. This suggests that the porosity of the particles alone is not responsible for the quenching of fluorescence of the microemulsion QD/silica particles.

DRIFTS spectra are shown in Figure 4.15 for arginine QD/silica, Figure 4.16 for commercial silica, and Figure 4.17 for microemulsion QD/silica particles along with their peak assignments. Of particular interest is the 961-980 cm⁻¹ peak assigned to Si-O stretch of silanol Si-OH groups. When we normalize the silanol peaks to the similar-intensity 800-812 cm⁻¹ Si-O framework peak, we find the intensity of the silanol peak to be 0.60 for commercial silica, 0.73 for arginine QD/silica, and 1.03 for microemulsion QD/silica. It is not clear from this data if the silanol groups are due to Q2 (which would have two OH per site) or Q3 (which would have one OH per site). As discussed previously, the presence of silanol groups on the surface of silica particles increases their reactivity. This increased reactivity may stem from an increased number of Q2 sites. We determine that the increased signal of the silanol peak is indicative of a greater number of
Q2 sites in the silica shell framework. This gives way to higher dissolution and porosity, leading to the quenching behavior we observed in Figure 4.6.

Optimization

**Dye and TEOS Concentrations:** Choosing the arginine dye/silica synthesis due to its stability, the concentration of dyes and TEOS used in the synthesis was optimized. These experiments are shown in Figure 4.11. Increasing the rhodamine 800 dye concentration up to $1.32 \times 10^{-3}$ M gave the highest fluorescence intensity. $1.36 \times 10^{-3}$ M was the ideal concentration of rhodamine 6G, as going above this concentration resulted in silica cores aggregating significantly upon addition of arginine to the reaction solution. Above this amount, the dye exhibited self-quenching and fluorescence intensity decreased. Using the fluorescence intensity of rhodamine 800 encapsulated silica, we determined an ideal TEOS concentration of $8.78 \times 10^{-5}$ M. Above and below this concentration, the fluorescence intensity decreased. We noted in subsequent DLS size measurements that the particle size did increase with increasing TEOS concentration: from 192 nm to 224 nm. At lower concentration of TEOS, we suggest that the silica shell layer is not thick enough to encapsulate and hold the dye molecules. It is not immediately clear why the thicker silica shell results in a decrease of fluorescence intensity.

**QD/Silica Synthesis: Influence of Arginine:** In Nann’s work, on which we base our microemulsion synthesis, the amount of ammonia used was found to influence the polydispersity and QD distribution of final particles. With increasing ammonia, the
reaction was more rapid, and the particles obtained were monodisperse and found to primarily contain one QD per particle. Because the arginine reaction involves a more dense, slowly forming shell, we varied the arginine concentration to determine if changing this would result in evenly distributed particles. With arginine increasing to $5.63 \times 10^{-3}$, and $1.13 \times 10^{-2}$ M, only large aggregates were observed (Figure 4.13c and d). When arginine concentration was decreased to $9.38 \times 10^{-4}$ M, particles did show improved morphology (Figure 4.13a), but these did not have stable fluorescence. The results of these studies are summarized in Table 4.1. The $2.81 \times 10^{-3}$ M arginine sample showed the highest quantum yield (25% vs 7-8%), and was the particle chosen for further biological studies.

Figure 4.12 shows the evolution of particle quantum yields over 48 hours during the arginine QD/silica coating using $2.81 \times 10^{-3}$ M arginine. We observed that the quantum yield of the arginine QD/silica particles decreased in the first hour of the reaction from 40% to 34%. From this point, the quantum yield increased up to 56% after 10 hours. The quantum yield decreases from this point through the remainder of the reaction, ending at 36% after 48 hours (further decreasing to 25% after washing).

Similar observations of increasing QY with silica coating have been made in the literature. CdTe QDs with an initial QY of 8-10% increased to 15% upon silanization. This is explained by the silica shell passivating the quantum dots much like in a typical core/shell QD synthesis. Multiple shell layers have been shown in literature to improve quantum yields and particle stability; CdSe particles with a complex 7-layer shell structure had a QY of 47.8% before and after microemulsion coating with silica, while
particles with simpler shell structures were quenched within 24 hours. A sample removed 20 hours into the reaction was set aside to observe, and was found to not have stable fluorescence, decreasing to 3% within 3 days after the reaction. It is likely that there is some time between 20 hours and 48 hours which gives a higher quantum yield and remains stable, but due to the sharpness of QY drop-off, this was not investigated further.

Characterization of Final Particles

Zeta Potential: Zeta potential vs. pH titrations shown in Figure 4.14 confirm that surface properties are not influenced by the encapsulated QDs or dyes. This is important because the behavior and fate of these particles in biological systems is known to be influenced by the surface charge. Our group has previously published results showing that negatively charged QDs quickly associated with cell surface scavenger receptors while the same QDs ligand-exchanged to be positively charged QDs did not. Any species other than silica present on the surface of the particles would shift the titration plot, particularly in the case of the positively charged rhodamine dyes. Literature value for IEP of silica is approximately pH 2, in agreement with our measurements of 1.91 for commercial silica, 1.70 for rhodamine 800/silica, and 1.34 for rhodamine 6G/silica. The QD/silica particles did not appear to cross the IEP.

TEM: Both dye/silica particles appeared similar, shown in Figure 4.18 a and b for rhodamine 6G/silica and rhodamine 800/silica respectively. Rhodamine 6G/silica
particles were 28 ± 11 nm and rhodamine 800/silica particles were 32 ± 15 nm. These particles ranged in size from 11 to 67 nm. QD/silica particles shown in Figure 4.18c were 29 ± 6 nm, ranging from 15 to 47 nm. QD/silica particles contained 0-5 QDs per particle, and efforts to make homogeneous single QD particles resulted in unstable fluorescence quantum yield. Single QD silica particles have been reported in the literature by Nann, and these were the basis of our microemulsion synthesis. However, as shown in Figure 4.6 and Figure 4.7, our QDs in this synthesis did not result in single-QD silica particles, and fluorescence was quenched. The range of these particles’ sizes was also wider than that of the arginine method; from 19 to 62 nm.

**Optical Spectra:** UV/Vis absorption and fluorescence spectra for all fluorophores before and after silica coating are shown in Figure 4.19. For all three fluorophores, the addition of a silica shell resulted in increased scattering interference, seen as the baseline increasing towards lower wavelength. This increase is also seen in the plain QDs and is characteristic of semiconducting materials. We note the appearance of dimer peaks in the UV-Vis spectra of both rhodamine 6G and rhodamine 800 (a and c). These peaks have been noted in literature, typically seen with increasing concentration. The rhodamine 6G H-dimer has been reported to form more readily in dense silica compared to more porous silica, and is non-fluorescent, accounting for the decrease in quantum efficiency we see after coating (20% coated vs 90% uncoated). While the appearance of these dimers is typically accompanied by a red-shift in the emission maximum wavelength, we note a red-shift for rhodamine 800 (711 nm to 716 nm), but a blue-shift for rhodamine 6G (552 nm to 543 nm). Blue-shift of rhodamine family dyes has been reported previously,
such as in the case of rhodamine B,\textsuperscript{45} but this is associated with a molecular change to its lactone form. In our case, the blue shift may be due to the propensity for rhodamine 6G to form H-dimers instead of J-dimers in more dense silica networks along with the fact that not all dimers will be perfectly aligned,\textsuperscript{37} allowing for a large enough population of weakly fluorescent H-dimers to cause an overall blue-shift in the emission spectrum. QD absorption and fluorescence was not significantly affected by the silica coating, shifting from 578 to 576 nm in the emission (part g).

Figure 4.20 shows the TCSPC lifetime measurements of rhodamine 6G and QDs with and without silica coatings (rhodamine 800 was not excited by our LED light source for this instrument and could not be measured). Rhodamine 6G lifetime of 4.1 ns agreed closely with a literature value of 4.08 ns\textsuperscript{29} while QDs majority contribution of 16 ns fell within the range typically reported in literature.\textsuperscript{39–41} After silica coating, the lifetime of rhodamine 6G decreased and required a 2-exponential fit: 3.5 ns and 1.0 ns. An early report on the fluorescence lifetime of rhodamine 6G bound to a silica surface also reported a 3.5 ns lifetime, adding that at higher levels of surface coverage they observed two distinct components with lifetimes of < 1 ns and > 2.5 ns.\textsuperscript{46} QD/silica particles also showed decreased lifetimes, with the major contributing lifetime decreasing from 16.1 ns uncoated to 12.3 ns after silica coating. Decrease in lifetime of QDs is generally attributed to an increase in non-radiative decay, due either to particle aggregation (leading to self-quenching) or surface modification.\textsuperscript{47,48} Both of these phenomena are present in our particles. Multiple QDs in each silica particle (Figure 4.18c) may be close
enough to quench each other, and the evolution of quantum yield during silica coating (Figure 4.12) indicates silica formation around the QD impacts exciton recombination.

Comparison to Literature Particles

**Dye/Silica:** Production of fluorescent dye-modified silica is a well-developed field. A 2012 review article by Bae, Tan, and Hong in Chemical Communications\(^49\) provides an overview of the current state of this research area. A large number of dyes have been conjugated to silica nanoparticles, primarily by covalent linkage. These particles have been used extensively in biological research, including in-vivo imagine in mice. Many of these studies unfortunately do not report quantum yield values which would allow for direct comparison to other particles. There is also a lack of information on the stability of the particles and their ability to retain dyes. Dawson reported dye leakage of covalently-linked FITC from Stober synthesized particles, due to the dissolution of the silica.\(^18\) This was remedied by utilizing arginine in place of ammonia. Rezwan published silica/rhodamine 6G/silica particles in which the dye was only electrostatically bound.\(^31\) These particles were stable for at least 10 days and showed no evidence of leakage.

In our attempts to replicate Rezwan’s Stober dye/silica with smaller core particles, we noted significant dye leakage, and as discussed previously this is attributed to greater reactivity of small silica nanoparticles. By adapting Dawson’s arginine method to this, we were able to produce small (<100 nm) silica nanoparticles with stable fluorescence which
did not require a covalent linkage step. Because our synthesis only relied on the positive
charge of the dye electrostatically attaching it to the silica cores, this could be generalized
to other dyes with positive charge, and we show that this synthesis is also successful
using the near-IR rhodamine 800 dye.

Quantum Dot/Silica: There are many reports of silica coated QDs. These are
summarized in Table 4.3. A 2001 paper by Alivisatos\textsuperscript{50} reports TOPO capped CdSe/ZnS
QDs silanized and solvent exchanged into water with QYs range from 5\% for 620 nm
emitting particles to 18\% for 544 nm emitting particles and stable fluorescence up to one
month. Nann’s microemulsion synthesis,\textsuperscript{14} which we base our microemulsion synthesis
on, reports particles with an even distribution of 1 QD per particle. No QY was reported
however. A subsequent paper utilized these same particles for the detection of glucose by
allowing peroxide access to QD surface via porous structure,\textsuperscript{34} indicating a propensity for
these particles to become quenched under certain conditions. Chang reported in 2008\textsuperscript{35}
that CdSe QDs with a complex 7-layer shell with a QY of 47.8\% were not affected by
addition of a microemulsion-synthesized silica shell, while the same particles with a less
complex shell were quenched. These were the highest QY QD/silica particles we noted in
literature. Xu\textsuperscript{32} reported uncapped CdSe with a poor quantum yield of 0.4\% were
increased to 1.4\% after a microemulsion addition of silica shell, but fluorescence was lost
over a period of 7 days. Rao\textsuperscript{33} reports particles based on Alivisatos’ method. These also
showed an increase in QY over time, from 10\% to 15\%, and were able to image these
particles in vivo in a mouse. Near IR CdHgTe/Silica particles with a QY of 16\% were
reported by Chen and Cui\textsuperscript{51} which were studied in an in vivo model and found to localize
within the liver and lungs. Chaniotakis has published the only biosilification of semiconductor QDs that we have found.\textsuperscript{52} CdSe/ZnS QDs conjugated to acetylcholinesterase were embedded in a poly-L lysine matrix and exposed to silicic acid formed by hydrolysis of TMOS with HCl, resulting in 64 nm sized silica particles with several QDs inside. These particles showed stable fluorescence in solution, but had a porous nature which allowed for use in detection of acetylcholinesterase activity. We note that, in contrast to the slow kinetics of Dawson’s dye biosilification (48 hours at 70°C), these particles were fully synthesized within 4 hours at room temperature.

We present a simple synthetic method, requiring no ligand exchange or complex shell formation, with a strong QY of 25%. This is higher than all mentioned here other than Chang’s 47.8% which required an extensive shell before particles were not quenched. We demonstrate that the arginine based silica synthesis is more robust, producing stable particles without a need for more complicated shell structures.

\textit{Biological Studies}

We have shown that our particles are internalized by both MS-H macrophages (Figure 4.21) and the intestinal epithelial cell line C2BBe1 (Figure 4.22). We chose this cell line because of its relevance to nanoparticle ingestion. Internalization into C2BBe1 cells indicates that particles can translocate out of the intestines, from which they may localize in other organs. Following this evidence, the particles were injected into the guts of mice via gavage to study the potential absorption of particles, and we noted presence
of particles in several tissues both in and out of the digestive system. In the digestive system we observed particles in the stomach, small intestines, cecum, and colon (Figure 4.23). We also observed particles in the kidney, lung, liver, brain, and spleen (Figure 4.24), indicating that these particles do absorb into the bloodstream and subsequently localize within several organs. We were not able to discern silica fluorescence in the liver with rhodamine 6G and rhodamine 800. This is attributed to autofluorescence of a greater number of blood cells present in the liver. We also noted in the in vitro cell studies that the rhodamine 6G/silica particles were more easily imaged in cells than QD/silica particles. This is contrary to what was expected from the quantum yields of these particles (20% for rhodamine 6G/silica and 25% for QD/silica).

We have noted studies tracking fluorescent silica particles in vivo following direct injection into the blood stream via the tail vein, but this would not be relevant to food safety if particles simply pass through the digestive system when ingested. We have shown here that particles leave the digestive system, indicating a need for further study of their bioaccumulation and potential toxicity via this route. Dekkers’ 2011 review article notes that nanosilica is present even in materials considered to be bulk silica, and oral exposure in mice of 1500 mg silica nanoparticles per kg body weight per day for 10 weeks resulted in liver toxicity, in agreement with our findings that particles escape the digestive system and localize within the liver among other organs. Bouwmeester found that commercially obtained food products contained nanosilica which aggregated in the gut of mice, and then deagglomerates once leaving the gut, indicating that gut epithelial cells are likely exposed to nanosilica when these commercial products are ingested.
previous study in our lab\textsuperscript{54} involving a simulated digestion mirrors these results, showing particle aggregation in stomach conditions (pepsin solution at pH 2) which then deagglomerated in intestinal conditions (neutral solutions of pancreatin and bile salts).

4.5. Conclusion

We show here that an arginine based silica shell synthesis is ideal for encapsulation of quantum dots and rhodamine-family dyes. Particles were strongly fluorescent and showed no dye leaching or fluorescence quenching within one week of exposure to biological conditions. Rhodamine 6G and QD silica particles had quantum yields of 20 and 25\%, respectively, and were both easily imaged in macrophage cells. Zeta potential titrations and IR spectroscopy both indicate that these particles are suitable for studying biological fate of similar sized silica particles. Particles were detected via fluorescence in the several organs of mice after oral exposure, indicating that some population of the silica nanoparticles escapes the digestive system following ingestion.

4.6. Future Directions

Having shown that silica particles clearly escape the digestive system and localize throughout the body, there are several additional directions to continue this research. For in vitro experiments, the rate of uptake of the silica particles into C2BBe1 or other relevant cell lines can now be studied using these fluorescently labeled particles. For in
vivo experiments, we can now determine the persistence or clearance rate of the particles in various tissues. The toxicity of the silica nanoparticles to the tissues that they accumulate in should also be examined. In addition to this, we showed in Chapter 2 that titania also survived our digestion treatment, and would internalize within C2BBe1 cells. Fluorescently functionalized titania particles should allow for similar studies to be done as we have shown with silica here.
4.7. References


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Figure 4.1. CdSe/CdS/ZnS QD, Rhodamine 6G, and Rhodamine 800 structures. Charged sites at neutral pH are indicated on Rhodamine 6G and Rhodamine 800.
Figure 4.2. Reaction scheme of ammonia-driven silica coating for a) dye/silica particles utilizing a Stober synthesis, and b) QD/silica particles utilizing a reverse microemulsion synthesis.
Figure 4.3. Reaction scheme of arginine-driven silica coating for dye/silica and QD/silica particles
Figure 4.4. Fluorescence Spectrum of Silica/Rhodamine 6G/Silica Particles Synthesized by Ammonia-Driven Synthesis. $\lambda_{ex} = 480$ nm
Figure 4.5. Dye leaching of rhodamine 6g from ammonia-drive r6g/silica. a) Change in fluorescence intensity at 4, 6, and 8 days after synthesis in PBS media. b) Fluorescence spectrum of PBS supernatant after centrifuging particles, indicating significant dye leakage from particles into the solution. $\lambda_{\text{ex}} = 480$ nm
Figure 4.6. Fluorescence quenching of silica coated QDs prepared by reverse microemulsion after 0, 1, and 3 days in PBS solution. $\lambda_{ex} = 375$ nm
Figure 4.7. TEM image of Ammonia-Driven QD/Silica particles indicating particle entrapment. Particle size $37 \pm 9$ nm ($n=51$). Scale bar represents 20 nm.
Figure 4.8. Arginine-Driven Rhodamine 6G/silica particles a) after initial washing, and b) after one week incubation in PBS solution. $\lambda_{ex} = 480$ nm
Figure 4.9. Rhodamine 800/silica particles a) after initial washing, and b) after one week incubation in PBS solution. Spectrum of supernatant in b) indicates no dye leakage. $\lambda_{ex} = 635$ nm
Figure 4.10. Fluorescence quenching of silica coated QDs prepared by arginine silica synthesis after 0 and 7 days in PBS. No quenching is observed. $\lambda_{ex} = 375$ nm
Figure 4.11. Dye and TEOS concentration optimization for rhodamine 800/silica particles. These particles were not sufficiently luminescent for quantum yield analysis in Quanta-Phi system. As-synthesized samples were observed with 10:1 dilution. $\lambda_{ex} = 635$ nm
Figure 4.12. Quantum yields of aliquots removed during arginine-driven QD/silica synthesis. $\lambda_{ex} = 480$ nm
Figure 4.13. Representative TEM images of arginine QD/silica particles synthesized for 48 hours with a) $9.38 \times 10^{-4}$ M, b) $2.81 \times 10^{-3}$ M, c) $5.63 \times 10^{-3}$ M, and d) $1.13 \times 10^{-2}$ M arginine. Primary particle sizes measured to be a) $22 \pm 3$ nm, b) $29 \pm 6$ nm, c) $29 \pm 5$ nm, and d) $31 \pm 8$ nm.
Figure 4.14. Zeta potential titration of silica cores, R6G/Silica, R800/Silica, and QD/Silica. Similar values for all three indicate that dye particles are completely encapsulated within silica shell. IEP values were 1.91 for commercial silica, 1.34 for R6G/silica, and 1.70 for R800/silica. QD/silica did not cross the IEP.
Figure 4.15. DRIFTS spectrum of arginine QD/silica particles. Peaks are assigned to silica structure, with additional signal from residual water and arginine.

Peaks (cm$^{-1}$):
- 800 Si-O
- 963 Si-O
- 1104 Si-O-Si (TO mode)
- 1206 Si-O-Si (LO mode)
- 1635 H-O-H (residual water)
- 1668 C=O (residual arginine)
- ~3400 O-H
Figure 4.16. DRIFTS spectrum of commercial silica particles. Peaks are assigned to silica structure, with additional signal from residual water.

Peaks (cm$^{-1}$):
812    Si-O
980    Si-O
1102   Si-O-Si (TO mode)
1204   Si-O-Si (LO mode)
1632   H-O-H (residual water)
$\sim$3400 O-H
3747   Silanol
Figure 4.17. DRIFTS spectrum of Stober QD/silica particles. Peaks are assigned to silica structure, with additional signal from residual water.
Figure 4.18. TEM micrographs of a) Rhodamine 6G/Silica, particle size 28 ± 11 nm (n=55) b) Rhodamine 800/Silica, particle size 32 ± 15 (n= 54) nm c) QD/Silica particles, particle size 29 ± 6 nm (n=50)
Figure 4.19. Optical Spectra. UV/Vis absorption spectra of a) rhodamine 6G, c) rhodamine 800, and e)QDs before and after silica coating. Fluorescence spectra of b) rhodamine 6G $\lambda_{ex} = 480$ d), rhodamine 800 $\lambda_{ex} = 635$ and f) QDs $\lambda_{ex} = 375$ before and after silica coating.
Figure 4.20. TCSPC Fluorescence Lifetime Spectra of a) Rhodamine 6G dye, b) Rhodamine 6G/silica particles, c) CdSe/CdS/ZnS Quantum Dots, d) Silica coated quantum dots. 455 nm pulsed LED light source, 1 MHz repetition rate.
Figure 4.21. MH-S macrophage cells were grown to confluence in an 8-chamber slide and treated for 24 hours with a 100 µg/cm² dose of a) QD/silica and b) rhodamine 6G/silica particles. Cells were fixed in 4% paraformaldehyde and stained with DAPI (blue; cell nuclei) and E-cadherin (red; junctions between cells) and analyzed by confocal fluorescence microscopy. Both particles are shown in green.
Figure 4.22. The intestinal epithelial cells C2BBe1 were grown to confluence in an 8-chamber slide and treated for 24 hours with a 100 µg/cm² dose of a) QD/silica and b) rhodamine 6G/silica particles. Cells were fixed in 4% paraformaldehyde and stained with DAPI (blue; cell nuclei) and E-cadherin (red; junctions between cells) and analyzed by confocal fluorescence microscopy. Both particles are shown in green.
Figure 4.23. Mice were administered optimized rhodamine 6G/silica particles orally every 24 hours for four days and organs were harvested three hours after the final administration. Confocal microscopy of frozen tissue sections are shown: a) stomach, b) intestine, c) cecum, and d) colon. Sections were stained with DAPI (cell nuclei; blue) and E-cadherin (junctions between epithelial cells; red). Rhodamine 6G/silica particles are green.
Figure 4.24. Mice were administered optimized rhodamine 6G/silica particles orally every 24 hours for four days and organs were harvested three hours after the final administration. Confocal microscopy of frozen tissue sections are shown: a) kidney, b) lung, c) liver, d) brain, and e) spleen. Sections were stained with DAPI (cell nuclei; blue) and E-cadherin (junctions between epithelial cells; red). Rhodamine 6G/silica particles are green.
Table 4.1. Quantum yields and average size measured by TEM of QD/silica particles synthesized using varying amounts of arginine.

<table>
<thead>
<tr>
<th>Arginine used (M)</th>
<th>Quantum Yield</th>
<th>Average Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$9.38 \times 10^{-4}$</td>
<td>8%</td>
<td>$22 \pm 3$</td>
</tr>
<tr>
<td>$2.81 \times 10^{-3}$</td>
<td>25%</td>
<td>$29 \pm 6$</td>
</tr>
<tr>
<td>$5.63 \times 10^{-3}$</td>
<td>7%</td>
<td>$29 \pm 5$</td>
</tr>
<tr>
<td>$1.13 \times 10^{-2}$</td>
<td>7%</td>
<td>$31 \pm 8$</td>
</tr>
</tbody>
</table>
Table 4.2. Summary of Final Particle Characteristics

<table>
<thead>
<tr>
<th>Particle</th>
<th>Average Size (nm)</th>
<th>IEP (pH)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>Quantum Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>R800/Silica</td>
<td>76 ± 19</td>
<td>1.70</td>
<td>716</td>
<td>--</td>
</tr>
<tr>
<td>R6G/Silica</td>
<td>78 ± 15</td>
<td>1.34</td>
<td>543</td>
<td>20%</td>
</tr>
<tr>
<td>QD/Silica</td>
<td>29 ± 6</td>
<td>--</td>
<td>576</td>
<td>25%</td>
</tr>
</tbody>
</table>
Table 4.3. Summary of literature QD/silica particles including synthetic method, QY of particles after coating, and stability. NR=not reported

<table>
<thead>
<tr>
<th>Reference</th>
<th>Synthetic Method</th>
<th>QY</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alivisatos 50</td>
<td>TOPO capped QD exchanged with MPS, hydrolyzed in base</td>
<td>5-18%</td>
<td>Stable for 1 month</td>
</tr>
<tr>
<td>Nann 14</td>
<td>Microemulsion. TOPO capped CdSe/ZnS</td>
<td>NR</td>
<td>Porosity gives rise to potential quenching 34</td>
</tr>
<tr>
<td>Chang 35</td>
<td>Microemulsion. CdSe QDs with 7-layer cap</td>
<td>47.8%</td>
<td>NR</td>
</tr>
<tr>
<td>Xu 32</td>
<td>Microemulsion. MA capped CdSe</td>
<td>1.48%</td>
<td>Quenched within 7 days</td>
</tr>
<tr>
<td>Rao 33</td>
<td>GSH capped CdTe. Silanization based on Alivisatos</td>
<td>15%</td>
<td>Increased intensity over 3 days in serum</td>
</tr>
<tr>
<td>Chen &amp; Cui 51</td>
<td>CdHgTe QDs coated with MPS in basic solution before adding sodium silicate</td>
<td>16%</td>
<td>Particles observed in-vivo after 36 hours</td>
</tr>
<tr>
<td>Chaniotakis 52</td>
<td>MUA capped CdSe/ZnS biosilanization via poly-L-lysine matrix and silicic acid</td>
<td>NR</td>
<td>Stable for 5 days</td>
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<tr>
<td>This work</td>
<td>MPA capped CdSe/ZnS in an arginine-driven Stober-type synthesis</td>
<td>25%</td>
<td>Stable for 7 days</td>
</tr>
</tbody>
</table>
Chapter 1


Chapter 2


Chapter 4


