A Novel Method for Bioanalysis of Dye Covalently Encapsulated in Silica Nanoparticles and Characterization of NIR Dyes for Future Approach

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A NOVEL METHOD FOR BIOANALYSIS OF DYE COVALENTLY ENCAPSULATED IN SILICA NANOPARTICLES AND CHARACTERIZATION OF NIR DYES FOR FUTURE APPROACH

by

KYLE EMER

Under the Direction of Gabor Patonay, PhD

ABSTRACT

Silica nanoparticles with dyes-encapsulated has been characterized by TEM and DLS, which were employed in biological applications with *A. castellanii*. Amoebae have pathogenic properties that can cause sight-threatening keratitis and granulomatous encephalitis. Phagocytosis of dye-covalently encapsulated in silica nanoparticles has shown promise *in vitro* and could be employed *in vivo* for detection. Time-dependent analysis of the SiNPs and *A. castellanii* displays micrographs of cellular components in the process of endocytosis and exocytosis. Alive and potentially deceased investigation of *A. castellanii* with SiNP uptake was evaluated to indicate detection with altered variables. Spectroscopic analyses were conducted on NIR dyes that will be essential for nanoparticle synthesis for *in vivo* and *in vitro* imaging.

INDEX WORDS: silica nanoparticles, *A. castellanii*, NIR dyes, TEM, DLS, confocal
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by

KYLE EMER

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

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in the College of Arts and Sciences

Georgia State University

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A NOVEL METHOD FOR BIOANALYSIS OF DYE COVALENTLY ENCAPSULATED IN SILICA NANOPARTICLES AND CHARACTERIZATION OF NIR DYES FOR FUTURE APPROACH

by

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Office of Graduate Studies
College of Arts and Sciences
Georgia State University
December 2015
DEDICATION

I would like to dedicate this thesis to my beautiful wife, Britney, and our kids, Brendon and Kylee. It takes a strong woman to make a strong man and Britney is definitely one of the strongest people I know. She’s been through more stressful and heartbreaking situations than people will see in their lifetime and her strength is what makes me cherish every moment with her. It may not seem like it at times but she knows I would do anything for her, and I know she would do the same for me. She is the mother of my children and the reason I try so hard. Without her, I wouldn’t know what real love was. Kylee, I remember the emotion I felt when you first came into this world. It’s unexplainable the depth of happiness that fulfilled me that day. You are a brilliant, young princess now, and I hope you keep striving to learn and to excel in all of your endeavors. Just remember when I’m away at school or working; I’m building you and your brother a foundation so that you can construct achievements of your own. Brendon, the first time I met you…I never thought I would be a worthy role model for you. Ten years later, I see that my accomplishments have reflected a positive outcome on your performance academically and yourself as a person. I am grateful to be your stepdad and to help guide you on your path to being a great man. Aria, you will soon enlighten us with your presence and I hope to be the father that any girl would want. I love you all.
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1.1 Introduction

1.1.1 Silica Nanoparticles and their applications

Nanochemistry is having an increasing attractiveness in the scientific world. Nanochemistry is the production of nanoparticles (NPs), nanotubes, nanowires, nanocrystals, and nanocomposites and how they interact in various living and non-living systems. The interest in NPs arises as they do not follow the laws of classical physics nor those of pure quantum chemistry. Mechanical, magnetic, catalytic, electronic, optical, and biological characteristics can be modified by nanoparticles. One of the exceptional properties of NPs is their nanometric size that can fill in gaps between bulky molecules. Size of NPs can range from 1 to 1,000 nm and can consist of biodegradable polymer, metals, or lipid constituents. The applications of nanoparticles include medicines, cosmetics, and many imaging techniques.¹ Nanoparticle-based drug delivery systems (DDS) have been utilized to improve human health by illuminating tumors, the brain, and other cellular functions while using medical imaging.²

Breakthroughs in the synthesis of silica nanoparticles (SiNPs) are beneficial when designing and developing a nanosized probe. Silica itself does not possess any properties for use in bioimaging but is known to be nontoxic. SiNPs can be modified with various organic groups and conjugated to biomolecules, which include monoclonal antibodies, DNA, or other ligands.³ These surface modifications allow (a) control of interaction of NPs and the biological environment, (b) tuning for specific binding to its target molecules, and (c) offer increased circulation times by evading the reticuloendothelial system (RES).⁴ This system is part of the
mammalian immune system and consists of phagocytes. Phagocytes are white blood cells that phagocytize or engulf foreign particles in several living organisms. Phagocytes are alerted by surface receptors of which molecules are toxic and which are beneficial to the organism. SiNPs are highly soluble in aqueous solutions due to the high concentration of silanol groups on the surface. This structural property is important for in vivo applications.  

Applications of multifunctional NPs can also be extended in photoinduced therapies such as photodynamic therapy, photomechanical therapy, and photothermal therapy. Photodynamic therapy is a well-known technique that uses photosensitizers to generate reactive oxygen species to irreversibly damage the potential pathological cells without harming the healthy cells. The issue with this is the cytotoxicity that may occur from the reactive chemical species. Modified SiNPs with iron oxide species can deliver desired treatment in vitro under near-infrared light irradiation, which will kill target cancer cells and has hopeful results to adjacent healthy cells. The most promising therapeutic method is photothermal therapy that consists of a photoabsorbing agent that converts radiation to heat. This method is convenient and promising in killing tumor cells because of its high efficiency and invasiveness. Laser-induced heating of selected tumor cells by inducing localized hyperthermia can prevent damaging healthy cells. Due to the highly soluble NPs with silanol groups, this treatment is more achievable. 

The SiNPs usually consist of a polymeric structure consisting of siloxan (-Si-O-Si-O-) and high concentrations of silanol (Si-OH) groups on the outer surface and are a type of colloidal metal oxide, which can be easily modified. Two methods utilized in the synthesis of these types of nanoparticles are the Stober method or the microemulsion method. There are two major classes of SiNPs: solid silica NPs (SSNPs) and mesoporous silica NPs (MSNPs). The MSNPs have many pores with a silica matrix and SSNPs have no pores. The MSNPs, with its empty
pores and channels, can be filled with various amounts of biomolecules and drugs as well as filling the gaps of molecules. The NPs can obtain new properties and capabilities by the modification and functionalization of its surface. The most important aspect of SiNPs is their surface modification can be utilized to achieve interactions with numerous bio-environments. Recent studies have shown that MSNPs have been internalized by both animal and plant cells successfully. These studies can extend to $^{19}$F magnetic resonance imaging (MRI), fluorescence imaging, and further depth on drug delivery. Controlled release of drugs from MSNPs pores is an excellent feature and can also prolong drug efficiency. This can be achieved by photocleavage, pH change, or biomolecules. Highly sensitive $^{19}$F magnetic resonance imaging agents can be encapsulated in the MSNPs with attached ligands for active targeting. This design is very promising for intracellular applications and may one day be used for oral consumption. Safety and potential toxicity concerns are incorporated with oral application of nanoparticles due to the increased effects of encapsulated drugs.

The properties of SiNPs are not just focused in biomedical applications but can also be used in polymer composites and green chemistry. Epoxy polymers are widely known as adhesives and when cured, the polymer is amorphous and highly-crosslinked. The epoxy assembly with SiNPs has both encouraging and undesirable properties that range from high modulus and failure strength to being brittle and poor resistance once a crack is initiated. When various silica nanoparticles weight percentages are implemented into the epoxy, the percent is the determining factor whether you achieve decent results from altered stress and compression tests.

When it comes to the field of green chemistry, chemists are attempting to find new approaches to use the least amount of solvent and produce sufficient yields. This can be
achieved from either biological systems or new catalytic techniques. Recent studies on sugars in the biphasic system have shown promise with SiNPs. The NPs are modified with amino and alkyl chains on the surface. These particles are used to create an emulsion in the biphasic system for sugar dehydration to 5-hydromethylfurfural (HMF). The outcome was a 10% HMF increase yield and 20% HMF increase yield for both fructose and glucose dehydrations. The SiNPs are then recycled and can be used for repeatable analysis. This is a type of reaction that utilizes the SiNPs as a catalyst and is just one of many applications of SiNPs.

1.1.2 Cyanine Dyes and NIR Dyes, their fluorescence occurrence and applications

Fluorescence light is a natural phenomenon that has been studied for years while employed in research. This phenomenon occurs when an organic or inorganic chromophore absorbs light and releases a photon. The molecule will excite from its singlet ground state, $S_0$, to its singlet excited state, $S_1$. In these states the electrons are still opposite spins. As the molecule relaxes from its excited singlet energy state, $S_1$, to its ground singlet energy state, $S_0$, the molecule can emit fluorescent light at a wavelength corresponding to the energy difference of the two states. The emitted photon is usually lower energy or has a longer wavelength. The molecule can sometimes excite to a higher singlet state, $S_{2+n}$, which can relax to the lower excited singlet state. This type of mechanism is called internal conversion. These transitions of energy levels can be well clarified by the Jablonski diagram in Figure 1.1.
Another form of emitted light is phosphorescence and can be seen by the naked eye as well as fluorescence, just depends on the wavelength. The lifetime ($\tau$) between phosphorescence ($10^3$ to $10^6$ s$^{-1}$) and fluorescence ($10 \times 10^9$ s$^{-1}$) is considerably distinctive. Phosphorescence occurs when an electron from the excited $S_1$ state changes spin and is now in the excited triplet state, $T_1$. When it relaxes to the $S_0$ state, the emitted photon takes a longer time to change its spin back to its original state so then possesses phosphorescence characteristics. The conversion between the $S_1$ to $T_1$ state is called intersystem crossing and usually is contributed interaction with other molecules or light.$^{15}$

Cyanine dyes are fluorescent molecules that are composed of a conjugated system. This system is comprised of a polymethine chain with an odd number of carbon atoms and two nitrogen heterocyclic rings. As seen in Figure 1.2, the n represents the number of methine between the heterocyclics: where n = 0, 1, 2, and 3 are monomethine, trimethine, pentamethine, and heptamethine, respectively. The color of the dyes is generated by electron propagation between the interaction of the polymethine bridge and heterocyclic ring system.$^{14}$ Varying the R-groups or heterocycles allows control of the chromophore, such as absorbance

![Jablonski diagram](image-url)
wavelength and fluorescence. The longer the chain of the polymethine chain, the longer the absorbance and emission wavelengths up to the desired near-infrared range for imaging. Different heterocyclic conjugation can also affect the desired wavelengths. In Figure 1.3, the most common heterocyclics that are implemented in cyanine dyes can be seen.

The applications of cyanine dyes vary through different types of imaging to solar cells. Fluorescence of cyanine dyes and other molecules can change due to pH, solvent, and light. For \textit{in vivo} imaging the desired wavelengths are in the near-infrared (NIR) range due to the low amount of energy. Energy is inversely proportion to wavelength so less the energy, higher the wavelength. The NIR spectral range is from 650-900 nm and offers an advantage over visible light range. In living systems, scattering and auto fluorescence are prominent and can potentially interfere with \textit{in vivo} imaging. Not only does NIR fluorescence imaging enhances the signal-to-noise ratio but also provides greater penetration into target tissues.
is due to the shorter wavelength and can enter tissues without being absorbed by biomolecules in that range.

1.1.3 Dye-doped & Dye Covalently Encapsulated Silica Nanoparticles

The progress of silica-based fluorescent nanoparticles for biological applications has increased in the last decade. These types of fluorescent probes or markers are essential in many different assays such as immunoassays and cytotoxicity assays. Several organic dyes are commercially available and are suitable for fluorescent labeling as well. The problem with these dyes includes a short Stokes shift, poor photostability, sensitive or quenching due to buffer, solubility problems, and photobleaching. Conducting Förster resonance energy transfer (FRET) applications, you want a longer Stokes shift so that the emission wavelength will distinctively be absent from the excitation wavelength so no overlapping occurs. This will reduce errors in calculating the desired assay. Even if these commercial dyes are readily available and cost efficient, the problem can still remain on the hydrophobicity, quenching in aqueous solution and irreversible photodegradation. The approach to encapsulate a large number of fluorophores per synthesized probe with a modifiable outer surface is essential because of the increased photostability of the fluorophores. There are two chemical ways to integrate organic dyes into silica nanoparticles: covalently bonding and electrostatic interactions. These ways can be achieve by the two main synthesis methods known as the Stöber and microemulsion methods. Both are sufficient techniques to encapsulate organic dyes into a silica nanoparticle. However, the Stöber method favors covalent bonding over electrostatic encapsulation.

1.1.4 Acanthamoeba Castellanii

Acanthamoeba castellanii is a small amoeba, an eukaryotic cell, that has been isolated in various sources such as soil, fresh water, dust, and air as well as from anthropogenic
ecosystems.\textsuperscript{22} \textit{A. castellanii} first was discovered in 1930 by Castellani while investigating a culture of \textit{Cryptococcus pararoseus}. The genus \textit{Acanthamoeba} was established in 1930 by Volkonsky and later in 1931, recommended three groups of amoebae based on their morphology. \textit{Hartmannella} are those with round smooth walled cysts, \textit{Glaseria} characterized by nuclear division in the cyst, and \textit{Acanthamoebae} had pointed spindles at mitosis with double-walled cysts and irregular outer layer.\textsuperscript{23} The morphology of \textit{A. castellanii} can differ while in the trophozoite form depending on its surroundings. They become larger in size when bacteria are present which in turn causes the \textit{A. castellanii} to clump together in groups of 10-39 cells.\textsuperscript{24} The two main phases of amoebae are the dormant cyst and active vegetative trophozoite. The life-cycle consists of a growth phase and two cellular processes known as encystment and excystment.\textsuperscript{25} When the cyst stage occurs the amoeba is resistant to extreme conditions such as severe temperature,\textsuperscript{26} desiccation,\textsuperscript{27} and antimicrobials.\textsuperscript{28-29} \textit{A. castellanii} may be in its protective state but they can not perform endocytotic processes, phagocytosis or pinocytosis. Phagocytosis is the internalization of large particles or microorganisms for food uptake through the plasma membrane.\textsuperscript{30-31}

They are also opportunistic protozoan pathogens that can lead to both sight-threatening keratitis and fatal granulomatous encephalitis. Granulomatous amoebic encephalitis is an infection of the central nervous system and keratitis can result in blindness and is very painful.\textsuperscript{24} Many features of \textit{A. castellanii} are still mysterious to this day.

\subsection*{1.1.5 Goal of this study}

The focus of the research is synthesizing silica nanoparticles with dyes and introducing the particles to \textit{A. castellanii}. Visualization and spectroscopic analysis of these factors will help future detection of the amoebae species and reduce the spread of pathogens they possess. Before
the dye-doped silica NPs are introduced to the amoebae though, characterization of the NPs by transmission electron microscopy (TEM) and dynamic light scattering (DLS) is required. Theoretically, the amoebae being incubated with the NPs will uptake the NPs by phagocytosis. Fluorescence microscopy should show the NPs inside and outside the amoebae after the experiment is completed. Further visualization will be accomplished by confocal microscopy, which will provide a thorough image and further analysis. These NPs and experimental analysis can be utilized for detection of amoeba so further pathogenic properties can be avoided.

1.2 Experimental

1.2.1 Instrumentation

All absorbance spectra were preformed either on Cary 3G UV-Vis Spectrophotometer (Agilent Technologies Incorporated, Santa Clara, CA) using 1cm polystyrene spectrophotometer cuvettes (Sigma-Aldrich, St. Louis, MO). All fluorescence spectra were preformed on LS 55 Fluorescence Spectrometer (PerkinElmer, Waltham, MA) using 1cm polystyrene fluorimeter cuvettes (Sigma-Aldrich, St. Louis, MO). Dynamic light scattering analysis was conducted on the NanoBrook 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY). Transmission electron microscopy micrographs were conducted by the Zeiss LEO 906E (Zeiss, Thornwood, NY). Nanoparticle separation required an Allegra 64R Centrifuge (Beckman Coulter Inc., Brea, CA). Amoeba and NPs were centrifuged at 3200 RPM on a Clay Adams Brand Compact II Centrifuge (Fisher Scientific, Pittsburgh, PA). Fluorescence and light micrographs were taking through the Nikon Eclipse E800 with the Zeiss AxioCam. The filter blocks used were a FITC block and a G-2A block and illumination with a mercury arc burner. All micrographs were taken at 400x magnification. Zeiss LSM 780 scanning laser confocal microscope was used for the confocal micrographs.
1.2.2 **Chemicals and Reagents**

All the following chemicals were produced by Sigma-Aldrich, St. Louis, MO:

- Rhodamine B isothiocyanate (mixed isomers), fluorescein isothiocyanate (isomer I, 90%), 1-hexanol (anhydrous, ≥99%), 3-aminopropyl triethoxysilane (≥98%), tetraethyl orthosilicate (99.999% trace metal basis), sodium phosphate (dibasic, 98%), sodium hydroxide (pellets, 97%, A.C.S. reagent), D-(+)-glucose (dextrose, minimum 99.5%), cyclohexane (anhydrous, 99.5%), Triton™ X-100 (laboratory grade), and potassium chloride (for molecular biology, ≥99.0%). Fisher Scientific, Fair Lawn, NJ, produced all the following chemicals: sodium chloride (certified A.C.S.), potassium phosphate (monobasic, certified A.C.S.), and ammonium hydroxide (certified ACS Plus). BD Biosciences, Advanced Bioprocessing, Sparks, MD, produced all the following chemicals: BD Bacto™ Proteose Peptone and Bacto™ Yeast Extract. Nanopure deionized water was produced by Dr. Wilson’s lab from Georgia State University.

1.2.2.1 **PY6 Broth**

Broth was prepared using 20g protease peptone, 1.0-2.0g yeast extract, and 3.0g dextrose mixed in 900 mL DI water. Phosphate-buffered saline (PBS) was prepared by adding 8.0g sodium chloride, 0.2g potassium chloride, 1.44g sodium phosphate, and 0.24g potassium phosphate. The pH was adjusted to 7.4 by adding sodium hydroxide pellets. Volume was adjusted to 1L and sterilized by autoclave. 100-mL of PBS was added to the PY6 broth to fulfill the 1L standard and sterilized by autoclave.

1.2.3 **Dye covalently encapsulated Silica Nanoparticle synthesis**

The following synthesis is an adaptation that utilizes the reverse microemulsion method. Fluorescein isothiocyanate (FITC) and Rhodamine B isothiocyanate (RBITC) were the dyes both used in separate synthesis. A known amount of dye is added to 1-hexanol to make
a 1 mM solution. Add 10 molar equivalents of 3-aminopropyl triethoxysilane (APTES) and allow stirring for 12 h in round-bottom flask. The reagents are air sensitive and need to be completed in a closed container in the dark. The micelle is then prepared using 15.0 mL cyclohexane, 2.60 mL 1-hexanol, 3.550 mL Triton™ X-100, 1.125 mL DI H$_2$O, and 0.140 mL ammonium hydroxide and allow stirring for 10-15 min to generate the microemulsion. After time elapsed, slowly add 1.00 mL of dye-APTES conjugate to the microemulsion and allow stirring for 30 min at 120 RPM. Finally, add 0.2 mL tetraethyl orthosilicate (TEOS) and allow stirring for 24 h. The dye-doped SiNPs are now achieved. The scheme below shows the reverse microemulsion synthesis.

![Scheme of dye-covalently encapsulated silica nanoparticles](image)

**Figure 1.4: Scheme of dye-covalently encapsulated silica nanoparticles**

SiNPs sample cleanup involves centrifugation in an Allegra 64R Centrifuge at 10,397 g for 2 h, then supernatant is discarded and pellet is resuspended in total of 16 ml MeOH, then centrifuged again. This process is repeated 3 times, and the third time the pellet is resuspended in 1 ml MeOH and distributed between preweighed 2-ml centrifuge tubes. 0.1 ml 40% w/v glycerol
in MeOH is added to each tube, and tube contents are blown down using nitrogen gas in a dry room temperature turbovap lv (Biotage). Tube + sample weights are taken and tube and glycerol masses are subtracted from the total to get NP sample masses. Glycerol masses are obtained by preparing blank tubes containing only 40% glycerol and equivalent volumes of MeOH.

1.3 Results and Discussion

1.3.1 Characterization of Silica Nanoparticles

The need for characterization is dire for the understanding of how and why the nanoparticles preform in different environments. The nanoparticle size and shape are desirable to predict whether or not the nanoparticle is paramount for an experiment. The complexes inside or outside the nanoparticle are particularly important as well. The fluorophore’s photostability study is essential for visual and fluorescence analysis. Continuous exposure to light can cause a fluorophore to become immobile to emit a photon, which in turn causes photobleaching.

1.3.1.1 Size Determination

1.3.1.1.1 Transmission Electron Microscopy

This type of microscopy uses electrons as a source instead of various wavelengths of light. The light microscopes are limited by the wavelengths of light. Electrons are known to have smaller wavelengths and are used to penetrate through its objective. TEM can create images that have a thousand times better resolution than light microscopes. Instead of traditional lenses to focus the light onto the object, electromagnetic lenses are used to focus the electrons down a vacuum as a thin beam. Electrons can bounce back but most will penetrate through and produce a shadowy image on a fluorescent screen. Various types of darkness in the images indicate the density of the object. In Figure 1.5, you can see darker shadows due to the
overlapping of the nanoparticles. Preparation of TEM samples is relatively easy but can cause aggregation once dried for analysis.

![Transmission Electron Micrographs of Rhodamine B SiNPs](image)

**Figure 1.5: Transmission Electron Micrographs of Rhodamine B SiNPs**

The aggregation of the RBNPs can be seen in Figure 1.5. Measurements of the diameter of the RBNP were conducted to provide a range, mode, and average sizes of these particles using the reverse microemulsion synthesis. The range of the RBNP’s diameter is from 50-80 nm. The average diameter of the RBNPs was 68.91± 7.71 nm. The average isn’t relative as the mode of the nanoparticles for this analysis. The reverse microemulsion synthesis has a stopping point at which it halts the spherical shape from enlarging. Even if the range is 50-80 nm and the average is 68.91 nm, the mode is the most abundant in the synthesis. The mode of the RBNPs had an abundant at 73.112 nm and second abundance at 70.968 nm.
1.3.1.1.2 Dynamic Light Scattering

The investigation of Dynamic Light Scattering of dye covalently encapsulated SiNPs in different medias is presented. This method uses the interference method of particles in Brownian motion, which uses incident laser light. The light scatters after coming in contact with the nanoparticles in colloid. The colloid consists of a solution with particles ranging from 1-1000 nanometers in size that usually do not aggregate and evenly distributed. The time it takes to conduct the analysis, fluctuations will occur in the scattered light intensity due to particle movement and will also cause fluctuations in the local density. These fluctuations are determined by the diffusion coefficient, which depends on the nanoparticle size. Einstein and Smolukhovskiy have an equation that describes the dependence of the diffusion coefficient from particle size if spherical shape is determined:

$$D = \frac{kT}{6\pi\eta R}, \quad (1)$$

where $D$ is the diffusion coefficient, $T$ is the temperature, $R$ is the radius of the nanoparticle, $k$ is the Boltzmann constant and $\eta$ is medium viscosity. Polydispersity correlates with single particle size mode are expected and a single exponential fit is applied with the width of assumed Gaussian distribution. Any value fewer than 20% for polydispersity its safe to accept that the sample of nanoparticles is monodisperse. There is also an equation that Smolukhovskiy determined that the assumption that each collision of nanoparticles makes them stick together or fast coagulation. The $t_k$ is the time of fast coagulation:

$$t_k = \frac{4\eta}{8n_0 kT}, \quad (2)$$

where $\eta$ is dynamical viscosity of liquid, $n_0$ is initial concentration of the system, $T$ is the temperature and $k$ is the Boltzman constant. This analysis doesn’t use the following equations to determine the time of coagulation, the coagulation decreasing factor, or the intensity of scattering.
of polarized incident light but are good to know for future dynamic light scattering studies. The following is the coagulation-decreasing factor and this is the equation for qualitative estimation:

\[ W \approx \frac{1}{2k}\exp\left(\frac{u_{max}}{kT}\right) \]  

(3)

where \( r \) is particle’s radius, \( 1/\kappa \) is the thickness of ion atmosphere, and \( u_{max} \) is the height of potential barrier. Using the previous equation and suppose that the coagulation time is \( W \) times smaller than \( t_k \), time of fast coagulation, its safe to assume:

\[ t = W t_k \]  

(4)

The intensity of scattering of polarized incident light is described by this formula derived from the Rayleigh theory:

\[ I_s = \frac{8\pi^4N\alpha^6}{\lambda^4r^2}\left|\frac{m^2-1}{m^2+2}\right|^2(1 + \cos^2\theta)I_i \]  

(5)

Where \( \alpha \) is the radius of the particle, \( \lambda \) is the wavelength, \( m \) is the correlation of refractive indexes between particle and medium, \( I_i \) is the intensity of incident light, \( \theta \) is proposed as the angle of scattering, and \( r \) is distance from the particle to the point of observation.\(^{35}\)

The following analysis was to determine the average diameter of my Fluorescein and Rhodamine B silica nanoparticles. The experiment was conducted in two different mediums to compared and contrast the variance of solvent and nanoparticle. The two separate mediums were methanol (MeOH) and deionized H\(_2\)O. For analytical investigation the following dynamic light scattering experiment was conducted in triplicate. In Figure 1.6-1.9, the graphs show a logarithmic representation of diameter versus intensity from the three consecutive runs. Table 1.1-1.2 displays the average diameter with FNPs in H\(_2\)O and MeOH. The average diameter in H\(_2\)O was 112.7 nm compared to the 96.0 nm in MeOH. Swelling of the particles are known to happen in more aqueous media. The polydispersity is less than 20% for the water analysis but the MeOH analysis shows a polydispersity of more than 20%. This indicates that the particles
are not uniformly sized in MeOH. The RBNPs were also analyzed with the same parameters as the FNPs. As seen in Table 1.3-1.4, the average diameter for RBNPs in water and MeOH are 159.7 nm and 131.3 nm respectively. The polydispersity did not exceed the 20% limit so it is safe to assume that the RBNPs are monodisperse.

The comparable results of Transmission Electron Microscopy and Dynamic Light Scattering of RBNPs can be examined further. The TEM sample was dried, which caused aggregation and could achieve an accurate analysis of the diameter. The DLS experiment indicated that the RBNPs were almost double in size when introduced into H₂O. The swelling occurs in more polar solvent than compared to MeOH and the polydispersity is low, which indicates little aggregation.
Figure 1.6: DLS Logarithmic Representation of the Diameter of FNPs in H₂O

Table 1.1: DLS Triplicate Run of FNPs in H₂O

<table>
<thead>
<tr>
<th>Run</th>
<th>Eff. Diameter (nm)</th>
<th>Half Width (nm)</th>
<th>Polydispersity</th>
<th>Baseline Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>118.8</td>
<td>40.8</td>
<td>0.118</td>
<td>9.2</td>
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<tr>
<td>2</td>
<td>112.1</td>
<td>37.8</td>
<td>0.113</td>
<td>9.2</td>
</tr>
<tr>
<td>3</td>
<td>107.1</td>
<td>38.4</td>
<td>0.128</td>
<td>9.2</td>
</tr>
<tr>
<td>Mean</td>
<td>112.7</td>
<td>39</td>
<td>0.12</td>
<td>9.3</td>
</tr>
<tr>
<td>Std. Error</td>
<td>3.4</td>
<td>0.9</td>
<td>0.004</td>
<td>0.1</td>
</tr>
<tr>
<td>Combined</td>
<td>112.2</td>
<td>39.3</td>
<td>0.123</td>
<td>9.5</td>
</tr>
</tbody>
</table>
Figure 1.7: DLS Logarithmic Representation of the Diameter of FNPs in MeOH

Table 1.2: DLS Triplicate Run of FNPs in MeOH

<table>
<thead>
<tr>
<th>Run</th>
<th>Eff. Diameter (nm)</th>
<th>Half Width (nm)</th>
<th>Polydispersity</th>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>42.2</td>
<td>0.179</td>
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<td>2</td>
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<td>44.7</td>
<td>0.224</td>
<td>3.3</td>
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<tr>
<td>3</td>
<td>93.7</td>
<td>42.5</td>
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<td>8.9</td>
</tr>
<tr>
<td>Mean</td>
<td>96</td>
<td>43.2</td>
<td>0.203</td>
<td>7.2</td>
</tr>
<tr>
<td>Std. Error</td>
<td>1.9</td>
<td>0.8</td>
<td>0.013</td>
<td>2</td>
</tr>
<tr>
<td>Combined</td>
<td>96</td>
<td>43.2</td>
<td>0.203</td>
<td>7.2</td>
</tr>
</tbody>
</table>
Figure 1.8: DLS Logarithmic Representation of the Diameter of RBNPs in H$_2$O

Table 1.3: DLS Triplicate Run of RBNPs in H$_2$O

<table>
<thead>
<tr>
<th>Run</th>
<th>Eff. Diameter (nm)</th>
<th>Half Width (nm)</th>
<th>Polydisperity</th>
<th>Baseline Index</th>
</tr>
</thead>
<tbody>
<tr>
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<td>9.9</td>
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<tr>
<td>3</td>
<td>154.7</td>
<td>60.4</td>
<td>0.152</td>
<td>8.1</td>
</tr>
<tr>
<td>Mean</td>
<td>159.7</td>
<td>59.9</td>
<td>0.141</td>
<td>9</td>
</tr>
<tr>
<td>Std. Error</td>
<td>2.6</td>
<td>1.8</td>
<td>0.009</td>
<td>0.5</td>
</tr>
<tr>
<td>Combined</td>
<td>159.7</td>
<td>59.5</td>
<td>0.139</td>
<td>9</td>
</tr>
</tbody>
</table>
Figure 1.9: DLS Logarithmic Representation of the Diameter of RBNPs in MeOH

Table 1.4: DLS Triplicate Run of RBNPs in MeOH

<table>
<thead>
<tr>
<th>Run</th>
<th>Eff. Diameter (nm)</th>
<th>Half Width (nm)</th>
<th>Polydispersity</th>
<th>Baseline Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>136.5</td>
<td>45.7</td>
<td>0.112</td>
<td>9.5</td>
</tr>
<tr>
<td>2</td>
<td>150.9</td>
<td>10.7</td>
<td>0.005</td>
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<td>3</td>
<td>112.4</td>
<td>55</td>
<td>0.24</td>
<td>10</td>
</tr>
<tr>
<td>Mean</td>
<td>133.3</td>
<td>37.1</td>
<td>0.119</td>
<td>9.6</td>
</tr>
<tr>
<td>Std. Error</td>
<td>11.2</td>
<td>13.5</td>
<td>0.068</td>
<td>0.2</td>
</tr>
<tr>
<td>Combined</td>
<td>131.3</td>
<td>47.1</td>
<td>0.129</td>
<td>10</td>
</tr>
</tbody>
</table>
1.3.1.2 Photostability

In this study, Rhodamine B and Fluorescein dye-covalently bounded SiNPs are compared to the commercial dye of both Rhodamine B and Fluorescein. The conditions for the experiment are determined by both light and dark analysis. Both sets were independently exposed to either a constant light source or kept in the dark for fifteen consecutive days. They were measured each day with the same blank to produce consistency. The light analysis required a special light source that mimicked nature sunlight and kept constant. The dark studying is completed to compare if sunlight affects the fluorophore or if solvent also is a factor. The solvent utilized in this study was PBS pH 7.4 with 2% DMSO to achieve solubility of dyes.

Figure 1.10: Photostability analysis of FNPs and Fluorescein in PBS pH 7.4 with 2% DMSO

Photostability for FNPs versus Fluorescein shows a trend in Figure 1.9 for both light and dark analysis. The fluorescein dye is known for photobleaching and will photodegrade at a faster rate. The FNP seem to be more stable when exposed to light for 15 days. This is helpful for
later light exposure for imaging. FITC when exposed to the fluorescence microscope light source, can photodegrade within minutes or even seconds.

Figure 1.11: Photostability analysis of RBNPs and Rhodamine B in PBS pH 7.4 with 2% DMSO

Photostability analysis between RBNPs and Rhodamine B in light and dark analysis can be shown Figure 1.11. Rhodamine and derivatives are very photostable compared to other commercial fluorophores. In Figure 1.10, a new trend is shown compared to the fluorescein study. The Rhodamine B and RBNPs are stable when exposed to light but seems to degrade due to the solvent. The Rhodamine B dye can see a pattern that the light exposure slightly photo degrades the fluorophore but can be indicated that the solvent effect is key.
1.3.2 Visualization and Fluorescence Intensity of dye covalently encapsulated silica nanoparticles and Acanthamoeba Castellani

1.3.2.1 Preliminary Analysis

Throughout this analysis, many parameters were taken into account to manipulate the \textit{A. castellanii} to uptake the various nanoparticles. Prior experimentation was unsuccessful due to working with living organisms. The experiment was relatively easy when conversing between scientific professionals and colleagues. The first setback was the auto-fluorescence of the \textit{A. castellanii}. Cell density plays an important role in controlling the amount of auto-fluorescence intensity. Due to each fluorometer having different light intensities, it is difficult to indicate a cell density that would work on all fluorometers. \textit{A. castellanii} is centrifuged to eliminate the broth that can be poured off. The \textit{A. castellanii} is then resuspended in nanopure-deionized water and testing with the fluorometer. After many attempts, the fluorescence of the \textit{A. castellanii} has to be taken before implementation of the SiNPs. The SiNPs fluorescence controls are indicated before employed with the \textit{A. castellanii}. The difference from the spectra can be used to compare whether or not the SiNPs were phagocytized by the \textit{A. castellanii}, attached to its outer membrane and/or do not interact. The second setback was the time needed to manipulate \textit{A. castellanii} to go from encystment to trophozite form. A two-hour incubation trial was conducted where \textit{A. castellanii} was introduced into fresh broth and incubated at 37 °C at 200 RPM. This is the optimal temperature and rotations to influence the amoebae to be in their feeding form. After the two hour period, the amoebae were centrifuged at 1,100g for 10 min. The broth was poured off and nanopure water with Fluorescein nanoparticles were introduced. The amoebae were incubated again for two hours at same temperature and rotations. After time elapsed, the amoebae were centrifuged again for 10 min. The supernatant was collected and the pellet was
resuspended with nanopure water. Both the supernatant and the amoebae/NP were analyzed by fluorescence to show a minor change in the intensities. The supernatant indicated an intensity of free FNPs in water and the amoebae intensity had an increase of intensity over the control intensity of the amoeba. This increase indicated that FNPs were collected in the pellet as well. Using light microscopy, the amoebae could be analyzed after the experiment to show that some of the amoebae were still encysted. This preliminary trial indicates that the time dependency of the incubation period was too short and needed to be extended.

1.3.2.2 Final Analysis

The experiment parameters were modified to give the amoebae more time and opportunity to uptake the various dye-encapsulated nanoparticles. Instead of the fresh broth and nanoparticle incubation lasting 2 h, each part was increased to a 24 h period. This manipulates the amoebae at a higher percentage to transform from their encystment to trophozite stage and also give a higher percentage of phagocytosis of dye-encapsulated nanoparticles. The following conditions were taken with a difference in dyes that were encapsulated. First, FNPs were used to see the difference in intensity. After the procedure, light and fluorescence micrographs were taken to have visual conformation of the amoebae and NPs. This was also conducted with the RBNPs and further visualization was completed by confocal microscopy.

In Figure 1.12, graphs (a) and (b) indicate the control intensities of the amoebae and the FNPs separate before they were incubated together. Graphs (c) and (d) are the intensities after the 24 h incubation of the amoebae and FNPs. As you can see in Figure 1.11, the increased fluorescence intensity between 490nm and 540nm correlates with the FNP fluorescence. This gives the assumption that the amoebae phagocytized the FNP and the supernatant is the free FNPs and water. Supernatant 6 is an outlier as the fluorescence is increasingly high where both
the amoebae and FNPs emit. After last centrifugation and during pouring off the supernatant, part of the amoebae was introduced into the supernatant 6. This experiment is conducted with six separate controls to show reproducibility.

Figure 1.12: Fluorescence Analysis of FNPs and *A. Castellanii*
Perkin Elmer LS-55, FLWinLab, Excitation: 350 nm, Slit Width: 5 nm, Scan Speed: 150nm/min. Solvent: 5 mL Nanopure DI H2O. (a) Six varying Amoebae controls before incubation. (b) Six varying FNPs before incubation. (c) Amoebae and Nanoparticle analysis after 24 h incubation at 200 RPM at 37°C and centrifuged at 3500 RPM for 10 min. (d) Supernatant was collect and analyzed.

Fluorescence intensities can only tell us that the containing solution emits a photon in the range that correlates with the amoebae and nanoparticles. Visualization is needed to see if the amoeba phagocytized the FNPs or the FNPs are free in the solution. Figure 1.13 shows the amoebae and FNPs in both light and fluorescence microscopy. These are the initial micrographs after the slides were made with no added solution. The amoeba and NPs can be seen in the light micrograph as well as the fluorescence micrograph. The dense white clusters in the light
micrograph correlates to the FNPs. The fluorescence micrographs can confirm with the increased light intensity in the identical areas. As these slides are not fixed, the *A. castellanii* is still active and can still perform biological processes.

Figure 1.13: Initial Light and Fluorescence Micrographs of Amoeba and FNPs
The differences between Figures 1.13 and 1.14 is the time elapsed between each micrograph. Figure 1.12 is the initial micrographs after the slides were completed and Figure 1.14 is a 24 h elapsed from the initial to see if anything different could be examined. In both figures, these are the same amoeba. In Figure 1.14, it is assumed that the amoeba realizes that the FNPs are not food and also can’t be digested. The increased fluorescent channels have not been seen in previous studies with nanoparticles or amoebae. The presumed analysis of the micrograph may indicate that the amoeba is performing exocytosis of the FNPs and is pumping the nanoparticles through channels for excretion. Amoebae are known for performing this with the mutualistic bacteria living internally. Another assumption is that the amoeba indicates that something foreign inside their membrane and need to convert into its cyst form to guard itself. Fluorescence microscopy still only shows a 2D image and it is not confirmed that the nanoparticles have actually penetrated the membrane. Analysis on both living and deceased amoebae is needed to confirm if the nanoparticles are readily adhering to the membrane or phagocytosis is desired.
The exact parameters were utilized when introducing a different dye-encapsulated nanoparticle. As you can see in Figure 1.15, the graphical representation mimics Figure 1.12. The RBNPs have a different wavelength than FNPs at which they excite and emit photons. The excitation wavelength and all other parameters were kept the same to show reproducibility. The
boxes within the graphs show the intensities where the RBNPs emit. The RBNPs have an emission wavelength that is outside the auto-fluorescence wavelength of the amoebae. This experiment gives a more achievable analysis when using fluorescence spectroscopy.

The light and fluorescence micrographs shown in Figure 1.15 are not as clear as Figure 1.13 with the cluster of nanoparticles. There is a cluster of RBNPs located on the bottom right of the amoeba and can be seen in both images. The increased intensity of the RBNPs cannot be seen and may indicate that the amoebae didn’t phagocytize much of them. It could also be assumed that the RBNPs are inside the amoeba and may have too much interference to be seen in the fluorescence micrograph. An abundance of bacteria is located outside the membrane of the
amoeba in the light microscopy image. The fluorescence filter helps eliminate the bacteria from being seen as interference.

Figure 1.16: Initial Light and Fluorescence Micrographs of Amoeba and RBNPs
The 24 h elapsed micrographs showed distortion within the images in Figure 1.17. The two images are the same slide but different amoeba in each. The light micrograph shows the channels but the fluorescence micrograph was not taken with this amoeba. The fluorescence micrograph also shows the channels of RBNPs that correlates to the 24 h elapse with the FNPs. This coincidence of the various NPs causing the similar structural occurrence needs to be further investigated. The correlation that should be considered is the alive and deceased analysis of the various NPs and *A. castellanii*. 
The living and deceased amoebae analysis was carried out with the same parameters as the previous amoebae and NP experiments. The only difference is that two controls of the amoebae are deceased by boiling them at 100 °C for 10 min. The two deceased controls were measured with fluorescence after the heating as seen in Figure 1.18. The following experiment will use either FNPs or RBNPs in both the alive or deceased amoebae to indicate if phagocytosis is needed. Deceased amoebae do not have the ability to perform biological processes so the
The likelihood of the nanoparticles being internal should not be seen. It is still possible for the NPs to adhere to the membrane of the deceased amoebae and will be there after the final centrifugation step. In Figure 1.17(d), the supernatant 1 and 2 correlate with RBNPs and supernatant 3 and 4 correlate with the FNPs.

**Figure 1.18: Fluorescence Analysis of Living and Deceased Amoebae and SiNPs.**
Perkin Elmer LS-55, FLWinLab, Excitation: 350 nm, Slit Width: 5 nm, Scan Speed: 150nm/min. Solvent: 5 mL Nanopure DI H₂O. (a) Two Living and Deceased Amoebae controls before incubation. (b) Two varying FNPs and RBNPs before incubation. (c) Amoebae and Nanoparticle analysis after 24 h incubation at 200 RPM, 37°C, and centrifuged at 3500 RPM for 10 min. (d) Supernatant was collect and analyzed.

Comparable visualization by fluorescence microscopy was used to determine the outcomes between the living and deceased amoebae. In Figure 1.19, the living amoebae have an increased fluorescence throughout its structure like previously seen in Figure 1.13. The deceased amoebae have a lower fluorescence within the image but it can be seen that the FNPs are
clustered on the membrane. This indicates that phagocytosis isn’t need for the detection of *A. castellanii*. The deceased amoeba also has a channel that was previously examined in Figure 1.13 after 24 h elapsed time. This could be that the amoeba was attempting to retreat into its cyst form during the increased temperature. Figure 1.20 shows the living and deceased amoebae with RBNPs instead. The living amoebae show an increase in fluorescence around aggregated RBNPs that can also be seen in the deceased amoebae as well. It is safe to assume that the NPs have terminal groups that can adhere to the surface of the amoeba membrane. The living amoebae have higher fluorescence due to the uptake of the RBNPs within its cellular structure.
Figure 1.19: Fluorescence Micrographs of FNPs with Living Amoebae (top) & Deceased (bottom)
Confocal microscopy was conducted to achieve the final visualization of the dye-encapsulated NPs and *A. castellanii*. It was established that the FNPs were both inside and outside the amoebae as seen in Figure 1.21. The amoebae were stained with DAPI, which should target the nucleus of the amebae. Instead this caused the commensal bacteria within the amoebae to be stained and also has an excitation wavelength similar to the FNPs due to DAPI. Modification of the spectral detector allowed for visualization of the nanoparticles. The brighter
green spots on Figure 1.21 that are agglutinating together are the FNPs. This enhanced microscopy has given confirmation FNPs being on the outer and inner portions of the amoebae.

Figure 1.21: Confocal Micrograph of FNPs and *A. castellanii*

Figure 1.22 also confirmed the successful integration of RBNPs within and outside the amoebae. The blue that is seen is the stained commensal bacterium, as the nucleus was not stained by the DAPI. The bright red large blots are the RBNPs agglutinating within the amoeba. Spectral modification of the instrument helped achieve the validation of this investigation.
Figure 1.22: Confocal Micrograph of RBNPs and *A. castellanii*

1.4 Conclusion

Successful characterization of the SiNPs by transmission electron microscopy, dynamic light scattering, and photostability were conducted. The confirmed visualization of the dye-encapsulated NPs and *A. castellanii* was fully achieved by confocal microscopy. The NPs can be seen on the interior and exterior of the amoebae, which indicates that the amoebae needed to phagocytize the NPs and the NPs may automatically adhere to the surface of the membrane. The changes in intensities between all parameters of the experiments confirmed that the NPs could be used for detection of *A. castellanii* and other amoebae species. The essentially usefulness for this detection would be the RBNPs. This is due to the less interference intensities in the range of biological auto-fluorescence. Utilizing a NIR dye for this analysis would be essential for lowering the interference almost completely. A NIR dye would emit in a range that is in the median of no biological auto-fluorescence for spectroscopic and visual analysis.
2 SPECTROSCOPIC DETERMINATIONS OF FLUORESCENT NIR IMAGING AGENTS FOR FUTURE APPLICATIONS

2.1 Introduction

2.1.1 Pentamethine cyanine dyes and its derivatives

Cyanine dyes are very essential in many biological applications especially in imaging. Pentamethine cyanine dyes usually absorb light in the 640-670 nm range and emit fluorescence in the near-infrared range around 690-720 nm. These ranges are essential for many biological elements. All living organisms have auto-fluorescence due to the biomolecular structures and they have the capability to absorb and emit light. These biological fluorophores can be avoided by utilizing pentamethine cyanine dyes and other near-infrared dyes. The visible region (400-600 nm) that is associated with majority of biomolecular auto-fluorescence can cause unwanted competition. This can make it hard to differentiate between signals and cause undesirable results. Near-infrared fluorophores have gained an increasingly interest and are known for their optical properties. They are relatively stable with high molar absorptivity coefficients and high fluorescence quantum yields. Many methods to utilize green chemistry in the synthesis of pentamethine cyanine dyes and various other polymethine dyes can be easily achieved with considerable yields. Pentamethine cyanine dyes and other polymethine dyes have also been used in solar cells. The power efficiency has show to increase with the dye and bilayer solar cell complex.

2.1.2 In vivo imaging and future advances

Major advances in biological imaging have grown substantially in the last decade. Some used more than others but all serve a purpose in scientific analysis. They all also have their
advantages and disadvantages depending on the parameters of approach. *In vivo* molecular imaging main development can fall into two categories, which contains positron emission tomography (PET) approaches and optical imaging modalities. In vivo means “within the living,” which can sometimes be confused with *in vitro* meaning “in glass.” Bioluminescence and fluorescence is the key for most biological imaging and can be challenging when investigating deeper into tissues and other biological materials. The fluorophores give off scattering light and makes it difficult to interpret the target tissues. Computer software programmers are creating various algorithms to battle this concern and also create more sophisticated maps of fluorophores inside living animals. Many alternative *in vivo* imaging techniques are also being used that do not use a fluorophore for exploration.

Radionuclide imaging and magnetic resonance imaging (MRI) are two examples of non-fluorescence *in vivo* imaging. Radionuclide imaging utilizes radioactive material or radioisotopes that are injected into the subject and is known to cause less radiation exposure than x-rays. A special gamma camera or scanner is used to capture the images and provides a non-invasive method for motoring functions of individual organs. This method has higher sensitivity, specificity, and the possibility of quantification of these alternations with different radiotracers. These tracers make it possible to indicate pathophysiological information in patients and can be used for clinical treatment guidelines. Studies for the detection of spinal metastases uses $^{99m}$Tc-labeled phosphates are used and known for their higher sensitivity than conventional radiographs. Risks are also noted in this non-invasive method and could cause radiation poisoning by excessive use.

MRI uses a strong magnetic field and radio waves to produce an image. It is known that applying appropriate frequency to the body can excite the hydrogen atoms in the water molecules
of tissues. This frequency will cause the hydrogen atoms or protons to emit a radio frequency that can be measured by a receiving coil. The frequency can be encoded to create an image by varying the magnetic field strength by the receiving coils. The magnetic field can also be unsafe for people who have pacemakers or any type of metal in or on the body. The magnetic field is increasingly strong and has caused fatalities. There has also been great promise in MRI applications and recently has been used to quantify the amount of iron that accumulates in the brain related to Alzheimer's disease.\textsuperscript{44}

Deployment of sound waves to structures to produce images or increase reaction rates is well known as an ultrasound, alternative variety of non-fluorescent approach of \textit{in vivo} imaging. The energy source of an ultrasound is very environmentally friendly with includes low energy requirements, low waste, and can enhance rate of reactions by many orders of magnitude.\textsuperscript{45} For \textit{in vivo} in humans, real-time ultrasound is regularly used for assessing pregnancy, fetal development, and results of treatment that could modify parameters.\textsuperscript{46-49} Early confirmation of pregnancy can be used as evidence for delayed development or growth retardation in animals.\textsuperscript{48} This confirmation has saved many lives, animal and human alike. Besides imaging, Doppler ultrasound has been used for measuring the direction and velocity of blood flow by using the reflect sound waves off blood cells to propose direction and using the shift in frequency transmittance to calculate velocity.\textsuperscript{47}

\textit{In vivo} imaging utilizes all different modalities to include x-ray imaging. This is the most popular in pre-diagnosis in the clinical setting. The more advanced type of x-ray imaging is x-ray computed tomography (CT). A CT imaging system process cross-sectional images of specific areas. Areas can range from anatomical, wood\textsuperscript{50}, steel\textsuperscript{51}, and numerous commercial
applications. CT is still the most often imaging used to detect suspected acute stroke and hemorrhaging in the brain. It is cost efficient and can help with fast diagnosis.

Optical fluorescence and bioluminescence imaging are known for their low resolution and possibilities of increase noise due to auto-fluorescence. Fluorescence imaging is usually performed in the range of visible to near infrared. Increased methods and understanding the molecular structure of fluorophores has broaden the imaging capabilities for use today. These fluorescence imagers operate using different light filters to analyze the desired wavelength. The near-infrared range helps eliminate potential noise from other tissues when examining deeper. The future imagers are employing many different in vivo imaging modalities into one machine for even further analysis.

2.1.3 Goal of this study

This study is to measure the molar absorptivities and quantum yields of several novel pentamethine cyanine dyes. The pKₐ determination will indicate pH in which these carboxylic mono- and di- acids are deprotonated and indicate a shift or change in absorbance. These NIR dye characteristics will be useful in biomolecular imaging with less interference from background fluorescence and scattered light. The future goal is to utilize the Stöber method for synthesis of novel NIR dye-encapsulated silica nanoparticles and used for increased visualization of in vivo and in vitro imaging.
2.2 Experimental

2.2.1 Instrumentation

All absorbance spectra were performed either on Cary 3G UV-Vis Spectrophotometer (Agilent Technologies Incorporated, Santa Clara, CA) using 1 cm polystyrene spectrophotometer cuvettes (Sigma-Aldrich, St. Louis, MO). All fluorescence spectra were performed on LS 55 Fluorescence Spectrometer (PerkinElmer, Waltham, MA) using 1 cm polystyrene fluorimeter cuvettes (Sigma-Aldrich, St. Louis, MO). All pK\textsubscript{a} determinations were conducted with a TitroLine alpha plus automatic titrator that was connected to a Dell computer and controlled by Titrisoft 3.0 software.

2.2.2 Chemicals and Reagents

Tyler Dost of Dr. Maged Henary’s group at Georgia State University synthesized the following pentamethine dyes: T-45, T-46, T-50, T-55, T-62, T-85, and T-87. Dimethyl sulfoxide (99.9%, for spectroscopy, ACROS Organics\textsuperscript{TM}). Rhodamine B (≥95%, HPLC, Sigma-Aldrich, St. Louis, MO). Acetate buffer pH 4.5 was used for pK\textsubscript{a} analysis, which required sodium acetate (certified A.C.S., crystal, Fisher Scientific, Fair Lawn, NJ) and acetic acid (glacial, EMD Millipore, Billerica, Massachusetts). 0.2 M Sodium Hydroxide (Pellets/Certified ACS, Fisher Scientific, Fair Lawn, NJ) was the titrant for pK\textsubscript{a} determination.
2.2.3 Method to determining molar absorptivity

To obtain quantitative information concerning dye interactions in DMSO, determining the molar absorptivity and quantum yield are two specifications that are usually retained. To obtain these analyses, quantification of the absorptive and emissive behaviors of the pentamethine cyanine dyes are desired.

Figure 2.1: Mono- and di- acid pentamethine cyanine dyes
Referring to Beer’s Law, the relationship between absorbance \( A \), concentration \( c \), path length of cell holder \( l \), and molar absorptivity \( \varepsilon \) is described.

\[
A = \varepsilon lc \quad (7)
\]

Spectroscopic measurements can be made to determine the molar absorptivity of a given substance: as the concentration of a solution increases, there should be a subsequent increase in absorbance. Furthermore, the absorbance can be plotted as a function of concentration wherein Beer’s Law can then be fit to a linear equation, thus yielding a straight line; the slope of which is the molar absorptivity.\(^{16}\) This can only be achieved if maximum absorbance units are less than 1.0 au.

### 2.2.3.1 Molar absorptivity sample preparation

A serial dilution of each dye was prepared in DMSO at concentrations ranging from 1.0 – 6.0 \( \mu \)M. Absorbance measurements of the standard dye were taken after novel dyes to ensure reliable controls are achieved. Each dye was analyzed simultaneously alongside Rhodamine B and replicated at least two times to ensure reproducibility. Microsoft Excel 2010 was used for all linear regression analyses from which the slope of the absorbance units as a function of concentration was obtained.\(^{53}\)

### 2.2.4 Method to determining quantum yield

The quantum yield of a compound is the rate of emissive processes compared to the rate of absorbed processes, or simply the number of photons emitted versus the number of photons absorbed. Majority of quantum yield determinations utilize the photon counting method or the comparative method. The comparative method is a simplistic approach that utilizes a standard compound with a known quantum yield with an absorbance and emission maximum wavelength
(\lambda_{\text{max}} \text{ and } E_{\text{max}} \text{ respectively}) near that of the unknown compound. The determination of the unknown quantum yield can be found according to:

\[
\phi_S = \phi_{STD} \left( \frac{\text{slopes}}{\text{slopes}_{STD}} \right) \left( \frac{\eta_S^2}{\eta_{STD}^2} \right) \tag{8}
\]

where \( \phi_S \) and \( \phi_{STD} \) are the quantum yields of the unknown and standard compound respectively and \( \eta \) is the index of refraction of the respective solvents. It is essential to have the relatively identical refractive index or same solvent for both the standard and unknown to have a more precise estimate.

### 2.2.4.1 Fluorescence quantum yield sample preparation

Serial dilutions of each dye for all absorbance measurements were prepared in DMSO at concentrations ranging from 1.0–6.0 µM and all fluorescence measurements were taken at concentrations diluted 10-fold from the absorbance concentration values. Suitable concentrations for each dilution were determined based on instrumental limits for both absorbance and emission measurements, wherein measured absorbance values did not exceed 1.0 absorbance unit and absorbance values for fluorescence samples were kept below 0.10 units to avoid self quenching. The comparative method was used for the determination of all quantum yield values as given in equation above, wherein the slope of the line obtained from the graph of the emission peak area as a function of absorbance units for each dye was used.

### 2.2.5 Protonation Studies

Determining the acid dissociation constants (pK\(_a\)) can be achieved by many different options. These include nuclear magnetic resonance spectroscopy, acid-base titration, amperometric titrations, fluorescence spectroscopy, and UV-visible spectroscopy. Amperometric titration is the type of titration that is used to find the equivalence point with
electrical current. The traditional method, acid-base titration, will be conducted to find the pKₐ’s of the mono- and di- acid pentamethine cyanine dyes. Knowing that these dyes are water-insoluble, 1 mL DMSO was added to each dye and then introduced to acetate buffer pH 4.5. A standardized solution of 0.2M NaOH was used as the titrant. Graphical representation of pH vs volume titrant added can aid finding the equivalence point. This point can be used to find the pKₐ of the pentamethine dyes by finding the total amount of titrant at the point. At ½ equivalence point, you will also have half the titrant. From this intersection at the ½ equivalence point, you can measure the pH that will correlate with the dye’s pKₐ.

2.3 Results and Discussion

2.3.1 Molar Absorptivities and Quantum Yields

The molar absorptivity of the pentamethine cyanine dyes was determined in DMSO. The dye’s behaved as expected with Beer’s Law as there was a linear increase in absorbance as the concentration of dye was increased but only if the absorbance is under 1.0 as seen in Figure 2.2. It was indicated that the Molar Absorptivity (ε) value for T-46 was 7.9E+04 M⁻¹cm⁻¹ at 650 nm as seen in Figure 2.3.

![Figure 2.2: Absorbance as a function of wavelength of T-46 from 0-6 μM](image-url)
Figure 2.3: Absorbance as a function of T-46 concentration

The quantum yield was calculated with Equation 8 above. Rhodamine B was used as a standard for all the pentamethine cyanine dyes. This is the simplest method of determining quantum yields and is relatively straightforward, as the quantum yield for Rhodamine B is widely known in many solvents. Solvents have different refractive indexes that can change the quantum yield and molar absorptivities of the fluorophore. The equation can correct for the various refractive indexes for quantum yield determination but can also cause greater error in the analysis. It is safe to run all the following standards and samples in the same solvent to avoid issues. The fluorescence experiment was a 10-fold dilution from the absorbance samples to make analysis more easily achievable. As you can see in Figure 2.4 and 2.5, the fluorescence intensity also shows a linear increase. Figure 2.6 indicates the two fluorescence intensities as a function of absorbance. This is used to determine quantum yield for two separate experimental runs. The average quantum yield ($\phi$) for T-46 was determined to be 0.15. The following Figures (2.6-2.17) display graphical representation analysis for Molar Absorptivites and Quantum Yields for dyes T-45, T-50, T-55, T-62, T-85, and T-87.
Figure 2.4: Fluorescence Intensity as a function of wavelength of T-46 from 0-0.6 µM

Figure 2.5: Fluorescence Intensity as a function of Absorbance of T-46
Figure 2.6: Absorbance as a function of T-45 concentration

Figure 2.7: Fluorescence Intensity as a function of Absorbance of T-45
Figure 2.8: Absorbance as a function of T-50 concentration

Figure 2.9: Fluorescence Intensity as a function of Absorbance of T-50
Figure 2.10: Absorbance as a function of T-55 concentration

Figure 2.11: Fluorescence Intensity as a function of Absorbance of T-55
Figure 2.12: Absorbance as a function of T-62 concentration

Figure 2.13: Fluorescence Intensity as a function of Absorbance of T-62
Figure 2.14: Absorbance as a function of T-85 concentration

Figure 2.15: Fluorescence Intensity as a function of Absorbance of T-85
**Figure 2.16:** Absorbance as a function of T-87 concentration

- Equation: \( y = 374030x - 0.0426 \)
- \( R^2 = 0.99799 \)

**Figure 2.17:** Fluorescence Intensity as a function of Absorbance of T-87

- Equation 1: \( y = 13725x + 534.16 \)
  - \( R^2 = 0.99593 \)
- Equation 2: \( y = 15434x + 542.7 \)
  - \( R^2 = 0.99288 \)
2.3.2 \( pK_a \) Determinations

All of the examined pentamethine cyanine dyes have either one or two \( H^+ \) that can dissociate from the carboxyl group on their terminal alkanes. These carboxyl groups are not conjugated to the system and will not affect the outcome on the absorbance or fluorescence. If these carboxyl groups were part of the conjugated system, it may cause a shift in the absorbance and fluorescence peaks. It is also known that oxidizing or reducing a fluorophore can cause the system to activate or deactivate fluorescence. In Figure 2.18, the blue line represents the amount of titrant added corresponding to the \( \text{pH} \) meter calculating the \( [H^+] \). The \( H^+ \) concentration is lowered due to the base added. The \( H^+ \) on the carboxyl group is more readily to dissociate when \( [H^+] \) is lowered. The spike in \( \text{pH} \) indicates the dissociation of the \( H^+ \) from the carboxyl group and the equivalence can be obtained. The equivalence point is the middle region of the increased spike in \( \text{pH} \). The red line indicates the first derivative taken from the data collected during the titration. The first derivative is graphical representation of \( \Delta \text{pH}/\Delta \text{Volume} \) vs Volume. The highest point corresponds to the maximum value of titrant added. Remember, that the \( \frac{1}{2} \) equivalence point is equal to where the \( \frac{1}{2} \) titrant volume corresponds. The end result is

<table>
<thead>
<tr>
<th>Dye</th>
<th>( \lambda_{\text{ex}} ) max (nm)</th>
<th>( \lambda_{\text{em}} ) max (nm)</th>
<th>Stokes' Shift (nm)</th>
<th>( \varepsilon ) (M(^{-1})cm(^{-1}))</th>
<th>( \phi )</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-45</td>
<td>649</td>
<td>665</td>
<td>16</td>
<td>7.31E+04</td>
<td>0.54</td>
</tr>
<tr>
<td>T-46</td>
<td>650</td>
<td>663</td>
<td>13</td>
<td>8.10E+04</td>
<td>0.15</td>
</tr>
<tr>
<td>T-50</td>
<td>683</td>
<td>696</td>
<td>13</td>
<td>4.97E+04</td>
<td>0.05</td>
</tr>
<tr>
<td>T-55</td>
<td>687</td>
<td>699</td>
<td>13</td>
<td>2.64E+05</td>
<td>0.09</td>
</tr>
<tr>
<td>T-62</td>
<td>652</td>
<td>668</td>
<td>16</td>
<td>2.20E+05</td>
<td>0.93</td>
</tr>
<tr>
<td>T-85</td>
<td>653</td>
<td>665</td>
<td>12</td>
<td>5.99E+04</td>
<td>0.42</td>
</tr>
<tr>
<td>T-87</td>
<td>691</td>
<td>701</td>
<td>10</td>
<td>3.68E+04</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 2.1: Average Molar Absorptivities and Quantum Yields of Pentamethine Dyes
indicating the pH at the ½ equivalence point and that will theoretically designate your pKₐ for your dye.

![Figure 2.18: Acid-Base Titration of T-45 in Acetate Buffer pH 4.5](image)

The second derivative was taken on a separate analysis to hopefully see two peaks. Two peaks will be implying two dissociations of H⁺, which is assumed in di-acid compounds. Dyes T-50, T-55, T-62, T-85, and T-87 all have the ability to dissociate two H⁺ ions. While conducting this experiment in triplicate to provide a more thorough analysis, no second peak was indicated when taking the second derivative. It is assumed that the dissociation constants for the two H⁺ ions are very similar.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Average pKₐ</th>
<th>Standard Deviation</th>
<th>Relative Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-45</td>
<td>5.09</td>
<td>0.02</td>
<td>0.40</td>
</tr>
<tr>
<td>T-46</td>
<td>5.08</td>
<td>0.02</td>
<td>0.36</td>
</tr>
<tr>
<td>T-50</td>
<td>5.07</td>
<td>0.02</td>
<td>0.30</td>
</tr>
<tr>
<td>T-55</td>
<td>5.11</td>
<td>0.02</td>
<td>0.31</td>
</tr>
<tr>
<td>T-62</td>
<td>5.14</td>
<td>0.01</td>
<td>0.23</td>
</tr>
<tr>
<td>T-85</td>
<td>5.17</td>
<td>0.04</td>
<td>0.84</td>
</tr>
<tr>
<td>T-87</td>
<td>5.17</td>
<td>0.20</td>
<td>3.81</td>
</tr>
</tbody>
</table>
2.4 Conclusion

The pentamethine cyanine mono- or di- acids were characterized by determining their molar absorptivities, quantum yields, and pK\textsubscript{a}’s. These dyes will be used for further spectroscopic and imaging studies by encapsulating into silica nanoparticles. These dyes are novel for fluorescence-type imaging and spectroscopic analysis due to the minimum fluorescence interference within biological systems. Also while being encapsulated, dye leakage will be minimized and will also minimize carcinogenic effects of dyes. Most dyes are known for being carcinogenic and having an alternate method to reduce tissue exposure would be essential for future imaging. These highly modifiable NIR nanoparticles can also be used for limitless bioanalytical assays with reduced background interference and reduced harmful exposure.
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