Tuning Upconversion Nanoparticles for Enhanced Photodynamic Therapy

Worcester Polytechnic Institute • Major Qualifying Project

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Abstract:

PDT is an emerging cancer treatment that combines both light and a photosensitizing agent to produce reactive oxygen in cells. Although upconversion nanoparticles (UCNPs) are attracting much attention for their potential biological application, there are still several drawbacks that limit its general clinical use such as limited tissue penetration depth and sever overheating effect. Consequently, there has been extensive nanoparticle surface modification research into the design of improved alternative photosensitizers aimed at overcoming these drawbacks. Therefore, the goal of this project was to enhance the surface modification of UCNP to demonstrate high upconversion luminescence efficiency, as well as have hydrophilic surface characteristics that are compatible with biomolecule. Thus, based on recommendations from the previous MQP research group, team conduct repeating experiments in alternative UCNPs with different laser power density (we used 800nm whereas the previous MQP used 980nm) and address the possible over heating concerns through in vitro tests by enhancing tissue penetration depth and optimize singlet oxygen production to kill cancer cell.

Key Words

ALA:	5-aminolevulinic acid
CW:	Continuous Wave
DI water:	Deionized water
EDC:	1-Ethyl-3-(3-dimethylaminopropyl)-carbodimide
FDA:	U.S. Food and Drug Administration
Hyd:	Hydrazine
Ln:	Lanthanide
MTT:	Methylthiazol Tetrazolium
NaYF4:	Sodium Yttrium Fluoride
Nd:	Neodymium
NIR:	Near Infra-Red
$NOBF_4$:	Nitrosonium tetrafluoroborate
PAA:	Polyacrylic acid
PBS:	Phosphate Buffer Saline
PDT:	Photodynamic Therapy
PpIX:	Protoporphyrin IX
PS:	Photosensitizer
ROS:	Reactive Oxygen Species
TEM:	Transmission Electronic Microscopy
Tm:	Thulium
UC:	Upconversion
UCNP:	Upconversion Nanoparticle
Yb^{3+}:	Ytterbium
$1O_2$:	Singlet Oxygen

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Chapter 1: Introduction

Cancer is the fifth leading cause of death in the United States. In 2014, approximately 1,665,000 new cancer cases and 585,000 cancer deaths are projected to occur in the U.S. alone (Siegel, 2014). Several treatment options include radiotherapy, chemotherapy, and surgery. These treatments subject the patient to harmful side effects due to the damage of healthy cells and may lead to another cancer in later life. Photodynamic therapy (PDT) has been investigated as a technique that combines light energy and drug therapy to actively target and monitor diseased tissue. PDT can consider as medical miniature tools by combining a systematic administration of a nontoxic photosensitizing (PS) drugs along with light to kill cancer cells. Over a certain amount of time the drug selectively accumulated in the cancer cells, then applying specific wavelength of light causes the drug to react with oxygen destroy the tumor cell. Since PS alone is nontoxic and ineffective so typical PDT treatments involve three components: the photosensitizer, oxygen and light source within the tissue (Zhang, 2014).

Lanthanide doped upconversion nanoparticles (UCNPs) are the luminescent trivalent ions which are able to convert low energy light to high energy light. These particles can be used in PDT to replace or to assist traditional organic dyes and ultra violet (UV) phosphors, because they are optically active to produce emission when excited. UCNPs-PDT is well suited for use in theranostics that can consequently increase patient survival, since it has a unique form of nonionizing radiation therapy that administered via light-absorbing substance, and has zero autofluorescence background to adjust the signal-to-noise ratio. In addition, UCNP's have great potential to contribute to deeper tissue penetration of nanoparticle excitation wavelengths and increased emission intensity and reduced light scattering, because UCNPs are nonbinding, and less

light scattering. Thus, lanthanide-doped UCNPs have attracted our attention to work on a team of students, academic advisor from WPI, and physician from UMASS Medical School to design and test UCNPs for PDT to maximize light penetration through biological tissue.

Lanthanide UCNPs refers to an anti-stokes type process that convert sequential absorption of NIR light to visible/UV light. Dr. Han's lab has patented a variety of novel nanoparticles for PDT with excitation peaks at both 800nm and 980nm. Because these particles must be synthesized in organic solvents, their surfaces contain toxic ligands that limit their use in cell therapy and drug delivery. Through functional group conjugation, we are able to replace toxic ligands with a hydrophilic coating that is coupled to 5-amino levulinic acid (5-ALA). 5-ALA is a precursor in the biosynthesis of protoporphyrin IX (PpIX) which is a photo excitable porphyrin with an excitation peak of 470nm. By delivering exogenous 5-ALA to cancer cells in vitro, the Han lab has demonstrated the ability to stimulate PpIX production and finally leads to formation of heme. After PpIX accumulates in the cell, the UCNP can be excited and converts the 800nm NIR light to a 470nm wavelength UV emission, which then activates PpIX. A red light emission at 670nm from the PpIX molecule is then responsible for the inducing a higher energy state to cellular triplet oxygen, which catalyzes the formation of singlet oxygen or reactive oxygen species (ROS). This excited state of the oxygen molecule then begins to denature cellular proteins and results in cell death.

Clinical applications of UCNP's have been limited due to the negative heating effects of high-powered lasers and the limited tissue penetration of low-powered lasers. Our work aims to investigate the heating effects and tissue penetration depth of the 800nm laser in comparison to the 980nm laser at FDA regulated 0.7 Watts/cm^2 power density for effective biological use. As a

number of studies have focused on tuning upconversion emission using core @shell architectures with two or more Ln^{3+} activators are best solution to regulate heating effects and apply for biological application. One major limitation of conventional UCNPs is the low conversion efficiency especially under a low-intensity laser excitation due to extremely weak absorption of sensitizer Yb^{3+} ion as well as having very narrow NIR absorption spectral window that cause serious overheating damage (Zhang, 2014). Therefore, we aimed to enhance the surface modifications of our particle by investigating alternative conjugation and/or drug delivery vehicles. We hypothesized that surface modifications of ALA can improve its cellular permeability, increased stability in physiologic pH and increased selectivity and limitation of side effects. The current process of hydrazine coating and ALA conjugation decreases luminescent intensity and increases particle size. Increased size makes targeted cellular uptake more challenging because smaller particles permeate cell membranes more easily. We tested the effectiveness of our UCNP delivery in vitro using HELA cells and an MTT assay to determine the effectiveness of our particle to cells. Pork tissue was used to test the heating effects of the 800nm laser compared to the 980nm laser.

Chapter 2: Background

Unlike normal cell growth and death, cancer cells continue to grow and form new abnormal cells that invade or grow into other tissues. In the cancer cells the damage DNA is neither repaired nor causes cell death like it should, therefore, the cell goes on making new cells. Cancer has many possible causes and ability to travel to other parts even get into the body's bloodstream or lymph vessels of the body where they can grow and form new tumors spreading is called metastasis. Although not all tumors are cancer (Unlike malignant tumors, benign tumors are not cancerous and they don't spread to the other part of the body), different types of cancer can behave very different rate and respond to different treatments. That is why people with cancer needs special attention and treatment to cure their kind of cancer.

Cancer is a 2nd most prevalent cause of death for all ages and, in 2010 was the leading cause of death for the people ages 40 – 79. According to Siegel, et al, in 2014 there were 1.6 million new cases of cancer and 580,000 deaths in United States (U.S.), which figures out to 1600 deaths per day (Siegel, 2014).. These risk estimates the lifetime risk of developing and dying from cancer that measure of how widespread cancer is in the U.S.

Cancer treatment is challenging because it is difficult to remove cancerous tissues without damaging healthy cells. There are various types of cancer treatments such as: chemotherapy, radiation therapy, or surgery. In chemotherapy, various chemicals are used to kill the targeted cancer cells using anti-cancer drugs, when injected into vein/bloodstream the drug travels to reach and destroy the cancer cells. In radiation therapy, high-energy radiation is used to kill the cancer cells by damaging their DNA. There are multiple transplantation surgeries used to help treat patients such as: bone marrow transplantation, peripheral blood stem cell transplantation and

childhood hematopoietic cell transplantation. However, because it uses high energy X-ray or particles to kill cancer cells and cancers don't usually have discrete tumors size or behavior, it is difficult to avoid side effects that include nausea, fatigue, and hair loss, vomiting, diarrhea, and loss of appetite when the radiation enters the body.

There are a lot of other side effects of chemotherapy and radiation such as: dental problems, fatigue, diabetes, endocrine problems, hypothyroidism, incontinence, infertility, memory problems, learning disabilities, lymphedema, neuropathy, organ damage, osteoporosis, general pain, premature aging effects, and sexual problems (MD Anderson Cancer Center, 2014).

There is another treatment for cancer which is known as Photodynamic Therapy (PDT) on which our project focused on. PDT is considered to be minimally invasive when compared with chemotherapy and radiotherapy because it allows for better detection and eradication of diseased tissue without harming healthy cells (Dai et al., 2012) since it uses special photosensitizer agents that act as drugs along with certain kind of light to kill cancer cells. Unlike radiotherapy, PDT is a form of nonionizing radiation therapy and it can be repeated many times at the same site with only short time of treatment. However; there are some limitation to this treatment as well such as it can only treat cancer up to 12mm of tissue depth and has higher heating effects which causes skin burning.

2.1. Photodynamic Therapy in Medicine

Photodynamic therapy (PDT) is a treatment that uses a drug called a photosensitizing agent or photosensitizer along with a particular light energy, which can target cancer cells and kill them (American Cancer Society, 2013). Photodynamic therapy has significant advantages compared to current treatments because it has no long-term side effects if use properly (Dai et al., 2012). It is

less invasive than surgery, it is easy to use, it can be targeted very precisely and it often costs less than many cancer treatments. Depending on the treatment area the photosensitizing agents are injected interstitially, intravenously, inhaled, or topically applied. Once the upconversion nanoparticle are injected, they can take 24-72 drug-to-interval hours (which is period of time between the drug given time to the light is applied) as shown in figure 1.

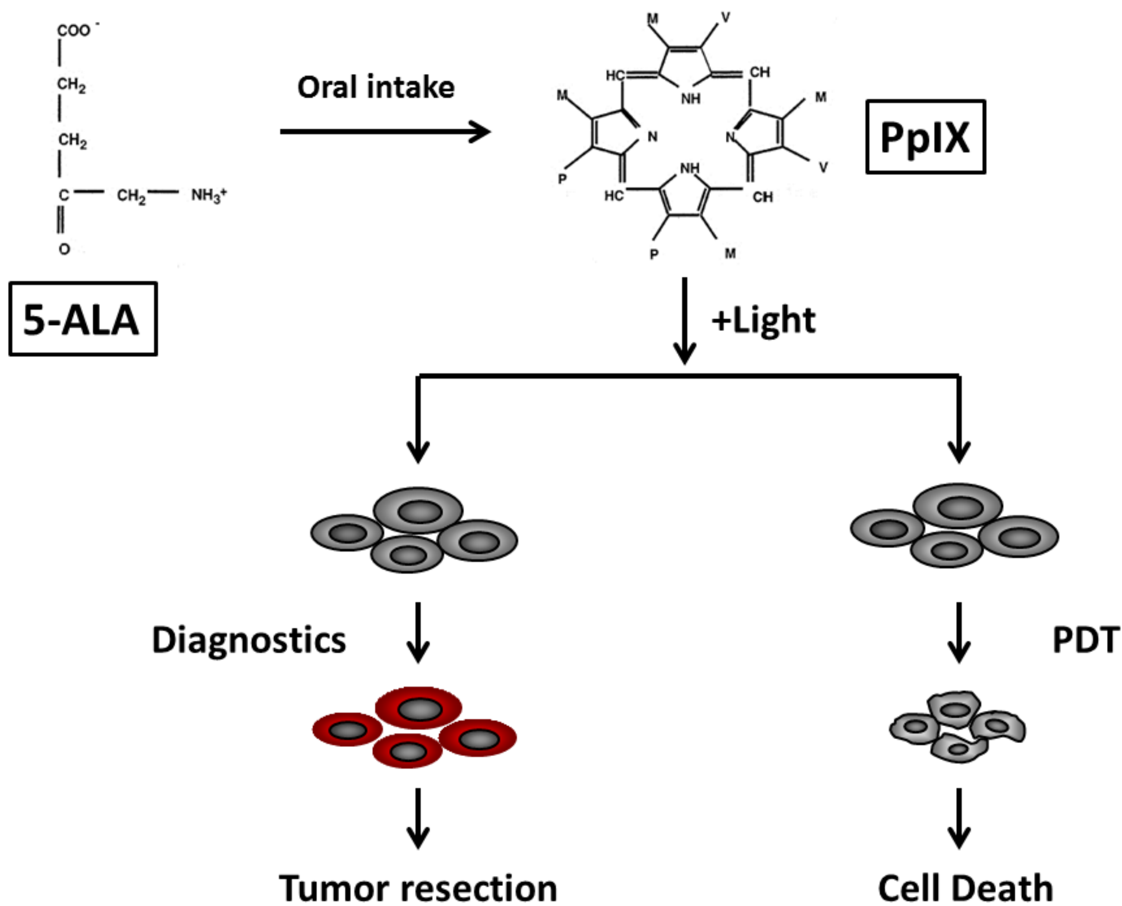

Figure 1: General Schematic of PDT mechanism

The use of NIR light excitation, and utilizes photo triggered chemical drugs (photosensitizes) to produce singlet oxygen ($1O_2$) to kill tumors. The successful PDT incorporates three main components that includes the photosensitizer, the light source, and the oxygen within

the tissue at the diseases site, killing the tumor cells. Photosensitizer is injected to cancer patient and NIR light is shine on it which covert ordinary oxygen to aggressive toxic singlet oxygen. Singlet oxygen is extremely toxic to cells but it is so reactive to that it burn itself almost insistently and only damage near spots where it was generated which is why this treatment is has less damaging to healthy tissues.

Even though PDT has shown great potential, it still has limitations as well. The main limitations for this application are tissue penetration depth of 1 millimeter which can only treat skin surface cancer due to lack of PpIX accumulation that reduce damage of deeper layers. There are some side effects to this treatment as well, such as severe toxicity kills the healthy tissues around tumor cells. Patients are also advised to not go under bright light for at least a week. Although there are some limitations to this treatment, it still has advantages over treatments such as chemotherapy, radiology, and surgery, which are expensive and not ideal for every cancer disease.

2.2. Nanoparticles in Medicine

As Chen et al. defined how to develop high-quality lanthanide-doped luminescent nanoparticles with adjustable crystalline phase, shape, and size is crucial for variety of potential medical applications such as drug delivery for cancer treatment and molecular imaging (Chen, 2014). Upconversion nanonparticle (UCNPs) have emerged recently as a type of nanoparticles. They are combination of doped-lanthanide elements. Continuous absorption of multiple photons on the lanthanide ions assist for effective energy transfer to facilitate photochemical reactions that help for advance drug delivery. They can absorb near infrared light (NIR) and convert this low energy light into high energy light and emit ultra violet light (UV) visible light. The mechanism

of the energy transfers within lanthanide UCNPs process is an important process for PDT since photosensitizer alone is nontoxic and ineffective.

There are various nanochemistry research to produce upconversion nanoparticles (UCNPs) in highly control manner such as hierarchical core/shell UCNPs, thermolysis strategy, Ostwald-ripening method, and hydro (solvo) thermal strategy. Our project focuses on the novel method of the core/shell UCNPs that can control the composition of phase and size. It is believed that the diameter of nanoparticles for cancer therapeutics should be in the range of 10 – 100 nm (Commission, E., 2006)). The nanoparticles should be accessible to and disseminated within tumors in order to be effective to kill tumors. It is possible to inject particles directly into tumors. However, particles may still be forced from cancerous tissue and enter circulation where they tend to accumulate in the liver (Duncan et al., 2010).

As PDT there is another application of nanoparticles in medical research for early cancer detection, screening, and use for therapeutic purposes for life-threatening diseases called Photoluminescence imaging (PL). High contrast cellar imaging using the engineered UCNPs can increase the saturation threshold to accomplish cell imaging with both high resolution and high contrast. The optical/magnetic nanoparticles application key factor in biomedical imaging is the particle size. For this application, the emission intensity and size of the nanoparticle are mutually dependent parameters, and in general, weak emission resulted from a smaller nanoparticle size (Wang, 2013). Wang et al. indicated that the surface modification of nanoparticles enable the cellular uptake efficiency more that could be used to image variety of cell lines through cellular endocytosis way in HELA cells (Wang, 2013). Hence, nanoparticles can be characterized by size, shape, and chemical composition.

The surface volume ratio of nanoparticles increase by reducing the size of the nanoparticle. Reduced size has been shown most effective for use in drug delivery (Wang, 2013). The chemical composition of the nanoparticle is also important and can affect the toxicity and cellular targeting. The reactivity of nanoparticles can be affected by the chemical absorption on the surface of nanoparticle. Shape is another factor that has to be considered to reduce the toxicity and enhance targeting. For example, nanotubes a few nanometers in diameter but with several micrometers length can leave the carcinogenic effects. Thus, the nanochemistry for controlled synthesis helps for PL imaging to demonstrate various animal imaging, testing the biocompatibility behavior of UCNPs as well as for theranostic applications.

Depending on the part of the body being treated or people with certain types of cancer, the photosensitizer agent is directly injected into the bloodstream of the vein and light is applied to the area that needs to be treated. This light cause the reaction to kill cancer cells. This is known as PDT and it can be used in people with certain types of cancer to help them live longer and improve their quality of life. Although several photosensitizing agents are currently approved by the US Food and Drug Administration (FDA) to treat cancers and studies have shown that PDT can work better than surgery or radiation therapy, however; it has few limitations. As American Cancer Society specified, the main limitation is, it can’t be used to treat malignant cancers, special precautions must be taken after the drugs are put in, because it can lead swelling and other possible side effects.

2.3 UCNP Structure

UCNP-based therapeutic applications have shown promising results in drug delivery and PDT through increasing the lipophilicity. The advantage of modification ALA-PDT improve

cellular permeability, increased stability pH concentration, increased selectivity of UCNP-photosensitizer into tumor, and enhance the rate of their enzymatic conversion into photoactive compounds (Malgorzata et al. 2013) Similarly, the Han lab has developed a novel class of cascade sensitized, lanthnide-doped, core-shell, 980nm excitation wavelength UCNPs for use in PDT, however; they had limitation of deep tissue penetration and higher heating effects. Then Han lab developed a novel class of cascade sensitized, lanthnide-doped, core-shell-shell, 800nm excitation wavelength USNPs for use in PDT. These particles use neodymium (Nd) as a photonnsensitizer, ytterbium (Yb) as a bridging ion, and thulium (Tm) as an activating ion. Fusing these lanthanide ions within a dielectric lattice, the Han lab has been able to tailor the emission wavelength of their novel UCNPs to overlap the absorption spectra of naturally occurring photosensitizer PpIX. This allows for luminescence upon laser excitation as well as activation of radical oxygen species (ROS) within cells. Changing the concentration a neodymium allows for emission wavelength tunability. Dr. Han's lab has studied the luminescent efficiency of various neodymium concentrations in order to optimize luminescent intensity that overlaps the emission spectra of PpIX (Shen et al., 2013). By delivering nanoparticles directly to the cancerous cells, the localized UV emission necessary to activate PpIX and catalyze singlet oxygen production can be induced noninvasively using a cheap NIR laser, which has minimal heating effects and damage to surrounding healthy tissue. The optimal transparency window for biological tissue penetration is spectral range of 750 – 1000 nm (Yang et al., 2014). After conducting research the team decided to experiment 800nm UCNPs.

2.4 Function

In order to accomplish effective drug delivery and enhance photodynamic therapy, the particle must be multifunctional. The specific functions of a successful product include intense

luminescence, targeted delivery, and significant PpIX and ROS production. Luminescence intensity is engineered by changing ion and ligand concentrations in a homogenous or heterogeneous core/shell/shell structure. We aim to utilize a heterogeneous UCNP core and fluoride based dielectric lattice. Surface engineering is necessary to remove toxic ligands and incorporate biocompatible targeting mechanisms in order to enhance drug delivery. ALA is a precursor to PpIX and has been shown to stimulate PpIX production, particularly in cancer cells. By increasing conjugation density of ALA to the surface of our particles, we are able to enhance PpIX production, resulting in intense luminescence and increased ROS production (Chen et al., 2014).

It has been shown that cancer cells express a variety of unique signaling mechanisms. The ALA uptake mechanisms of various cancer cells have been studied using ALA derivatives and esters (Rodriguez et al., 2006). ALA is taken up by Beta transport mechanisms such as Gat-2 and Gat-3 (Rodriguez et al., 2006). Different modifications to the ALA molecule result in different uptake mechanisms (Rodriguez et al., 2006).

2.4.1 Polymer Chemistry/Ligand Exchange

Lanthanide based UCNP's are hydrophobic and can dissolve in the nonpolar solvents but not in biological buffer or aqueous solution, which limits their applications for PDT (Wang, Cheng, & Liu, 2013). It is necessary to change the surface of UCNP's from hydrophobic to hydrophilic, making them more water-soluble so they can be used in bio-analytical applications. The surface of the nanoparticles can be modified using a ligand exchange method. In this method the replacement of hydrophobic ligands occurs with hydrophilic ligand without significantly affecting the chemical and optical properties of UCNPs (Chen et al., 2014). After becoming hydrophilic, the

polymers become more stable, avoiding aggregation and precipitation during application. One way to make the nanoparticle hydrophilic is the conjugation of hydrazine. Hydrazine is hydrophilic, inorganic compound, stable in solution and carries a positive charge.

2.4.2 Protoporphyrin IX (PPIX)

Protoporphyrin IX (PpIX) is produced in cells via the heme synthesis pathway from the substrate of ALA. In addition to being able to target the UCNPs ferrochelatase converts the PpIX into heme. Ferrochelatase is typically overexpressed in cancer cells. The heme synthesis pathway is usually under tight control with feedback inhibition by heme controlling the synthesis of ALA. PpIX is commonly used for photodynamic therapy, and for tumor detection because it can emit light energy to produce singlet oxygen, which kills the cancer cells. PpIX is known to be an excellent photosensitizer because of its minimal photo-damage effects. ALA is an FDA approved photosensitizer and ALA-derived PpIX methods have been clinically used in PDT.

After conducting the research we decide to use 800nm excitation laser which emits blue light and has shorter wavelength and higher frequency. The purpose of using blue light is that it has is brighter emission and produce more PpIX than red light which will kill more cancer cells.

Chapter 3: Objectives

In this chapter we will discuss our goals for this project and how we plan to target each element. Last year MQP team were able to made a core-shell UCNPs particle to make PDT better. The goal of their project was to use red visible light using 980nm laser to activate ALA to produce singlet oxygen. There particle enhanced light penetration of 1.2cm in pork tissue and was great to use in photodynamic therapy. However; they had limitation of higher heating effects, less emission. After conducting research on PDT, UCNPs and pervious MQP we discovered that there is need for enhanced treatment for PDT using UCNPs. Thus, our goal was to investigate 800nm laser which has brighter emission and investigate heating effects of 800nm and 980nm LED lasers at the clinically approved power density of 0.7 watt/cm^2 has less heating effects.

3.1 Initial Client Statement

Following is the initial statement was provided by our client.

> *"Investigate coating mechanisms that will increase the solubility and tissue penetration depth of hydrophobic UCNP's while maintaining high upconversion (UC) efficiency and minimizing surface related deactivations in order to utilize UCNP's for photodynamic therapy (PDT). Specifically, to kill HeLa cells, in vitro, using a visible or wavelength UCNP core and photosensitizer doped shell structure which absorbs emitted photon energy to activate the formation of reactive oxygen species (ROS) in surrounding cells resulting in apoptosis."*

Afterward more research was conducted on PDT and UCNPs as well as meetings were arranged with clients to better understand the need and expectation from our client.

3.2 Objectives

After conducting research team discovered a lot of information regarding UCNPs and PDT which then was used to lay out the objectives of in a list based on current limitation with 980nm UCNPs. After analyzing the client statement and researching UCNPs objectives were made as shown below.

- Brighter blue light
    - More than $5*10^7$ A.U
    - Brighter emission than red light
- Particle size
    - Diameter between 30-50nm
    - After conjugation diameter between 35- 70nm
- Safety and biocompatibility
    - Cellular survival rate before radiation treatment
    - Reduce laser heating effects 800nm vs. 980nm
- Enhanced MTT assay
    - Cell viability of >50%

After organizing the list of objectives they were broken down into detail objectives. We wanted our blue light to be produce brighter emission $>5*10^7$ A.U because brighter emission will have higher wavelength. Diameter of UCNPs particle size should be under 30-50nm and after conjugation it should be between 35-70nm because as research shows cancer cells can uptake those cells easily. Have less heating effects than 980nm using FDA approved density for patient safety. Finally, cell killing above 50% which will prove our project is success.

3.3 Final Client Statement

After having meetings with client we came up with this final client statement as shown below.

> *"Investigate the heating effects of the 800 and 980nm LED lasers used to activate lanthanide doped UCNP's for photodynamic therapy (PDT). Specifically, study the cell viability and free radical production of HeLa cells, in vitro, using NIR-to-UV UCNPs which activate PpIX to catalyze the formation of reactive oxygen species (ROS) in surrounding cells resulting in cell death."*

3.4 Project Strategy

After final client statement our project goals were clear since we had limited budget we prioritized biocompatibility as an important goal. Our project will utilize blue light emission (470nm) from the UCNP to activate the photosensitizer PpIX; thus, the system must efficiently produce a bright, effective blue light in the presence of PpIX. Bodily tissue has an optimal transparency window of 700 – 1100 nm. Since 470nm does not fall within the optimal transparency window we must use a UCNP that enables the deeper penetrating 800nm or 980nm light to be converted to the 470nm wavelength necessary to activate PpIX, which catalyzes oxygen radicals. The stability of the UCNP system will be considered with respect to consistent hydrazine coating and ALA conjugation density, which also contribute to cell uptake and ROS production. To check the size and consistency of UCNPs Transmission electron microscopy (TEM) will conducted and to check the cell viability team will conduct MTT assay test. Finally, the 800nm excitation is hypothesized to reduce the heating effects on surrounding tissue compared to the 980 nm excitation wavelengths for which laser heating effects will be experimented.

Chapter 4: Alternative Design

4.1 Requirement Analysis:

The ability to fine tune UCNP material properties is what makes them an attractive area of research for photodynamic therapy. The ability to alter nanoparticle chemical and optical properties is vast but is also limited. Certain design changes can have negative side effects and research is necessary to determine the dependence between variables like size, shape, optical properties, and toxicity. We are focusing on the laser heating side effects and particle toxicity and their dependence upon size and optical properties. We are then concerned with optical properties and their dependence on particle composition and lattice structure. Particle composition is again, a vast area of interest but our research is limited to a select combination of lanthanide doped UCNPs. The structure is dependent upon the material used which is limited to a dielectric fluoride host lattice.

As mentioned in earlier chapter, it is proven by previous MQP group that 980nm excited UCNP can be used for PDT but they have few limitations. The major limitation of the most commonly used Yb^{3+} -sensitized UCNPs are physically fixed excitation band at 980 nm that cause maximum absorption peak of water molecules and increase the heating effects. The purpose of our project was to come up with the 800nm UCNP which can decrease the heating effect and enhanced the cell viability. In order to make 800nm UCNP effective for PDT by overcoming the limitations, it was necessary for the team to do research that how 800nm UCNP can be conjugated to enhance the brightness of the particle, lowering heating effects and enhancing cell viability.

There are a variety of molecules within the human body that are sensitive to light and the UV photons can manipulate the functions of biomolecules for onsite drug release via effective

photo activation. The pro-drug stimulation and photo-excitation of PpIX at 470nm has been FDA approved for the treatment of pre-cancerous lesions of the face and neck. Research into using up-conversion nanoparticles as transducers to convert the deep tissue penetrating NIR light to UV photocatalyst is necessary to determine if this photodynamic therapy is effective in terms of possible negative heating effects of the laser along with nanoparticle toxicity to cells. Even though NIR-UCNPs has vital role for the biomedical research with excellent light penetration depth, it requires that precise light control and sufficient energy activated photosensitizer can lose its energy by emitting fluorescence as well as generate ${}_1O^2$.

Molecules within human tissue absorb, reflect, and trap light energy causing it to be reflected or scattered at the skin surface which can be used for photodynamic therapy. In order to enhance the effectiveness of this mechanism we must better understand the optimal wavelength for tissue penetration with limited side effects such as unwanted heat, pain, or collateral tissue damage. To properly evaluate, it was essential for the team to perform research on Dr. Han's novel nanoparticles compositions and related methods for surface modification that enable the upconverting luminescence to optimize the efficient excitation of 800 nm. One of the advantage of working on this sensitized tri-doped UCNP is a biocompatible activation and emission wavelengths that can be altered based on the molar composition while the particles brightness can be controlled through epitaxial growth and allotropic phase transfer. Both of these changes is a single crystal soft film coating applications as semiconductor and the element structure are bonded together in a different manner. Therefore, if we can better understand the tissue material properties and photodynamics, then we are one step closer to achieving deep tissue disease treatment via the combination of two inactive components, visible light and a photosensitizing drug.

If we can better understand the optimal conditions for safe, optical tissue penetration, the particles can be tuned to produce a desired emission wavelength from the desired activation wavelength, such as that necessary to photocatalyze PpIX to produce reactive oxygen species. It is therefore not only critical to understand the effects of the lasers on tissue but also the effects of nanoparticle energy transduction within the cell or extracellular space. This requires an extensive knowledge of system components and the alternatives available. Material science and engineering are key to identifying the necessary changes to increase the effectiveness of the Han lab's approach to photodynamic therapy.

4.2 Alternative Designs:

Many aspects of the final design can be controlled during synthesis.

Polymorphism is physical property that can be controlled during the synthesis and refers to the two atomic lattice crystal structures provides a matrix to bring these centers into optimal positions. Synthesizing the alpha phase particle is the first step in the 2- step thermolyisis method. The alpha-to-beta phase transition is achieved by cleaning, drying, and dispersing in solvents where it is heated to 300 C and cooled at a rate of 15 C per minute. The alpha particles occupy a (BCC) cubic space lattice while the beta particles occupy a hexagonal (HCP) structure. Last year's MQP team was able to show that using an alternative CaF2 ceramic host lattice in the synthesis of alpha phase nanoparticles increases their efficiency while a NaYF4 host lattice is most suitable for efficient beta particles. There are a variety of alternative dielectric-ceramic host lattices available when considering research into nanoparticle synthesis including, LaF3, YF3, Y2O3, or LaPO4. An ideal host lattice for this application must be optically transparent, reduce lattice surface defects, and be dielectric. The beta particle has been shown to be the brighter. However, the beta

particles require more materials, result in a lower yield of end product, and have a tendency to agglomerate during synthesis, resulting in undesirable particle size.

One of the most flexible components of our nanoparticle is the molar concentration of dopant precursors. Because the HCP crystal lattice is a close-packed structure, there is no room for dopant molecules to occupy free space between the atoms so they instead occupy a variety of defects in the dielectric crystal lattice. Changing the concentration of dopant precursors lies at the heart of UCNP technology. Altering the concentration allows for the UCNPs photodynamic properties such as activation and emission wavelength, transduction efficiency, and also degree of difficulty in synthesis. For example, our particles utilize a core that is theoretically occupied by 100% dopant molecules, this characteristic makes our particles difficult to synthesize but the core is able to produce brighter blue light emission.

4.3 Final Design:

Given the variety of technical parameters involved with our nano-scale transduction mechanism, we identified each parameter and how it functions with respect to our sponsors objectives. Last year's MQP team was able to produce positive results that demonstrate substantial singlet oxygen production under 1.2cm of pork tissue using UCNPs as transducer. The goal of their project predominantly focused on to enhance the light penetration and reach deeper tissue using the UCNPs as transducer that convert 980nm NIR to red visible light and activate ALA to produce singlet oxygen. Adding a second epitaxial shell to the nanoparticles has been shown to increase luminescence intensity by decreasing emission deactivations caused by crystal surface structures limit the light penetration through tissues makes this treatment ineffective in the treatment of bulky tumors. The drawback of this approach is the increase in size associated with

the added epitaxial shell that cause light hypersensitivity and inefficiency in the management of metastatic tumors.

In addition, nanoparticles with diameters less than 50 nm have been shown to be most effective at entering the cell. Since the cellular uptake mechanisms are not well understood, we intended to compare the cell viability of the core-shell-shell particle with the core-shell particle and then, determine whether the negative effects of size would outweigh the positive effects of increased luminescence intensity on cell death. The increase in particle size also provides a larger, and more uniform surface area which could potentially serve to increase the total number of binding sights for ALA which induce PpIX accumulation.

A variety of ALA uptake mechanisms have been under research to help enhance the effectiveness of PDT. The best strategy was for coating UCNPs with polymer shells for their protection, functionalization, conjugation, and for biocompatibility (Beyazit et al.) Thus, if the final diameter of the ALA-Hyd-PAA-UCNP is less than 100nm, it could be injected directly into tumor tissue where ALA can be released within the cell or possibly in the extracellular space taken by the cell through a variety of transport mechanisms. There are multiple approaches to sub-dermal or deep tissue photodynamic therapy. As Malgorzata et al. suggested that ALA pharmacokinetics is essential to design most effective ALA-PDT to enhance its permeability through the lipid networks in the stratum corneum. One of the techniques being implemented in Dr. Hans's lab was the applications of lanthanide based up-conversion nanoparticles as photo-excitable delivery vehicles, with the FDA approved pro-drug ALA strategically conjugated to the surface.

Last year's MQP group used a core@shell (@ is a popular way to say a core shell nanostructure, and we speak @ as "at". A@B usually means that A is the core and B the shell)

particle α-$NaYF_4$:Yb80%Yb,2%Er@CaF_2 which emits red light at 980 excitation. They were able to show that increasing the Yt concentration of particles results in an increase in red emission while an increase in Nd will result in an increase in blue emission while also tailoring the excitation wavelength to 800nm. So, we used core@shell@shell UCNP in our project: $NaYbF_4$:0.5%Tm @$NaYF_4$:30%Nd @$NaYF_4$ as shown in figure 2. Our particles were synthesized in organic solvents at 300 ^{o}C using a modified two step thermolysis method which leaves hydrophobic ligands present on the particle surface. Tm nanoparticles were shown to produce UV or blue light emissions while Er particles have been shown to produce green or red particles. The excitement associated with recent nanoparticle research has contributed greatly to the variety of dopant combinations and the effects they have on and particle efficiency and resonant energy transfer or the ability to fine tune the activation or emission wavelengths of UCNPs for PDT.

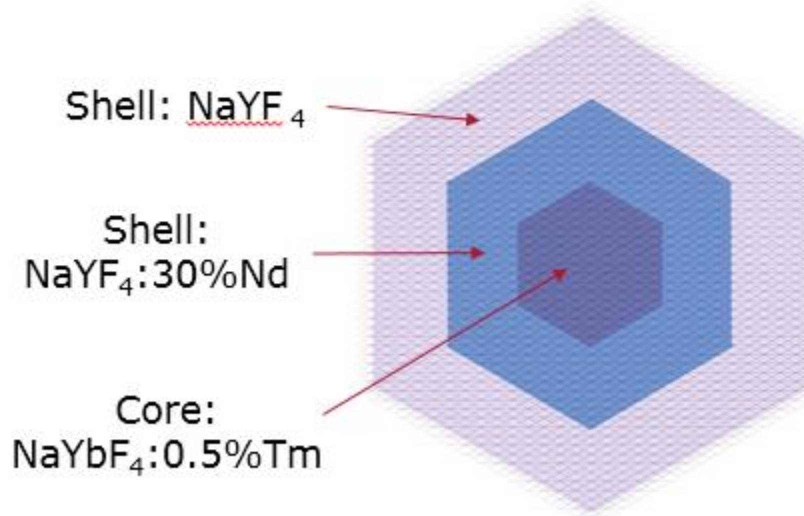

Figure 2: Upconversion Nanoparticle Diagram

We began our experiments by chemically exchanging the toxic oleic acids ligands with an optically transparent and biologically inert PAA coating. We did this by first thoroughly rinsing the particles, followed by an $NOBF_4$ treatment, and then mixing in excess PAA solutions. Then the particle were conjugated to hydrazine to make our UCNP hydrophilic. Lastly, we were able to complete ALA conjugation as shown in figure 3.

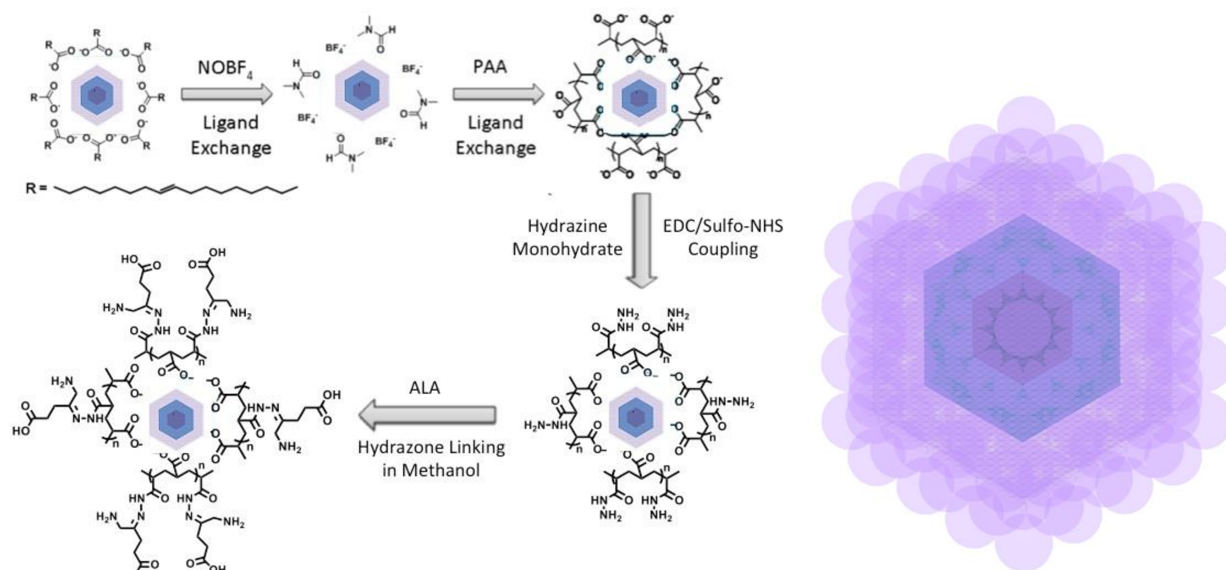

Figure 3: Left side: The process of Conjugation 5-Aminolevulinic acid on $NaYbF_4$*:0.5%Tm @*$NaYF_4$*:30%Nd @*$NaYF_4$*, On right side: Conjugated UCNP*

Lanthanide doped upconversion nanoparticles have the potential to trigger subcutaneous tumor cell death. ALA is conjugated to a PAA coated UCNP and delivered to HELA cells in vitro. A pH sensitive hydrazine linkage allows for ALA to be released upon entry into the cell. ALA it is then converted to PpIX by the mitochondria and released into the cytosol. PpIX is a photosensitive molecule capable of catalyzing free radicals from molecular oxygen within the cell when exposed to UV light. Since UV light does not travel well through tissue, upconversion nanoparticles are used to convert, tissue-penetrating-NIR-light to PpIX-activating-UV-light. The overexpression of

PpIX normally found in cancer cells, combined with the ALA induced PpIX stimulation, creates a localized sensitivity to the upconversion UV emission, produce singlet oxygen which results in localized tissue death upon irradiation as shown in the figure 4.

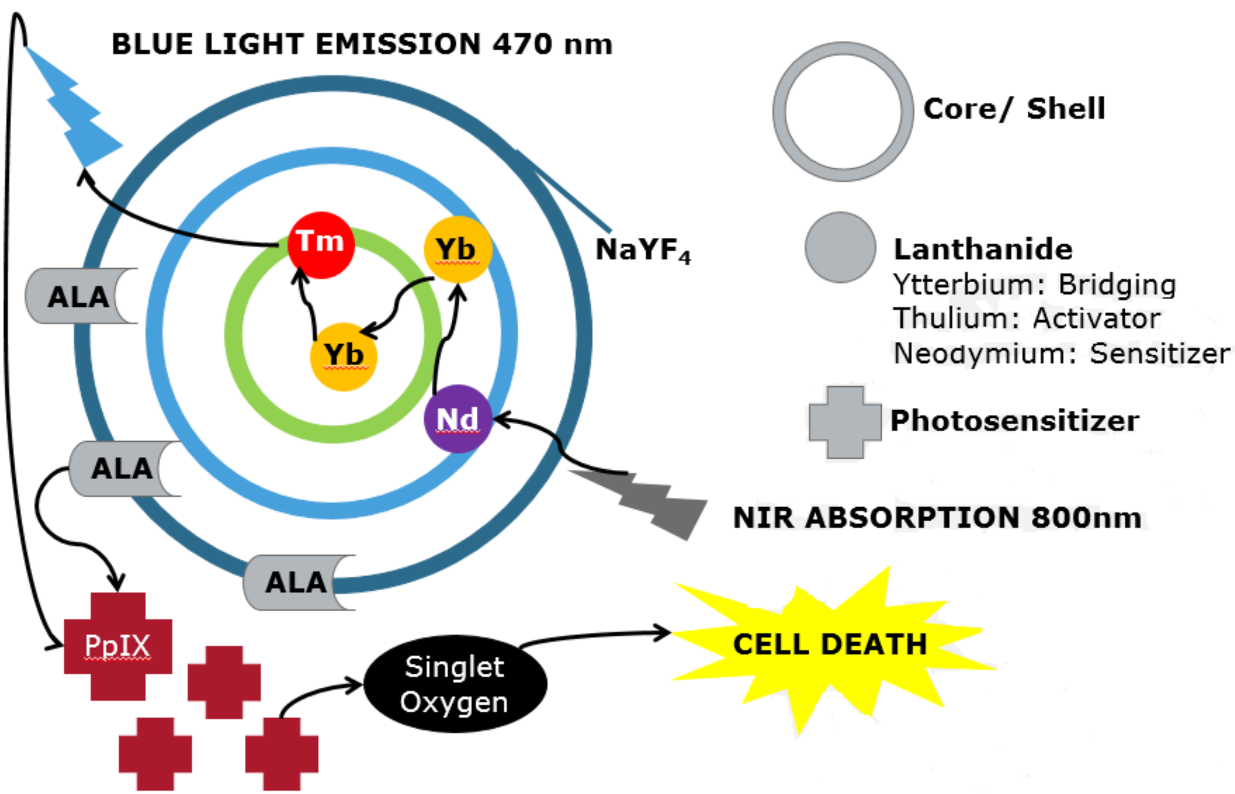

Figure 4: Schematic of final design components and its work mechanism

The photosensitizer that our UCNP transduction mechanism utilizes is the naturally occurring PpIX molecule which has a range of activation wavelengths between 470nm-640nm which falls towards the UV end of the visible spectrum. If we can determine at which wavelength PpIX excitation produces the most effective MTT results, we can then adjust our dopant concentration in order to produce the optimal emission wavelength upon excitation. Our core shell-shell particle utilizes an emission wavelength of 470 nm which is a blue colored visible light. There are a variety of photosensitizer molecules available when designing a UCNP for PDT mechanism.

Many approaches bind or conjugate the photosensitizer directly to the nanoparticle which can then be delivered and activated as a single unit. Organic dyes have been shown to be effective at PDT delivery but also demonstrate toxic effects and increase particle size.

Chapter 5: Design Verification

In order to address if our UCNP have consistent size, well conjugated, enhanced heating effects and MTT assay, we looked into some experiments.

5.1 Transmission Electron Microscopy (TEM)

It is necessary to keep the size of UCNP consistent and within the range of 30 – 65nm, so UCNP's can be up-taken by cells easily. Transmission electron microscopy (TEM) technique was used to image UCNPs to visualize the consistency of the size of the UCNP in which a beam of electrons is transmitted through an ultra-thin specimen, and interact with the specimen when it passes through. This technique was used three times, first to visualize size of core $NaYbF_4$:0.5%Tm, secondly the size of core and shell $NaYbF_4$:0.5%Tm @$NaYF_4$:30%Nd and lastly for core/shell/shell $NaYbF_4$:0.5%Tm @$NaYF_4$:30%Nd @$NaYF_4$ as shown in figure 5 a, b, & c.

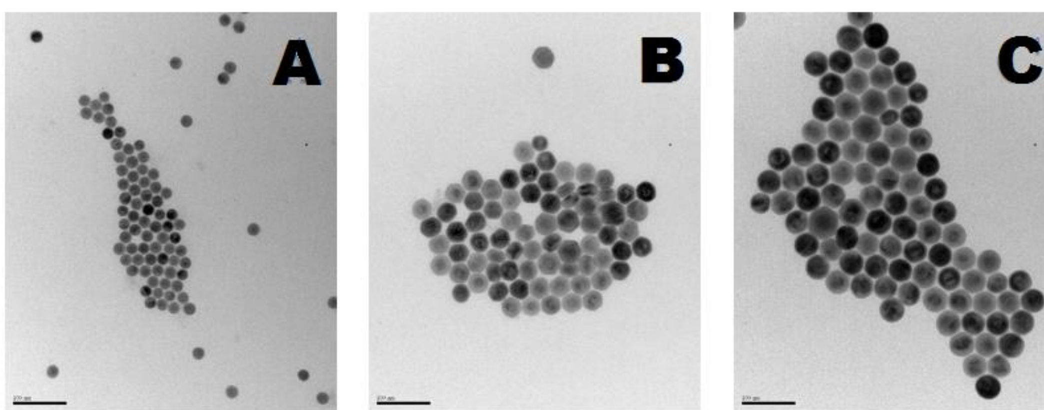

Figure 5: TEM images of core@shell@shell particle (a), Core: $NaYbF_4$: 0.5%Tm (b), Core@shell: $NaYbF_4$:0.5%Tm@$NaYF_4$:30%Nd (c), Core@shell@shell: $NaYbF_4$:0.5%Tm@$NaYF_4$:30%Nd@$NaYF_4$ to see the consistency of size at 100nm scale bar.

The TEM results showed that our final core@shell@shell nanoparticles possess a uniform size of ~50nm with a narrow size distribution, as shown in Figure c. This size of UCNP is appropriate for tumor accumulation.

5.2 Fluorometer

The fluorometer was used to compare the emission spectra of the uncoated UCNP's to the PAA-UCNP's in hexane to see the effect of PAA to the UCNPs. The measurements provided the following data using the background total intensity as measure of signal to noise ratio in 0.85W/cm^2 power density and that influenced by factors like the quality of the optics and scattered light in the system. We discovered that the PAA-UCNP's maintained a maximum emission peak at 475nm (Soret Band) with 3 smaller emission peaks (Q Bands) at 449nm, 360nm and also 512nm both in water and Hexane as shown in figure 6. It proves that our UCNP emit 475nm blue light when shine an 800nm near infrared light and the role of NIR-to-blue visible light transducer for endogenous PpIX.

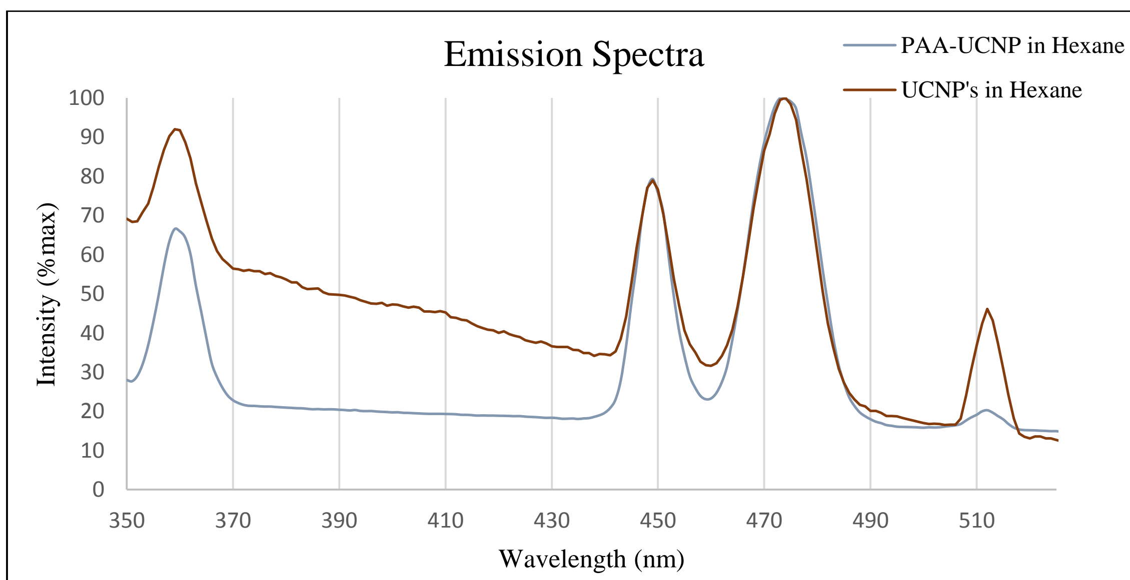

Figure 6: Emission Spectra of PAA-UCNPs in Hexane and UCNPs in Hexane both in 10mg/mL

5.3 Methylthiazol Tetrazolium (MTT) Assay

Methylthiazol Tetrazolium (MTT) assay was performed to verify the toxicity of ALA-UCNPs to HELA cells, which mean that UCNP minimally toxicto healthy cells. HELA human cervical cancer line was chosen to conduct this experiment and Antibiotic-free DMEM+10% FBS was used to maintained the cultures by avoiding bacteria. All assays were performed under sterilized hood and incubated at 37 ^oC and 5% Carbon Dioxide (CO_2). All cells count were determined by using disposable hemocytometer.

$1x10^4$ HELA Cells were plated in a 96-well microtiter plate and incubated for 37 ^oC with 5% carbon dioxide (CO_2). The experiment was setup for three controls, 1) Cells with 5µl ALA-UCNP, 2) Cells with 10µl ALA-UCNP, 3) Just cells. Cells in first four columns of plate were exposed to 100µg/mL of ALA-UCNPs, cells in next four column were exposed to 200µg/mL of ALA-UCNPs and cell in last four columns were not exposed to UCNPs (growth control). After four hours two wells of each group was irradiated with CW 800nm laser diode at $0.7W/cm^2$ for 15 minutes then incubated the plate for another 24 hours. Cells were washed with PBS and then were labeled with 12mM solution of MTT in PBS for 4 hours. Lastly the media was aspirated and replaced with 50µl DMSO and plate was read at 540nm absorption to determine the formazan absorption.

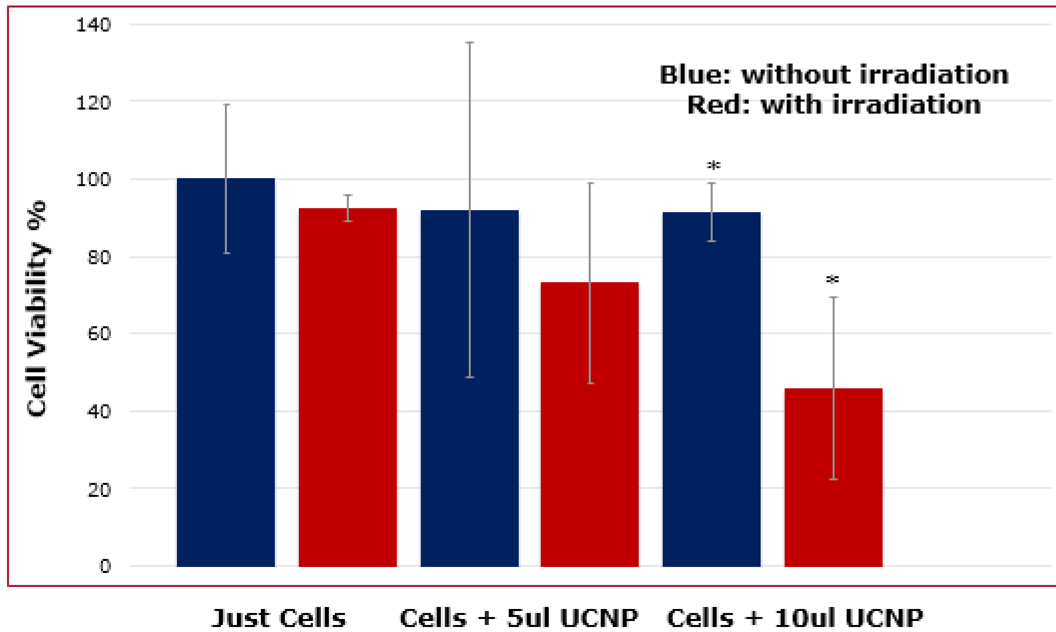

Figure 7: Cell Proliferation Assay (MTT): Where HELA cells were exposed to 100µg/mL ALA-UCNPs, 200µg.mL ALA-UCNPs and nothing (growth control) and irradiated with CW 800nm light at 0.7W/cm^{2}power density.

Our results showed that irradiation without adding UCNP to cells or UCNPs without irradiation are non-toxic to cells. On the other hand, when UCNPs were exposed to cells and irradiate the cells then UCNPs became toxic to cells. The results showed 200µg/mL ALA-UCNP killed almost 54% cells after 15 minute irradiation, 100µg/mL ALA-UCNP killed almost 22% cells after 15 minute irradiation while other controls had minimal cytotoxicity. The statistical analysis showed that there is significant difference between 200µg/mL ALA-UCNP without irradiation and 200µg/mL with irradiation. This difference proved that UCNP without irradiation are minimal toxic to HELA cells.

5.4 Heating Effect Experiment

Since our project focused on improving the current UCNP design by changing the excitation wavelength we set up experiment to compare the 800nm and 980nm lasers and determined which is more effective for PDT based on heating effects. Our experiment was performed on 1.5cm thick pork tissue that was stored in a 0 ^{o}C refrigerator and allowed to sit out in room temperature for half an hour. Prior to testing a microscope was used to set this experiment. The microscope was modified by taking out one lens and by fixing the laser in lens place and aligned in a way to pass through the pork tissue as shown in figure 8. On the side of the microscope the IR temperature sensor was adjusted which can measure temperature of the pork repeatedly with +/- 0.2C precision and accuracy.

Figure 8: Microscope set up to test heating effect of 800nm & 980nm laser

Modifying allowed us to precisely control light exposure and reduce atmospheric attenuations for repeatable measurement. When pork tissue was completely thawed it was

placed on the microscope glass plate. After making sure that everything is fixed and placed correctly multiple pork tissue were irradiated with both 800nm and 980nm on three different power densities 700mW/cm^2, 1000mW/cm^2, and 1300mW/cm^2 respectively for 5 minutes and temperature was record via the IR temperature sensor.

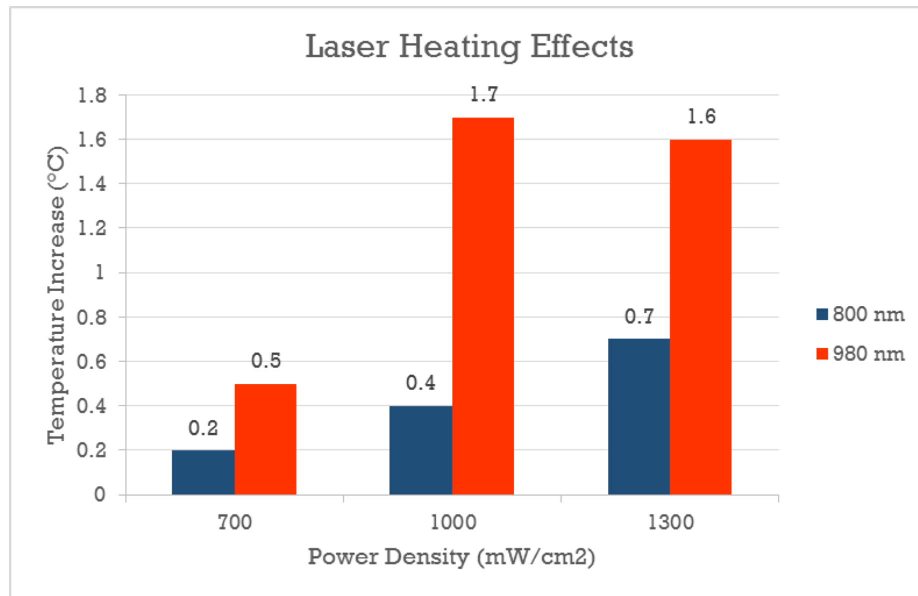

Figure 9: Heating effect data of both 800nm and 980nm laser at three different power densities 700mW/cm^2, 1000mW/cm^2, and 1300mW/cm^2 showed the average temperature increase of the pork tissue based on all measurements taken during each time period.

The 700mW/cm^2 power density is the FDA approved power density because it shows minimal heating effects. Our data shows that the 980nm laser has a significantly higher heating effect on pork tissue than the 800nm laser on all the power densities as shown in figure 9. As the power density of the lasers were increased, the gap between the temperatures differences also increased which illustrates higher power density also increase the heating effects.

Chapter 6: Discussion

Like other cancer treatments such as chemotherapy radiotherapy and surgery there is one treatment called Photodynamic therapy (PDT) on which our project was concentrated on. Upconversion nanoparticles (UCNP) are currently being used with PDT for cancer treatments and have shown promising results (Mahmoud El-Rifai, 2014). It is proven that UCNP with 980nm laser can be used in PDT for cancer treatment from previous MQP; however, there were few limitations with 980nm laser such as higher heating effects in the tissue and less tissue penetration (Mahmoud El-Rifai, 2014). The project was mainly focused on showing that *NaYbF4:0.5%Tm@NaYF4:30%Nd@NaYF4* particle has less heating effects, enhanced MTT essay by increasing blue light emission through photodynamic therapy use with our UNCPs, which activates $NaYbF_4$:0.5%Tm@$NaYF_4$:30%Nd@$NaYF_4$ the photosensitizer drug 5-Aminolevulinic Acid (ALA). From previous MQP we added another shell of neodymium this shell was added to beta phase because it cause higher energy transfer and emits brighter blue light which has shorter wavelength and higher frequency. The team conducted variety of experimetns to confirm that UNCPs has low toxicity when exposed to cells and the blue emission has higher intensity than red emission by using $NaYbF_4$:0.5%Tm@$NaYF_4$:30%Nd@$NaYF_4$ and the ALA-UCNPs conjugation has therapeutic potential.

The team conducted experiments to evaluate the cell viability, heating effects, blue emission light and size with ALA-UCNPs conjugated particles to use core@shell@shell UCNPs as an activator and drug carrier for ALA these were compared to previous MQP.

After discovering that our UCNPs has best blue emission, which synthesized by team as discussed in appendix (I,II,III,IV) ALA-conjugated *$NaYbF_4$:0.5%Tm@$NaYF_4$:30%Nd@$NaYF_4$.* ALA-UCNPs were expected to be phagozytized by the cells during MTT assay. While hydrazine-UCNP in endosome gets activated by NIR light which causes PpIX to produce singlet oxygen ($1O_2$) and eventually kills cancers cells. Lastly, we designed a closed experiment where no air can pass through and we can set it on a constant temperature because our temperature measurement utilizes an IR laser we have to make sure that there was no possibility of measurement interference from NIR excitation laser. Once the team had established optimal blue emission PDT effect of UCNPs heating effects of laser was desired. The team conducted a test on pork tissue to verify the heating effects as shown in figure 8. This test was conducted to prove that heating effects of 800nm is less than heating effects of 980nm which shows that there will be less damaging and burning of skin will be caused.

Blue emission is very important because in PDT photosensitizer drug is activated by blue emission the team used ALA as a photosensitizer drug. Brighter blue emission light is important for PDT because it will activate the UNCPs which will induce ALA release and ALA will induce PpIX accumulations.

The team wanted to check the size of the UCNPs which was ~50nm as shown in the figure 5c from transmission electron microscope (TEM) the size requirement fits the average range size which can be uptaken by cancer cells easily. We decreased the concentration of Yb^{3+} because it is shown that 80% of Yb^{3+} has higher heating effects which decreased the heating effects. To get brighter blue emission we added another shell of

neodymium which is shown in figure 2. After the ALA conjugation, UCNPs size was 65nm which is still in acceptable range because it can still be used in human and tumors cells still can uptake it, as TEM showed that particles formed a uniform pattern and ALA-UCNPs distribution in water.

One of the important experiment team conducted was Methylthiazol Tetrazolium (MTT) Assay for different reasons firstly to check the toxicity of UCNPs (cell viability) and secondly to check the photosensitizer drug ALA to evaluate the cell for which HELA cells were used. In figure 7 cell viability is shown where ALA-UCNPs exposure to cells and with 15 min irradiation shows 54% viability, UCNPs were exposed to cells 4 hours before the test was conducted under CW 800nm 0.7W/cm^2 excitation, it shows that UCNPs are non-toxic to cells without irradiation which shows 800nm UCNPs has excellent cell viability.

6.1 Economics

Photodynamic therapy treatment using UCNP on larger perspective, cancer treatment is one of expensive treatment in medical field which cost up to $100,000 for a year of treatment (*Levy, 2013*). Despite the high cost of this treatment still does not show high efficient in most of cancer cases. After the research on UCNP with 800nm laser, we hypothesize that PDT with UCNP has promising and less expensive treatment than existing treatments because the material for UCNPs and ALA are not expensive due to the tumor targeted nature of ALA.

6.2 Societal Influence

Whenever a treatment is in the process of getting approved not only its benefits are seen but also its side effects are considered. After conducting the research team looked if it has any effects to environment. One of the important aspect is disposal of from manufacturing the material and as well as therapy itself. Research shows there is no harm to environment plants or animals while using Yb^{3+} because it water-soluble but can have mild-toxicity to water animals which is why monitoring the disposal process is important but it does not require any permit or license (*Ytterbium Oxide, 2010*).

6.3 Political Ramification

Cancer is well known diseases which has taken everybody's attention globally. Medically many research are taking place to have a treatment which can be used globally. Since there are many factors which causes cancer such as tobacco, diet, genetic, chemical radiation etc.

6.4 Ethical Concern

The goal of team was to provide an alternative treatment for cancer by using PDT with AL-UCNPs which has potential to provide patients with minimal side effects as well as less expensive than existing treatments. Patients' needs to inform how treatment works and limitation and risk of treatment are discussed such as minimum damaging of skin or tissues and not 100% of tumor cells will be killed. To insure the pain level and risks in vivo studies should be done before introducing to clinical trial.

6.5 Health and Safety

One of the main goal of this project was to design a treatment which is safe for patient for which we considered toxicity and heating effects in vitro studies. As results shows this treatment will have less damaging of skin and kill more cancer cells. Safety is major goal for everyone which is why we accomplished a PDT with ALA-UCNP treatment which will kill only cancer cells with minimal damaging near healthy tissues with less damage to skin. This drug is designed to have minimal side effects to nearby healthy tissues and organs. The treatment should be noninvasive so the patient does not suffer a lot of pain.

6.6 Manufacturability

Our project is easily reproducible its one week process to synthesize the particle, after one week if it's made correctly it can be used for cancer treatment. Since the project goal was not to look in to how it should be injected severe studies can be done on this matter. Since cancer is growing disease it should be affordable for people all over the world so everyone can take it benefits. Our UCNPs is better because it has great size, less heating effects and enhanced cell viability also it is less costly than existing treatments and has less side effects.

Chapter 7: Final Design and Validation

Cancer is a deadly disease, and which has various treatments but those treatments have limitations so an effective and safe treatment is needed. PDT shows promise for the treatment of cancer. The 980nm laser was already proven to be use for PDT to treat cancer but there were limitation such as less absorbance, and higher heating effects which can cause skin damaging and burning. Therefore, the team designed an 800nm upconversion nanoparticle conjugated with 5-aminolevulinic acid with photodynamic therapy to treat cancer with less heating effect and enhanced cell viability to make it more effective for cancer therapy.

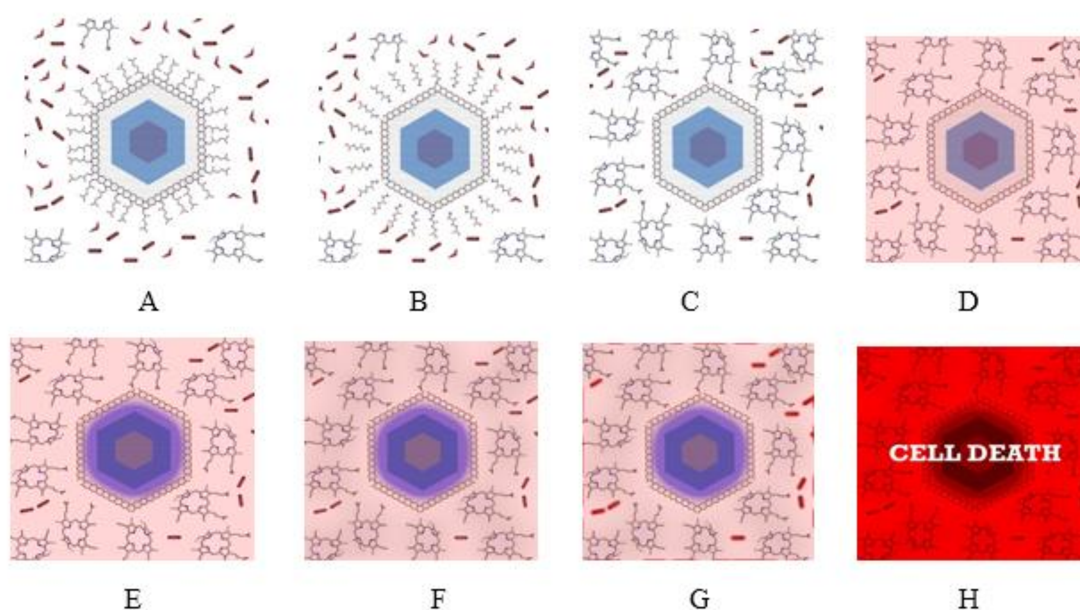

Figure 10: Schematic of final design mechanism A)Cellular Uptake, B)pH induced ALA release, C)ALA induced PpIX accumulation, D)NIR laser excitation, E)470nmUCNP emission, F)PpIX 470nm absorption, G)PpIX photocatalyzes triplet oxygen, H)Singlet Oxygen causes cell death

In photodynamic therapy, cells uptake the UCNP, pH induced the UCNP and they release ALA, which induced PpIX accumulation. When NIR laser shined on UCNP, they emit visible light. PpIX absorbs this light and photocatalyze the singlet oxygen which eventually kills the cancer cells. The mechanism of final design is shown in figure 10. Several experiments were performed to validate the final design.

7.1. Polymer coating and pro-drug conjugation.

The team verified the translucent properties of ALA and PAA and determine how much they reduce the emission intensity of our particles and compare them with the 980 nm particle to determine if there is any difference. In order to emit blue light and enhance the brightness of light the team used extra shell of neodymium around the UCNP which sense the 800nm light and helps to emit brighter blue light. Blue light have shorter wavelength and high frequency which help to produce more PPIX. The team successfully synthesis the core@shell@shell UCNPs and did the conjugation. Firstly, UCNPs were conjugated to NOBF4 to remove the original capping ligand on the hydrophobic UCNPs, because the ion NO+ can replace the rare earth ions and coordination ligands. In the next step, UCNP were synthesis by PAA for ligand exchange. Because it has excellent properties such as hydrophilicity, and non-toxicity it is more stable in water and as a capping agent PAA allowed for nanoparticles with bright emission in aqueous systems. Thirdly UCNPs were conjugated with hydrazine. The reason of using hydrazine is that it is positively charged, more stable in biological fluid and hydrophilic. Lastly, the particles were conjugated with ALA which help to induce PPIX accumulation.

7.2 Cell viability assay

The toxicity and effectiveness of the UCNPS with 800nm laser irradiation were verified by doing MTT Assay. The team verified in this experiment that the irradiation without UCNP or UCNP without irradiation are minimally toxic to cells. Also, two different concentrations 100µg/ml and 200µg/ml of ALA-UCNPS were compared when irradiated with 800nm laser for 15 minutes. The result of this experiments illustrate that UCNPs with irradiation are toxic to cells.

7.3 Heating effects experiments

Based on the fact that our cells are ~75% water and that water has an extinction coefficient that is 20X less at 800nm than 980nm (United States of America, Massachusetts Patent No. WO?2014?116631, 2014). To test the heating effects of the 800 and 980 nm lasers pork tissue which has a greater heating effect. Various power densities were used for this experiment in which FDA approved 700 mW/cm^2 was included to test their effectiveness. The team determined that the 980 nm has a higher heating effect than 800 nm, so skin burning and damaging can be lessened by using 800nm laser.

Chapter 8: Conclusion and Recommendations

As we discussed in previous chapters, cancer is leading cause of death in U.S therefore, the current viable option to treat cancer is using PDT, which is an effective preventive treatment. Photodynamic therapy (PDT) with topical 5-aminolevulinic acid (ALA) is increasingly employed for cancer treatment and is combined with both light and a photosensitizing agent to produce reactive oxygen in cells. The aim our project focused on the ALA derivation to enhance its permeability through considering the alternative designs for biocompatibility and test various in vitro parameters that influence of tissue temperature on PpIX accumulation. The team hypothesize that surface modification are favorable for compound to reach the target site and improve the penetration depth based on material physical, chemical, or biological characteristics. Our major challenge was to alter the Yb^{3+} sensitized UCNPs since the peak absorption of Yb^{3+} ions excited at 980nm as a result, the cells and tissue withhold on this higher laser radiation and concurrently induce heat damage.

Therefore, for design selection using the transmission electron microscopy (TEM) experiment showed that our final core shell/shell nanoparticles have uniform diameter, approximately 50nm in size. This modification confers the uniform particles size that enable to distribute the ALA-UCNPs in water that help to use for biological purpose. The UCNPs invention are preferably configured as *Core@shell@shell: $NaYbF_4$:0.5%Tm@$NaYF_4$:30%Nd@$NaYF_4$* with a biocompatible 800nm excitable property that employ Nd^{3+} as photosensitizer and Yb^{3+} as bridging ions that give a strong blue upconversion emissions without phot bleaching (Han, 2014).

Our team used similar experimental approach as the previous MQP team to test in vitro activity using biocompatible calcium fluoride (CaF_2) shell UCNPs. Therefore using selected tested method, we were able to compare the uncoated UCNPs with PAA-UCNPs both within water and hexane. The coated PAA-UCNPs achieved a maximum emission peak at 475nm in water and 450nm in hexane. This indicates that two different conjugated sample were cheeked to see their usability in different environment through TEM and fluorometer experiment. Therefore, the results shows that for uniform particle size (50nm) under the 800nm NIR laser the coated PAA-UCNPs can emit enough blue brightness at nearly $5.58*10^7$ A.U. Thus, the team proves the advantage of ALA derivatives over PAA-UCNPs can mainly enhance the rate of their enzymatic conversion into photoactive compounds to reach the target site as well as improved skin penetration.

Therefore, to verify the team's design accomplishment we performed MTT assay if the ALA working effectively as cancer treatment through conducted acceptable particle dosage and see cell death by applying the 800nm wavelength laser exposure. MTT assay was performed to show that only the UCNPs without irradiation are not able to kill that much cells and our data show that almost 100% viability after 15minute of exposure for 800nm wavelength laser. However, 10µl ALA-UCNP showed about 54% cell viability on the same MTT assay for 15 minutes. Thus, this MTT assay proves theory that ALA conjugated UCNPs had the most cell died using the 800nm laser shine with 30% Nd^{3+}. Thus, the ease of surface modification enable the team to use the right size of nanoparticle to create the cytotoxicity assay, which acceptable in physiological testing.

Although substantial overheating may induces cell and tissue damage, some heating is needed to promote PpIX production from ALA in cells and in tissue. Also, because the

sensitizers absorb NIR photons and then transfer the energy to the activators, the temperature dependence of PpIX production may be used for improvement of PDT whether accumulation of PpIX can be selectively enhanced by minimizing the overheating effects. Therefore, since the team hypothesized that the 980nm laser, typically used to trigger the Yb^{3+} sensitized upconversion process, we wanted to substitute to 800nm to sensitized Nd^{3+} by building the core/shell/shell structure to ensure successive energy transfer between the mixtures of lanthanide elements. As preliminary in vivo test, the team performed on 1.5cm thick pork tissue to compare the heating effects between 980nm laser, which is strongly absorbed by water in biological tissue and could cause sever overheating effect whereas 800nm laser results confirmed that local overheating effect is greatly minimized. We tested on the pork tissue both 800nm and 980nm on the three different power densities and thus, we can conclude that our observation promises $Nd^{3+} \rightarrow Yb^{3+}$ as bioprobes for both in vitro and in vivo imaging under 800nm irradiation.

8.1 Recommendations

The main problem with the current PDT treatment is limited penetration depth due to inefficient NIR wavelength to activate ALA and limited accumulation of PpIX in the underlying stroma that may damage of deeper layer or the PpIX fluorescence not able to observed deeper tissue. Unfortunately, this set of experiments did not include the testing for tissue penetration depth and oxygen availability test due to lack of experimental laboratory resources and time. However, we recommend the following further modifications and tests such as further modifications of ALA-PDT will improve the cellular permeability, because ALA is a polar molecule and has significant role in improving its cellular permeability, increased stability in physiologic pH, increase

selectivity. Second, investigate the necessity of Nd^{3+} by increasing the concentration under excitation of 800nm not only well away from peak absorption of water, but also essential to minimize overhear heating effects. Last, identify additional factors that affect ALA pharmacokinetics to design effective ALA-PDT through detail studies of molecular mechanics of cytotoxicity are required to implement in vivo test.

APPENDIX

Appendix 1: NOBF4 Ligand Exchange

1. Get a fresh 20mL vial with a white cap
2. Using the 1000µL pipette labeled ODE and a clean blue pipette tip, pipette 1mL of $NaYbF_4$:0.5%Tm@$NaYF_4$:30%Nd@$NaYF_4$ nanoparticles into the empty vial. Do this quickly because the hexane likes to drip all over the place.
3. Using the same pipette and more fresh tips, pipette 4 mL of hexane into the same 20mL vial with 1mL nanoparticles.
4. Using the same pipette and more fresh tips, pipette 5 mL of DMF into the same 20mL vial with 1mL nanoparticles and 4 mL hexane. You will see that the DMF and hexane are immiscible.
5. Find the 20mL white capped vial on top of the vacuum oven filled with stir bars with the words "$NOBF_4$ Hexane" on them. Take a pair of forceps/tweezers and wash them in hexane over the organic solvents dump. Then, use the tweezers to transfer one stir bar from the "$NOBF_4$ Hexane" vial to your vial with nanoparticles, hexane, and DMF.
6. Place your vial with a stir bar, nanoparticles, hexane, and DMF under a fume hood on a magnetic stirrer (best to use the hood across from the vacuum oven. Use the magnetic stirrer which is small, blue, and from VWR, not the ones from Corning). Close the vial with the white cap and turn it up the maximum stir speed. Stabilize the stirring by moving the vial to the center of the stirrer. You should see the hexane and DMF forcibly mixing.

7. Get a disposable scoop under the hood, carefully open the vial with nanoparticles while it's stirring. With the NOBF4 in one hand and the scoop in the other while keeping everything under the hood, open the NOBF4 bottle. You might see the contents start smoking--that's okay.
8. Now, scoop .2g of the NOBF4 and add it to the vial with the nanoparticles stirring. Since you can't measure the NOBF4 outside the hood, you have to guesstimate what .2g looks like. After the NOBF4 is added to the vial, immediately close both the vial and the NOBF4 bottle tightly. You should see the nanoparticle solution turn green and blue, maybe with some green gas on top.
9. Let the reaction sit overnight!

Appendix II: Exchange anionic ligands with PAA on UCNP

1. Take the UCNPs+hexane+DMF+NOBF4 mixture that has been stirring overnight and stop the mixing and open it under the hood. Let the gas escape. Get a 50 mL conical tube. Using a magnet to keep the stir bar in, pour the contents of the vial into the conical tube.
2. Find the isopropanol spray bottle. Wash the sides of the vial with around 5mL isopropanol and pour the isopropanol into the hexane/DMF/UCNPs/NOBF4 mixture. When you now mix the "yesterday"+isopropanol, you should see it swirl and clarify. Wash the vial again with another 5mL for a total of 10mL isopropanol in the conical tube (a total 20 mL volume since the initial mixture was 10 mL)
3. Close the conical tube and keep swirling it until you might see a small precipitate. Create a water balance for it and centrifuge it on the countertop centrifuge next to the vacuum oven at maximum (7000) speed for about 10 minutes.

4. Take the conical out. If the pellet looks white and homogeneous, you can do the next step. Otherwise, decant the supernatant, redissolve in 10mL DMF using the sonicator, and centrifuge again.
5. Decant the supernatant and redissolve in 10mL DMF using the sonicator.
6. Prepare a 10 mL 10mg/mL Poly (acrylic acid) (PAA)/DMF solution in a 50 or 100 mL single-necked round bottom flask. Dissolve in the sonicator.
7. Add a stir bar from the bottle labeled "PAA DMF" above the vacuum oven and place the flask in the oil via the clamp on top of the red hot plate in the fume hood behind the one where you stirred the UCNPS with NOBF4.
8. Adjust the red hot plate settings to 80°C and 1000 rpm.
9. Once the temperature is 80°C and it is stirring, use a 1000 μL pipette to 1 mL by 1 mL pipette the 10 mL UCNP/DMF solution into the flask with stirring PAA/DMF solution at 80°C. Seal the flask and let it stir with heat overnight.

Appendix III: Hydrazine Conjugation

1. Take the PAA-UCNPs in DMF that have been mixing at 80°C overnight and stop the mixing and heat. Use a Kim wipe to wipe away the oil from the bottom of the flask as you take it out of the clamp and into a holder. Using a magnet to keep the stir bar in, decant the contents of the flask equally into 2 fresh 50mL conicals.
2. Wash the insides of the flask with around 5mL hexane using the spray bottle. Swirl the flask around and decant again into 1 conical. Wash the flask with 5mL hexane again and decant into the same conical tube. The hexane and PAA-UCNPs in DMF should not mix.
3. Wash the sides of the flask with around 5mL isopropanol using the spray bottle.

Swirl the flask around and decant again into the same conical. Swirls around the tube and you should see the two solvents mix (and sometimes get very turbid). Wash the flask with 5mL isopropanol again and decant into the same conical tube. Keep swirling the conical until you see a small precipitate. The volume of the tube should be 30mL (10 mL PAA-UCNPS/DMF, 10mL hexane, 10mL isopropanol).

4. Repeat steps 2-3 for the other conical with the other 10mL of PAA-UCNPS in DMF.
5. Balance your two conicals with isopropanol and ask Xiang for help to spin it at 11,000 rpm for 30 minutes at 20°C in the big departmental centrifuge. He will teach you how to use it so you can do it by yourself in the future. Keep a timer for the 30 minutes.
6. Immediately after the centrifuge stops, take the tubes out and bring it to the dark room. Test and see using the pen laser whether the nanoparticles have collected in the pellet and there is not much in the supernatant. If there is still some nanoparticles in the supernatant, centrifuge again. Otherwise, continue.
7. Decant the DMF/hexane/isopropanol into the organic solvents dump and flip over the tubes on towels, Let all the solvent trickle down the side and onto the towels.
8. Afterwards, us a 1000µL micropipette to add 10mL of DI water to each tube. Close the tubes and sonicate until the pellet is dispersed in the water. The water might/should be much more turgid, and that is okay. Check it with the laser and you might/will see it significantly dimmer and grainier, but that is okay.
9. Balance the tubes and centrifuge them at 11000 rpm for 30 mins. Again, afterwards, check with a laser and if it's done decant the supernatant.

10. Repeats steps 8-9 two more times, washing the PAA UCNPs in water. Finally, resolubilize in 5mL PBS. Of that 5mL sample, take 1 mL and save it in the very small black-topped glass vial.
11. Afterwards, if when you shine the laser into the solution there still seems to be a granular look to the luminescence, centrifuge the two tubes in the counter top centrifuge at max speed for less than 10 seconds and check the supernatants. If you can still see a lot of luminescence but without the grains, save the supernatant and dispose of the pellet. If you see nothing, resolubilize the pellet and centrifuge for less amount of time/at slower speed. If you still see the granular pieces, repeat this again.
12. Once you have decently-bright non-granular PAA UCNPs dissolved in 4mL PBS, transfer the solution to a clean white capped vial and add a fresh stir bar to it. Place it on one of the small VWR blue magnetic stirrers under a hood and turn it up to max speed. Stabilize the stirring by centering the vial.
13. Find the EDC in the -20°C freezer and the sulfo-NHS in the low humidity cabinet. Weigh 50mg of EDC and 25 mg of sulfo-NHS on the same weigh paper on the analytical balance. Transfer the EDC and sulfo-NHS to the stirring PAA-UCNPS/PBS solution by folding the paper and pouring it in to the vial and trying to remove anything that stuck to the paper. Let the PAA-UCNPs react with the EDC and sulfo-NHS for 2 hours.
14. After 2 hours, take the contents of the vial and decant it into a clean 50mL conical (keep the stir bar in with a magnet). Save the vial with the stir bar and close it under the hood. Create a water balance for the conical you just filled and centrifuge the

tube for 30 mins at 11000 rpm at 20°C. Afterwards, immediately check the pellet and supernatant (you know what I mean), decant, and resolubilize in 5mL PBS using a sonicator.

15. Transfer those 5mL PBS to the vial you were stirring the nanoparticles and EDC/sulfo-NHS in before and continue mixing on the same stirrer.
16. Now comes the dangerous part :) Using an automatic propipetter (the one that has to plug in to the outlet) and a 5mL glass graduated pipette, add 5 mL hydrazine monohydrate to the vial of EDC/sulfo-NHS activated UCNPs in PBS. This hydrazine stuff is used in rocket fuel and is highly reactive. Wear gloves and keep everything under the hood. Try not to make it touch any plastic and close it tightly after you're done. You might notice that the contents of the vial will get a bit warm after you add the hydrazine, which is a good sign.
17. Let it react overnight.

Appendix IV: ALA-Coupling

1. Take the UCNPs that have been mixing with hydrazine, EDC, and sulfo-NHS overnight and place them in the 4°C fridge (under the pH meter) for a second. Meanwhile, get a fresh 50mL conical and water balance. After 5 mins in the fridge, take the vial out and using a magnet to keep the stir bar in, decant the contents into the fresh conical.
2. Make a water balance for the conical and centrifuge it 11,000 rpm, 30 min, 20°C. Immediately after the 30 mins, go and take the tube out and check to make sure the supernatant has no UCNPs in it with both the pen laser and the big laser (around .5

amps). If there is still some, centrifuge for another 20 mins. Otherwise, quickly decant the supernatant into the sink and wash down with the tap running.

3. Resolubilize the Hyd-UCNPs in 5mL of DI water.
4. Repeat steps #2-#3 two more times to make absolutely sure there is no free hydrazine on the UCNPs by washing it multiple times.
5. After the last wash, find the bottle in my desk which says “methanol and acetic acid”. Pipette 5mL of that into the conical to resolubilize the UCNPs. If it’s not that soluble, that’s okay. Keep going with it and try to disperse it as best as you can.
6. Repeat Step 2 and 5 once more to make sure there is no water and only methanol and acetic acid in the solution.
7. After you have 5mL of Hyd-UCNPs in the “methanol and acetic acid” solution in a conical tube, transfer the contents of the tube into a fresh white-capped vial. Add a stir bar and place it on a medium-high speed stirrer.
8. Find the bottle in my desk which says “ALA in methanol”. While the Hyd-UCNPs are stirring, add 4mL of this solution with a pipette to the vial. Afterwards, cover in aluminum foil and let it react overnight.

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