Near infrared contrast agents for theranostic applications

Er, Ronald Shi Tao

2019

Er, R. S. T. (2019). Near infrared contrast agents for theranostic applications. Master's thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/105640>

<https://doi.org/10.32657/10356/105640>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**NEAR INFRARED CONTRAST AGENTS FOR
THERANOSTIC APPLICATIONS**

ER SHI TAO, RONALD

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

2019

NEAR INFRARED CONTRAST AGENTS FOR THERANOSTIC APPLICATIONS

ER SHI TAO, RONALD

SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfillment of the requirement for the
degree of Master of Science

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

19 JUN 19

.....
Date

A handwritten signature in black ink that reads "Ronald". The letter "R" is large and stylized, with a long horizontal stroke that extends to the right. The "o" is a simple circle, and the "n" and "a" are connected. The "l" is a simple vertical stroke. The "d" is a simple circle with a vertical stroke. The signature is written in a cursive, flowing style.

.....
ER SHI TAO, RONALD

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it of sufficient grammatical clarity to be examined. To the best of my knowledge, the thesis is free of plagiarism and the research and writing are those of the candidate's except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

19 JUN 19

.....
Date

.....
Assoc. Prof. Xing Bengang

Authorship Attribution Statement

This thesis **does not** contain any materials from papers published in peer-reviewed journals or from papers accepted at conferences in which I am listed as an author.

19 JUN 19

.....

Date

A handwritten signature in black ink that reads "Ronald". The letter "R" is large and stylized, with a loop at the top. The "o" is a simple circle. The "n" and "a" are connected, and the "l" is a simple vertical stroke. The "d" is a simple circle with a vertical stroke. The signature is written in a cursive style.

.....

ER SHI TAO, RONALD

Abstract

In recent years, there has been a shift towards personalized medicine which is evidenced by the Food and Drug Administration (FDA) approvals^[1]. One of the key principles of personalized medicine is the use of diagnostic tools such as imaging techniques to provide accurate information about the medical condition in order to prescribe the right treatment for the right person at the right time and dose. However, there are limitations to the current imaging techniques to achieve this aim, such as poor image resolution, high cost, and potential side effects. Optical imaging (OI) that uses near-infrared (NIR) light promises a minimally-invasive method of gaining accurate information by virtue of its deep penetration depth without background and biosafety issues. The aim of this thesis is to explore some of the NIR light responsive theranostics that are used in current diagnosis and therapeutics, such as NIR phototherapy and NIR-II fluorescence imaging. In particular, making use of the up- and down-photon converting features of lanthanide-doped nanoparticles (LNPs) not only achieves sensitive NIR optical sensing but also enables efficient therapeutic effects against deep-tissue disorders.

Our first project introduces a NIR theranostic platform that was fabricated by Upconversion Nanoparticles (UCNPs) and the cyanobacteria, in which core-shell UCNPs, NaYF₄:Yb/Tm (30:0.5)@NaYF₄, are prepared *via* a hydrothermal method. It is subsequently modified with an amino-silica shell, followed by conjugating it to cyanobacteria, *S. Elongatus*. It is known that blue light is able to activate the photosynthesis pathway that is capable of oxygen gas (O₂) generation thereby facilitating the management of ischemia or hypoxic damages. Such a promising NIR light-powered photosynthesis platform will allow the effective therapeutics in deep tissues and boost the phototheranostics into a brand new field. The second project presents a novel nanodiagnostics for ratiometric redox luminescence imaging in the second NIR window (NIR-II), which was fabricated by the energy transfer integration of Cerium ion (Ce³⁺)-

sensitized LNPs, NaCeF₄:Nd/Yb/Er(1:20:1). This type of LNPs uses the excitation of NIR light at 808nm while the emissions are at 1064 and 1530 nm. Upon the addition of an oxidant (e.g. hydrogen peroxide, H₂O₂), the emission intensity at 1530 nm would be reduced due to the oxidation of Ce³⁺ to Ce⁴⁺ which hinders Er³⁺ emission in the photon energy transfer process. Meanwhile, it may not affect the 1064 nm emission associated with Nd³⁺. Thus, the redox status could be determined by the ratio of Em₁₀₆₄/Em₁₅₃₀. However, the preliminary data showed that both of 1064nm and 1530nm emissions are quenched by H₂O₂. Future work to understand the mechanism of the redox-induced energy transfer inside LNPs will be necessary to allow for the development of more sensitive ratiometric NIR-II imaging probes of H₂O₂ as well as the understanding of the oxidative stress implications in living conditions.

In summary, the development of the NIR contrast agents is able to provide broad perspectives for both bioimaging and therapeutic applications in sensitive and deep tissue resolutions. We believe that the works presented in this thesis may serve as an archetypal system to encourage innovative theranostics not only for pathological assessments but also benefit for the precise intervention of disorders.

Acknowledgements

I will like to acknowledge my supervisor, Associate Professor Xing Bengang for the opportunity to come under his apprenticeship to further research in the area of imaging and theranostics. In the research year, he had rendered guidance to encourage critical thinking and challenge the status quo. I sincerely thank him for his kind understanding with the part-time study arrangement.

I will also like to acknowledge Dr. Zhang Wen Min, Dr. Chong Haolun, Dr. Wu. Xiangyang, Dr. Ng Shue Mei, Ms. Germaine Kwek and Mr. Wang Zhi Min for the kind support and collaboration for the past research year. It is also their patient guidance that had further brought clarity to the research.

I will also like to thank Dr. Chang Yong Qin, General Manager of Singapore Radiopharmaceuticals Pte. Ltd, who had encouraged me to take the step to undertake this research degree during my term as a full time employee. Without his encouragement, this research will not have commenced.

I will also like to thank my family, colleagues from Roche Singapore Technical Operations, and friends who had supported me throughout this period, taking the extra mile to ensure that I am well-rested.

Table of Contents

Abstract	1
Acknowledgements	3
Table of Contents	4
Chapter 1 Introduction.....	6
1.1 The importance of theranostic contrast agent in current context	6
1.2 The emergence of optical imaging.....	9
1.3 Hypothesis	11
1.4 Methodology	12
 Chapter 2 Types of Contrast Agent used in NIR imaging.....	 13
2.1 Broad Introduction to NIR Contrast Agent	13
2.2 Infrared Fluorescence proteins as contrast	14
2.3 Organic Dyes as contrast agents	14
2.4 Lanthanide Nanoparticles Contrast	15
2.4.1 Upconversion Nanoparticles (UCNP)	15
2.4.2 Downconversion Nanoparticles (DCNP)	17
2.5 Comparison of NIR Contrast Agent	18
 Chapter 3 Phototherapy of NIR contrast agent.....	 21
3.1 UCNP-conjugated cyanobacteria therapeutic system	21
3.2 Materials and Method	22

3.3	Results and Discussion	27
3.4	Conclusions	31
Chapter 4	NIR-II deep tissue imaging of NIR contrast agent	32
4.1	Ratiometric redox luminescence imaging in the second NIR window	32
4.2	Materials and Method	33
4.3	Results and Discussion	35
4.4	Conclusions	37
Chapter 5	Conclusion and Future Work	38
	References	39

Chapter 1: Introduction

1.1 The importance of theranostic contrast agent in the current context

Hippocrates noted in his writing that “*It is far more important to know what person the disease has than what disease the person has*”. This statement has profound significance and implication in the development of medical science. The statement encapsulated the two different fundamental philosophies of medicine. The former phrase “*what person the disease has*” is anchored and focused towards the *patient* that the disease had impacted while the latter phrase “*what disease the person has*” is anchored and focused towards the *disease* that the patient is suffering. The ancient Greek physician understood the significance of different approaches and their implication. This quote by Hippocrates has become one of the key guiding principles in recent years as the industry moves towards “personalized medicine”. In the developmental years of the medical science, the approach that is adopted by the medical community is heavily based on the latter where it aims to increase the knowledge and understanding of the disease condition that is independent of the patient’s inherent condition. Building on the understanding of the disease condition, physicians will then appropriate the treatment accordingly. This can be observed as the “one-size-fits-all” approach that had been adopted in the early years. One of the examples of the effect of this approach is the development and use of β -lactam antibiotics such as Penicillin G^[2]. Since its discovery in the mid-1940s, it has been widely used as the key drug against bacterial infections. Today, we also observed that there is a crunch of available antibiotics in the market as many patients who used antibiotics frequently had grown a dependency on the drug. This dependency led to the body being acclimatized to the use of the drug and causes the need for stronger antibiotics when a variant strand of bacteria attacks the body. Furthermore, it is known that β -lactam antibiotics are typically effective with gram-positive bacterial infections while being ineffective against gram-negative bacterial infections. This is one of the key challenges faced by the medical community

as there is a need to search for alternatives to antibiotics or stronger antibiotics to treat infections. In view of such a trend in the drug resistance, clinical scientists are therefore charged with a mission to search for alternatives. It has to change to a more personalized approach. This draws us back to the ancient Greek physician former phrase where its anchors on the patient that the disease has impacted. This philosophical approach requires the use of combined knowledge to understand the patient's inherent condition in relations to the disease. *W. Ken Redekop* ^[3] summarizes this approach as the use of combined knowledge about a person to predict treatment response, disease prognosis and disease susceptibility. The framework that Redekop provided forms the fundamental basis for diagnosis and treatment. This had led to the emerging branch of medicine – Theranostics.

Theranostics is hence important in the movement towards this new philosophical approach. It is the capacity of drugs that serves as both diagnostic and therapeutic activities ^[4]. This dual-effect of theranostic positions itself favorably amid the movement towards personalized medicine and steer away from the “one-size-fits-all” approach. The design of theranostics is typically more complicated due to the need for both diagnostic and therapeutic properties in the same setting. Development of technology had attempted to actualize the theranostic properties of drugs, especially in the field of molecular imaging. One of the most classic examples of theranostic is the use of radio-iodine (I-131) in the treatment of Thyroid Cancer. It utilizes the unstable nuclei which emits beta-particles that are able to kill cancerous cells. As it undergoes radioactive decay to emit a beta-particle, the excited state of the daughter nuclide emits a gamma-radiation that allows machines equipped with a gamma camera to detect the presence of the uptake of I-131 in the body. Hence, this allows I-131 to serve as both a diagnostic and therapeutic agent ^[5]. However, the drawback with this approach is the use of

ionizing radiation which can cause secondary tumor generation and poses health concerns. The dosing and the safety of the drug become essential when considering this approach.

Clinical scientists are hence required to search for non-toxic approaches where the use of ionizing radiation is mitigated to the lowest. These had pointed many clinical scientists towards alternatives such as Magnetic Resonance Imaging (MRI) where the use of ionizing radiation is eliminated. In MRI, superconducting magnets are used and contrast agents are required to obtain better resolution of the images. This requires the use of magnetic metals such as Gadolinium to obtain spatiotemporal images of the body. This approach has provided a diagnostic approach but it has limited therapeutic effects. In recent years, clinical scientists have attempted to actualize the therapeutic effect through the use of magnetic nanoparticles where it is able to release of drug upon the application of a magnetic field. This allows for the actualization of theranostic properties. However, this approach is costly due to the need for superconducting magnets which comes from specialized equipment for the theranostic application.^[6]

It is then important to search for even more effective methods that possess theranostic effects while mitigating the negative impact of ionizing radiation and high cost. Optical Imaging (OI) has emerged as an alternative to the current imaging techniques that is able to perform the dual properties of theranostics. It uses Ultra-violet (UV) visible light which is non-toxic and easily accessible virtually anywhere. This technique is founded on the fluorescence or bioluminescence for the reconstruction of images. In the evaluation of optical imaging, it is considered a better option for physicians as it can be observed by the naked eyes. It also lowers the cost as the light source can be readily available. However, this technique is not without

limitations. It has issues pertaining to the penetrating depth of UV-visible light as well as health issues that arise from the use of UV light.

1.2 The emergence of Optical Imaging (OI)

OI had emerged to overcome the limitations of ionizing radiation as well as the lowering of cost. It uses light in the electromagnetic spectrum as the irradiation source and undergoes energy transitions that emit fluorescence of light in the visible range which allows for its imaging capability. OI works on the operating principle of absorption of photons that corresponds to the allowable transition between the Highest Occupied Molecular Orbital (HOMO) and the Lowest Unoccupied Molecular Orbital (LUMO). Depending on the non-radiative transitions, the de-excitation of the electrons from the LUMO to the HOMO emits photons that are observed as fluorescence. This fluorescence can show the position of the tissue of interest and the contrast agent moiety is synthesized to identify specific receptors in the body. This technique typically uses highly conjugated organic dyes that close the gap between the HOMO and LUMO due to delocalization resonance effect. Hence, the fluorescence is typically in visible range which makes it observable in the naked eyes. Another mechanism that it undertakes is the Forster Resonance Energy Transfer (FRET) where the incident light is absorbed by an organic dye and the energy is transferred to another organic dye. The transferred energy undergoes a non-radiative transition to emit a wavelength in the visible range when both dyes are in close proximity. This allows the visualization of the reactions between non-bonding components. One of the key applications is in image-guided surgery. This application uses a contrast agent known as 5-aminolevulinic acid (5-ALA) which elicits the synthesis and accumulation of fluorescent porphyrins which can be visualized under a modified neurosurgical microscope in white light illumination.^[7] This can be applied towards surgery that requires total resection of tumor cells, especially in the not easily accessible region of the

body. However, this technique is limited by the short fluorescence lifetimes as well as the tissue absorption in the visible region. Hence, it is typically performed in the optical window which is also known as the tissue transparent window where the light scattering and tissue absorption is the least. Due to these limitations in the applications, the design of contrast agents for optical imaging is essential to resolve issues in optical imaging which will be discussed in Chapter 2.

The significance of contrast agents is found in its application. In the most conventional optical imaging types, highly energetic photons such as UV light are used. In recent developments, visible light has also been employed. Such imaging techniques are used by surgeons who visualize the location of tumor mass through the detection of fluorescence from the contrast agent. Hence, this allows for the detection of tumor cells at non-accessible location that are intended to be resected during the surgical procedure. However, the use of UV light also has its limitations that are observed in its application. Some of its limitations include weak penetration depth, high autofluorescence, and low signal-to-background ratio.^[13] It can also potentially induce photoreaction in biomolecules that can harm healthy living cells. According to studies, the penetration depth of UV light is approximately 1mm which is unable to reach the dermal vessels level where the blood vessels are located. It is in that basis that UV is used for open surgical procedures for the locating of the tumor cells. Visible light is a similar truncation of the UV light methodology. It uses readily available light and it is able to fluorescence as soon as it is in contact with it. It provides a cheaper option in locating the tumor cells. Many researchers had developed a contrast agent that uses UV light over the years. In the most recent publication in 2018, Liu and his team synthesized a contrast agent known as nanoparticles that are applied to optogenetics^[8]. It uses UV light and activates the membrane proteins for its opening and closing and hence manipulating the Calcium trafficking activities across the membrane. This application had a significant impact as it changes the method of

diagnosis and treatment to be administered to the patients by physicians. However, highly energetic photons are susceptible to a process known as reactive oxygen species (ROS) production. This is one of the known causes of cancer and the advisory to the public that it is not advisable to be exposed to UV light for a prolonged period of time. There are three classifications of UV light which are UV-A, UV-B and UV-C which are characterized by the position of the maximum peak. The highest energetic photon which is UV-C is able to provide the energy for bond cleavage in the protein and causes an oxidation of the oxygen. This product of this electron transfer causes the formation of free radicals such as superoxide anion, hydroxyl radical ion. These free radicals are then reactive to bind with other proteins or cells which causes the disintegration of the cells. As a result, it kills healthy cells and in the case where apoptosis of the damaged cells is not repaired, it results in a mutation and the creation of uncontrolled cell cycle and hence generation of cancer cells. In regards to this note, many researchers had looked for alternatives. It was observed that another electromagnetic wave known as the NIR is able to overcome some of the challenges of the UV-based optical contrast agents.

1.3 Hypothesis: NIR light contrast agents are able to apply for theranostic applications

In the previous sub-chapters, it is noted that personalized medicine requires a paradigm shift from the “one-size-fits-all” approach to personalized medicine. This personalized medicine vision can only be achieved through a comprehensive data collection and understanding of both the patient medical condition as well as the disease condition that is able to make a better and informed decision of the treatment. Having considered the current technologies that are available to medical practitioners, it is hence important to devise a new method for theranostic application. Having understood that OI is an emerging technology and the limitation for the use of UV or visible light in the imaging technique, it is therefore, a need to source for a non-

toxic light source without compromising the information is able to obtain. In this thesis, a novel contrast agent is synthesized using nanoparticles that use NIR to achieve its theranostic applications.

1.4 Methodology: Synthesis of NIR light contrast agent and use for therapy and diagnostic

In this thesis, it will be segmented into a few categories. Firstly we will evaluate the types of NIR imaging contrast agent and the advantage and the disadvantages of each contrast agent. Secondly, we will explore some of the applications of the contrast agent such as conjugating with cyanobacteria to produce oxygen for Myocardial Ischemia Infraction and using lanthanide nanoparticles for NIR-II imaging. Lastly, we will do a summary of the contrast agent in this thesis and evaluate it for its possible applications.

Chapter 2: Near-Infrared Light (NIR) Imaging Contrast Agent

2.1 Broad Introduction of the types of contrast agents that are used in optical imaging

In the previous chapter, we discussed the emergence of OI and its advantages and disadvantages. It is noted that NIR light contrast agents are significantly favored over its UV counterparts. This is primarily due to its greater penetration depth, greater signal-to-background ratio, low autofluorescence and high resistance to photobleaching. There are three main types of contrast agent that are currently available, namely organic dyes, infrared fluorescence proteins, and nanoparticles. These contrast agents have different mechanisms of action that are able to fluorescence for imaging. For example, Infrared Fluorescence Protein (IFP) may require a chromophore such as Biliverdin which is able to emit fluorescence upon conformational changes to the protein structure. In other types of mechanism types such as organic dyes, it requires a highly conjugated chemical structure that is lower the energy gap between the HOMO and LUMO to allow for the fluorescence of light in the visible region. Each types of contrast agent and mechanisms share a common factor which is the energy gap that is inherent in the chromophore or fluorophore that emit signals to be detected in the visible region. Each of these contrast types had some limitations as well such as competitive binding with Haem-derived compounds and serotonin toxicity for IFP and organic dyes respectively. In the recent development of NIR light contrast agents, there is an emerging class known as lanthanide nanoparticles (LNPs) which leverages on the inherent “ladder-type” energy levels in the trivalent lanthanide ions for the fluorescence of light. This unique property of LNPs allows for a specific color to fluorescence and allows for applications beyond imaging but also therapy.

2.2 Infrared Fluorescent Protein as Contrast

There are two main types of IFP that are used in *in vivo* imaging, namely IFP and iRFP. IFP proteins are derived and engineered from *Deinococcus radiodurans* while iRFP (infraRed Fluorescent Proteins) are derived and engineered from *Rhodopsuedomonas Palustris*. Both IFP and iRFP bind to Biliverdin which is a chromophore at either Cys15 or Cys256 or both.^[9] The ones that bind to Cys256 showed a blue-shift effect while the ones that bind to Cys15 showed a red-shift effect upon irradiation with NIR light. Irradiation of Biliverdin-incorporated IFP with NIR light causes isomerization and conformation change of the backbone of the protein which leads to a fluorescence output that is specific to the protein. However, there is a competition between Biliverdin and haem-derived compounds such as Protoporphyrin IX that can lead to a decrease in fluorescence intensity. However, this does not change the position as the emission is due to conformational changes of protein backbone due to the binding of Biliverdin and hence it is characteristic to the protein. This application is limited to whole-cell labels for non-invasive NIR imaging and is useful in whole-body imaging.

2.3 Organic Dyes as Contrast agents

Organic dyes that are approved by the FDA for intra-operating imaging application is indocyanine green (ICG) and methylene blue^[10]. These dyes are organic molecules that consist of highly conjugated systems. These highly conjugated systems lower the energy gap between the HOMO and the LUMO. As a result, the absorption of the light is shifted towards the visible light region and it is able to undergo non-radiative process prior to the emission of light. The Stokes shift for the organic dyes is about 20nm. This indicates that the detection requires a detector that is able to detect NIR light. Despite the good application of ICG and Methylene blue in intra-operative applications, there are also toxicity issues that are associated with ICG and methylene blue. The former is incompatible with kidney patient due to the decomposition

of ICG to toxic materials upon irradiation while the latter is a monoamine oxidase inhibitor which can cause serotonin toxicity at low doses. This drives the development of more fluorophores such as Cy5.5 and polyvinylpyrrolidone-conjugated iridium complex which is able to respond to the tumor microenvironment for hypoxic cells.

2.4 Lanthanide Nanoparticles

2.4.1 Upconversion Nanoparticles (UCNP)

Lanthanide nanoparticles are one of the most versatile contrast agents that are available for NIR light imaging. One of the reasons for its versatility is because of the ladder-like energy levels in the 4f-5d transitions. This ladder-like energy levels allows for non-radiative transitions and emit light of a particular wavelength. For example, a Tm (III) co-doping will cause a blue emission while an Er (III) co-doping will cause a green emission. This difference in emission is attributive to the difference in the energy levels in each lanthanide and the choice of lanthanide makes it simpler for the tuning of the emissions. This versatility of the lanthanide nanoparticles positions itself well in two forms of imaging in the visible region as well as the NIR-II window. In the former, the lanthanide undergoes a process known as Energy Transfer Upconversion (ETU) where energy is transferred from the “activator” ion to the “emitter” ion. This ETU process allows an additional transition to the higher energy levels and emits a light of shorter wavelength. This makes the application of NIRF plausible. This is especially so when lower energetic NIR photons are used as the irradiation source and fluorescence in the visible region. This fluorescence in the visible region is widely applied in image-guided surgery where surgeons inject these nanoparticles into the parts on the open field. It then switches off the light in the operating theatre to allow for the impacted tumor cells to “light up” and perform a total tumor resection procedure instead of a partial tumor resection based on the field of sight. The limitation for this procedure is the autofluorescence that is derived from the cell upon

irradiation and may potentially give a false-positive result on the illumination. Despite that, it is still widely used as it is able to visualize the non-accessible area of the tumor cells.

Figure 1: Energy transfer mechanism in $\text{NaYF}_4: \text{Yb/Tm}$ and $\text{NaYF}_4: \text{Yb/Er}$ ^[11]

As shown in Figure 1, the energy at $^2\text{F}_{5/2}$ on the Yb^{3+} ions undergo ETU to $^1\text{G}_4$ of Tm^{3+} . Electrons at the $^1\text{G}_4$ energy level de-excite to the ground state and emit blue light at 460nm. Some electrons de-excite to the $^3\text{F}_4$ energy level and emit red light. These electrons can undergo another excitation from the irradiation beam to the $^3\text{F}_2$ energy level before de-exciting and emit red light. This process is known as the PA mechanism. The last mechanism is exemplified in the $\text{NaYF}_4: \text{Yb/Er}$ energy level diagram. The excited energy level of Yb^{3+} is similar to the energy level of $^4\text{I}_{11/2}$ of the Er^{3+} . These electrons at the $^4\text{I}_{11/2}$ can get excited once again by the irradiation source and excite to $^4\text{I}_{7/2}$ energy level. This is a process known as ESA. As illustrated in Figure 1, there are a few characteristic of UCNP that fulfill its aim to emit a photon in the UV-visible range. The first characteristic is the activator ion which is Yb^{3+} that absorbs the NIR photons. The second characteristic is the emitter ion which is either Tm^{3+} or Er^{3+} . The emitter ion has to be an ion that has “ladder” type energy levels. This allows for both up- and down-conversion that allows for the tuning of the observed color. However, the

limitation of fluorescence is the lifetimes. Hence, in our experiment, we synthesized a core-shell-shell particle ($\text{NaYF}_4@\text{NaYbF}_4:\text{Tm}@\text{NaYF}_4$) that is aimed to confine the energy within the inner-shell and enhance the blue light emission that is derived the emission of Tm^{3+} .

2.4.2 Downconversion Nanoparticles as contrast

The versatility of LNP had enabled its application for imaging in the NIR-II region. This window is characterized by the strong water absorption peak but low autofluorescence signal. The lanthanide nanoparticles used for the design in this region are primarily targeted to perform a downconversion. This is typically observed in most energy transition where it undergoes non-radiative transition and emits light of a longer wavelength. The NIR-II window is also associated with deep tissue imaging primarily attributive to its deeper penetration depth that is derived from using a light source of longer wavelength. It is circumvented typically with polymers which are able to alter its structural conformation that typically overcomes the strong absorption peak. The polymers used are typically long chains such as PEG which has electrons delocalization and this electron donating group are able to lower the energy gap between the HOMO and LUMO. As a result of this reduction of the energy gap, it is able to avoid the strong water absorption peak.

Figure 2: Schematic energy transfer mechanism of $\text{NaCeF}_4:\text{Yb/Er}$ ^[12]

NIR light is absorbed and excites electrons from the $^2F_{7/2}$ to $^2F_{5/2}$ on the Yb^{3+} ion. The energy is transferred to electrons at $^4I_{11/2}$ energy level of Er^{3+} . At this juncture, there are two approaches for the electrons, both in the UC and DC which can also be known as Downshifting (DS). Spontaneous DC transitions occur from $^4I_{11/2}$ to $^4I_{13/2}$ before transitions to the ground state and emit a photon of wavelength at 1530nm. In this DCNP, there is a process known as phonon-assisted non-radiative relaxation that is facilitated by Ce^{3+} ion. The energy gap in Ce^{3+} ($^2F_{5/2} \rightarrow ^2F_{7/2}$) corresponds to the spontaneous DC transitions ($^4I_{11/2} \rightarrow ^4I_{13/2}$). This assisted the DC transition on Er^{3+} as energy is absorbed by the Ce^{3+} ions. The population of electrons at the $^4I_{13/2}$ energy level enhances the emission of photons at 1530nm.

2.5 Comparison of Contrast Agent

The contrast agents evaluated are the infrared fluorescence proteins, organic dyes, and nanoparticles. The mechanisms of these contrast agents can be summarized in the table below.

Table 1: Comparison of Contrast Agent and Mechanisms

Contrast Agents	Mechanism
Infrared Fluorescence Proteins	Conformation changes that led to fluorescence
Organic Dyes	Excitation and emission mechanism
Nanoparticles	Energy transfers between the activators and the emitters

As summarized in table 1, the mechanisms of each type of contrast agents are different and it requires different synthesis steps. The fluorescence proteins are limited as it is based on the conformational changes. One of the key advantages of isomerism and conformational changes

is the reversibility of this change and it is able to work as a photo switch. It is indeed important for a photo switch in contrast agent that is able to allow for evaluation in both commission and omission of the signal. This helps to evaluate background signals or interpret results when there is an omission of signals. However, this fluorescence protein is limited in its signal due to the autofluorescence signal within the NIR-I region. The naturally occurring fluorescence of cells can interfere with the signals to give a false-positive result. Organic are similar in terms of the detection with infrared fluorescence proteins. It works based on the energy gap between the HOMO and LUMO of the molecule. This type of mechanism works well when it evaluates the system. For example, the irradiated light can give a signal based on the interactions between the antigen and markers. In the event that there is a correct match, it is able to give a fluorescence signal that is able to be interpreted as a positive result.

Lanthanide nanoparticles are one of the newest classes of contrast agents. This uses lanthanides co-doped nanostructures that have absorption of lower energy photons but is able to upconvert the energy to higher energetic photons. The key advantage of using nanoparticles is the allowable transition that leads to both upconversion and downconversion properties. In the UCNP type mechanism, it is able to visualize the system and in the DCNP type mechanism, it is able to image deeper tissues. However, the limitation of DCNP is the strong absorption of water within the NIR-II region which makes it require a water-dispersible coating and conjugation with a phospholipid or liposomes. Hence, UCNP and DCNP are good contrast agents that are versatile by the choice of the activator and emitter. For example, 808nm irradiation is possible when Nd^{3+} is added as a dopant and an emission wavelength can change from 1400nm to 1530nm when the emitter is changed from Tm^{3+} to Er^{3+} . It is also important to have good control over the size and morphology of the nanoparticle due to the Ostward Ripening process. In this new nanotechnology, the size of the particles plays a critical role in

confining the energy and enhancing the emission. The reaction conditions are critical as a longer synthesis time can lead to a more thermodynamic product over a kinetic product that is driven by the Ostward Ripening process. Hence, evaluating nanoparticles against the fluorescence proteins and organic dyes, the nanoparticles are versatile as the signal position can change with the co-doping of different emitters into the system. Similar to other contrast agents, there are safety concerns regarding its applicability in biological system. The inherent nature of lanthanide is considered non-toxic as the LD₅₀ of lanthanide ions in the form of chlorides and nitrates are in the range of 10mg/kg to 1100mg/kg. ^[15] These high LD₅₀ values suggest that the use of trivalent lanthanide ions is safe and can be used for biological applications. It is also noted that only chronic exposure to lanthanide compounds may lead to conditions such as pneumonitis and acute inflammation. However, this toxicity is not applicable to UCNPs as it is embedded in a dielectric matrix, and cannot diffuse into cells unless it undergoes decomposition *in situ* which exposes the lanthanide ions to the phospholipids bilayer. In conclusion, LNPs are positioned favourably with minimal toxicity to biological systems and has great potential as an emerging class of contrast agents.

Chapter 3: Phototherapy of NIR contrast agent

3.1 UCNP-conjugated cyanobacteria therapeutic system

Upconversion nanoparticles are not only applicable in fluorescence imaging but it is also possible to use the fluorescence light for therapeutic applications. The fluorescence light, which is tunable through the selection of the emitter ions, is able to activate microorganisms which are sensitive to light within the blue and UV region. The UV light, in contrast, is more energetic than blue light and can kill microorganism through the breaking of the double strand 2'-deoxyribonuclease (DNA). Blue light, on the other hand, is lower in energy and is able to provide energy for other biological applications. In our experiment, we design an innovative biologic system that uses UCNP to activate photosynthetic cyanobacteria (*S. Elongatus*) to produce oxygen as a therapy method for Ischemia-Reperfusion injury. Ischemia-Reperfusion injury is one of the leading known causes of death. It is a condition where there is a lack of supply of nutrients and oxygen to the muscles and tissues. In the lack of oxygen and nutrients to the tissues, apoptosis of the cell begins. This is especially so for the cardiac arrest where there is a lack of oxygen and nutrient supply to the brain, causing the brain tissues to die quickly and hence enter into a brain dead condition. In such a case of Ischemia, the sole remediation is timely reperfusion of oxygen into the cell so that the cell death can be delayed. One of the examples of such timely reperfusion is the execution of cardiopulmonary resuscitation (CPR) procedure where oxygen is blown into the lungs and the compression circulates the oxygenated blood into the body so as to delay cell death. It is noted that there is also reperfusion injury that is due to the anaerobic metabolism interruption and causes production of reactive oxygen species (ROS) upon reperfusion. The free radicals that are produced are reactive to kill healthy cells and cause damage to the cell. In this experiment, we designed an innovative system where we use near-infrared light (NIR) to activate nanoparticles that exhibited upconversion properties. The emitted light (blue) is then used to activate photosynthetic cyanobacteria (*S.*

Elongatus) to produce oxygen supply to the muscles and cell to delay cell death. The mechanism of action can be illustrated in Scheme 1.

Figure 3. Illustration of the NIR upconversion-cyanobacteria photosynthetic system against cardiac oxygen-glucose deprivation

3.2 Methods

The addition of the amino-silica shell to the UCNP allows for conjugation with photosynthetic cyanobacteria. The characterization of the nanoparticle can be performed using Transmission Emission Microscopy (TEM) for the size of the synthesized particle. A spectroscopic method is employed to characterize strategy employed for this design is the use of an upconversion nanoparticle ($NaYF_4$: Yb/Tm). The selection of the lanthanide allows for an irradiated light at 980nm and an emission at 430nm. A core-shell strategy is employed to confine the energy transfer within the core and the surface is modified with the position at which it emits. In the conjugation with cyanobacteria, a fluorescence protein (FITC) is used to characterize the conjugation via confocal microscopy. A Dynamic Light Scattering (DLS) analysis was also performed to determine the average size of the particles. The results are summarized in Figure 4.

Preparation of NaYF₄: Yb/Tm (30:0.5) core nanoparticles:

Yttrium (III) Acetate hydrate (172.9mg), Ytterbium (III) Acetate hydrate (126.7mg) and Thulium acetate hydrate (17.3mg) was dissolved in 3mL of Methanol and was subsequently added to a round bottom flask containing 6mL of Oleic Acid and 7.5mL of Octadec-1-one with a stirring speed of approximately 900 rpm. The reaction mixture was heated to 100°C to remove methanol under vacuum. The temperature was raised further to 130°C to allow the reaction under nitrogen gas to proceed to form Lanthanide (III) oleate complexes for 30 minutes. It was lowered to 50°C and Ammonium Fluoride (133.3mg) dissolved in 5mL of Methanol containing sodium hydroxide (100mg) was added. The temperature of the reaction flask was raised to 100°C to remove methanol under vacuum. Subsequently, it was heated to 290°C for 2 hours. It was cooled naturally to room temperature and precipitated with EtOH. The particles were centrifuged at 6000 rpm for 5 minutes thrice.

Preparation of NaYF₄: Yb/Tm (30:0.5) @NaYF₄ core-shell nanoparticles:

Yttrium (III) Acetate hydrate (182.236mg) was dissolved in 3mL of Methanol and was subsequently added to a round bottom flask containing 6mL of Oleic Acid and 7.5mL of Octadec-1-ene with a stirring speed of approximately 900 rpm. The reaction mixture was heated to 100°C to remove methanol under vacuum. The temperature was raised further to 130°C to allow the reaction under nitrogen gas to proceed to form Yttrium (III) oleate complexes for 30 minutes. It was lowered to 50°C and NaYF₄ core nanoparticles were added to the reaction mixture. The reaction mixture was heated to 90°C to remove hexane. After completion of removal of hexane, the reaction mixture was lowered to 50°C and ammonium fluoride (133.3mg) dissolved in 5mL of Methanol containing sodium hydroxide (100mg) was added. The temperature remained at 50°C for 30 minutes to allow for the coating to occur. The temperature of the reaction flask was raised to 100°C to remove methanol under vacuum.

Subsequently, it was heated to 290°C for 2 hours. It was cooled naturally to room temperature and precipitated with EtOH. The particles were centrifuged at 6000 rpm for 5 minutes thrice.

Preparation of NaYF₄: Yb/Tm (30:0.5) @NaYF₄@dSiO₂ nanoparticles:

NaYF₄@NaYbF₄: Tm@ NaYF₄ core-shell-shell nanoparticles (5mg) was dissolved in 20mL of hexane. The nanoparticles are added into a round bottom flask containing 5mL of Triton X-100, 5mL of 1-hexanol and 850μL of deionized water with a stirring speed of approximately 900 rpm. Triethylorthosilicane (TEOS, 2 μL) and 3-aminophenyltriethylsilicate (APTES, 8 μL) was mixed in 200 μL of hexane. The mixture was added dropwise to the reaction flask with a stirring speed of 1000rpm. The mixture was stirred for 4 hours prior to the addition of aqueous ammonia (25%, 200 μL) into the reaction mixture and stirred for an additional 43 hours. The mixture was washed with Ethanol and centrifuged at 8000rpm for 9 minutes. The nanoparticles are dispersed in 10mL of deionized water.

Figure 4. Preparation and characterization of UCNPs. A) Scheme of UCNPs preparation and modification. B, C) TEM images of OA-coated core-shell UCNPs and $\text{UCNP}@\text{SiO}_2\text{-NH}_2$, respectively. D) Average hydrodynamic diameter distribution of the prepared $\text{UCNP}@\text{SiO}_2\text{-NH}_2$ (48 ± 1 nm). E) Upconversion luminescence spectra and a photograph of $\text{UCNP}@\text{SiO}_2\text{-NH}_2$.

Figure 4 shows the method of synthesis and the characterization of the novel nanoparticles for its applications. The synthesis method employed is established literature that uses hydrothermal conditions in the UCNP synthesis and microemulsion conditions in the surface modification with silica-coating as shown in Figure 4(a)^[14]. The TEM images in Figure 4(b) showed that

the particles are uniformed at around 40nm. This method was slightly modified to hold at the particle growth temperature of 290 °C for 2 hours instead of the typical 1.5 hours. This is to ensure uniform particle formation, as well as the completion of the reaction as 1.5 hours, seems to give non-uniform particles which give a mixture of alpha and beta type nanoparticles. While it does not have a significant impact on the application, it is important to have uniformed size and shape. In the microemulsion method for silica-coating, it is noted that the amount of Tetraorthoethylsilicate (TEOS) and (3-Aminopropyl) triethoxysilane (APTES) are critical in the silica-coating efficiency as well as the thickness of the coat. In the standard microemulsion method, 8uL of TEOS and 2uL of APTES was added to the reaction mixture before the addition of aqueous ammonia (25%) to polymerize the silica coat. However, it was noted during the synthesis procedure that the 8uL and 2uL combination did not yield good coating and gives a coating of 1-2nm. However, in another experiment, we tripled the quantity of the TEOS and APTES to 24uL and 8uL respectively. TEM images were taken for the coat and it was observed that there were more uniformed silica-coat with a thickness of 5nm. This thickness is sufficiently good for application for subsequent application as shown in Figure 4(c). A luminescence spectrum was taken for the UCNP and a double peak at 460nm was observed. This is consistent with the transition from the 1G_4 and 1D_2 energy levels as shown in Figure 4(e).

3.3 Results and Discussions

It was noted during the synthesis of the core-shell nanoparticles, the duration where it holds at 290°C is critical. In established literature employing hydrothermal strategy, it reported a hold time of 1.5hours. However, for this time duration, it was noted that the particles contain a mixture of alpha and beta particles. This is indicative of an incomplete reaction for the epitaxial growth of the nanoparticles. At 2 hours, we observed that there are lesser smaller particles and its size are more homogeneous and uniformed.

Surface modification of the UCNP is critical in improving its biocompatibility as the nanoparticles synthesized are soluble in organic solvents such as cyclohexane. Hence, a microemulsion was performed with surfactant (Triton X-100) and a co-surfactant (1-hexanol) to allow both aqueous water, silicates as well as the nanoparticles to mix. It was noted that the reaction time, amount of TEOS and APTES used are critical in terms of the thickness of the shell as illustrated in Table 2. This reaction is slow and requires aqueous ammonia to catalyze the reaction. This causes the silica shell to be formed and hence the production of water-soluble nanoparticles. A further modification to the established literature was to increase in the dose of TEOS and APTES that alters the thickness of the silica shell. We attempted to use the combination of 8uL of TEOS and 2uL of APTES, the silica-shell was unable to be seen under the TEM. Considering the slow reaction in the polymerization of the silica-shell, the duration for the reaction was extended from 12 hours to 43 hours. The combinations of the TEOS and APTES, as well as the duration of the reaction, are summarized below.

Table 2: Comparison of TEOS, APTES and Reaction time on Silica shell thickness

Experiment No	Amount of TEOS	Amount of APTES	Reaction Time	Remarks
1	8	2	12	No product
2	8	2	43	1nm thick
3	24	8	72	5nm thick

In table 2, it was noted that the reaction time was critical in the use of polymerization that is evidenced from the reaction time in Experiment 1 and 2. We performed the third experiment with thrice and amount of TEOS and APTES as well as the increase in reaction time, we were able to obtain a 5nm thickness of silica-shell. This indicates that the quantity of the TEOS and APTES impacted the thickness of the shell coated.

After the successful synthesis of the silica-coated UCNP, it is important to test the adsorption capability of the silica-coated UCNP to cyanobacteria. This is based on the electrostatic attraction between the positively charged amino-group on the surface as well as the negatively charged phosphoryl group on the outer membrane of *S. Elongatus*. In order to validate and visualize the interaction between the charge variants, a fluorescence organic dye molecule, FITC is first tagged to the silica-coated UCNP. Control of the cyanobacteria was taken when excited in the red and green channel. However, on the addition of the UCNP tagged with FITC to the cyanobacteria green fluorescence from the FITC as shown in Figure 5(a). This indicates that the electrostatic adsorption is successful and the FITC fluorescence.

Preparation of NaYF_4 : Yb/Tm (30:0.5) @ NaYF_4 @ dSiO_2 conjugated with cyanobacteria

Cultured cyanobacteria were the first harvest by centrifugation thrice with BG-11 medium. Subsequently, the cyanobacteria (20 μL) is added to the silica-coated UCNP (20 μL) for held for 30mins. The conjugated cyanobacteria were evaluated using TEM and confocal imaging as shown in Figure 5 below.

Figure 5. Fabrication of UCNP-cyanobacteria based NIR photosynthesis platform. A) Schematic illustration of UCNP@SiO₂-FITC preparation and the cyanobacteria labeling by electrostatic adsorption. B) The TEM image of cyanobacteria labeled with UCNP@SiO₂-NH₂ (Scale bar: 0.5 μM). C) Confocal images of cyanobacteria labeled with UCNPs@SiO₂-FITC. Red channel: Ex.640 nm, Em. 660 nm; Green channel: Ex. 488 nm, Em. 512 nm. Scale bars: 20 μM.

As shown in Figure 5(b), the UCNP is able to be labeled on the cyanobacteria. The UCNP are distributed across the cyanobacteria. In a control experiment, excitation of the cyanobacteria is used at 640nm. At this wavelength, FITC is not activated to emit green fluorescence light. On the electrostatic adsorption and excitation wavelength of 488nm, the green fluorescence light observed corresponds to the presence of FITC. In this case, the superimposed images of the green and red channels showed that the position at which the cyanobacteria exhibited red fluorescence is consistent with those when FITC gives a fluorescence. This shows that the labeling of UCNP to the cyanobacteria is successful. This successful labeling of the UCNP to the cyanobacteria shows promising results for the applications for Ischemia-reperfusion experiments. It also suggests that the cyanobacteria conjugated UCNP remains integral. In addition, TEM images shown in Figure 5(b) shows that the structure of the cyanobacteria is

intact and does not show any deformities. Hence, it is plausible to conclude that the UCNP does not have any cytotoxicity impact to the cyanobacteria. Furthermore, in a review article^[15], there was a report of the use of NaYF₄:25% Yb³⁺, 0.3% Tm³⁺ with a SiO₂ bioconjugation shell in an IV administration with virtually no tissue deposition of after 24 hours post injection. This suggest there is no toxicity to the cells of interest as well as to cyanobacteria. In terms of phototoxicity, the Ultraviolet (UV) light produced upon the irradiation of Near-infrared (NIR) light are negligible. This is because cyanobacteria are photosynthetic in nature and has the capability to overcome UV toxicity, in the form of UV-induced Reactive Oxygen Species (ROS), using UV absorbing compounds such as mycosporine-like amino acid (MAAs) and scytonemin (SCY). Hence, toxicity of UCNP to cyanobacteria is also minimal. In order to understand its toxicity in an *in vivo* application, it is important to establish if there are any condition in the environment that may kill the cyanobacteria. Jeffrey *et. al*^[16] publication in 2017 had reported strong evidence that the cyanobacteria can be kept alive. It was reported that *S. Elongatus* can be cultured with rat cardiomyocytes under physiological conditions. It showed that at physiological conditions, cyanobacteria are able to grow. In the tissue oxygenation experiment, the rodents are injected with *S. Elongatus* had a 25-fold increase in oxygen levels while the untreated with *S. Elongatus* only had a 3-fold increase in oxygen levels. This further provide evidence that the conjugated cyanobacteria can be kept alive in its potential application.

3.4 Conclusion

The experiments conducted above demonstrated that UCNP, which is a NIR contrast agent, is able to function as a therapeutic drug. This is performed through surface modification with a silica-coating and subsequently uses the electrostatic charges on the surface to the label to the cyanobacteria. The confocal images demonstrated the successful labeling of the UCNP to the cyanobacteria and bring both in close proximity for the energy transfer. Despite the lack of oxygen-reperfusion experiments, the successful labeling shows that it a potential treatment drug that can be controlled with an external NIR light source for the controlled oxygen-reperfusion. However, more oxygen-reperfusion studies are required to further understand and evaluate its effectiveness in supplying oxygen in an ischemic condition.

Chapter 4: NIR-II deep tissue imaging of NIR contrast agent

4.1 Ratiometric redox luminescence imaging in the second NIR window

In our experiment, we synthesize a sub-10nm nanoparticle that undergoes downconversion properties in the NIR-II window. This particle undergoes a phonon-assisted downconversion transition that enhances the emission at 1530nm. This is because the energy gap in the Ce^{3+} ions is approximately the same as the transition of Tm^{3+} . This energy transition is enhanced as the Ce^{3+} serves to absorb the energy and causes a population of photons at the desired energy level. As a result, the emission at 1530nm is enhanced. In our experiment, we leverage on this energy transition to consider if a redox imaging can be performed. Redox imaging is critical in the biological systems to detect the presence of reactive oxygen species (ROS) as well as mitochondrial redox. In both cases, these are associated with metastasis tumor cells as well as cancer-promoting species. In this particular design, we design a robust nanoparticle that is able to correlate to the redox condition in the biological system.

Figure 6. Illustration of the principle of LNP for ratiometric redox imaging in the NIR-II window. The LNP is modified with the hydrophilic polyethylene glycol (PEG).

In the scheme shown in Figure 6, the design uses an Nd (III) lanthanide as an internal standard due to the emission observed at 1064nm, a characteristic emission of Nd (III). The operating principle of this design leverages on the energy level of Ce (III). As previously mentioned, Ce(III) serves as a phonon-assisted transition for the observation of Er (III) emission peak at 1530nm. In the presence of oxidizing agent such as ROS, the Ce in the core-shell is oxidized

from Ce(III) to Ce(IV). This oxidization causes the energy level to be lowered as the nucleus attracts more strongly to the valence electrons. Hence, the energy gap in Ce(IV) is no longer similar to that of the Er(III) transition. This subsequently does not allow for the phonon-assisted transition for the emission of 1530nm. Hence, the emission at 1530nm will be diminished. In similar reducing conditions, the 1530nm will be reappeared and cause it to be a good photo switch. In order to have a real application to this system, it is important to have an internal standard. Nd(III) was hence employed as the internal standard due to its capability to be irradiated at 808nm and its emission at 1064nm. While it is known that the Er(III) emission will reduce, the Nd(III) peak will also reduce corresponding as well. The ratio of the Nd(III) and Er(III) will then be able to quantify the oxidized state of the biological system.

4.2 Materials and Methods

In our experiment, we synthesize NaCeF₄: Nd/Yb/Er (20/1/0.5) to study the energy transfer mechanism.

a) Preparation of NaCeF₄:Nd/Yb/Er(1:20:0.5) core nanoparticles:

Cerium (III) Acetate (234.8mg), Neodymium (III) Acetate hydrate (32.1mg), Yttrium (III) Acetate hydrate (53.2mg) and Erbium (III) Acetate hydrate (17.2mg) was dissolved in 3mL of Methanol and was subsequently added to a round bottom flask containing 5mL of Oleic Acid, 10mL of Octadec-1-ene and 5mL of Oleylamine with a stirring speed of approximately 900 rpm. The reaction mixture was heated to 100°C to remove methanol under vacuum. The temperature was raised further to 130°C to allow the reaction under nitrogen gas to proceed to form Yttrium (III) oleate complexes for 30 minutes. It was lowered to 50°C and Ammonium Fluoride (133.3mg) dissolved in 5mL of Methanol containing sodium hydroxide (100mg) was added. The temperature of the reaction flask was raised to 100°C to remove methanol under

vacuum. Subsequently, it was heated to 290°C for 2 hours. It was cooled naturally to room temperature and precipitated with EtOH. The particles were centrifuged at 6000 rpm for 5 minutes thrice.

b) Preparation of NaCeF₄:Nd/Yb/Er(1:20:0.5)@ NaCeF₄ core-shell nanoparticles:

Cerium (III) Acetate (234.8mg) was dissolved in 3mL of Methanol and was subsequently added to a round bottom flask containing 5mL of Oleic Acid, 10mL of Octadec-1-ene and 5mL of Oleylamine with a stirring speed of approximately 900 rpm. The reaction mixture was heated to 100°C to remove methanol under vacuum. The temperature was raised further to 130°C to allow the reaction under nitrogen gas to proceed to form Yttrium (III) oleate complexes for 30 minutes. It was lowered to 50°C and core nanoparticles are added. The reaction mixture is heated to 90°C to remove hexane. The reaction mixture was lowered to 50°C and Ammonium Fluoride (133.3mg) dissolved in 5mL of Methanol containing sodium hydroxide (100mg) was added. The reaction mixture was allowed to stir for 30 minutes. The temperature of the reaction flask was raised to 100°C to remove methanol under vacuum. Subsequently, it was heated to 290°C for 2 hours. It was cooled naturally to room temperature and precipitated with EtOH. The particles were centrifuged at 6000 rpm for 5 minutes thrice.

4.3 Results and Discussions

Figure 7. Characterization and H_2O_2 response of the LNP. A) The TEM image of LNPs. B) Downconversion luminescence (DCL) spectra of LNPs with or with OA coating. C) The H_2O_2 response of PEG-modified LNPs. $\text{Em}_{1060}/\text{Em}_{1530} = 2.76$ (before H_2O_2 treatment), $\text{Em}_{1064}/\text{Em}_{1530} = 4.81$ (after H_2O_2 treatment). D) Downconversion luminescence spectra of LNPs with different contents of the Er^{3+} -doping.

In Figure 7(a), the TEM images of the LNPs are synthesized. The nanoparticles in this case are smaller in size and less uniform than that of the UCNPs. This is primarily due to a process known as Ostward Ripening Process. Ostward Ripening is the process where smaller particles are redissolved and form larger particles. This process is driven by the energetics and the thermodynamically products are formed with greater reaction time. In this reaction, we performed the synthesis with the use of oleylamine. Oleylamine has a significant role to play in the nucleation and growth process. It slows down the Ostward Ripening process and prevents

the formation of bigger particles. In our experiments, we also observed that without the use of Oleylamine, the particles formed are bigger in size but did not show the favored emission position upon irradiation with 808nm light. With the use of Oleylamine and reaction condition of 2 hours, we are able to obtain a more uniformed particle which is approximately 10-15nm. In Figure 7(b), we also observed that the OA-LNP particles that are tested in cyclohexane solvent showed a distinct peak at 1530nm while the Ligand-Free-LNP particles that are tested in water showed no peak at the 1530nm. This is primarily due to the choice of solvent used. As ligand-free-LNPs do not have OA capped on the surface, their solubility is significantly better in water. As a result, upon testing in water, it is noted that in the NIR-II window, the emission evidenced by the OA-LNP are absorbed by the strong absorption of water at 1500nm. In view of the limitation of the strong absorption of water in the NIR-II window, we conjugate the LNP with amphipathic polymer like Polyethylene glycol (PEG). It increases the solubility in the buffer as well as the detection of the Er (III) emission position as shown in Figure 7(c). The emission shows that on the treatment of Hydrogen Peroxide, there is a reduction of the Er(III) emission. With the choice of Nd(III) as a lanthanide to be co-doped, there exists an internal standard at 1064nm. Taking the ratio of the Er(III) and Nd(III), it is possible to quantify the oxidation that has occurred. The ratio of Nd(III)/Er(III) emission has changed from 2.76 to 4.81. This shows that in an oxidizing environment, Ce^{3+} is oxidized to Ce^{4+} causing a decrease in the Er(III) emission. With the significant increase in the ratio, the difference in intensity can be used to correlate the oxidizing condition. However, more works have to be done to show the correlation between the oxidizing environments against the intensity to establish a calibration curve for quantification. It is noted that there is a reduction in Nd(III) emission at 1064nm. This is due to the smaller energy gap in Ce^{4+} after oxidation which also undergoes a phonon-assisted transition that correspondingly decrease in similar intensity of Nd(III) and

Er(III) transition. However, it does not impact Nd(III) as an internal standard for ratiometric analysis.

4.4 Conclusion

In summary, we concluded that NIR-II window is a good platform for deep tissue imaging, redox imaging and dynamic monitoring of the redox condition. However, it also has significant challenges such as the strong water absorption that can be circumvented by performing a surface modification with a polymer such as PEG. Xialian *et. al*^[12] had reported the use of the constructed nanoparticles for *in vivo* bioimaging which uses a phospholipid as the bioconjugate to the nanocrystals. The experiment is performed over a 24h period with diminishing emission signal. This suggest that the integrity of the nanocrystal *in vivo* is intact for approximately 24h. Hence, the LNP is stable for its potential *in vivo* applications for redox imaging. The decrease in intensity of Er(III) emission on the treatment of an oxidant demonstrates the omission of phonon-assisted transition. This is consistent with the hypothesis that it is attributive to the oxidation of Ce (III) to Ce (IV) ions. Another challenge in this design is the simultaneous reduction of Nd(III) emission intensity at 1064nm as well after treatment with an oxidant. Hence, further works are required to establish the mechanism of oxidation of the core-shell nanoparticle for effective quantitative application of LNP in NIR-II imaging using ratiometric analysis. Despite the lack of understanding of the mechanism of oxidation of the nanoparticle, the ratio of the Nd (III) Emission at 1064nm and Er (III) emission at 1530nm are significantly large for their application in the ratiometric analysis of the redox condition of the biological system. It has theranostic applications in both NIR-II imaging and redox imaging.

Chapter 5: Conclusion and Future Work

In summary, we had explored the various types of NIR imaging contrast agent that is able to perform its role in the NIR-I and NIR-II imaging region. It was noted that the use of up- and down-conversion nanoparticles are able to achieve its aims in the developing of NIR contrast agent that is able to enhance NIR-II imaging and exhibit therapeutic properties. In the first project, we demonstrated that NIR contrast agent is able to play a role in therapy especially when electrostatically labeled with cyanobacteria. This is definitely a promising application for timely reperfusion in Ischemia conditions and reduces the impact of Ischemia-reperfusion injury due to delay reperfusion that may generate more ROS to cause more cell death. In the second project, we demonstrate that LNPs that utilize the down-conversion features to perform NIR-II imaging and the ratiometric analysis using this property can be further explored to obtain more information about the redox condition in the biological system. Both projects had demonstrated that there is potential in the use of LNPs for theranostic application. The minimal toxicity and stability of LNPs had positioned itself favourably as good and plausible contrast agent for *in vivo* applications. Although the development of a contrast agent that is able to perform both therapeutic and diagnostic capability is not shown within the projects of this thesis, it is able to demonstrate that LNPs are good NIR contrast agent that is able to have therapeutic capabilities such as the case with cyanobacteria. It is definitely promising that further development of LNPs will be able to achieve this aim of using a single NIR contrast agent to serve theranostic applications and hence actualize the vision towards personalized medicine.

References

1. Personalized Medicine Coalition. *Personalized Medicine at FDA: 2018 Progress Report*. Washington D.C, 2018
2. Mariya, Lobanovska and Giulia Pilla, Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future?, *Yale Journal of Biology and Medicine*, **2017**, 90, 135-145
3. W. Ken Redekop, Deirde Mlads. The faces of personalized medicine: A Framework for understanding its meaning and scope, *Value in Health*, **2013**, 16, S4-S9
4. S. Jeelani *et. al.* Theranostics: A treasured tailor for tomorrow, *Journal of Pharmacy and Bioallied Sciences*, **2014**, 6, S6-S8.
5. Edward B. Silberstein. Radioiodine: The Classic Theranostic Agent, *Seminars in Nuclear Medicine*, **2012**, 42, 164-170
6. Vijay *et. al.* Magnetic Resonance Imaging: Principles and Techniques: Lessons for clinicians, *Journal of Clinical and Experimental Hepatology*, **2015**, 5, 246-255
7. Chensu Wang *et. al.* Optical Molecular Imaging for tumor detection and image-guided surgery, *Biomaterials*, **2018**, 157, 62-75
8. Shuo Chen *et. al.* Near-Infrared Deep Brain Stimulation via upconversion nanoparticle-mediated optogenetics, *Science*, **2018**, 359, 679-684
9. Olesya V. Stepanenko *et. al.* Interaction of Biliverdin Chromophore with Near-Infrared Fluorescent protein BphP1-FP Engineered from Bacterial Phytochrome, *International Journal of Molecular Sciences*, **2017**, 18, 1009
10. Shihong Li *et. al.* Near Infrared fluorescent imaging of brain tumor with IR780 dye incorporated phospholipid nanoparticles, *Journal of Translational Medicine*, **2017**, 15, 18
11. Sen Liang *et. al.* A user-friendly method for Synthesizing High Quality NaYF₄:Yb, Er (Tm) Nanocrystals in Liquid Paraffin, *Journal of Nanomaterials*, **2011**, 2011, 302364

12. Xialian Lei *et. al.* Intense near-infrared-II luminescence from NaCeF₄: Er/Yb nanoprobe for *in vitro* bioassay and *in vivo* bioimaging, *Chemical Science*, **2018**, 9, 4682-4688
13. Linna Lyu *et. al.* Near-Infrared light-mediated rare-earth nanocrystals: recent advances in improving photon conversion and alleviating thermal effect, *NPG Asia Materials*, **2018**, 10, 685
14. X. Ai *et al.* Synthesis of Core-shell Lanthanide-Doped Upconversion Nanocrystals for Near-Infrared Light-Mediated Bioapplications, *Journal of Visualized Experiment*, **2017**, e56416
15. Anna *et al.* Upconverting nanoparticles: assessing the toxicity, *Chemical Society Review*, **2015**, 44, 1561-1584
16. Jeffrey *et. al.* An innovative biologic system for photon-powered myocardium in the ischemic heart, *Science Advances*, **2017**, e1603078