Selective Heating of Multiple Nanoparticles as a New Strategy for Controlled Release Applications

by

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B.S. Chemical Engineering
Michigan State University, 2002

Submitted to the Department of Chemical Engineering in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY

May 2009

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Abstract

Utilization of nanoparticle heating for controlled release application was proposed and its feasibility was explored. The proposed method was formulated by realizing that biomolecule–nanoparticle conjugation is heat sensitive and both their dimensions are in the same length scale. This exploration centered on showing the proof of concept that conjugated biomolecules can be released from the nanoparticle surface in a controlled manner by heating the nanoparticles via external energy sources. The selectivity of the multiple releases was also investigated.

Two mechanisms of nanoparticle heating were explored. The AC magnetic heating of magnetic nanoparticles has limitation due to its low-power energy delivered to nanoparticles. The irradiation of femtosecond laser pulses on the absorbing gold nanorods provides the answer to this limitation due to the very high-power of energy delivery through these ultrashort pulses.

We developed gold nanorod surface customization technique to enable DNA–nanorod conjugation, thus turning gold nanorods into nanoscale carriers. Pulsed laser excitation in resonance with their absorption peaks can heat and melt the nanorods. This is exploitable for controlling the release of DNA oligonucleotides conjugated onto the nanorod surface. Nanorods with different aspect ratios absorb light at different wavelengths and thus can be excited independently. We have successfully demonstrated the selective releases of two distinct DNA oligonucleotides, where each is released from a different type of nanorod. This was accomplished by the laser excitation at two different wavelengths corresponding to both of the nanorods’ absorption peaks. The releases were very selective, efficient, and externally tunable by adjusting the laser fluence. The released DNA oligos were still functional. This concept is expandable to beyond two species. Its thiol conjugation chemistry is versatile and capable of high loading. With these advantageous factors, this proof of concept of selective multiple triggered releases from gold nanorods have a great potential as a new strategy for multiple controlled released application.

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Acknowledgments

There are too many people contributed one way or another to my graduate study experience. Below is just my attempt to list as many people as I can think of during the thesis writing.

First and foremost, I am very very grateful for the opportunities, guidance, support, and advises that my advisor Assistant Professor Kimberly Hamad-Schifferli gave me throughout my study. I also like to thank her for giving me so much freedom to think, formulate, and execute any research ideas. Without these, I wouldn’t be able to achieve the proposed research goals for my thesis. It has truly been a very profound research and learning experience.

I am also very grateful to my co-advisor Professor Paula T. Hammond and my thesis committee Professor T. Alan Hatton and Professor Angela M. Belcher for their very helpful comments and feedback throughout my thesis development.

I also like to thank our collaborators Professor Andrei Tokmakoff and his graduate students in assisting me with the laser experiments, which is the essential part of my thesis. I also like to thank Assistant Professor Krystyn Van Vliet and the MIT CMSE for letting me use their equipments.

It has been a great pleasure to know and learn from my labmates, especially SunHo and Marie-Eve throughout these years. I owe them many thanks. A few undergraduate students, Aey, Stefan, and Ivan have assisted me very well with my laborious experiments. I like to thank them for their contributions. I wish them all the best for their future endeavors.

I also like to thank the people in my personal life circle. I am very thankful to my family for their mental support throughout my 12-year stay in the US. I am very grateful for my father who gave me so much freedom at my early age; thus he never imposed his life perspective on me, which enable to me to be more open minded. I am extremely grateful for my girlfriend Alice who stayed on my side throughout my struggles and for being a good listener to my random
thoughts. I will also miss thoughtful discussions with my friends, especially Raga, Fritz, and Rezy.

I also like to thank MIT for teaching me a couple important life lessons. I learned that open-mindedness is not always directly proportional to intelligence. I also come to realize that the truth is more important than happiness, because the truth will outlast happiness. Thank you, MIT.
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Chapter 1

Introduction

1.1. Motivation

Nature has managed to produce multitude biological molecules that can perform numerous tasks with high degree of precision and efficiency. This diversity of biomolecules typically composes a very complex biological system with its robust multi-layered control system. In an effort to harness the engineering of nature, many scientists and engineers are working not only to characterize this biological system but also to develop a method for direct manipulation of the biomolecules. This is important because in any attempt to understand the natural function of any biomolecule, one typically performs elimination processes at multiple levels. Therefore it is understandable that there is always the need for more methods to manipulate or control these biomolecules.

On the other hand, nanoscale material (nanoparticles) has gained a lot of interest in the past decades, due to their unique tunability of their optical, magnetic, electrical, thermal, and chemical properties. These nanoparticles (NPs) are typically about the same length scale of the biomolecules, such as proteins and DNA. Soon enough researchers started to explore the means to interface between the NPs and the biomolecules. This leads to many fundamental studies attempting to understand the
nature of these biomolecules using the nanoparticles as their tools.\textsuperscript{12, 13} These nanoparticles can be tuned appropriately for different purposes. They can be synthesized small enough to be used as probes for understanding the structure of biomolecules. They can also be synthesized to be large particles that can carry a high number of biomolecules on their surface.

Acknowledging that there is always the need for more ways to control biomolecules, we are motivated to develop a better and simpler method to control biomolecules by utilizing nanoparticles. Realizing that most biomolecules are heat sensitive in term of their structures, activities, and conjugations (with NP); we proposed an idea of controlling the biomolecules by increasing their local temperatures. Since both biomolecule and NP are typically in the same length scale, we then proposed the utilization of NP as the local heat sources. The main hypothesis is basically exploring the energy delivery mechanism from external sources to increase the temperature of NP which is conjugated to or incorporated with biomolecules. In turn this will elevate the temperature of the biomolecule; which then alter its structure, activity, or even their conjugation with NP itself. So if we can control the delivery of energy to heat up the NP, then we can control the biomolecules directly with NP. Therefore this method will not rely on physiological environment as control mechanism.\textsuperscript{14-16} We are interested in one of the simplest form of control of biomolecules; controlled release of the conjugated biomolecules from their NP counterpart.

The next stage of the hypothesis is utilizing different NPs to control the releases of different biomolecules. This will show the selectivity and multiplicity of this method. More specifically we are motivated by the potential application of our method for
enhancing the efficacy of combination therapy. Combination therapy or the use of multiple drugs has been sought for improving treatment efficacy of diseases such as malaria,\textsuperscript{17} cancer,\textsuperscript{18} and HIV.\textsuperscript{19} Though proven to be effective, the differences in the chemical properties (such as molecular weight, solubility) and pharmacokinetics of the components in a drug mixture can create challenges for loading, delivery, and release of multiple drugs.\textsuperscript{20} Even if a pre-determined synergistic ratio is encapsulated in a carrier, this ratio may not be maintained at a target upon delivery or during release. Typically, the timing of the release of each species is crucial for drug efficacy, as has been observed for tumor treatment.\textsuperscript{18} Therefore, for effective combination therapy, release rates of each drug must be controlled independently. Current solutions involve complex systems such as polymer multilayer\textsuperscript{21} or sophisticated bioMEMS implants.\textsuperscript{22,23} Nanoscale carriers have gained attraction, but achieving different release windows for each drug in a mixture requires engineering intricate architectures.\textsuperscript{18} Extending all of these strategies beyond two species or even changing the order of release is problematical. Clearly, an effective method to externally control release of each species independently and actively would ultimately lead to optimization of combination therapies. Therefore, in this thesis we like to show that our proposed method can be potentially effective for this particular application; by showing the proof of concept of our idea in utilizing multiple types of NPs to independently control the release of different biomolecules.
1.2. Objectives

In order to show the proof of concept of our hypothesis, we need to accomplish four specific aims from our investigation:

1. Explore methods for delivering energy to the NP from external energy sources, in order to heat up the NP.
2. Show the proof of concept that we can selectively heat different types of NPs.
3. Develop systems that incorporate NP and biomolecule (nano-bio systems)
4. Utilize these nano-bio systems to show that we can selectively release multiple biomolecules via the selective heating of different types of NPs.

1.3. Strategies

We investigated two methods of delivering energy into the NPs from external energy sources in order to elevate their temperature. They are alternating current (AC) magnetic field heating of magnetic NPs and pulsed laser photothermal heating of gold nanorods (NRs). The first mechanism typically utilizes the localized magnetic NPs in human tissues (e.g. tumor tissue) to transfer energy from external AC-magnetic field into heat. This heat will enable the destruction of malignant tissue where the particles are localized. The study of magnetic fluid hyperthermia was started in 1957 by Gilchrist et al., utilizing micrometer magnetite particles. But, not until 1993, when A. Jordan et al. used single domain NPs, showed that NPs gave much superior heating than multi-domain micrometer particles. We are interested in study the heating rate of different types of magnetic NPs. The difference in the magnetic properties of different types of magnetic
NPs will govern their heating profiles. We hope to exploit the differences in their heating profile for selective heating between two different magnetic NPs. In turn, we use this selective heating for controlling the selective release of different biomolecules.

On the other hand, gold,\textsuperscript{26,27} NPs have been exploited for both passive and active targeted delivery.\textsuperscript{28,29} In the case of gold NPs, their surface chemistry has been proven to be chemically versatile for loading biomolecules and optimizing physicochemical parameters.\textsuperscript{26,30} Gold nanorods (NRs) have also become attractive for biological applications due to their optical properties.\textsuperscript{31,32} Pulsed laser excitation in resonance with their longitudinal surface plasmon resonance (SPR\textsubscript{long}) can heat NRs locally to high temperatures,\textsuperscript{33} inducing melting. This triggered melting is exploitable for controlling the release of biomolecules conjugated to the NRs.\textsuperscript{34} Since SPR\textsubscript{long} is tunable by changing NR aspect ratio (AR),\textsuperscript{6,35} NRs with different ARs can be excited independently at different wavelengths. If different NRs are conjugated to different molecules, this strategy could be utilized for orthogonal triggered release of multiple species. Therefore we hope to show that this strategy can also serve well to our hypothesis.

1.4. Thesis Outline

The investigation of AC magnetic field heating of different types of magnetic NPs is presented in Chapter 2. In the following chapter (Chapter 3), we show our method of loading magnetic NP and drug model into lipid vesicles. The NP-lipid vesicle system was then used to study the feasibility of controlled release of the drug model via AC magnetic field heating. This study and its limitation are presented in Chapter 4. This chapter also
discusses how the photothermal heating mechanism may overcome such limitation. In Chapter 5 we show our synthetic capability of producing two different types of gold NRs. We then show that they can be excited independently and thus selectively heated (and melted) by exposure to femtosecond laser pulses at their corresponding absorption wavelengths. This investigation is presented in Chapter 6. In Chapter 7 we show that we can load different type of DNA oligonucleotide onto each type of gold NR (NR act as nanoscale carrier). Therefore we can study the release selectivity of different DNA oligonucleotides from two different types of gold NRs when they are selectively excited (melted). This selectivity study is presented in Chapter 8. Finally the thesis concludes with the discussion of our experimental results, the limitations and advantages of our proposed method, and how it can potentially be useful for the proposed controlled release application (Chapter 9). This chapter also includes some recommendations for further studies.
Chapter 2

Magnetic Field Heating of Nanoparticles

Magnetic nanoparticles (NPs) have been investigated for a variety of biomedical applications. These applications range from magnetic separation of biomolecules, magnetic carriers for drug delivery, magnetic resonance imaging (MRI) contrast enhancement, to hyperthermia. Magnetic NPs have some unique properties which make them very attractive for these applications. First, their sizes (from 10-100 nm) make them much smaller than a cell (10-100 μm) or virus (20-450 nm), but close to a protein (5-50 nm) or a gene (2nm wide and 10-100 nm long). Due to their size and solubility, it enables them to get closer to, tagged, labeled, or bound onto any of these biological entities. Second, since they are magnetic and the ability of magnetic field to penetrate into human tissue, therefore they can be remotely affected or controlled by external magnetic field. Third, these particles can response resonantly to time-varying magnetic field, which in turn, it enable the transfer of energy from the excited field to these particles.

Hyperthermia utilizes all of the above attractive properties of magnetic NPs, especially the third property. This application utilizes the localized magnetic NPs in
human tissues (e.g. tumor tissue) to transfer energy from external AC-magnetic field into heat. This heat will enable the destruction of malignant tissue where the particles are localized. This story of magnetic fluid hyperthermia already started in 1957 by Gilchrist et al.\textsuperscript{24}, where they used AC magnetic field to heat up the micrometer magnetite particles which have been injected into the subserosa of the intestine of dogs. Numerous other researchers have followed up the investigation in this area. But, not until 1993, when A. Jordan et al.\textsuperscript{25} used single domain NPs, showed that NPs gave much superior heating than multi-domain micrometer particles even at lower field amplitudes.

2.1. Magnetic Fluid Hyperthermia

In order to appreciate and get a better picture on the idea of using localized heating mechanism to kill malignant cell in tumor tissue, we need to go back and learn about its history. The development of the concept started from the idea of using elevated body temperature to cure illness and all the way to magnetic fluid hyperthermia. Of course, this was also followed by evolving principle of the heating mechanism along the way.

2.1.1 History of Hyperthermia

The history of hyperthermia dates back to around 3000 BC when the Egyptians used a “fire drill” to burn away a tumor in the breast and in India people used steam bath to cure variety of illness.\textsuperscript{36} In today’s terminology, these practices of tissue destructions at temperature of higher than 47°C are called “thermoablation”. The term of
“hyperthermia” it self is coined to an application of temperature increase in range of 41 to 46°C onto malignant cells in cancer tissue. Nowadays, hyperthermia has replaced thermoablation due to the undesired systemic side effects of thermoablation. The compelling biological rationale of this hyperthermia concept is mainly on the differences of vascular and blood flow system between tumor and normal tissues. Typical tumor tissue has poor and disorganized vascular and blood flow system in comparison to normal tissue. This deficiency has two implications. First, poor blood circulation in tumor tissue means that it has poor heat dissipation if the tumor may become hotter. Second, it is known that tissue with poor blood circulation will result in nutrient deficiency and lower pH around that tissue region. Both of these implications make tumor cells viable to killing by hyperthermia. The essential challenges for the hyperthermia application is providing sufficient enough heat and it has to be localized around the tumor and leaving the surrounding tissue unaffected.

There have been a numerous techniques in the history of hyperthermia, but they mainly categorized into three techniques, whole body, regional and localized hyperthermia. With whole body hyperthermia, thermal energy is introduced into the body and maintained the temperature at 42°C for a few hours. This can be done by non-invasive methods such as hot air, hot wax, infrared or radiofrequency radiation, invasively by heating the blood extra-corporeally. Uniformity in temperature can be achieved but the maximum permissible temperature is relatively low. With the regional hyperthermia a large portion of your body (trunk or whole limbs) where the tumor located, are heated either invasively or non-invasively. While with the localized hyperthermia, only a smaller volume of the tissue local to the tumor is heated. Both
regional and localized hyperthermia can be performed invasively by the usage of non-ionizing electromagnetic waves or ultrasound, or invasively by using interstitial implants inserted into the body cavities.  

### 2.1.2 Current Status of Magnetic Fluid Hyperthermia

The latest and current method in localized hyperthermia is involving the usage of ferromagnetic or ferrimagnetic material confined within the treatment area and exposed to external oscillating electromagnetic field. Instead of using electric (E-) field as for most of the interstitial hyperthermia method, magnetic (H-) field dominant system is used to inductively couple the energy to the lossy medium of the ferromagnetic particles. These confined ferromagnetic particles dissipate energy from the applied H-field in the form of energy through various kinds of energy loss mechanisms, such as eddy current loss, hysteresis loss and relaxation losses, which will be discussed in details in the following subsection. The first study of magnetic fluid hyperthermia (MFH) was conducted by Gilchrist et al. in 1957.²⁴ They injected micrometer size of magnetic particles into the subserosa of dog intestine and observed a high concentration of magnetite localized in particular lymph nodes near the injection site. Then they used an excised lymph node containing 47mg of ferrite per gram of tissue, an AC H-field intensity of 15 - 20 kA/m and frequency of 1 – 2 MHz. They observed a temperature increase of 4.7°C/min. Gilchrist et al. continued their work in this MFH method for the next decade since his first reported study. The trends in their experiment setup are decreasing frequency and increasing H-field amplitude, with their rationale of reducing the heating of normal tissue.³⁷,³⁸ In 1979, Gordon et al.³⁹ for the first time used a narrow
distributed dextran magnetite to treat mammary tumor bearing rats. They used AC H-field with frequency of 450 kHz, but unfortunately no field strength was given. In 1993, A. Jordan et al.\textsuperscript{25} studied the comparison between large multidomain particles and single domain particles in their potential for MFH using specific absorption rate (SAR) as a quantitative measurement. SAR will be discussed in details in subsection 2.1.4. In the same year, DCF Chan et al.\textsuperscript{40} discussed a method of colloidal magnetic iron oxide synthesis and its superiority in SAR measurement. And in the past few years there is even stronger surge of interests in using ferrofluids for the hyperthermic treatment of human cancers.

2.1.3 Magnetic Field Heating Mechanism

Instead of reviewing all the heating mechanism for all type of hyperthermia techniques, this subsection will only review the current technique (magnetic fluid hyperthermia) which is relevant to this proposal. We know that the particles dissipate energy from the applied H-field in the form of heat. The three known mechanisms of this heat dissipation will be described in details in following subsections. Depending on their material properties, NPs can utilize either one or any combination of those mechanisms.

1. Eddy-Current Loss

When we place a conductive (magnetic or non magnetic material) material in a (AC or DC) magnetic field, the magnetic flux moves, the motion of magnetic lines cutting across the conductor forces the free electrons in the conductor to move, producing
current. This is called induced current, because the process is an induction where there is no physical connection between the source of magnetic field (e.g. magnet) and the conductor.

When we place a conductive material in a varying magnetic field (AC magnetic field), the currents are induced on the conductor, which flow in swirls or eddies within the solid mass of the conductive material. These are called eddy currents. The heating due to eddy currents is the same as the current flowing through a light bulb filament or an electric resistance heater. Basically the current flow through a resistance \(R\) and the heating is simple \(I^2R\) heating. M.T. Thompson discussed further details, complications and theory to quantify this loss mechanism.

2. Hysteresis Loss

Hysteresis describes the phenomenon of the lagging behind of response of the magnetic flux, \(B\) in a material when the applied magnetizing field (force), \(H\) is increased or decreased, as shown in Figure 2.1. This phenomenon can be physically explained by the magnetic domain wall motion and magnetic moment rotation which occur when a magnetizing force is being applied. As the magnetizing force is decreased or change direction these wall motion and moment rotation is not perfectly elastic or reversible, meaning that they lags the increase or decrease of magnetizing force. This phenomenon can be viewed as an internal friction. The work done by the magnetizing force against this internal friction produces heat. This energy wasted in form of heat is called hysteresis loss. Hysteresis loss can be measured by vibrating sample magnetometer (VSM) or
superconducting quantum interference device (SQUID). The hysteresis loss can be estimated approximately in the case nearly rectangular hysteresis loop by

\[ P_{\text{hys}} = P_{\text{hys}} f M_s H_c \]  \hspace{1cm} (2.1)

where \( P_{\text{hys}} \) is a constant factor, \( f \) is the field frequency [Hz], \( M_s \) is the saturation magnetization [T], and \( H_c \) is the coercivity [A/m]. Since coercivity depends on the particle dimension,\(^4\) therefore the power loss \( P_{\text{hys}} \) clearly depends on both frequency and the dimension of the particles.

![Hysteresis Loop](image)

**Figure 2.1.** Hysteresis Loop, B-H curve as a response to alternating magnetizing force \( H \).

3. **Relaxation Losses**

Superparamagnetics are magnetic particles which volumes are small enough that the thermal energy is considerably larger enough to affect the magnetization of each particle which results in random orientation of the magnetic moments of particles.
Therefore in the absence of an external field, these particles have no magnetization. In the present of an applied magnetic field, we need to take into consideration the relaxation time of the system due the thermal energy effects on the magnetization process. For our frequency range of the applied AC magnetic field, there are two types of relaxations to be considered:

1. **Brownian Relaxation**: With this Brownian mechanism, the magnetic moment seems to be locked in the crystal axis of particle, therefore the entire particle rotate in alignment with the direction of magnetic field. The Brownian relaxation time constant is given by:

   $$\tau_B = \frac{8\pi\eta R_H^3}{k_B T}$$

   (2.2)

   where: $\eta$ = solvent viscosity [Pa·s], $R_H$ = hydrodynamic radius of the particle, $k_B$ = boltzman’s constant and $T$ = temperature.

2. **Néel Relaxation**: at higher frequency, the Brownian motion is essentially frozen out, the magnetic moment start to rotate within the particle. This second mechanism is call Néel relaxation. The Néel relaxation time constant is given by:

   $$\tau_N = \tau_0 \exp \frac{KV}{k_B T}$$

   (2.3)

   where: $\tau_0$ = the natural gyromagnetic frequency ($\tau_0 \sim 10^9$ s), $K$ = anisotropy constant [J/m$^3$], and $V$ = Volume of the particle.

These two mechanisms compete at certain frequency, while one will dominate over the other at certain range of frequency. When both exist, the effective relaxation time will be:
\[ \tau_{\text{eff}} = \frac{\tau_N \tau_B}{\tau_N + \tau_B} \]  (2.4)

The power losses to one or both of these relaxation mechanisms can be calculated by: \( P = \frac{(mH\omega\tau_{\text{eff}})^2}{2\pi k_B TV (1 + \omega^2 \tau_{\text{eff}}^2)} \)  (2.5)

where: \( m = \) the particle magnetic moment \([J/T]\), \( H = \) the external magnetic field strength \([T]\) or \([A/m]\), \( \omega = 2\pi f \), and \( f = \) the frequency of the applied H-field \([Hz]\). This equation clearly shows that the power losses depends the material properties \((m)\), frequency \((\omega = 2\pi f)\) and particle dimension \((V = \frac{4}{3}\pi a^3)\).

### 2.1.4 Specific Absorption Rate (SAR)

C.K. Chou\(^8\) first adopted the terminology of specific absorption rate (SAR), which is commonly used in bioelectromagnetics research as a form to quantify the rate of energy deposition in tissue, into the hyperthermia study. For the in vitro study of magnetic fluid hyperthermia, SAR \([J s^{-1} kg^{-1}]\) quantifies the amount of energy converted by magnetic particles from H-field into heat per unit time and mass. The in vitro SAR of magnetic fluid is usually determined from the time-dependent calorimetric measurement. **Figure 2.2 (a)** shows the typical experiment setup of in vitro SAR measurement. The SAR of magnetic fluid is usually determined by the “rate of temperature rise” method. **Figure 2.2 (b)** shows the typical temperature profile of time-dependent SAR measurement. It is determined by the initial linear temperature rise after switching on the magnetic field.\(^8\)
\[ SAR = c \frac{dT}{dt} \]  \hspace{1cm} (2.6)

where \( c \) = the specific heat capacity of the solution [J g\(^{-1}\) K\(^{-1}\)], and \( \frac{dT}{dt} \) = the temperature increase per time [K/s]. The \( c \)-value of the solution is defined as the weighted mean between the magnetic particles and the solvent (e.g. \( c \) of water = 4.118 J g\(^{-1}\) K\(^{-1}\)).

As it is described earlier that there are a few mechanism to how magnetic particle dissipates heat. The magnitudes of all of these mechanisms depend on frequency \( f \), magnetic field strength \( H \), and a frequency-dependent characteristic constant of a material \( k \). Therefore the SAR values should also indicate their dependency on these variables. Their dependency can be approximately described by:

\[ SAR \approx kf^n H^m \]  \hspace{1cm} (2.7)

While most researchers\(^{25,48}\) reported in very much of agreement that the value of \( m \) equal to 2, but \( n \) varies slightly. Jordan, A. et al.\(^{25}\) reported that \( n = 1 \), while Chan, D.C.F., et al.\(^{48}\) reported that \( n \) varies from 1.1 to 1.5. In general, indeed the SAR values depend on the frequency and amplitude of the applied AC H-field. The differences in the dependency of SAR values on frequency may be due the differences in the experimental setups (such as how good is the thermal insulation of the sample).
2.2. Selective Heating of Multiple Nanoparticles

Magnetic NPs have been getting a lot of interests from the scientific community due to their size- and material-dependent properties. As mentioned earlier in the introduction, we propose to develop a method to independently address more than one type of NP by selective magnetic field heating. We can accomplish this by studying the NP heating dependence on the frequency of magnetic field by varying NPs' material and
size. First we need to conduct theoretical analyses for feasibility study. We then need investigate the heating rate profile of NPs of different magnetic materials as a function of frequency and determine if there are optimum heating rates for a narrow frequency range. Furthermore, the dependencies on the amplitude of the applied H-field are also equally important, and will also be investigated. SAR measurement discussed earlier is an ideal tool for these experimental explorations.

2.2.1 Theoretical Exploration

We mainly interested in smaller superparamagnetic NPs because their sizes are in the same order of magnitude with biomolecules such as DNA and proteins. The relaxation losses (Equation 2.2 – 2.5) are the primary mechanism of magnetic heating of the superparamagnetic NPs. The power loss equation (Equation 2.5) shows that magnetic field heating is a function of the material, NP size, and the field characteristics. We illustrate that tuning of all of these parameters is necessary to find conditions under which multiple types of NPs can be heated independently.

In order to investigate the feasibility of our propose study, the dependence of the power loss equation on these parameters is explored. The material dependence of power losses is reflected by the parameter $m$ and $K$ in equation 2.5 and 2.3 respectively. For the spinel ferrite MFe$_2$O$_4$ ($M = \text{Fe, Co, Mn}$) NPs,$^{49}$ the magnetic moment per particle, $m$ strongly depends on the type of transition metal ion, $M^{2+}$ occupying the tetrahedral and octahedral positions. The anisotropy constant, $K$ highly depends on the type of crystal structure and the degree of crystallinity of the NPs. Both $m$ and $K$ also have a strong dependence on NP size. Therefore the material and size dependence of the power losses
are intertwined. Figure 2.3 shows a plot of equation 2.5 as a function of NP size for a given field of 0.01 T and frequency of 3 MHz for CoFe$_2$O$_4$, Fe$_3$O$_4$, and γ-Fe$_2$O$_3$, and MnFe$_2$O$_4$. The heating shows strong size dependence for each of the materials, which has been observed experimentally.$^{25}$

![Figure 2.3. Power loss equation as a function of nanoparticle size for a field of 0.01 T and frequency of 3 MHz for CoFe$_2$O$_4$ (dashed line), Fe$_3$O$_4$, (solid line) and γ-Fe$_2$O$_3$ (dotted line) and MnFe$_2$O$_4$ (points and line).](image)

The frequency dependence of the magnetic field heating based on the power loss equation is plotted in Figure 2.4 for 14 nm CoFe$_2$O$_4$ (dashed line), 15 nm Fe$_3$O$_4$ (solid line), 15 nm γ-Fe$_2$O$_3$ (dotted line), and 12 nm MnFe$_2$O$_4$ (points and line) with an applied field of 0.01 T. The power dissipated by the NPs increases with frequency and then plateaus at higher frequencies. This general shape has been observed experimentally in the literature.$^{25,50}$
Figure 2.4. Power loss equation as a function of frequency at 0.01 T for 14 nm CoFe$_2$O$_4$ (dashed line), 15 nm Fe$_3$O$_4$ (solid line), 15 nm $\gamma$-Fe$_2$O$_3$ (dotted line), and 12 nm MnFe$_2$O$_4$ (points and line).

Independent heating of these four types of NPs is possible if field strength $H$ is also tuned. Figure 2.5 shows the power loss equation where four different field strengths are applied at different frequency ranges. 14 nm CoFe$_2$O$_4$ (dashed line) can be heated preferentially over the 15 nm Fe$_3$O$_4$ (solid line), 15 nm $\gamma$-Fe$_2$O$_3$ (dotted line), and 12 nm MnFe$_2$O$_4$ (points and line) at any frequency below 20 kHz and at any field strength. 15 nm Fe$_3$O$_4$ can be heated selectively if one applies field strengths of 1 mT at a frequency of 1 MHz. 15 nm $\gamma$-Fe$_2$O$_3$ can be heated selectively if one applies field strengths of 0.35 mT at a frequency of 20 MHz. Finally, 12 nm MnFe$_2$O$_4$ can be heated preferentially at frequencies above 100 MHz if the field strength is 0.13 mT. This shows that according to the power loss equation, tuning the parameters of NP size, material, field frequency and field strength, one can preferentially heat one type of NP over the others.
2.2.2 Experimental Results

Magnetic Nanoparticles

We also tested this theoretical hypothesis experimentally. We performed magnetic field heating study of two different types of NPs, iron oxide and magnetic Au/Fe NPs. Aqueous Fe$_3$O$_4$ NPs were purchased from FerroTec (Product name: EMG 705). The NPs were at a volume fraction of 3.9% and used without modification. TEM imaging by JEOL 2010 FEG Analytical Electron Microscope (Figure 2.6 (a)) confirmed that NPs were well dispersed and not aggregated, with \( <d> = 12.5 \text{ nm} \pm 3.4 \text{ nm} \) (Figure 2.6 (a)). Size analysis was done utilizing ImageJ.$^{51}$

Figure 2.5. Power loss equation as a function of field frequency and field strength for 14nm CoFe$_2$O$_4$ (dashed line), 15nm Fe$_3$O$_4$ (solid line), 15nm γ-Fe$_2$O$_3$ (dotted line), and 12nm MnFe$_2$O$_4$ (points and line).
Fe/Au bimetallic NPs were synthesized in water by a simple modification\textsuperscript{52} of a standard synthesis for gold NPs\textsuperscript{53}. In brief, HAuCl\textsubscript{4} and FeCl\textsubscript{3} were reduced in aqueous solution simultaneously in the presence of citric acid, tannic acid, and sodium carbonate at $T = 50^\circ$C to nucleate Au NPs doped with Fe. The NPs were functionalized with the ligand bis(p-sulphonatophenyl) phenylphosphine dihydrate, dipotassium salt (BPS) by ligand exchange. The NPs were purified from reagents and excess ligand by multiple precipitations with NaCl and centrifugation. They were re-suspended in water and further purified by agarose gel electrophoresis. \textbf{Figure 2.7 (a)} shows a TEM image of the sample deposited from water solutions onto ultra thin holey carbon coated copper grids (Ted Pella). The TEM image reveals that the NPs are well separated and not present in aggregates. In addition, the NPs resulting from this synthesis are relatively monodisperse. A distribution of $<r> = 3.9\pm0.5$ nm (histogram, \textbf{Figure 2.7 (b)}) was determined through analysis of TEM images containing approximately 175 NPs. The Fe content of the NPs was 1.8%, as measured by elemental analysis in STEM.
**Experimental Setup**

The first challenge for performing SAR measurement is how to generate AC magnetic field with strong enough amplitude to heat up the NPs and to characterize this field. This subsection addresses these issues. A typical simple system to generate AC magnetic field requires at least a signal generator, an amplifier, and an inductor coil, as shown in Figure 2.8. As the current \( I \) flow through the copper wire of the coil, by Ampere’s law a uniform magnetic field inside the solenoid coil can be approximated to be:\(^{41}\)

\[
B = \frac{NI}{l} \quad \text{(as long as } l \gg d \text{)} \tag{2.8}
\]

where \( N \) = the number of turns of the coil, \( l \) = the length of the coil, and \( d \) = the diameter of the coil.
Figure 2.8. A typical system for generating AC magnetic field.

**Coil Design**

A handmade inductor coil can easily constructed using an insulated copper wire. This copper wire usually graded by its diameter which will have a certain value of resistivity, $\rho$ [ohms/meter]. The coil’s DC resistance is simply:

$$R_{DC} = \rho L \quad (2.9)$$

where $L$ = the total length of copper wire used to construct the coil. There is also an additional resistance due to the non-uniformity of magnetic field distribution in the wire (skin effect) and the induction effect of the neighboring magnetic field of each wire turn in the coil (proximity effect). This resistance value goes up with higher frequency, and can be approximated by: \(^{54}\)

$$R_{AC}(f) = \left(2.61 \times 10^{-7}\right) \sqrt{fp_r} \quad (2.10)$$

where $f$ = frequency [Hz], $\rho_r$ = relative resistivity (compared to copper = 1), and $D$ = wire diameter. The total resistance can be approximated by: \(^{54}\)

$$R(f) = \left((R_{DC})^2 + [R_{AC}(f)]^2\right)^{1/2} \quad (2.11)$$
As current $I$ flows through the coil, overcomes the total resistance $R$, there will be some losses in form of heat. These losses are usually known as copper losses, and can be calculated by:

$$P = I^2 R$$  \hspace{1cm} (2.12)

As we want to selectively heat up the NPs by the magnetic field, we need to avoid the heat propagation from the coil to the NP solution. Therefore we need to have a mechanism to withdraw the heat from copper losses away from the sample inside the coil. Figure 2.9 shows the schematic of our system to address this issue. This proposed solution utilizes the circulating water bath to continuously withdraw the heat from copper losses and some insulating layer to further isolate the sample.

**Figure 2.9.** Water-cooled coil system to selectively deliver H-field to the sample.

**RF Test Measurements**

*H-field Characterization.*

To the best of our knowledge, there is not a commercial H-field probe to measure magnetic field at wide range of high frequencies. However there are current probes that are suitable for our purposes to measure the current flowing in the coil at high frequencies. The current probe (Tektronix Inc.) that we used simply measures the
magnetic field around the current-carrying wire to back-calculated the current amplitude. With a known current flow, we can then estimate the H-field strength.

**RF-immune Temperature Probe.**

Since the sample is placed inside the coil to get maximum H-field, therefore to measure the temperature we need to place the probe inside the H-field environment. Generally, thermocouples are made of metal, which will be affected by H-fields, invalidating the temperature reading. Fortunately, there are non-metal fiber optic temperature probes which are small in dimension and flexible. This is very advantageous for our temperature (SAR) measurements. This probe (Luxtron Corp.) essentially utilizes a fluorescent material with temperature dependent optical properties. This material is placed at the tip inside the probe, which is in thermally equilibrium with the outside temperature. Inside the probe there are also a bundle of optical fibers for excitation and emission measurements of the fluorescent material. This probe was utilized for all the SAR temperature measurements.

**Typical Experimental Setup**

The actual experimental setup combine the two concepts illustrated in Figure 2.8 and 2.9. It was similar to those used in the other literatures for hyperthermia evaluation. Current was supplied to a water-cooled coil of 25 turns, inside of which the sample was placed. Sample volume was typical around 100 µl. The temperature of the sample was measured as a function of time by a fluorescent temperature probe (Luxtron). Currents were supplied by a signal generator (Hewlett Packard) amplified through a 100W...
amplifier (Amplifier Research), in the frequency range of 100 kHz to 100 MHz. The field strength was calculated by measuring the current using a high-frequency current probe (Tektronix) and an oscilloscope (Agilent). Custom software (written in LabView) controlled the application of the signal and also measured the field strength and temperature. Due to the mismatch of the impedance, the current in the coil was obtained by combining frequency dependence circuit simulations in PSPICE (Personal computer Simulation Program with Integrated Circuits Emphasis) with the actual readings of the current probe.

**SAR Measurements – Results and Discussion**

SAR Measurement is an ideal tool for studying the heating rate of NP solution at certain range of frequency. We conducted a series of SAR measurement for two types of NPs mentioned above, Fe$_3$O$_4$ and Fe/Au NPs. They were suspended in water with concentration of 1.19 mg/ml and 14 mg/ml for Fe$_3$O$_4$ and Fe/Au NPs respectively. Figure 2.10 shows the typical heating profile these NPs solutions when they are exposed to AC magnetic field. An alternating magnetic field at given approximate field strengths (green line) at frequency of 1 MHz and 40 MHz for Fe$_3$O$_4$ and Fe/Au NPs respectively, were applied between $t = 100$ s and 400 s. Upon the application of the field, the temperature of NP solution increased; while the control experiments without NPs in the solution show no temperature increases (blue line). The SAR value of NP solution is determined by the initial linear temperature rise of a fluid measured after switching on the magnetic field (Equation 2.6). The initial slope of the temperature increase was typically obtained from the temperature profile (such as in Figure 2.10) by fitting the rise to an
exponential function based on the analytical solution of the boundary value problem for \( T(t) \) of the sample [20]. The estimated value of the initial slope was calculated by differentiating the resulting exponential function with respect to time and solving at time equal to the time when the field first turned on. The heat capacities \( (c) \) of the NP solutions were approximated by using a weighted average of heat capacity of the NP \( (c_{NP}) \) and water \( (c_{water}) \).

**Figure 2.10.** SAR measurements. Upon the application of the magnetic field (green line), the temperature of (a) \( \text{Fe}_3\text{O}_4 \) (brown line) and (b) \( \text{Fe}/\text{Au} \) (red line) nanoparticle solution increased, while the controls (blue lines) stayed at room temperature.

The SAR value was measured and calculated for a given field strength and frequency. Therefore, we conducted a series of SAR measurement at various field strengths and frequencies to test our theoretical hypothesis mention earlier in this chapter. **Figure 2.11** shows the plots SAR values or power loss as a function of the applied magnetic field strengths for both \( \text{Fe}_3\text{O}_4 \) and \( \text{Fe}/\text{Au} \) NP solutions. SAR has been shown empirically to be a function of field strength \( (H) \) to \( H^m \) and frequency \( (f) \) to \( f^n \) (Equation 2.7) where the values of \( m \) and \( n \) are experimentally determined. **Figure 2.11** also shows this dependence on field strength (brown square for \( \text{Fe}_3\text{O}_4 \) NPs and red square for \( \text{Fe}/\text{Au} \) NPs) can be fitted to the \( m^{th} \) order power-law curve. In the case of \( \text{Fe}_3\text{O}_4 \) NPs the
value of $m \sim 2$ (black line); while for the Fe/Au NPs the value of $m \sim 2$ at low field strengths (blue line), but at higher field strengths, $m \sim 3$ (green line). Third-order power-law $H$ dependences are typical of Rayleigh losses.\(^{55}\) Previous work has shown that second order losses are indicative of superparamagnetic samples and third order losses for ferromagnetic samples.\(^{50}\) The phenomenon of switching from $m = 2$ to $3$ field dependence has been observed in magnetic hysteresis of magnetosomes\(^{56}\) but this is not described by the power loss equation, which only shows a square law field dependence. Since we also observe this behavior, it suggests that hysteresis losses may play a role in the heating of the Fe/Au NPs. Therefore, power losses alone cannot describe the field-dependent behavior of these particles.

![Figure 2.11.](image)

**Figure 2.11.** Power loss as a function of field strength for (a) Fe$_3$O$_4$ (brown square) and (b) Fe/Au (red square) nanoparticle solution. The plot was fitted to the $m^{th}$ order power-law curve for both Fe$_3$O$_4$ (black line) and (blue line and green line).

In order to assess our hypothesis and to compare to the theoretical assessments, we performed SAR measurement at a range of frequencies ($f$). Figure 2.12 shows power loss per particle as a function of field frequency for both Fe$_3$O$_4$ and Fe/Au NP. Both plots seem to follow relaxation losses trends, where it is a square law dependent on frequency.
at lower frequencies, but frequency independent above some threshold. This behavior is similar to our theoretical assessment. The same have also been observed experimentally for Fe₃O₄ NPs. It should be noted that the frequency dependence at which SAR plateaus is extremely sensitive to the value of the anisotropy constant used in the calculation. Thus theoretical calculations can be used only as an approximation of the trends.

**Figure 2.12.** SAR values or heating rate of Fe₃O₄ (brown square) and Fe/Au (red square) nanoparticles as a function of frequency.

**Figure 2.13.** SAR values or heating rate of Fe₃O₄ (brown square) and Fe/Au (red square) nanoparticles at (a) low and (b) high frequency ranges.
2.3. Summary

In summary, we have reviewed extensively the concept of magnetic heating of nanoparticles and how we plan to exploit this for our hypothesis. We have also studied the size and material dependence of magnetic field heating of nanoparticles. Independent heating of multiple types of nanoparticles is theoretically possible by exploiting the size and material dependence of the power loss equation. Preliminary results from experiments on two different types of nanoparticles show that independent heating has feasible potential.
Chapter 3

Loading Magnetic Nanoparticles into Lipid Vesicles

In the previous chapter we have shown the feasibility of selective heating of multiple magnetic nanoparticles (NPs) both theoretically and experimentally. The end goal of our hypothesis is to utilize this selective heating for potential selective multiple controlled release application. In order to study the feasibility for this application, we need to build system that can incorporate both the NPs and biomolecules and have the mechanism to release the molecules when the NPs heat up. We proposed a system of mixture of two different thermosensitive vesicles each contain different types of NPs and molecules to be released (Figure 3.1). By appropriate tuning of the applied magnetic field, we can selectively heat certain type of NPs, thus disrupts certain vesicle which then release the contained molecules. We utilized lipid vesicles (liposomes) and fluorescent dye as thermosensitive vesicles and the models for biomolecules respectively. In this chapter we report a morphological study of NPs in large unilamellar lipid vesicles. Phase behavior of this NP-lipid system was studied to maximize the loading of NPs into vesicles.
3.1. Why Liposome – Nanoparticle System

Liposomes have attracted interest for drug delivery applications because of their ability to encapsulate and release payloads.\textsuperscript{57} Because their composition is similar to cell membranes and their surface chemistry can be modified for specific targeting, they have unique biological capabilities.\textsuperscript{58} For example, they have been used to target tumor sites and release anti-cancer drugs.\textsuperscript{59} Encapsulation of NPs in liposomes is desirable for applications such as triggered heating by hyperthermia, drug delivery,\textsuperscript{60-65} enhancing MRI,\textsuperscript{66} imaging by fluorescent NPs,\textsuperscript{67} and photothermal therapy.\textsuperscript{68} Liposomes are self-assembled vesicles of amphiphilic lipid molecules. The lipid bilayer which forms the vesicles wall is temperature sensitive and can be “melted” at certain temperature, thus
induce leakage/release of the payload. This liposome phase transition temperature can be tuned by adjusting length of the lipid molecules. This why liposome is the ideal system for our purpose to study controlled release induced by magnetic heating of encapsulated NPs (Figure 3.1)

Currently, numerous preparation methods of lipid vesicles exist. Encapsulation of hydrophilic moieties is achieved by introduction to the aqueous phase so that they can be spontaneously captured inside the liposomes during vesicle formation. Approaches that increase the internal liposome volume have been pursued for encapsulation of large amounts of payloads. The reverse-phase evaporation method by Szoka et al. has been successful in creating large unilamellar vesicles (LUV) with a large internal aqueous space, and has been used to make liposomes that encapsulate molecules, proteins, DNA, and NPs. The mechanism of encapsulation is hypothesized to occur when lipid vesicles self-assemble from a collapsed gel-like state, enclosing the payload into vesicles.71, 76

For many applications of NP-encapsulated liposomes, high density encapsulation is desirable. Hyperthermia and drug delivery both benefit from high NP loading. Also, magnetic resonance contrast enhancement relies on NP clustering and requires that the NPs be fairly close together, where inter-particle distances are on the order of only a few NP diameters. For our hypothesis described earlier, it is also desirable to have high loading of NP into liposomes in order to generate enough heat to disrupt the vesicle and release the load. Unfortunately, it is generally difficult to achieve uniformly spherical liposomes with high loading of NPs, and often low yields result. Recently, Chen et al. reported on average only 3 QDs per liposome of d~50-300 nm. Martina et al.
reported ~60 Fe$_2$O$_3$ NPs/100nm liposome. While liposome formation by itself is well understood, phase diagrams have not been constructed in the case of an encapsulant with significant volume fraction is present. It is expected that the presence of NPs, especially at high concentrations, will appreciably affect liposome phase diagrams, such as for block copolymers. Therefore, understanding NP-lipid morphologies is necessary to optimize NP encapsulation.

We performed a systematic study of the phase behavior of di-palmitoyl phosphatidylcholine (DPPC) with 12nm Fe$_3$O$_4$ NPs, with the goal of maximizing the NP encapsulation in DPPC vesicles. TEM imaging was used to qualitatively analyze resulting morphologies and quantify NP-loaded liposome size distribution. We are able to synthesize liposomes that are densely packed with NPs. Varying lipid and NP concentration results in different NP-lipid morphological structures. We find that high NP concentrations perturb the phase diagram in such a way that it effectively increases the concentration of the lipid. Key parameters for producing high yield NP loaded liposomes are determined.

### 3.2. Liposome Preparation Method and Characterization

**Materials**

Aqueous Fe$_3$O$_4$ NPs were purchased from FerroTec (product name: EMG 705). The NPs were at a volume fraction of 3.9 % and used without modification except dilution. TEM imaging (Figure 2.6 (a)) confirmed that NPs were well dispersed and not
aggregated, with $<d> = 12.5 \text{ nm} \pm 3.4\text{nm}$ (Figure 2.6 (b)). Di-palmitoyl phosphatidylcholine (DPPC) was purchased from Avanti Lipids. All other chemicals were purchased from Sigma-Aldrich.

**Liposome Preparation Method**

To produce liposomes with large internal volume and thus potential for high loading, we used a slightly modified version of the reverse evaporation method\(^\text{71}\) (protocol in Figure 3.2). 20, 40, or 60 mg DPPC was dissolved in 12ml of a v:v = 1:1 mixture of chloroform and isopropyl ether in a 50 ml round-bottom-flask that was cleaned using Avanti Procedure.\(^\text{84}\) This is referred to as the “organic solution” (panel 1). 1.5 ml of 3.9, 1.95, 0.98 or 0.39 vol% of Fe$_3$O$_4$ NP solution and 1.5 ml of 20mM calcein at pH 7 were added into the organic solution (referred to as the “mixture,” panel 2). Ar (g) was bubbled through the mixture to remove any oxygen.

The mixture was sonicated in a bath sonicator at $T = 50^\circ\text{C}$ for 5 min to produce a water-in-oil emulsion (Figure 3.2, panel 3). The mixture was cooled slowly and sat for >30 min. Stability of the emulsion was confirmed before proceeding. The organic solution was removed from the emulsion slowly using a rotary evaporator at $T=50^\circ\text{C}$ with $P = -11$ to -14 inHg for 2.5 hr (panel 4). At this point, most of the solvent had been removed, forming a gel which adhered to the flask wall (panel 5). Note that the operating temperature was well above the $T_m$ of DPPC (41.5$^\circ\text{C}$). Other REV methods to prepare DPPC vesicles\(^\text{69}\) and magnetoliposomes\(^\text{73}\) have used $T = 20-25^\circ\text{C}$ and 42$^\circ\text{C}$ respectively, while others use $T = 45-50^\circ\text{C}$.\(^\text{67, 75, 85}\) Here, sample repeatability was greatly improved by
operating at \( T = 50^\circ C \), significantly higher than \( T_m \) of DPPC. Therefore, all data presented on samples here had a preparation temperature of \( 50^\circ C \).

The vacuum in the flask was broken and purified deionized water was added to the gel (or continuous phase water, CPW\(^{86}\)) to replenish any that was lost during evaporation, or for further dilution to achieve certain final concentrations. The amount of CPW varied from synthesis to synthesis, and may also depend on the evaporator setup. Pressure was further reduced at increments of \(-1\) inHg/5min until reaching \( P = -22\) inHg. At this point the gel had completely collapsed, forming lipid vesicles (Figure 3.2, panel 6). This pressure was maintained for another 15 min to remove traces of solvent. The resulting aqueous solution was allowed to sit undisturbed for at least 3 hrs before deposition on a TEM grid for imaging.

**Purification by centrifugation**

To remove aggregates, the solution was first centrifuged at 100 g for 5 min and the supernatant retained. The solution was then centrifuged at 200 g for 5 min to remove un-encapsulated materials. The supernatant containing non-encapsulated NPs and calcein was discarded, and the desired product in the precipitate was re-suspended in 20 mM TES buffer solution (pH 7). Centrifugation was repeated twice, resulting in a purified solution of NP-encapsulated liposome vesicles.
Imaging and Sizing

Samples were imaged by TEM (JEOL 2011). Samples were deposited on holey carbon grids (Ted Pella) directly from solution. Images were taken at least within 3 days. Size analysis was done by Image J.\cite{51}

![Figure 3.2. Method for NP encapsulation into lipid vesicles via REV method. Lines: lipid, dark gray: organic solvent, light gray: water, circles: NPs, stars: calcein.](image)

3.3. Results and Discussion

Concentration Effect

By varying the lipid and NP starting concentrations, and the amount of added CPW, we studied the effect of lipid and NP concentration on resulting lipid/NP morphology. There is a limit to increasing the lipid concentration, because too high of a
l lipid concentration will result in the inability of the gel \( \text{(Figure 3.2, panel 5)} \) to collapse and form vesicles; this remains stuck to the flask wall and is not recoverable. In contrast, starting lipid concentration must be high enough to produce a stable water-in-oil emulsion \( \text{(Figure 3.2, panel 3)} \).

\textbf{Figure 3.3 (a)} shows the range of lipid and NP concentrations explored. \textbf{Figure 3.3 (b)-(f)} show images of the corresponding morphological features. The lipids are unstained so dark areas in the image correspond to NPs. These images were taken of the original solutions at least 3 hrs after preparation, without purification by centrifugation. These morphologies are identical to those observed for the lipid-only system \( \text{(Figure 3.4)} \). At the highest NP concentration (\(~100\) mg/ml, squares in \textbf{Figure 3.3 (a)}), perforated bicelles are formed \( \text{(Figure 3.3 (b))} \). The NPs are dispersed in the bicelle sheet that has pores (resembling a slice of Swiss cheese). Some of these bicelle sheets contain features of spherical lipid vesicles (resembling bubble wrap, Appendix A). A mixture of both was commonly observed. Others have proposed the structural model of these perforated bicelles (or perforated lamellar phase). 87,90-93

Phase behavior studies of lipid-only systems \( \text{(Figure 3.4)} \) find that spherical LUV and multilamellar vesicles (MLV) are formed at low lipid concentrations, while the perforated lamellar phase occurs at high concentrations. 87,89 Clearly, the NP concentration is high enough to affect the lipid phase diagram significantly, and changes the resulting phases. For example, increasing NP concentration at a fixed lipid concentration, the resulting morphology moves from HNLV to perforated lamellae. This effect is similar to increasing lipid concentration in lipid-only systems. For example, we can obtain the perforated bicelles phase at low lipid concentrations simply by increasing
the NP concentration. Interestingly, similar effects have been observed for lipids in the presence of large molecular weight hyaluronan, indicating that introduction of large non-lipid species can favor formation of lamellae. Varying lipid concentration from 15-40 mg/ml has little effect on the resulting phase (squares in Figure 3.3 (a)).

**Figure 3.3.** (a) Plot showing lipid and NP concentrations used. TEM images of the corresponding morphologies: (b) holey bilayer sheet; (c) mixed bilayer sheet with NP-loaded vesicles; (d) mixed empty and NP-loaded vesicles; (e) mostly HNLV; (f) mostly LNLV.

For a fixed lipid concentration of 23-27 mg/ml, decreasing NP concentration from 101-105 mg/ml results in different morphologies. At [NP] = 54-58 mg/ml, (diamonds, Figure 3.3 (a)) a mixture of perforated bicelles and NP-loaded vesicles resulted (Figure 3.3 (c)). Decreasing [NP] to 20 mg/ml (triangles in Figure 3.3 (a)) yields a mixture of
empty vesicles and NP-loaded vesicles (Figure 3.3 (d)). In the image, unloaded vesicles appear as large empty circular structures. Finally, at [NP] = 12-13 mg/ml (circles, Figure 3.3 (a)) mostly spherical vesicles with high loading of NPs are formed (Figure 3.3 (e)), or high density NP loaded vesicles (HNLV). Figure 3.5 shows a high resolution image of a HNLV ~200 nm in diameter, which contains a spherical mass of NPs at high density (Figure 3.5 (a)). Resulting morphologies seem to be independent of lipid concentration for [NP] >13 mg/ml.

![Phase Diagram](image)

**Figure 3.4.** The phase diagram of typical phosphatidylcholine (PC) and PC+PG (phosphatidylglycerol). Three phases are depicted and the phase transition temperature is 35°C. $C_{lp}$ is concentration of lipids. Reprinted with permission from the American Chemical Society.\(^7\)

At lower NP concentrations (5-13 mg/ml), changing lipid concentration varies vesicle loading and size (circles and stars in Figure 3.3 (a)). Lower lipid concentrations yields smaller, low density NP loaded vesicles (LNLV) (Figure 3.3 (f)). At [lipid] = 21-23 mg/ml the obtained morphology is predominantly HNLV (Figure 3.3 (e)) while at
[lipid] = 7-9 mg/ml it is predominantly LNLV (Figure 3.3 (f)). Figure 3.5 (b) shows a higher resolution image of a LNLV ~100 nm in diameter, which appears to have fewer layers of NPs encapsulated. This could be due to the fact that at lower lipid concentrations, only smaller vesicles can be formed on average.

Since it is desirable to produce high yield NP encapsulation into liposomes, the predominantly HNLV sample (Figure 3.3 (e)) was purified by centrifugation to remove un-encapsulated NPs (method and characterization, above). The resulting solution contained only uniformly spherical HNLV with no free NPs (Figure 3.6 (a)). Size distribution measured by TEM was d = 225 nm ± 67 nm (Figure 3.6 (b)).

Figure 3.5. TEM Images of (a) high density NP loaded vesicles (HNLV) and (b) low density NP loaded vesicles (LNLV)
Kinetic Effect

Incomplete vesicle formation has been observed in molecular dynamics simulations, where incomplete closure of the vesicle results in a water pore in the membrane. Because the bilayer vesicle is not sealed, the hydrophilic contents can spill out.\textsuperscript{95} We observed instances of this phenomenon where vesicles which did not reach complete closure, and NPs can be seen leaking out (Figure\ 3.7). This image is of the same sample in Figure\ 3.3 (e) and Figure\ 3.6, except the aliquot was withdrawn within 30 min after liposome preparation, as opposed to >3 hrs. This agrees with studies of the kinetic rate of micelle to vesicle transition of other lipid systems, which find that timescales for growth of disk-like intermediate micelles to closed vesicles is typically hours.\textsuperscript{96, 97} Dynamic light scattering experiments on egg lecithin suspensions after
sonication have shown that complete transformation from planar disks to closed vesicles was observed after 3 hrs.\textsuperscript{98}

Figure 3.7. (a) TEM image of predominantly HNLV solution sampled within 30min after liposome preparation shows the majority of vesicles have incomplete closure; (b) High resolution TEM images from (a) showing the incomplete vesicle closures where the NPs spill out.

**Other Effects**

Centrifugation for long times (>10min) or at higher speed (≥1000g) induced vesicle fusion (Figure 3.8). Others have studied the fusion phenomenon of lipid vesicles during storage which shows the apparent dependence on the duration of storage time and temperature.\textsuperscript{99-102}
3.4. Summary

In summary, the resulting morphology of a lipid/NP system is strongly dependent on NP and lipid concentrations. This study has enabled the production of large spherical lipid vesicles that encapsulate 12 nm Fe₃O₄ NPs at high yield (HNLV). The presence of NPs perturbs the phase diagram, with the effect of increasing the effective lipid concentration. Key factors for HNLV formation include an operating temperature of 50°C, higher than the lipid transition temperature. Sufficient time for complete vesicle formation is necessary (~hrs). Future studies will assess liposome stability, finer size control, and applicability for controlled-release.
Chapter 4

The Limitation of Magnetic Field Heating

In the previous chapter we have shown our extensive study of resulting morphologies of NP-lipid system. We have shown that we can tune the concentration of both NPs and lipid to obtain the morphology of high density NP loaded vesicles (HNLV). In this protocol, we also incorporated dye molecules (calcein) into the lipid vesicles. Calcein molecules will serve as molecule/drug release model. We hope that when we heat up the NPs inside the lipid vesicle via magnetic field heating, the heating will disturb the lipid bilayer and calcein molecules will leak out of the vesicles (as illustrated in Figure 3.1). In this chapter, we explore the feasibility and the limitation of this proposed control release mechanism.

4.1. Control Release via Magnetic Field Heating

In order to study the feasibility of control release application via magnetic field heating, we followed the protocol in the previous chapter to prepare high density NP loaded vesicles (HNLV). Calcein molecules were also incorporated into the vesicles. Purification of HNLV from the excess lipid molecules, NP, and calcein molecules were
performed prior to the control release study. The purification were done via centrifugation technique described in the previous chapter to ensure insignificant or minimum amount of free calcein molecules in the solution prior to the control release study.

First we need to show that calcein molecules were incorporated inside the HNLV. We used the previously reported method\textsuperscript{103} of using detergent Triton X-100 for solubilization of the lipid bilayer, which lead to the vesicle deformation. This treatment resulted in releasing any encapsulated materials. We applied this treatment to our purified HNLV solution to deform the vesicles thus releasing NP and calcein molecules. We then performed centrifugation to separate the NPs from the released calcein molecules in the supernatant. Fluorescence spectroscopy was used to scan the supernatant for evidence of the released calcein molecules. Figure 4.1 shows the fluorescence spectra of the supernatants from the purified HNLV solutions with (solid line) and without (dash line) Triton X-100 treatment. The spectra show clearly that calcein molecules were released from the vesicle due to the Triton X-100 treatment.

![Fluorescence spectra of supernatants from the purified HNLV solution after Triton X-100 treatment (solid line) and without the treatment (control) (dash line).](image)

**Figure 4.1.** Fluorescence spectra of supernatants from the purified HNLV solution after Triton X-100 treatment (solid line) and without the treatment (control) (dash line).
We also need to show that applied heat can disturb (melt) the lipid bilayer, thus enable the release of calcein molecules out of the vesicles. These control experiments were done by bulk heating (at certain temperature for 5 minutes) of the purified HNLV solution which then immediately followed by then centrifugation to separate the NP from the released calcein molecules in the supernatant. The released calcein were then quantified with fluorescence spectroscopy. Figure 4.2 shows the fluorescence spectra of the supernatants of the purified HNLV solutions after bulk heating treatments. It clearly shows that it required the bulk heating treatment temperature higher than the lipid transition temperature ($T_m$ of DPPC = 42°C) to deform or melt the lipid bilayer of vesicles and thus enable the release of the encapsulated materials (calcein molecules).

![Figure 4.2](image)

*Figure 4.2. Fluorescence spectra of supernatants from the purified HNLV solution after bulk heating treatment at temperature of 25°C (*dash line*), 50°C (*dot line*), and 55°C (*solid line*).*
Finally, we investigated feasibility of control release application via magnetic field heating. The idea is by applying AC magnetic field onto the purified HNLV solution we hope to heat up encapsulated NP to deform or melt the lipid bilayer of the vesicle and thus enable the release of calcein molecules (Figure 3.1). After applying the magnetic field onto the purified HNLV solution, centrifugation was immediately performed to separate the NP from the released calcein in the supernatant. Figure 4.3 shows the fluorescence spectra of the supernatants with or without the magnetic field treatment. The magnetic field was generated at frequency of 1 MHz with the amplitude of 3000 A/m. The spectra clearly show almost no release of calcein molecules resulted from the magnetic field treatment. Numerous other attempts at different frequencies and amplitude of the magnetic field also indicated no release of calcein due to the magnetic field treatment. This seems to indicate that magnetic field treatment unable to elevate the local...
temperature of the HNLV to high enough temperature that allows the deformation of the lipid bilayer and thus releasing the calcein molecules. These results seem to contradict other published result. This contradiction is due to the fact that their experiments were done with high concentration of magnetic NPs which cause the global temperature increase of the solution rather than the actual local temperature of the NP.

4.2. The Limitation of Magnetic Field Heating

In order to understand why magnetic field heating unable to produce significant temperature increase of the HNLV, we need to investigate the limitation of magnetic field heating of magnetic NPs. Since it is currently impossible to probe directly the actual local temperature of a single NP, most researchers study the magnetic field heating by monitoring the bulk temperature increase of a high concentration solution of magnetic NPs in an insulated sample container (Figure 2.2.(a)). Unfortunately this temperature increase is due to the accumulation of energy dissipated by the high concentration of the NPs to the surrounding medium inside an insulated sample container. Therefore, without the insulation and/or at low concentration of NP, there will be no bulk temperature increase of the solution. This is simply because the energy accumulated by a single NP rapidly dissipated to its surrounding medium due to its high surface area to volume ratio.

In order to understand more about this limitation, other researcher has conducted theoretical heat transfer approximation for the magnetic field heating of magnetic NPs. In these theoretical calculations, a single NP is modeled as a spherical heat source with constant power density (P) and radius (R) surrounded by medium with
homogeneous heat conductivity. To simplify the derivation, continuous temperature profile were assumed at the interface between the NP and the surrounding medium. In other word, it assumed negligible heat contact resistance at the interface. Assuming large heating time, the equilibrium spatial temperature increase as a function of the distance \( r \) from the centre of the spherical particle, described in the following equations:

\[
\Delta T(r) = \frac{R^2 P}{6k^N} \left[ 1 - \left( \frac{r}{R} \right)^2 \right] + \frac{R^2 P}{3k^M} \quad r < R
\]

\[
\Delta T(r) = \frac{R^2 P}{3k^M} \left( \frac{R}{r} \right) \quad r > R
\]

Where \( k^N \) and \( k^M \) are the thermal conductivities for NP and medium respectively.

The power density (P) in this calculation is nothing but the experimental SAR value in magnetic fluid hyperthermia study discussed earlier in chapter 2. Utilizing the reported values of SAR, these theoretical calculations came to a conclusion that there is only negligible temperature difference between the NP and the surrounding medium. Heat was just instantaneously dissipated from the NP to the surrounding medium due their high surface area to volume ratio. These researchers estimated that with the same power density, the particle has to be as big as 6 mm in diameter to generate temperature difference of 10 K. This clearly shown that utilizing magnetic NP as a heat source to generate local temperature increase by mean of magnetic field heating is not feasible.

Our attempt to increase the size of our system by encapsulation of magnetic NPs into the liposomes though proven to be effective but it did not solve heating limitation problem. Any attempt to increase the size of the NP-loaded liposomes is proven to be unsuccessful. It is also not practical, considering the amount of NP materials needed to increase the size of NP-loaded liposomes.
The other limitation of the magnetic field heating of magnetic NPs is its selectivity of heating multiple different NPs. Although we have shown theoretically (Figure 2.4) and experimentally (Figure 2.12 and Figure 2.13) that each magnetic NPs reach its optimum heating rate at different frequency range, the heating is not completely independent because at high frequency all the NPs are at their optimum heating rates. This selectivity may be improved by adjusting the magnetic field amplitude at different frequency range (Figure 2.5); but the feasibility of this approach is still unproven.

4.3. Alternative Heating Mechanism

Since nano size particles is the key component for our hypothesis, increasing their size up to the millimeter range is not an option. Therefore the only option left to be considered for generating a local temperature increase around the NPs is to increase the NP power density as the heat source. This increase has to be in the range of a few orders of magnitude to generate significant temperature increase. The next step is then to figure out the mechanism to deliver such high power density to NPs so that they can convert it to heat.

Recently, other researchers have managed to deliver very high power density of energy pulsed onto the gold NRs.\textsuperscript{[107-110]} This delivery mechanism was accomplished by irradiating the gold NR sample with energy from femtosecond laser pulses at the wavelength corresponding to the absorption wavelength of the gold NRs. Since the pulse duration is only a few femtosecond, therefore the power density could be in order of mega to gigawatts. With this high power density, femtosecond laser pulses can be utilized
to heat up or even melt the gold NRs. Figure 4.4 show the TEM images of gold NRs before and after the exposure with femtosecond laser pulses. It shows the NRs were melted and shape transformed to more energetically favorable spherical form. This heating mechanism of gold NRs can be an ideal solution for our hypothesis.

![TEM images of gold nanorods (a) before and (b) after exposure to femtosecond laser pulses.](image)

**Figure 4.4.** TEM images of gold nanorods (a) before and (b) after exposure to femtosecond laser pulses. Reprinted with permission from the Annual Review of Physical Chemistry.

### 4.4. Summary

In this chapter we have shown that our proposed heating mechanism for magnetic NP has failed to generate local temperature increase around the NP; therefore unable to be utilized for our purpose of controlled release applications. We also discussed the theoretical explanation of this physical limitation of magnetic field heating of magnetic NP. From the understanding of this limitation, we then considered a new heating mechanism by irradiation with femtosecond laser pulses. The laser exposure at the wavelength corresponding to the gold NRs absorption wavelength can heat up and even melt the NRs. We proposed to explore this mechanism for our hypothesis of utilizing different types of NP for controlled release applications. For the remaining chapters in
this thesis, we will discuss the application of this new proposed heating mechanism for our proposed hypothesis. The discussion will started with the synthesis and selective melting of different types of gold NRs. We then discussed the surface modification and DNA conjugation of the gold NRs. Finally, we will also discuss the feasibility of this mechanism for selective multiple controlled release applications.
Synthesis of Gold Nanorods

Synthesis methods of noble metal NPs, such as colloidal gold NPs have been long
known for their use for staining glass. Scientific investigation of these methods did not
start until late 19th century by Michael Faraday. In the recent years there are massive
works in perfecting the synthesis method to produce metal NPs at the precise sizes. This
is mainly because the particles dimension governs their optical properties. The
morphology of these particles also governs their optical properties. The optical
absorption of gold nanospheres changes slightly with the increasing of their diameters.
An increase in NP diameter from 9 nm to 99 nm results in the red-shift of the absorption
peak by only 58 nm. On the other hand if we introduce anisotropy to the particle, such
as anisotropy growth of gold nanorods (NRs), their optical properties change significantly
with their dimensions and aspect ratios. Increasing the aspect ratio of gold nanorods can
result in red-shift of their longitudinal surface plasmon resonance peak in the order of
hundreds of nanometers. This key element of gold NRs’ properties that make them
very suitable for our purpose; the need of multiple different NPs with different sizes,
morphologies, and optical absorptions.
Generally, there are three methods to synthesize gold NRs via wet chemistry.\textsuperscript{115} The first one is template method, which is basically electrochemical deposition insides nanoporous polycarbonate or aluminum template membrane. The second method is electrochemical method which is basically a simple two-electrode electrochemical system. The gold sacrificial anode is used as the source for the gold NR formation at the platinum electrode with the help of hexadecyltrimethylammonium bromide (CTAB) and small amount of tetradecylammonium bromide as rod-inducing co-surfactants. The last method, seeded growth method is the least difficult method with the highest yield. It uses gold nanosphere as nucleation site for anisotropy growth with the help of CTAB surfactants or binary co-surfactants. For the purpose of this study, we only explore the last method. There are several classifications of this method. For our purposes, single surfactant synthesis was performed to produce shorter capsule-like gold NRs (nanocapsules).\textsuperscript{116} The binary co-surfactants synthesis is performed to produce higher aspect ratio gold NRs and followed by fast reduction step to create bone-shape morphology (nanobones).\textsuperscript{117, 118} The remaining of this chapter discusses in details the synthesis protocols for producing these two types of gold NRs.

5.1. Gold Nanocapsule Synthesis

Lower aspect ratio (AR) capsule-like gold NRs (nanocapsules) are synthesized according to the single surfactant seed-mediated growth method by Murphy.\textsuperscript{116} This synthesis method is typically divided into two steps, the seed preparation and the growth of NRs. The only surfactant used for these steps is hexadecyltrimethylammonium bromide (CTAB). All the chemicals used for this synthesis were bought from Sigma-
Aldrich. **Figure 5.1** illustrated both the seed preparation step and the growth step of the gold NRs.

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**Figure 5.1.** Seed preparation and nanorod growth steps in gold nanorod synthesis. Reprinted with permission from the American Chemical Society.
Step 1. Gold seed preparation step. This step is basically rapid reduction of gold ion to instigate nucleation for generating small gold nanospheres in the present of CTAB surfactants. Typical protocol: 7.5 ml of 0.2 M CTAB solution was mixed with 0.25 ml 0.01 M HAuCl₄ in a beaker. While the solution was vigorously stirred, 0.6 ml of ice-cold 0.01 M of NaBH₄ was added and the solution turned brownish yellow. Vigorous stirring continued for another 2 min and then it was kept undisturbed at room temperature.

Step 2. Growth step of nanorods. Slow reduction of gold ion in the growth solution slowly deposits gold atoms onto the added seeds in anisotropy way resulting in elongation of the seeds to form gold nanocapsules. Typical protocol for growing nanocapsules: 10 ml of 0.01 M of HAuCl₄ was added into 237.5 ml 0.1 M CTAB in a glass bottle, and the solution turned orange. 1.5 ml of 0.01 M AgNO₃ was added to the solution, followed by gentle mixing. 1.6 ml of 0.1 M ascorbic acid was added into the solution, followed by gentle inversion until the solution turned colorless. 2 ml of seed solution was gently added to the growth solution. The solution sat on the bench undisturbed overnight, during which it turned reddish brown.

The NR growth rate was found to level off within about 12 hours. Figure 5.2. (a) shows that absorption profile of the nanocapsule solution. The longitudinal absorption peak (Grey line) is around ~800 nm; nicely overlap with the 800 nm laser profile (blue line) with very minimum overlap with the 1100 nm laser profile (red line). JEOL 2000 was used to take TEM images of these particles. Figure 5.2 (b) shows the capsule-like morphology of the nanocapsules. ImageJ was used for size analysis of these TEM images. Figure 5.3 shows the statistical distributions from these analyses. The nanocapsules’ dimension is approximately ~11 nm × 44 nm, with <AR> = 4.0.
Figure 5.2. (a) Absorption profile and (b) TEM Image of gold nanocapsule solution.

Figure 5.3. Size analysis of gold nanocapsules. Statistical distributions of (a) the diameter, (b) the length, and (c) aspect ratio of the nanocapsules.

5.2. Gold Nanobone Synthesis

Higher aspect ratio gold NRs are synthesized according to the binary co-surfactant seed-mediated growth method by El-Sayed. These co-surfactants are hexadecyltrimethylammonium bromide (CTAB) and benzyldimethylhexadecylammonium chloride (BDAC). Similar to nanocapsule synthesis, this synthesis method is also divided into two steps, the seed preparation step and the NR growth step. After the growth rate levels off, we introduce additional step,
fast reduction of the remaining gold ions in the growth solution, to generate bone-like morphology onto the both ends of the NRs (nanobones).117

**Step 1. Gold seed preparation step.** Typical protocol: 7.5 ml of 0.2 M CTAB solution was mixed with 2.5 ml 0.001 M HAuCl₄ in a beaker. While the solution was vigorously stirred, 0.6 ml of ice-cold 0.01 M of NaBH₄ was added and the solution turned brownish yellow. Vigorous stirring continued for another 2 min and then it was kept undisturbed at room temperature.

**Step 2. Growth step of nanorods.** Typical protocol: 125 ml of 0.001 M of HAuCl₄ was added into a mixture of 50 ml of 0.3 M CTAB and 75 ml of 0.3 M of BDAC (benzyldimethylhexadecylammonium chloride) in a glass bottle, and the solution turned orange. 5 ml of 0.004 M AgNO₃ was added to the solution, followed by gentle mixing. 1.5 ml of 0.1 M ascorbic acid was then added into the solution, followed by gentle inversion until the solution turned colorless. 0.25 ml of seed solution was gently added to the growth solution. The solution sat on the bench undisturbed overnight, during which it turned reddish purple. The resulting product was high aspect ratio gold NRs.

**Addition step. Growth step for dogbone feature.** In order to turn these NRs to nanobones, 4.63 ml of 0.1 M ascorbic acid was added into 250 ml of the NR solution, followed by gentle mixing. Reactions were left undisturbed at room temperature. After ~3 hrs the solution turned blue, indicating formation of nanobones.

The nanobones should have aspect ratio higher than nanocapsules therefore their absorption peak should be at higher wavelength than the nanocapsules’. **Figure 5.4. (a)** shows that absorption profile of the nanobone solution. The longitudinal absorption peak (Grey line) is around ~1100nm, nicely overlap with the 1100nm laser profile (red line)
with very minimum overlap with the 800nm laser profile (blue line). It also has very minimum absorption around ~800nm; therefore it has very minimum overlap with the nanocapsule absorption peak (Figure 5.2. (a) Grey line). These are very crucial for our objective of selective excitation between these two types of gold nanorods. JEOL 2000 was used to take TEM images of these particles. Figure 5.4 (b) shows the bone-like morphology of the nanobones. Image J was used for size analysis of these TEM images. Figure 5.5 shows the statistical distributions from these analyses. The nanobones’ dimension is approximately were ~17 x 89 nm with <AR> = 5.4.

**Figure 5.4.** (a) Absorption profile and (b) TEM image of gold nanobone solution.

**Figure 5.5.** Size analysis of gold nanobones. Statistical distributions of (a) the diameter, (b) the length, and (c) aspect ratio of the nanobones.
Figure 5.6. (a) Absorption profile and (b) TEM image of mixture solution.

Figure 5.7. Aspect ratio distribution of the mixture solution

5.3. Summary

TEM images and size analyses of both nanocapsules and nanobones show their differences in morphology, size and aspect ratio. Figure 5.6. (a) shows the absorption profile of the mixture solution containing both nanocapsules and nanobones. There is very minimal overlap of the SPR_{Long} peaks and distinct separation between the two SPR_{Long} peaks, each corresponding to different type of gold NRs. This will enable us to
excite the mixture of two types of NRs independently at two different wavelengths corresponding to their $\text{SPR}_{\text{Long}}$ peaks. Figure 5.6. (b) shows TEM image of this mixture solution. It clearly shows the distinction between nanocapsules and nanobones in terms of their sizes and morphologies. This will enable us to use TEM images for qualitative characterization of the mixture after laser irradiations two different wavelengths independently, corresponding to their $\text{SPR}_{\text{Long}}$ peaks. Figure 5.7 shows the aspect ratio distribution of the mixture solution. The mixture solution has broader distribution due to the combination of both nanocapsules and nanobones distributions. Therefore, we can also use the aspect ratio distribution analysis to quantitatively characterize the mixture after laser irradiations two different wavelengths independently, corresponding to their $\text{SPR}_{\text{Long}}$ peaks.
Chapter 6

Photothermal Heating of Gold Nanorods

The phenomena which based on conversion of optical energy into heat are commonly known as photothermal heating. Photoacoustic spectroscopy is considered the earlier application of this idea of optical energy can be absorbed selectively by certain materials and eventually converted into thermal energy; this photothermal heating effect is detected by an indirect acoustic method. The more recent application of this photothermal phenomenon is photothermal therapy; a medical application which use certain dye that can absorb specific band of optical energy and then convert to thermal heating for selective killing of the targeted cells.

The recent advancement of the photothermal therapy is the use of gold NPs. There are a few advantages of using gold NPs as the absorbers. Due to its high absorption coefficient, the use of gold NPs requires less laser power to reach the same threshold temperature than the conventional dye absorbers. Gold NPs can easily be conjugated with antibody for active targeting; increasing the selectivity of the photothermal effects to just on the targeted cells. The wavelength of the light can also be customized by adjusting the size or shape of the gold NPs to tune their maximum absorption peaks.
The ability to customize the wavelength of the incoming optical energy is very beneficial because the biggest challenge in photothermal therapy is the selectivity of the treatment. To ensure that the photothermal effect selectively destroying the targeted cells without affecting the nearby cell, tissues, or hemoglobin, the absorption wavelength of the absorbers have to be tuned to the wavelength where most biological entities will not absorb. This wavelength band of light (650 nm – 900 nm) where hemoglobin and water have minimum absorption is often referred as tissue optical window (Figure 6.1). This leads to the most recent advancement of photothermal therapy, which is the use of gold nanorods (NRs) as absorbers. Gold NRs are ideal absorbers because they can be synthesized to have their absorption peaks within the tissue optical window. Their absorption wavelength can be varied widely by adjusting their aspect ratio (Figure 6.2). Gold NRs also share the same other advantages as gold NPs have.

![Image of absorption spectra](image)

**Figure 6.1.** Tissue optical window. This window is ideal for in-vivo application because of the minimal light absorption of hemoglobin (>650 nm) and water (<900 nm). Reprinted with permission from the Nature Publishing Group.
Photothermal heating of gold NRs can be accomplished by a variety of light sources. Other researchers have shown that local temperature of the gold NRs can be elevated by irradiation with continuous wave laser, nanosecond-pulsed laser, or femtosecond-pulsed laser. With the same energy per pulse, the shorter the pulse the higher the NRs local temperature increase. On the other hand, if the pulse duration is shorter that heat dissipation time scale, the increased temperature can not be maintained. Contrary to pulsed laser, the increased temperature can be maintained during the continuous wave irradiation. For the purpose of our hypothesis, the following discussion will focus more on the use of femtosecond-pulsed laser for photothermal heating of gold NRs.

Earlier studies on photothermal heating of gold NRs by femtosecond-pulsed laser irradiation focused on how the irradiation induces shape transformation (melting).\textsuperscript{33, 108, 110} These studies encompass investigations such as energy threshold to induce complete melting, how the structural transition occurs, and what kind time scale of this transition. Some of the recent works in this area involve computational work to model the heat dissipation around the gold NRs.\textsuperscript{123} We will discuss these topics all together in this section.

There are multiple processes occurring with the gold NRs during the femtosecond-pulsed irradiation. First, the free electrons (electron gas) of the NRs absorb the photons from the laser pulse during the pulse duration. The NR (lattice) temperature increases due to the result of electron-phonon scattering; reaching a thermal equilibrium between the electrons and lattice. As the particle temperature increases, heat dissipation to the surrounding medium starts to occur through phonon-phonon interactions. The actual time scale of these processes depends on the NR dimension, laser pulse duration, and laser pulse intensity.\textsuperscript{123}

Femtosecond pump-probe absorption spectroscopy study has been done to probe the actual time scale of these processes.\textsuperscript{108} A sample is hit by some pump pulse which excites the NRs in the sample. After an adjustable time delay, a probe pulse hits the sample, and its transmission is measured. By monitoring these transmission signals of the probe pulses at various time delays, it is possible to obtain information about the NRs as
a function of time after excitation by the pump pulse. Selective excitation of the electron
gas of the NRs induce the transient bleaching of both the transverse and longitudinal
surface plasmon resonance bands and the existence of transient absorption band due to
broadening of both plasmon resonance bands.\textsuperscript{124, 125} El-Sayed group utilized pump-probe
spectroscopy method to monitor this phenomenon to study the time scale of the electron-
phonon and phonon-phonon relaxation times. The observed these timescale to be around
$\sim 1 - 4 \text{ ps}^{107, 113}$ and $\sim 100 \text{ ps}^{33, 107}$ for electron-phonon and phonon-phonon relaxation
times respectively. El-Sayed group\textsuperscript{108} also used the pump-probe spectroscopy method to
monitor the permanent bleaching of NR absorption around 700-800nm (longitudinal
surface plasmon resonance (SPR\textsubscript{long}) peak of the NR) due to induced melting and shape
transformation of NR into spheres after high energy pump pulse excitation. They
observed that the time scale of the NR melting is at least 30-35ps. It is significantly
shorter than the time scale for the NR heat dissipation to surrounding medium; thus the
NR increased temperature is maintained long enough for the melting process to complete.

\textbf{Figure 6.3} shows absorption spectra of gold nanorods solution exposed to 100 fs laser
pulses with energy of 40 $\mu$J as a function of exposure time.\textsuperscript{107} \textbf{Figure 6.4} shows TEM
images of the gold nanorods before and after exposure of 100 fs laser pulses with energy
of 40 $\mu$J for 7 min.\textsuperscript{107} It resulted in shape transformation from nanorods to spheres

The El-Sayed group has performed simple absorption analysis and
thermodynamics calculation based on bulk values to estimate the threshold energy to melt
a single gold NR. They estimated that it takes an average of $\sim 60 \text{ fJ}$ to melt a single NR
with dimension of 44 x 11 nm.\textsuperscript{109} Using the same approach, they also estimated the
lattice (NR) temperature as a function of laser fluence (\textbf{Figure 6.5}).\textsuperscript{33} Based on these
estimations and their experimental data, they concluded that the threshold for complete melting of the gold NRs with 100fs laser pulses is about 0.01 J/cm².33

Figure 6.3. Absorption spectra of gold nanorods solution exposed to 100 fs laser pulses with energy of 40 µJ as a function of exposure time (arrow direction: increase of exposure time). Reprinted with permission from the American Chemical Society.107

Figure 6.4. TEM images of the gold nanorods: (a) before and (b) after exposure of 100 fs laser pulses with energy of 40 µJ for 7 min. Reprinted with permission from the American Chemical Society.107
Recently, other researchers\textsuperscript{123} have also performed computational work to better estimate the surface temperature of NRs, surrounding water temperature, and the heat transfer relaxation time scales. They used two-temperature model to estimate the electron and lattice temperatures. Heat diffusion equation was used to describe the heat transfer from the lattice to the surrounding medium. They also used thermal interface conductance to estimate the coupling efficiency at the particle/water interface. The NR with dimension of 48 x 14 nm\textsuperscript{2} exposed to femtosecond laser pulses with fluence of 4.7 J/m\textsuperscript{2} and pulse duration of 250 fs. These parameters were chosen to avoid reaching melting temperature of gold and critical temperature of water. Considering the length scale of the problem, uniform temperature was assumed across the NR. They show temperature distribution of the surrounding water around the NR at 70 ps after the irradiation. Water temperature reaches to about 580K at the mid-length of the NR; while the NR temperature is uniformly at 1079K. Thermal penetration depth is about 7 nm and
20 nm at 50 ps and 500 ps after the irradiation respectively. They also learned that thermalization of the electrons is instantaneous compared to the electron-phonon and phonon-phonon relaxation time scales. Thermal equilibrium between the electron and the lattice was approximately 50 ps after the pulse. Negligible temperature difference at the interface between NR surface and adjacent water was reached at approximately 500 ps after the pulse.

6.2. Selective Melting of Two Types of Gold Nanorods by Femtosecond-pulsed Laser Irradiations

The melting phenomenon of gold NRs upon the exposure of the femtosecond laser pulses with high enough of laser fluence can be used as characterization tool for our hypothesis. Since our goal is to use selective heating of multiple types of gold NRs for selective multiple controlled release application, we can conceptually prove this concept by showing that selective melting of multiple types of the gold NRs is achievable. To perform this study, we need to synthesize two different types of gold NRs with different sizes and morphologies to enable quantitative and qualitative analyses via TEM imaging and absorption spectroscopy.

The Gold Nanorods: Nanocapsules and Nanobones

NR synthesis protocols were discussed in details in Chapter 5, they were designed produce two different types of NRs with distinct ARs, morphologies, and SPR$_{long}$ that overlapped with each of the laser excitation wavelengths. Short NRs, “nanocapsules,”
were \(\sim 11 \text{ nm} \times 44 \text{ nm}\), with \(<\text{AR}> = 4.0\) (Figure 6.6 (d)) and \(\text{SPR}_{\text{long}}\) at \(800 \text{ nm}\) (Figure 6.6 (b), black), coinciding with the short wavelength excitation at \(\sim 800 \text{ nm}\) (\(\lambda_{800}\)). Long NRs were bone-shaped (Figure 6.7 (d)), presumably due to preferential deposition at the ends from the excess reducing agent.\(^{117}\) “Nanobones” were \(\sim 17 \times 89 \text{ nm}\) with \(<\text{AR}> = 5.4\) and \(\text{SPR}_{\text{long}}\) at \(\sim 1100 \text{ nm}\) (Figure 6.7 (b), black), coinciding with the long wavelength excitation at \(1100 \text{ nm}\) (\(\lambda_{1100}\)). Size analysis was done utilizing ImageJ.\(^{51}\) Mixtures allowed distinction between their populations by both the AR and morphology (Figure 6.8 (d)). TEM sizing of a mixture exhibited broader AR distribution due to overlapping peaks at 4.0 and 5.4 (Figure 6.8 (a), black). The 1.5 – 2.0 AR peaks were from the small presence of synthesis byproducts (spheres, cubes, and stars). Spectral overlap of nanocapsules and nanobones at both excitation wavelengths was minimal (Figure 6.8 (b), black).

**The Laser Experimental Setup**

Laser irradiation was achieved using pulsed femtosecond lasers. For the 800 nm irradiation, the 82 MHz output of a Ti:sapphire oscillator (Tsunami, Spectra-Physics) is amplified at 1 kHz by a Ti:sapphire regenerative amplifier (Spitfire, Spectra-Physics) pumped by the doubled output of a Q-switched Nd:YLF laser (Empower, Spectra-Physics). The system produces 50 - 475 \(\mu\text{J}\), with duration of 100 fs centered at \(\sim 800 \text{ nm}\) at a 1 kHz repetition rate. Spot size was 6 mm. In a typical experiment, 50 \(\mu\text{L}\) of sample in 3x3 mm quartz cuvette was exposed to laser for 60 s.
Figure 6.6. Melting nanocapsules. (a) AR histogram of nanocapsules exposed to irradiation at 800 nm at 1.68 mJ/cm² (blue) and 1100 nm at 1.73 mJ/cm² (red), and unexposed (black). (b) optical absorption spectra of nanocapsules upon exposure to λ₈₀₀ irradiation. Fluence (mJ/cm²): 0 (black), 0.18 (red), 0.27 (green), 0.35 (blue), 0.70 (cyan), 1.06 (pink), 1.41 (orange), 1.68 (olive). (c) optical absorption of nanocapsules upon exposure to λ₁₁₀₀ irradiation. Fluence (mJ/cm²): 0 (black), 0.33 (red), 0.51 (green), 1.02 (blue), 1.39 (cyan), 1.73 (pink). TEM images of (d) unexposed nanocapsules sample, (e) of λ₈₀₀ irradiated sample with a fluence of 1.68 mJ/cm², and (f) λ₁₁₀₀ irradiated sample with a fluence of 1.73 mJ/cm².
Figure 6.7. Melting nanobones. (a) AR histogram of nanobones exposed to irradiation at 800 nm at 1.68 mJ/cm² (blue) and 1100 nm at 1.73 mJ/cm² (red), and unexposed (black). (b) Optical absorption spectra of nanobones upon exposure to 800 nm irradiation. Fluence (mJ/cm²): 0 (black), 0.18 (red), 0.27 (green), 0.35 (blue), 0.70 (cyan), 1.06 (pink), 1.41 (orange), 1.68 (olive). (c) Optical absorption of nanobones upon exposure to 1100 nm irradiation. Fluence (mJ/cm²): 0 (black), 0.33 (red), 0.51 (green), 1.02 (blue), 1.39 (cyan), 1.73 (pink). TEM images of (d) unexposed nanobones sample, (e) of 800 nm irradiated sample with a fluence of 1.68 mJ/cm², and (f) 1100 nm irradiated sample with a fluence of 1.73 mJ/cm².
The 1100 nm was generated via a homebuilt two-stage BBO/KNbO\(_3\) optical parametric amplifier pumped with the output of a Ti:Sapphire multipass amplifier (Femtolasers: 30 fs, 1 kHz, 800 nm). Although the OPA is optimized for the production of 3\(\mu\)m light, \(-2.6 \text{ - } 13.6\) \(\mu\)J pulses of 1100 nm light were generated, the difference frequency between 800 nm and 3 \(\mu\)m, is generated by the OPA and used for the experiments. The generated \(\sim\)1100nm has duration of 45 fs/pulse with repetition rate of 1 kHz. Spot size was 1 mm. In typical experiments, 50 \(\mu\)L of sample in 3\(\times\)3 mm quartz cuvette were continuously mixed with pipette tip while being exposed to laser for 60 s.

After 800 nm or 1100 nm laser exposure, samples were diluted with their corresponding solvents, 150 \(\mu\)L of 10 mM CTAB solution. UV-vis-NIR absorption scans were performed to monitor the change of the absorption profile due the shape transformation (melting). TEM imaging was also used to qualitatively confirm the melting and perform quantitative size analyses.

**Nanorod Melting and Its Selectivity**

Laser irradiation of gold NRs at SPR\(_\text{long}\) caused melting,\(^{33, 34}\) accompanied by a shape transformation to spheres. We studied the fluence dependence of nanocapsule and nanobone melting by monitoring absorption. \(\lambda_{800}\) irradiation of nanocapsules caused the SPR\(_\text{long}\) to decrease in intensity and blue-shift with increasing fluence, while the \(\sim\)520 nm peak increased (Figure 6.6 (b)). This suggested that melting transformed nanocapsules into shorter rods and spheres. TEM images of nanocapsules after \(\lambda_{800}\) irradiation (Figure 6.6 (e)) and size analysis showed the AR distribution shift to lower values (Figure 6.6 (a), blue), supporting shape transformation to spheres. To show that melting of
nanocapsules requires matching irradiation wavelength to the SPR_{long}, we irradiated nanocapsules at 1100 nm. The absorption spectrum was unchanged (Figure 6.6 (c)), demonstrating no significant effect. TEMs of nanocapsules after \( \lambda_{1100} \) irradiation (Figure 6.6 (f)) were also unchanged, and size analysis showed little effect on the AR distribution (Figure 6.6 (a), red).

The melting of the nanobones also required the matching of the irradiation wavelength to their SPR_{long}. When nanobones were exposed to \( \lambda_{1100} \) irradiation, the 1100 nm peak decreased and blue-shifted with increasing fluence, while the \( \sim \)520 nm peak increased (Figure 6.7 (c),). TEM imaging after \( \lambda_{1100} \) irradiation confirmed a shape transformation into shorter “candy-wrap” or \( \phi \)-shaped particles and spheres (Figure 6.7 (f)). Size analysis confirmed an AR shift to lower values (Figure 6.7 (a), red). \( \lambda_{800} \) irradiation had essentially no effect, as evidenced by no significant change in absorption scans (Figure 6.7 (b)). TEM after \( \lambda_{800} \) irradiation (Figure 6.7 (e)) and size analysis showed no significant changes in the AR distribution (Figure 6.7 (a), blue), indicating no shape transformation occurred.

We also selectively melted either the nanocapsules or nanobones when both were present in a mixture. The absorption scan had peaks at 800 and 1100 nm due to the presence of both species, and TEMs and AR histograms showed both populations (Figure 6.8 (a), black and Figure 6.8 (d)). \( \lambda_{800} \) irradiation caused the 800 nm peak to decrease, leaving the 1100 nm peak relatively unaffected (Figure 6.8 (b)). After \( \lambda_{800} \) irradiation, fewer nanocapsules were present relative to nanobones. Spheres appeared, resulting from the nanocapsule shape transformation (Figure 6.8 (e)). The AR peak at 4.0 decreased in intensity, and peaks at 3.0 or less increased, suggesting melting to form
Figure 6.8. Selective melting of nanocapsule/nanobone mixtures. (a) AR histogram of nanocapsules-nanobones mixture exposed to irradiation at 800 nm at 1.68 mJ/cm² (blue) and 1100 nm at 1.73 mJ/cm² (red), and unexposed (black). (b) optical absorption spectrum of mixture after $\lambda_{800}$ irradiation. Fluence (mJ/cm²): 0 (black), 0.18 (red), 0.27 (green), 0.35 (blue), 0.70 (cyan), 1.06 (pink), 1.41 (orange), 1.68 (olive). (c) optical absorption spectrum of mixture after $\lambda_{1100}$ irradiation. Fluence (mJ/cm²): 0 (black), 0.33 (red), 0.51 (green), 1.02 (blue), 1.39 (cyan), 1.73 (pink). TEM images of (d) unexposed mixture, (e) of $\lambda_{800}$ irradiated mixture with a fluence of 1.68 mJ/cm², and (f) $\lambda_{1100}$ irradiated mixture with a fluence of 1.73 mJ/cm².
spheres and shorter NRs (Figure 6.8 (a), blue). However, the peak at 5.4 was relatively unchanged. These results support that \( \lambda_{800} \) irradiation melted only the nanocapsules but not the nanobones. When the mixture was exposed to \( \lambda_{1100} \) irradiation, the 1100 nm peak decreased with increasing fluence (Figure 6.8 (c)) while the 800 nm peak increased, presumably due to shape transformation of nanobones into \( \phi \)-shaped NRs, which are expected to absorb at wavelengths lower than 1100 nm.\(^{126}\) TEM imaging after \( \lambda_{1100} \) irradiation (Figure 6.8 (f)) showed that the nanobones disappeared, with primarily nanocapsules, spheres, and \( \phi \)-shaped NRs remaining. The AR peak at 5.4 decreased while peaks at 4.0 or less increased, indicating nanobone shape transformation (Figure 6.8 (a), red). Evidently, \( \lambda_{1100} \) irradiation affected only the nanobones and not nanocapsules. Thus, laser irradiation could selectively melt each species in a mixture, corroborating single-type NRs melting studies.

### 6.3. Summary

Gold NRs can be heated up and even melted by exposure of femtosecond laser pulses with high enough energy at the wavelength corresponding to the NRs’ absorption peak. We have shown that tuning the laser fluence, we can govern the degree of this shape transformation. On top of it, we have also shown that we can selectively melted two different types of NRs by matching the laser irradiation wavelength to their absorption peaks. These two observations provide the crucial desired properties for our hypothesis. They are the tunability and selectivity for the potential multiple controlled release application.
Chapter 7

Surface Modification and DNA Conjugation

In the last chapter we have shown the key properties of photothermal heating of gold nanorods (NRs) that can potentially be desirable for our hypothesis in multiple controlled release application. Before we can apply this heating phenomenon for the controlled release application, we need a technique for loading the potentially-released molecules onto the gold NRs as the carriers. We decided to use DNA oligonucleotides for our drug model molecules and utilize the well known gold-thiol conjugation for loading the DNA oligos onto the surface of gold NRs. Unfortunately, the resulting synthesized gold NRs have their surface decorated by the bilayer cationic surfactants, Cetyltrimethylammonium bromide (CTAB). Not only that this surfactant is known to be toxic, the overall positively charged of the NRs induces aggregation with the negatively charged DNA oligos during the incubation. Thus the synthesized gold NRs are proven to be problematic for bioconjugation. Therefore the first section of this chapter will cover the NR surface customization technique that we develop to enable DNA
conjugation, which will be discussed on following section. The materials covered in chapter were previously published in Langmuir\textsuperscript{129} and ACS Nano\textsuperscript{130}.

### 7.1. Ligand Exchange and Surface Customization

In the recent years, there are tremendous efforts in incorporating nanotechnology (especially NPs) into biological applications and biomedical devices. Because of their stability and size uniformity, inorganic NPs have been widely developed for these purposes. Due to their tunable optical properties, metallic NPs especially gold NRs have recently become very attractive for many biological applications such as gene delivery,\textsuperscript{34} cell imaging,\textsuperscript{32} and photothermal therapy.\textsuperscript{131} However, their ligand functionalization has been problematic for conjugation chemistries. Gold NR synthesis results with a double layer of cationic surfactant, CTAB on their surface (NR-CTAB) for passivation. This has been proven to be very problematic for bioconjugation due to non-specific adsorption of DNA, cytotoxicity,\textsuperscript{127, 128} aggregations, and their long-term stability. These factors have severely limited the use of gold NRs for biological applications, especially when compared to gold NPs (spherical form).\textsuperscript{132} In order to overcome these limitations such as minimizing the non-specific adsorption during the bioconjugation to prevent aggregation, ligand exchange technique is a must for surface customization. This technique must produce NRs with uniform charge distribution to enable gel electrophoresis to assay conjugation and DNA confirmation on the NR surface. There are previous reports of NR ligand exchange techniques which separately permit conjugation to an antibody\textsuperscript{32, 133-135} or gel electrophoresis.\textsuperscript{136} However, there has not yet been a NR ligand exchange
technique that can enable ligand customization, biofunctionalization, and gel electrophoresis. We have also tried these previously reported methods with very limited success.32, 133-136 Due to the importance to this problem, we decided to develop our own technique. We believe that by solving this problem, the resulting solution, customizable ligand chemistry would broadly enhance the versatility of gold NRs in biological applications.

The previously reported ligand exchange techniques involve a very simple protocol of removing excess surfactants from the resulting synthesized nanorods and then followed by simple incubation with the new ligands with or without sonication to avoid aggregation.32,133-136 We develop a new technique with a very different approach with the previously reported works. This new approach involves “round-trip” phase transfer of the NRs for ligand customization and further DNA functionalization. Figure 7.1 shows the schematic of our ligand exchange technique. The protocol is basically a two-step ligand exchange. First, aqueous to organic phase transfer to strip the CTAB surfactant with dodecanethiol (DDT) and followed by the organic to aqueous phase transfer to replace DDT with negatively charged mercaptocarboxylic acid ligands. Resulting NRs are stable in physiological buffers and exhibit narrow bands in gel electrophoresis. DNA functionalization is straightforward, with minimal non-specific adsorption on the NR.
Figure 7.1. Schematic of nanorod ligand exchange protocol and DNA conjugation.  

We used the capsule-like gold NRs for this ligand exchange study. They were synthesized by literature methods. TEM imaging is used to determine the size of NR-CTAB; their mean dimension is 43.5 ± 11.9 nm (Figure 7.4 (a)). Centrifugation was typically used to concentrate the synthesized NR-CTAB solution prior to the ligand exchange protocol. The first step in the round-trip ligand exchange protocol utilizes aqueous-to-organic phase transfer.  

1ml of NR-CTAB solution at high concentration (2-5E-8 M) in water was put into contact with 1ml of pure dodecanethiol (DDT) (Figure 7.1, Left Image). After addition of 4ml of acetone, the NRs were extracted into organic DDT phase by swirling the solution for a few seconds, upon which the aqueous phase became clear, indicating that no NRs remained (Figure 7.1, Middle Image).

The second step in ligand exchange protocol involves organic-to-aqueous phase transfer to bring back the NRs back to aqueous phase with the desired ligands on their
surface. This phase transfer protocol follows the previously reported methods for gold NPs and quantum dots. First we need to remove the excess DDT by centrifugation technique. The DDT coated NR (NR-DDT) in the excess DTT phase was diluted with toluene (1X) and an excess of methanol (5X), then spun down, and resuspended in 1 ml toluene by brief sonication. The purified NR-DDT in toluene was then added all at once to 9 ml solution of 0.01 M mercaptocarboxylic acid (MCA) in toluene at elevated temperature while vigorously stirred. Three types of MCA ligands were used; they are mercaptohexanoic acid (MHA), mercaptoundecanoic acid (MUDA), and mercaptohexadecanoic acid (MHDA). The corresponding operating temperatures were 95°C for MHA and 70°C for MUDA and MHDA. The stirring and reflux continued until visible aggregation of the NRs was observed; typically within 2 - 15 min. The solution was then allowed to settle and cool to room temperature. The resulting aggregation indicates that NRs were successfully coated by new MCA ligand; where the resulting NRs (NR-MCA) are insoluble in toluene. The aggregates were then washed once or twice with toluene via decantation, then once with isopropanol to deprotonate the carboxylic acid. The aggregates spontaneously redispersed in 1X TBE and were no longer soluble in toluene (Figure 7.1, Right Image), suggesting residual DDT on the NR surface is minimal.

Once the NR-MCA are resuspended in 1x TBE buffer, they could have their MCA coating optimized, be ligand exchanged with another species, or conjugated to DNA (Figure 7.1). We incubated NR-MCA with 1 mM MCA in H₂O or a H₂O/ethanol mixture for further optimizing the MCA coating. We also performed further ligand exchange of NR-MHA by incubating them in 1 mM aqueous solution of PEG-thiols with
various molecular weights (HS-PEG\textsubscript{356}, HS-PEG\textsubscript{1000}, and HS-PEG\textsubscript{5000}). Pegylation of gold NPs are known to increase the NP stability in the physiological environments during the in-vitro and in-vivo experiments. Lastly we conjugated NR-MHA with fluorescently labeled thiolated DNA 40mers (5' HS-TTTTT TTTTT TTTTT TTTTT TCGGC CCGTA TAATT- TMR 3') using the charge screening method for DNA functionalization of Au NPs.\textsuperscript{144}

The width of the longitudinal surface plasmon resonance can be used to directly probe the stability and aggregation of NRs. UV-vis spectra of ligand exchanged NRs with MCA show shifts of the longitudinal plasmon with no significant broadening, indicative of no aggregation (Figure 7.2 (a)). The peak shift is linear with chain length (Figure 7.2 (a), inset) as expected from the change in refractive index of the MCA coating with increasing alkyl chain length.\textsuperscript{145-147} The longitudinal plasmon of NRs functionalized with PEG-SH (Figure 7.2 (b)) also showed no significant broadening. We found that the ligand exchanged NRs were stable even after 3 or 4 months of storage at high concentration (~2E-8 M), and the plasmon peaks exhibited no significant changes in peak width or position (Figure 7.3).

Heating gold NPs near the boiling point of the solvent in the present of surface-active ligands such as alkanethiols over certain period of time may result in reduction in average size of the particle due to digestive ripening.\textsuperscript{148} TEM imaging was used to probe any change in size of the NR dimensions upon ligand exchange (Figure 7.4). TEM size analysis determined mean dimensions of 43.2 x 11.8 nm for ligand-exchanged NRs, NR-MHA (Figure 7.4 (b)). In comparison with the original synthesized NR-CTAB (Figure 7.4 (a)), there is no indication of any significant size change due to the ligand exchange
protocol. We also performed size analysis on other types of ligand-exchanged NRs (NR-MUDA, NR-MHDA, NR-PEG$^{356}$, NR-PEG$^{1000}$, and NR-PEG$^{5000}$) and observed significant size change (Appendix B). We also observed no evidence of aggregation of the ligand-exchanged NRs.

Figure 7.2. UV-vis spectra of NRs upon ligand functionalization. (a) UV-vis spectra of NR-CTAB (black), NR-MHA (red), NR-MUDA (blue), and NR-MHDA (green), inset, longitudinal surface plasmon peak as a function of ligand length for SH-(CH$_2$)$_n$-COOH; (b) UV-Vis spectra of NR-MHA (black) before and after functionalization with PEG-SH, NR-PEG$^{356}$ (red), NR-PEG$^{1000}$ (green), NR-PEG$^{5000}$ (blue).

FTIR spectroscopy was performed to confirm and probe the nature of the ligands on the surface of gold NRs before and after the ligand exchange. FTIR spectroscopy profile of purified NR-CTAB (Figure 7.5 (a), black) showed peaks at 958 cm$^{-1}$ (arrow, 1) due to the quarternary amine stretch of CTAB (Figure 7.5 (a), green). The FTIR spectroscopy profile of purified NR-MHA (Figure 7.5 (b), red) exhibited a COO- stretch (1585 cm$^{-1}$, arrow, 2), shifted from the COOH stretch (1690 cm$^{-1}$, arrow, 3) for MHA.
alone (Figure 7.5 (b), blue), which is due to deprotonation of carboxylic acid of the MHA monolayer on the NR surface. NR-MHA lack a S-H stretch (2613 cm\(^{-1}\), arrow, 4) but still have a C-S stretch (706 cm\(^{-1}\), arrow, 5); indicating no free MHA molecules and suggesting that the existing MHA molecules bound to the NR surface via sulfur-gold bond.\(^{149-152}\) Similar observations can be inferred for other ligand-exchanged NRs (Appendix B).

![Figure 7.3](image)

Figure 7.3. UV-vis spectra of ligand exchanged NRs right after exchange (solid line) or after 3-4 months storage at high concentration (dashed).\(^{129}\)
Figure 7.4. TEM Images and size analyses of (a) NR-CTAB and (b) NR-MHA.¹²⁹
Figure 7.5. FTIR spectra of (a) cetyltrimethylammonium bromide, CTAB (green) and NR-CTAB (black) before ligand exchange. (b) mercaptohexanoic acid, MHA (blue) and NR-MHA (red) after ligand exchange.

We also performed gel electrophoresis to confirm the ligand exchange and probe the mobility differences between the resulting ligand-exchanged NRs. Gel electrophoresis was performed with 0.5% agarose gel in 0.5X TBE (Figure 7.6). NR-CTAB aggregate in buffer, and do not move from the well (Lane 1). NR-MHDA (Lane 2), NR-MUDA (Lane 3), and NR-MHA (Lane 4) all run in the positive direction, indicating that the resulting ligand-exchanged NRs are negatively charged, thus confirm the ligand exchange. The slight increasing trend in mobility is most likely due to a decrease in the hydrodynamic radius (RH) of the NRs as a result of decreasing alkyl chain length; considering the TEM images (Figure 7.4 and Appendix B) shows no significant physical size change resulting from the ligand exchange protocols. NR-PEG356 (Lane 5), -PEG1000 (Lane 6), and -PEG5000 (Lane 7) all ran in the positive direction, which
could be due to residual MHA on the NR surface. Mobility decreased with increasing PEG chain length, most likely due to the increase in RH with longer PEG. Furthermore, bands of all of the ligand functionalized NRs were narrow, exhibiting clear mobility shifts with surface functionalization, enabling reliable quantitative Ferguson analysis to find hydrodynamic radius\textsuperscript{136,153} and \( \zeta \)-potential.\textsuperscript{154}

![Image of gel electrophoresis](image)

1. NR-CTAB
2. NR-MHDA
3. NR-MUDA
4. NR-MHA
5. NR-PEG\textsubscript{356}
6. NR-PEG\textsubscript{1000}
7. NR-PEG\textsubscript{5000}

**Figure 7.6.** Gel electrophoresis (0.5% agarose in 0.5X TBE) of before (Lane 1: NR-CTAB) and after ligand exchange (Lane 2: NR-MHDA, Lane 3: NR-MUDA, Lane 4: NR-MHA, Lane 5: NR-PEG\textsubscript{356}, Lane 6: NR-PEG\textsubscript{1000}, Lane 7: NR-PEG\textsubscript{5000}).\textsuperscript{129}

### 7.2. Gold Nanorod – DNA conjugation

Any attempt to conjugate DNA oligonucleotides onto the surface of synthesized NR-CTAB will result in instantaneous aggregation due to the formation of network between the positively charged NR-CTAB with negatively charged string of DNA oligos. On the other hand; the negatively charged ligand exchanged NRs can be easily conjugated with DNA oligos via charge screening method.\textsuperscript{144} For example, after ligand
exchange the resulting NR-MHA was incubated with fluorescently labeled thiolated DNA 40mers (5’ HS-TTTTT TTTTT TTTTT TTTTT TCGGC CCGTA TAATT- TMR 3’) (SH-DNA); we called this solution as “incubation reaction”. We also incubate NR-MHA with fluorescently labeled non-thiolated DNA; we call this solution as “incubation control”. **Figure 7.7** shows the UV-Vis spectra of before and after DNA conjugation. DNA conjugation (**Figure 7.7, red solid line**) did not broaden or shift the longitudinal SPR significantly relative to the NR-MHA peak (**black dash line**); indicating no aggregation after DNA conjugation. Furthermore these UV-Vis spectra of the NR-DNA conjugate was taken 7 days after the conjugation was performed, indicating its long-term stability. Gel electrophoresis was performed to confirm the NR-DNA conjugation via Au-S bonding. NR-DNA conjugates of the incubation reaction (**Figure 7.8, Lane 3**) ran slower relative to NR-MHA (**Lane 1**), indicating a $R_H$ increase. Bands are narrow enough to permit Ferguson analysis of the NR-DNA. The narrow band also indicates no aggregation and a uniform charge distribution. The NRs of the incubation control solution exhibited no significant mobility shift, indicating minimal non-specific adsorption (**Lane 2**). The fluorescent of free DNA band of the incubation reaction solution (**Lane 3**) decreases in intensity compared to a control sample of equal concentration of the thiolated DNA by itself (**Lane 5**) while the non-thiolated DNA does not (**Lane 2 vs Lane 4**), also supporting covalent attachment. Fluorescence spectroscopy also indicated NR-DNA conjugation. Supernatant fluorescence decreased (**Figure 7.9 (a), red solid line**) from its original value (**red dash line**), indicating that DNA was removed from solution by conjugation to the NR. Nonthiolated DNA showed a smaller decrease (**Figure 7.9 (a), black line, solid vs. dash line**), indicating that covalent conjugation was
favored over non-specific adsorption. NR concentration was estimated using the extinction coefficients of the longitudinal plasmon band peak, \( \varepsilon = 4.6 \times 10^9 \text{ M}^{-1} \text{cm}^{-1} \). Fluorescence spectra quantified \( \sim 41 \text{ DNA/NR.} \)

**Figure 7.7.** DNA conjugation. UV-vis spectra of NR-MHA (mercaptohexanoic acid; black dash line), NR-MHA + non-thiolated DNA (black solid), NR-MHA + thiolated DNA (red line). 129

**Figure 7.8.** Gel electrophoresis (0.5% agarose in 0.5X TBE) of DNA functionalization of NR-MHA (UV image), **Lane 1**: NR-MHA, **Lane 2**: NR-MHA incubated with non-thiolated DNA, **Lane 3**: NR-MHA incubated with thiolated DNA, **Lane 4**: non-thiolated DNA, **Lane 5**: thiolated DNA. All DNA was functionalized with a 3’ TMR. 129
Gel electrophoresis can also be used to purify the DNA conjugated NRs (NR-DNA). NR-DNA bands had lower mobility than free DNA band therefore they were not at the same position as the free DNA bands in the agarose gels (Figure 7.8, lane 2 and 3). Thus the NR-DNA bands were free from the unconjugated DNA; these bands can be cut to extract the purified NR-DNA sample. The extraction method typically involved of dissolving the gel bands in 1xTBE buffer overnight and followed by centrifugation with 0.2μm filtered tube to separate the NR-DNA from the gel. Incubation of purified NR-DNA with mercaptohexanol (MCH) can displace the conjugated DNA; which then can be quantified by fluorescence spectroscopy.\textsuperscript{156} Typically, purified NR-DNA sample were incubated in 1 mM MCH overnight, displacing the DNA from the NRs. Displaced DNA was then separated from the NRs by centrifugation and quantified by fluorescence spectroscopy (Figure 7.9 (b), red solid line). As a control, a fluorescence scan was taken of the supernatant of the same NR-DNA solution without MCH treatment (red dash line). The scan was taken 9 days after the conjugation, indicating there was no significant detachment of DNA from NR-DNA conjugates over this period of time. The gel-purified NRs incubated with nonthiolated DNA was also treated with MCH and its supernatant exhibited no significant fluorescent peak (black solid line), indicating minimal non-specific adsorption. Quantification by fluorescence spectroscopy determined the conjugation ratio of approximately 28 HS-DNA/NR. This DNA loading translates to approximately 2 pmol DNA/cm\textsuperscript{2}, which is the same order of magnitude for DNA conjugation with gold nanowires\textsuperscript{157} or nanoparticles.\textsuperscript{158}
7.3. Summary

In this chapter we have shown that we can successfully performed ligand exchange of gold NRs via round-trip phase transfer method. The ligand exchange protocol can varied to enable ligand customization and straightforward DNA conjugation. Ligand-exchanged NRs were proven to have uniform charge distribution; enabling their
long-term stability in physiological buffers and quantitative analysis by gel electrophoresis. The straightforward DNA conjugation with the ligand-exchanged NRs will enable us to conjugate multiple DNA each onto different type of gold NRs for our hypothesis. This method for modifying the surface chemistry of gold NRs will also enhance their versatility in biological applications such as therapy, sensing, and imaging.
In the previous chapter we have shown that we can customize the ligands on the surface of gold nanorods (NRs) to enable straightforward DNA conjugation. In this chapter we will discuss the development of a system of two types of NR-DNA conjugates for the purpose of our hypothesis; selective release of multiple DNA oligonucleotides from different types of gold NRs. Figure X.1 show the schematic of the hypothesis. We then discuss how we prove the selective release hypothesis by selective melting of different NR via utilization of two different femtosecond laser with pulses center at two different wavelengths, each corresponding to different gold NRs’ absorption peak. The materials covered in chapter were previously published in ACS Nano.130

8.1. A System of Two Types of NR-DNA Conjugates

In the introduction chapter we have discussed the need for drug carrier strategy that can enable selective multiple drug delivery. An ideal method would involve the
ability to externally control the release of each drug independently. As mentioned earlier, we proposed the utilization of selective heating different types of NPs to release multiple types of DNA oligonucleotides, each conjugated to different NPs. In the DNA - gold NR system, we propose the utilization of two different types of gold NRs (Chapter 5) which were tuned two have two different absorption peaks (longitudinal surface plasmon resonance) with minimal overlap when they are mixed. This property will enable selective excitation by laser irradiation (Chapter 6). The two different types of gold NRs were also tuned to have distinct difference in their sizes, aspect ratios, and morphologies. These will enable qualitative and quantitative analyses after laser irradiation (Chapter 6). In Chapter 6 we have also shown that pulsed laser excitation in resonance with the NRs’ longitudinal surface plasmon resonance ($\text{SPR}_{\text{long}}$) can heat NRs locally to high temperatures, inducing melting. Since $\text{SPR}_{\text{long}}$ is tunable by changing NR aspect ratio (AR), we have also shown that the two types of NRs (nanocapsules and nanobones) with different ARs can be excited and melted independently at different wavelengths corresponding to their $\text{SPR}_{\text{long}}$. This triggered melting is exploitable for controlling the release of biomolecules conjugated to the NRs.\(^3\) If different NRs are conjugated to different molecules, this strategy could be utilized for orthogonal triggered release of multiple species. Here we demonstrate selective release of two distinct DNA strands from two different NRs by matching laser excitation wavelength to the NRs’ $\text{SPR}_{\text{long}}$ (Figure 8.1).

**DNA Conjugation**

A system consisted with two types of DNA conjugated gold NRs was used to test our hypothesis, as illustrated in Figure 8.1. Two different NRs, nanocapsules and
nanobones (Chapter 5), were ligand exchanged with mercaptohexanoic acid (MHA). The two different ligand-exchanged NRs were then conjugated to two different thiolated 40mers DNA, each labeled with different fluorophore and thus distinguishable. These 40mers DNA oligonucleotides had the same sequence of 5’ HS-TTTTT TTTTT TTTTT TTTTT TTTTT TTTTT TCGGC CCGTA TAATT 3’; each fluorescently labeled at the 3’ ends with either FAM (FAM-DNA-SH) or TMR (TMR-DNA-SH) (both were purchased from Sigma Aldrich) were conjugated to nanocapsules and nanobones respectively via gold-thiol covalent bond (Figure 8.2). The DNA-NR conjugation was achieved following charge screening protocols.\(^{129, 144, 158}\) Charge screening (salt-aging) was necessary to compensate for electrostatic repulsion between the NRs and DNA. Sodium dodecyl sulfate (SDS) surfactant was used to increase the stability of NRs during the salt-aging process. First, FAM-DNA-SH and TMR-DNA-SH were reduced by tris[2-carboxyethyl] phosphine (TCEP) with TCEP:DNA ratio of 100:1. Then nanocapsules or nanobones in the concentration range of 5-10 nM were incubated with the reduced FAM-DNA-SH or TMR-DNA-SH, respectively, in 10 mM phosphate buffer with 0.3% SDS concentration. DNA to NR ratios were 200 FAM-DNA-SH/nanocapsule and 400 TMR-DNA-SH/nanobone. After 3 hrs of incubation, charge screening was performed with salting buffer of 0.6 M of NaCl, 0.3 % SDS in 10 mM phosphate buffer. 8 μL of the salting buffer were added to the 200 μl of the conjugation solution every 30 min followed by 10s sonication. This step was repeated for total 5 times, which was then followed by overnight incubation.
Gel Electrophoresis

Two methods were used to confirm NR-DNA conjugation. First, gel electrophoresis was used to observe mobility changes to assay any change in the hydrodynamic radius of DNA-conjugated NRs. Gel electrophoresis was performed with 0.5 % agarose gels in 0.5× TBE (Figure 8.3). Glycerol was used for loading the samples to ensure the NRs stayed in the wells prior to traveling in the gel matrix. Nanocapsules ran toward the positive electrode, indicating a negative charge from the mercaptohexanoic acid ligand coating the surface (Lane 1). Nanocapsules incubated with thiolated FAM-labeled DNA 40mers (FAM-DNA-SH) were retarded, indicating a larger hydrodynamic radius due to conjugation (Lane 2). The UV image of the gel (Figure 8.3(b)) showed that the free FAM-DNA-SH band after conjugation (Lane 2) was dimmer than the equal-concentration free FAM-DNA-SH alone (Lane 3), also supporting conjugation to nanocapsules, which quench fluorescence. Nanobones showed similar results for thiolated TMR-labeled DNA 40mers (TMR-DNA-SH). The TMR-DNA-nanobones band (Lane 5) was retarded compared to nanobones alone (Lane 4), and the free TMR-DNA-SH band (Lane 5) was dimmer than the equal-concentration free TMR-DNA-SH alone (Lane 6). These results confirm DNA conjugation to both species.

Fluorescence Spectroscopy

The second method to confirm the conjugation was quantification of the DNA loading onto the NR surface. This was accomplished by two methods. First, we quantified the free unconjugated DNA via fluorescence spectroscopy of the fluorophore labels (FAM for nanocapsules and TMR for nanobones) with or without NRs present.
This was done by collecting the supernatants after centrifugation of the conjugation solution. The second method was accomplished by chemical displacement of the conjugated DNA using literature methods. Briefly, purified NR-DNA conjugates were incubated in 1 mM MCH overnight; displacing the DNA from the NRs. Free displaced DNA was separated from the NRs by centrifugation and quantified by fluorescence spectroscopy. Both methods gave similar estimates of the DNA loadings of approximately 114 DNA/nanocapsule and 284 DNA/nanobone.

**Figure 8.1.** Schematic overview of the selective release. Laser irradiation of DNA-conjugated nanocapsules (blue ovals) and nanobones (red bones) are exposed to $\lambda_{800}$ irradiation (left), which melts the nanocapsules and selectively releases the conjugated DNA (labeled by FAM (green triangles)). Exposure to $\lambda_{1100}$ irradiation (right) melts the nanobones, selectively releasing the conjugated DNA (labeled by TMR (orange stars)).
Figure 8.2. DNA-nanorod thiol conjugation. 40mers DNA oligonucleotides with the same sequence of 5’ HS-TTTTT TTTTT TTTTT TTTTT TTTTT TCGGC CCGTA TAATT 3’, fluorescently labeled at the 3’ ends with either FAM (FAM-DNA-SH) or TMR (TMR-DNA-SH) were conjugated to nanocapsules and nanobones respectively via gold-thiol covalent bond.

Figure 8.3. Gel assay of DNA-nanorod conjugation. (a) white light and (b) UV images of gel electrophoresis. Lane 1: nanocapsules, 2: FAM-DNA-SH + nanocapsules, 3: FAM-DNA-SH, 4: nanobones, 5: TMR-DNA-SH + nanobones, 6: TMR-DNA-SH. Dashed lines indicate positions of wells. Positive direction indicated.
8.2. Selective Controlled Releases

Finally, the mixture of purified FAM-DNA-nanocapsules and TMR-DNA-nanobones was laser irradiated for selective release (Scheme on Figure 8.1). We used the same laser experimental setup as for the selective melting study in Chapter 6. After 800 nm or 1100 nm laser exposure, samples were diluted with their corresponding solvent, 150 µL of 1× TBE. Samples were then centrifuged at 16,100 g for 5 min to remove the NRs and collect the supernatants. Fluorescence spectroscopy was used to quantify the released DNA in supernatants. We quantify the fluorescence intensity of FAM due to FAM-DNA released from nanocapsule and TMR due to TMR-DNA released from nanobones.

After λ800 irradiation, the supernatant fluorescence at 520 nm increased with fluence (Figure 8.4 (a), solid lines), illustrating FAM-DNA release. However, increased fluorescence at 580 nm was much lower, indicating insignificant TMR-DNA release (dashed lines). Therefore, λ800 irradiation could selectively release FAM-DNA from nanocapsules, while leaving TMR-DNA-nanobones undisturbed. At fluences <1.00 mJ/cm², FAM-DNA release was selective, reaching ~70% while TMR-DNA release was ≤ 10%, where 100% was the amount released by MCH treatment (Figure 8.4 (b)). For fluences > 1.00 mJ/cm², release of TMR-DNA from nanobones did increase, while FAM-DNA release was saturated.
Figure 8.4. Selective release - $\lambda_{800}$ irradiation. (a) Fluorescence spectra of supernatant after $\lambda_{800}$ irradiation, FAM-DNA peaks (solid lines) and TMR-DNA peaks (dashed lines). Fluence (mJ/cm$^2$): 0 (black), 0.18 (red), 0.27 (green), 0.35 (blue), 0.70 (cyan), 1.06 (pink), 1.41 (orange), 1.68 (olive). (b) % released of FAM-DNA (green triangles) and TMR-DNA (orange stars) as a function of $\lambda_{800}$ laser fluence.
Figure 8.5. Selective release - $\lambda_{1100}$ irradiation. (a) fluorescence spectra of supernatant after $\lambda_{1100}$ irradiation. FAM-DNA peaks (solid lines) and TMR-DNA peaks (dashed lines). Fluence (mJ/cm$^2$): 0 (black), 0.33 (red), 0.51 (green), 1.02 (blue), 1.39 (cyan), 1.73 (pink). (b) % released of FAM-DNA (green triangles) and TMR-DNA (orange stars) as a function of $\lambda_{1100}$ laser fluence.
When the mixture was exposed to $\lambda_{1100}$ irradiation, supernatant fluorescence at 580 nm increased (Figure 8.5 (a), dashed lines), while intensity at 520 nm was negligible (solid lines), illustrating TMR-DNA release with no significant FAM-DNA release. TMR-DNA release was selective, reaching 50 – 60% while FAM-DNA release was < 10% (Figure 8.5 (b)). Therefore, $\lambda_{1100}$ irradiation could selectively release TMR-DNA from nanobones, while leaving FAM-DNA-nanocapsules undisturbed. Evidently, the NRs undergo a shape transformation at these fluences (Figure 6.10 – 6.12), which probably induces release due to gold-thiol bond dissociation.\textsuperscript{160,161} We observed that re-adsorption of the released DNA back onto the melted gold NRs after a long period of time (3 months) was minimal (Figure C.1, Appendix C). We also observed that the laser irradiation had no effect on the fluorescence of the FAM-DNA and TMR-DNA (Figure C.2, Appendix C).

We confirmed that released DNA was still functional and could hybridize to a complement. Released DNA was incubated with DABCYL-functionalized DNA complements (5’ DABCYL-AATTATACGGGCG 3’), and hybrid formation confirmed by melting curves. Dequenching of FAM at 520 nm (Figure 8.6 (a), green) and TMR at 585 nm (Figure 8.6 (b), orange) was monitored with increasing temperature. Both curves were characteristic of functional hybrids, with $T_m$’s coinciding with that of the original (control) DNA ($T_m = 42^\circ C$) (Figure 8.6 (a), black and Figure 8.6 (b), black). Original (control) DNA is the original source DNA without going through NR conjugation and laser irradiation.
Figure 8.6. Melting Curve of (a) original (control) (black) and released (after $\lambda_{800}$ irradiation of 1.68 mJ/cm$^2$) (green) DNA-FAM and (b) original (control) (black) and released (after $\lambda_{1100}$ irradiation of 1.73 mJ/cm$^2$) (orange) DNA-TMR.

8.3. Summary

First, we have shown that we can effectively load different DNA oligonucleotide onto different type of gold NRs. The DNA-NR conjugation can be confirmed and quantified with gel electrophoresis and fluorescence spectroscopy. We then successfully prove our hypothesis by demonstrating the selective release of two distinct DNA oligonucleotides from two different NRs via selective laser-induced melting of NRs. This selectivity was accomplished by selective irradiation of two different NRs with femtosecond laser pulses at two different wavelengths corresponding to their longitudinal absorption peak ($\text{SPR}_{\text{long}}$). Because laser fluence governs the degree of NR melting, yield, and specificity of DNA release, these controlled releases are externally tunable. Releases were efficient (50-80%) and the released oligonucleotides were still functional. This proof of concept is potentially a powerful method for multiple-drug delivery strategies.
Chapter 9

Conclusions

9.1. Summary

As mentioned in the introduction chapter of this thesis, its main objective is an exploration in formulating a new concept of controlled release of biomolecules by utilizing the NPs. Due to the heat sensitive nature of the biomolecule conjugation with NPs and the fact that both of them are in the same length scale, the main idea behind this exploration is utilizing the heating of the NPs to govern the release of the conjugated biomolecules. There are four specific aims of our exploration. They are to explore methods for delivering energy to the NP from external energy sources in order to heat up the NP, to show that we can selectively heat different types of NPs, to develop bio-nano system, and to use this system for feasibility study of our idea of selective controlled release of multiple different types of biomolecules via selective heating of different types of NPs. For the first specific aim, we investigated two NP-heating mechanisms; the magnetic field heating of magnetic NPs and the photothermal heating of gold NRs. We investigated feasibility of each of these two mechanisms in fulfilling the three remaining specific aims.

We discuss extensively the theoretical prediction and experimental results of our magnetic field heating study in Chapter 2. Independent heating of multiple types of NPs
is theoretically possible by exploiting the size and material dependence of the power loss equation. Preliminary results from the bulk magnetic field heating (SAR) experiments of two different types of magnetic NPs (Fe$_3$O$_4$ and Au/Fe NPs) show that independent heating has feasible potentials. Each type of NPs reaches the optimum heating rate at different frequency range of the applied magnetic field. Therefore it is exploitable for selective heating between the two types of magnetic NPs by choosing the appropriate frequencies.

We have also developed a protocol for synthesizing lipid vesicles that can be loaded with high number of Fe$_3$O$_4$ NPs and dye molecules as drug model (Chapter 3). We accomplished this by systematically study the phase behavior of the lipid-NP system. At certain concentrations of lipid and NPs, it is possible to obtain the lipid vesicle morphology and the maximum loading of the NPs into these vesicles. We showed that by elevating the solution temperature (bulk heating) we can disturb the vesicle morphology (induce lipid phase transition) and thus release the encapsulated dye molecules. Unfortunately, exposure to the magnetic field does not produce the same effect. This is due to the physical limitation of the magnetic field heating experiment; low-power energy delivered to the NP to produce no significant local temperature rise with respect to the ambient surrounding medium (Chapter 4). Therefore magnetic field heating is not feasible for our proposed controlled release application.

The physical limitation of the magnetic field heating of magnetic NPs led us to the next exploration of the second heating mechanism. In agreement with other researchers, we showed that we can heat up and even melt gold NRs via photothermal heating by exposure to the femtosecond laser pulses. Since one of the specific aims in our
objective is to show that we can selectively heat up different type of NPs, we first show our gold NRs synthetic capability in Chapter 5. We were able to synthesize two different types of gold NRs; they are distinct in size, aspect ratio, and morphology. Their absorption profiles had very minimal overlap; therefore we were able to selectively excite them by femtosecond laser pulses at two different wavelengths corresponding to their longitudinal surface plasmon resonance peaks ($\text{SPR}_{\text{long}}$). This selective excitation enabled us to selectively heat up (even melt) the two different types of gold NRs (Chapter 6).

We then showed that we can effectively load different DNA oligonucleotide onto different type of gold NRs (Chapter 7). Ligand exchange of the NRs was required prior to conjugation to avoid aggregation. We developed the ligand exchange protocol to enable customization of the overall charge of the NRs and the thickness of the surface ligand monolayer. The DNA-NR conjugation can be confirmed and quantified with gel electrophoresis and fluorescence spectroscopy. We then successfully proved our hypothesis (the final specific aim) by demonstrating the selective release of two distinct DNA oligonucleotides from two different NRs via selective laser-induced melting of NRs (Chapter 8). This selectivity was accomplished by selective irradiation of two different NRs with femtosecond laser pulses at two different wavelengths corresponding to their $\text{SPR}_{\text{long}}$. Because laser fluence governs the degree of NR melting, yield, and specificity of DNA release, these controlled releases are externally tunable. The release yield was very high; up to $\approx 80\%$ of the load can be released. DNA melting study also showed that the released DNA oligonucleotides were still functional.
9.2. Conclusions

Finally, we have shown that our idea of utilizing the selective heating of different types of NPs for selective multiple controlled released applications is very feasible. We formulated our proof of concept through accomplishment of a sequence of four specific aims. We accomplished all by utilizing the photothermal heating of gold NRs via irradiation with femtosecond laser pulses. Pulsed laser excitation in resonance with their absorption peak can heat and melt the NRs; this is exploitable for controlling the release of biomolecules conjugated to the NRs. NRs with different aspect ratios absorb light at different wavelengths thus can be excited independently. We have successfully demonstrated selective release of two distinct DNA oligonucleotides; each released from different type of NR. This was accomplished by the laser excitation at two different wavelengths corresponding to the NRs’ absorption peaks. The releases were very selective, efficient, and externally tunable by adjusting the laser fluence. The released payloads (DNA oligos) were still functional.

Limitations

Even though the proof of concept of our hypothesis is very promising, we believe that our concept is not immune to certain limitations. The first limitation is the consistency of the NR synthesis. Although NR synthesis recipe has been widely published, the repeatability of these recipes is very sensitive to the quality of the chemicals used for the synthesis.\textsuperscript{119} Thermal degradation may also be an issue if payloads are loaded to close to the NR surface.\textsuperscript{162} This can be overcome by utilizing spacer molecules between the NR surface and the active loads; thus further study on utilizing the
appropriate spacer is a must. In the much bigger picture, the toxicity of NPs in general has to be extensively investigated before any inorganic NPs can be practical for in-vivo biological applications. The femtosecond laser setup itself is also known to be expensive.

**Advantages**

Although our concept can not escape from certain limitations, we still believe that our proof of concept is still fundamentally sound and we hope to inspire other researchers to investigate more or utilize it for different applications. We are hopeful in many ways due to the fact that our concept exploits the very fundamental material property of gold NRs; the independent of absorption profile of NRs with different ARs. With appropriate tuning of the NR synthesis, this concept is expandable to beyond two species. Since conjugation requires only standard thiol conjugation, it is potentially applicable to a wide range of molecules. NRs have relatively large surface area and the capacity to loads hundreds of molecules, and ~80% of the payload can be released. NR surface are also chemically versatile, with customizable coatings, and others have demonstrated active targeting by decorating the NRs with moieties such as antibodies and cell receptor ligands. These advantageous factors clearly indicate that our proof of concept of selective triggered release from gold NRs is potentially a powerful technique for improving controlled release applications such as multiple drug delivery strategies; the very problem that motivated our thesis work.
9.3. Further Studies

Effect of the spacer length

In our study of controlled release of DNA oligonucleotides gold NRs due to heating/melting of the NRs, we used 40mers DNA oligonucleotides (5’ HS-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCGGCCCGTA TAATT 3’, fluorescently labeled by FAM or TMR at the 3’ ends) with the first 26 poly-T sequence acts as a spacer. This spacer is used to ensure that the remaining (essential) part of oligonucleotides (the later 14 sequence) undamaged by the potential thermal degradation due to the heating of the NRs. To check its functionality, we hybridized this essential part with its complement. The complement was functionalized with a 5’ DABCYL (5’ DABCYL- AATTATACGGGCCG 3’) to quench the FAM and TMR in the hybridized state. Melting curves were obtained in a temperature controlled Peltier module of the fluorescence spectrometer, where the increase of fluorescence of either FAM or TMR was monitored as a function of increasing temperature (Figure 8.4 (b) inset and Figure 8.5 (b) inset).

Further study to investigate on whether this spacer can be damaged by the photothermal heating is certainly necessary to better understand the fundamental heating profile around the NRs. One possible experimental investigation would be varying the length of the spacer and then investigate whether the remaining (essential) part of the DNA is still functional. This study will also tell us on how far away from the NR surface the essential payload needed to be loaded to guarantee that when they are released they will be functional.
Surface customization for cell uptake

In order for our proposed controlled release method to be feasible for in-vivo experiments, we must first study the cellular uptake of the NRs. Other researchers have conducted some preliminary works in maximizing the NRs cellular uptake while maintaining the cell viability.\textsuperscript{163-165} We hope that our NR ligand exchange method which enables NR surface customization\textsuperscript{129} will inspire others to utilize our method to further optimize the efficiency of the uptake. In the hope that we will get a better understanding on the fundamental concepts such as which surface customization will work better for certain application.

Nanorod stability in the in-vivo environment

In the potential in-vivo study, before the NRs arriving at the targeted site or even during the incubation will cells, we need to ensure that they will not aggregate in the physiological environments. Systematic study on the NR stability in the physiological environments is a must prior to further in-vivo investigation. Long-term stability may be achieved by sophisticated NR surface customization. Our preliminary study indicates that neutral charged NRs is very stable in protein-rich media (Figure 9.1). Figure 9.1 shows the absorption scan of different charged NRs incubated in 1x TBE (control) and albumin-rich media at room temperature for 1hr and 37°C for 22hr. The disappearance (broadening) of the NRs’ absorption peak is due to their aggregation. The neutral charged NR seems to be stable (not aggregate) for a long period of time (22hr) and even at high temperature of 37°C. Alternatively, we may also need to design microscale carriers for delivering these NRs onto the targeted sites or even improving the cellular uptake.
Figure 9.1. Gold nanorods stability in albumin-containing media. Absorption spectra of (a) positively charged nanorods (NR-CTAB), (b) negatively charged nanorods (NR-MHA), (c) neutral charged nanorods (NR-PEG5000) after 1hr incubation at room temperature, and (d) neutral charged nanorods (NR-PEG5000) after 22 hr incubation at 37°C in 1x TBE (dash line) and albumin-containing media (solid line).

In-vivo selective release

Once we understand some of the fundamental concepts mentioned above, we can then perform the in-vivo study to investigate selectivity of our controlled released method. We can also learn whether our method can improve the efficacy of combination therapy treatments; the very problem that motivated our thesis work. Comparing with other methods\textsuperscript{18,21-23} is also a must for benchmarking the practicality of our method.
Appendix A

TEM Images for Lipid-Nanoparticle System

Below are additional high resolution TEM images; supporting figures for chapter 3.

Figure A.1. High resolution of Figure 3.3. (b) features perforated bicelles/lamellar phase.
Figure A.2. High resolution of Figure 3.3. (c) feature perforated bicelles/lamellar phase with NP-loaded vesicles
Figure A.3. High resolution of Figure 3.3. (d) feature empty and NP-loaded vesicles
Figure A.4. High resolution of Figure 3.3. (e) feature mostly HNLVs
Figure A.5. High resolution of Figure 3.3. (f) feature mostly LNLVs
Figure A.6. High resolution TEM image of “bubble wrap” like lamellar phase.
Figure A.7. High resolution TEM image of Figure 3.5. (a)
Figure A.8. More TEM images of the same purified HNLV solution as in Figure 3.5. (a); also used for size distribution analysis in Figure 3.5. (b).
Figure A.9. More TEM images of the same purified HNLV solution as in Figure 3.5. (a); also used for size distribution analysis in Figure 3.5. (b).
Appendix B

Supporting Figures for Gold Nanorod Ligand Customization

Below are supporting figures for chapter 7.

![TEM image and size analyses of (a) NR-MHDA and (b) NR-MUDA.](image)

Figure B.1. TEM image and size analyses of (a) NR-MHDA and (b) NR-MUDA.
Figure B.2. TEM image and size analyses of (a) NR-PEG$_{356}$, (b) NR-PEG$_{1000}$, and (c) NR-PEG$_{5000}$. 
Figure B.3. FTIR spectra of (a) MHDA (black) and NR-MHDA (red), (b) MUDA (black) and NR-MUDA (red), (c) PEG_{356} (black) and NR-PEG_{356} (red), (d) PEG_{1000} (black) and NR-PEG_{1000} (red), and (e) PEG_{5000} (black) and NR-PEG_{5000} (red),
Appendix C

Control Experiments for Selective Release Study

Below are supporting figures for Chapter 8.

The graph below shows the fluorescence spectroscopy scans of supernatants from the mixture of both types of DNA-gold nanorods after 800nm laser irradiation at 1.41 mJ/cm$^2$, one with the centrifugation (to remove the nanorods) performed immediately after the irradiation (black line) and the other was 3 months after (red line). We observed minimal re-adsorption of the released DNA back onto the melted gold NRs.

![Graph showing fluorescence spectroscopy scans](image)

**Figure C.1.** Re-adsorption of the DNA after laser irradiation.
Figure C.2. (a) below shows the fluorescence scans of FAM-DNA (solid line) and TMR-DNA (dash line) before (black line) and after (blue line) 800 nm laser irradiation at 0.35mJ/cm² for 60 s. Figure C.2. (b) below shows the fluorescence scans of FAM-DNA (solid line) and TMR-DNA (dash line) before (black line) and after (red line) 1100 nm laser irradiation at 1.73mJ/cm² for 60 s. These show that laser irradiation has minimal or no effect on the fluorophores.

Figure C.2. Effect of (a) 800nm and (b) 1100nm laser irradiation on the fluorescence of FAM and TMR.
References


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