Development of photo-responsive liposomes for biomedical applications

Malathi Mathiyazhakan

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NANYANG
TECHNOLOGICAL
UNIVERSITY

**DEVELOPMENT OF PHOTO-RESPONSIVE
LIPOSOMES FOR BIOMEDICAL APPLICATIONS**

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SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING

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DEVELOPMENT OF PHOTO-RESPONSIVE LIPOSOMES FOR BIOMEDICAL APPLICATIONS

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A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2017

NANYANG TECHNOLOGICAL UNIVERSITY
SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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Malathi Mathiyazhakan

March 2017.

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ABSTRACT

This thesis presents the development of photo-responsive liposomes for light controlled drug delivery and therapy. Various clinically successful liposomal formulations have been developed and commercialized over the years, but limited success has been achieved in improving their therapeutic efficacy, due to the lack of proper triggering mechanism to release the encapsulated drugs. Liposomes responsive to external stimuli are gaining distinct attention due to the ease of handling and reliability. Amongst various external stimuli, light is capable of providing unparalleled and precise control on location, time and dosage of the drug released. Additionally laser based therapy are clinically adaptive and cost effective for treating superficial tissues. In this light, we demonstrated the facile syntheses of photo-responsive liposomes by associating spherical gold nanoparticles (AuNPs) or branched gold nanostars (AuNSs) with temperature sensitive liposomes. Firstly, the mechanism of drug release from photo-responsive liposomes using pulsed lasers as the light source was studied. AuNPs were encapsulated within the liposomes by a conventional thin film hydration method and were responsive to visible light. When irradiated at 532nm, the AuNPs absorb the light and convert to heat, generating a cavitation surrounding the AuNPs due to the temperature gradient. The cavitation traverses as pressure waves and this photoacoustic (PA) effect caused by AuNPs disrupts the liposomes to release the encapsulated model drug calcein. Secondly, to generate liposomes responsive in the near infrared (NIR) region, AuNSs were deposited onto the liposomes with PA peak at NIR range (690 nm) and NIR triggered drug release was demonstrated. To shift the responsiveness of the

liposomes to longer wavelengths, NIR responsive liposomes were synthesized by an *in situ* diffusion controlled reduction method. AuNSs were simultaneously formed as the liposomes were synthesized with the optical absorbance peak at 803nm. This method also improved the encapsulation of AuNSs thereby enhancing the responsiveness of the system. The light irradiation was achieved using a pulsed laser diode (PLD) photoacoustic tomography (PAT) system. Light controlled drug release from the NIR-responsive liposomes was then evaluated on B16 F10 mouse melanoma cells, by delivering anticancer drugs such as Paclitaxel and Doxorubicin. The potential of the liposomes as contrast agents for PA imaging also was demonstrated using the PLD-PAT system. Photo-responsive liposomes exhibited improved PA contrast compared to that of blood. Although AuNSs have been characterized and has been used for biomedical applications in the recent past, association of the AuNSs with liposomal systems has not been explored so far. The AuNS-liposomes hybrid systems improves the efficiency of the liposomes in terms of improved drug delivery and can also be used in multimodal imaging applications. The use of light as an external stimuli to initiate drug release offers precise control over the location, time and dosage of the drug released, with maximized specificity and minimal side effects. In addition, the unique spectral properties and size dependant optical tunability of AuNPs offers the advantage of initiating the drug release at desired wavelength. Nevertheless, it is important to investigate and optimize the laser parameters inorder to achieve safe and effective control on the drug release. The light controlled release ability, therapeutic features and imaging capability of photo-responsive liposomes make them promising drug delivery system to skin, eyes and mucous membranes.

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- [5] **Mathiyazhakan, M.,** Upputuri, P. K., Sivasubramanian, K., Dhayani A., Vemula P. K., Pramanik, M., Xu, C.* Liposomes Containing Gold Nanostars for Photoacoustic Imaging and Controlled Drug Release. 6th International Conference on Metals in Genetics, Chemical Biology and Therapeutics, Bangalore, India, February 2016.
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LIST OF ABBREVIATIONS

AMF	Alternating Magnetic Field
ANSI	American National Standards Institute
AuNP	Gold Nanoparticle
AuNR	Gold Nanorod
AuNS	Gold Nanostar
CT	Computed Tomography
DDAB	Dimethyldioctadecylammonium (Bromide Salt)
DDS	Drug Delivery Systems
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DOPE	Dioleoylphosphoethanolamine
DOTAP	1,2-Dioleoyl-3-Trimethylammonium-Propane
DOX	Doxorubicin
DPPC	1,2-Dipalmitoyl-Sn-Glycero-3-Phosphocholine
DPPE	1,2-Dipalmitoyl-Sn-Glycero-3-Phosphoethanolamine
DR	Dehydration- Rehydration
DSC	Differential Scanning Calorimetry
DSPE	1, 2-Distearoyl-Sn-Glycero-3-Phosphoethanolamine
EMF	Electromagnetic Field
FBS	Fetal Bovine Serum
FDA	Food And Drug Administration
FESEM	Field Emission Scanning Electron Microscopy

FT	Freeze – Thaw
GALA	Glutamic Acid-Alanine-Leucine-Alanine
GFP	Green Fluorescent Protein
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HGN	Hollow Gold Nanoshells
HRTEM	High Resolution Transmission Electron Microscopy
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
LCST	Low Critical Solution Temperature
LDPE	Low-Density Polyethylene
MPPC	1-Palmitoyl-2-Hydroxy-Sn-Glycero-3-Phosphocholine
MSEF	Metallic Surface Enhanced Fluorescence
NIR	Near-Infrared
NP	Nanoparticle
OA	Oleic Acid
OCT	Optical Coherence Tomography
OPO	Optical Parametric Oscillator
PAI	Photoacoustic Imaging
PAT	Photoacoustic Tomography
PBS	Phosphate Buffer Saline
PDI	Poly Dispersity Index
PFC	Perfluorocarbon
PLD	Pulsed Laser Diode
pNIPAAm	Poly-N-Isopropylacrylamide
PTX	Paclitaxel

REV	Reverse Phase Evaporation
RGD	Arginylglycylaspartic Acid
SEM	Scanning Electron Microscopy
SERS	Surface Enhanced Raman Scattering
SPR	Surface Plasmon Resonance
TEM	Transmission Electron Microscopy
TFH	Thin Film Hydration
UST	Ultrasound Transducer
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Background and Scope

With the advent of nanotechnology, novel techniques of diagnosis, therapeutics and imaging emerged in the field of biomedicine. Multimodal nanoparticles have become the prime focus of research for diverse applications ranging from NPs based sensing, therapy and diagnosis.¹⁻³ Amongst various applications of nanotechnology in biomedicine, delivery of therapeutics to a specific target site remains the most important in the global market.⁴ The traditional delivery methods such as oral and intravenous injection suffers from limitations such as systemic toxicity, short circulation half-life, poor stability of drugs *in vivo* and insolubility in water.⁵ Meanwhile, the size, shape and high surface to volume ratio of the NPs along with some of the unique properties of the material itself renders them advantageous over conventional drug delivery. An ideal nanocarrier must remain in circulation for longer time, evade rapid renal clearance from the body, protect the therapeutic against non-specific interactions from the plasma proteins, highly stable in the plasma, reduce systemic toxicity and increase the bioavailability of the drug at the target site.⁶ The need to design such nanocarriers arises from the fact that some of the drugs used to treat life-threatening diseases such as cancer are highly potent and results in systemic toxicity in high dosages. Use of nanocarriers to deliver such highly toxic drugs will ensure localized delivery and at the same time severe side effects can be lowered to a great extent. Various nanocarriers such as carbon based materials (Carbon nanotubes, Graphene), hydrogels, microspheres, liposomes, micelles, Quantum dots, nanoshells, and polymeric systems have been used in the recent past, to achieve drug delivery.⁷ Several versatile surface modifications have been made to the nanocarriers to

achieve each or few of these characteristics to facilitate optimal drug delivery at target site. While some of these carriers such as carbon nanotubes and magnetic nanoparticles have entered the clinical trials, most of them are still in their preclinical stages of development and their biocompatibility and patient compliance are under review. However, liposomes have been the most successful carriers clinically, with over 20 liposome based therapeutics either in market or in various phases of clinical trials.⁸

Liposomes being the bio-mimics of the lipid bilayer structure, exhibit excellent biocompatibility. While ensuring facile cellular uptake, liposomes also provides room for both hydrophilic and hydrophobic therapeutics and surface modifications to render stealth or targeting abilities. For instance, surface modifications with the polymer poly-ethylene glycol (PEG) renders stealth properties to the NPs and increases the plasma half-circulation time.⁹ With optimal drug accumulation and reduced toxicity being immediate need, the first generation liposomes were simple consisting of the carrier and the therapeutic. As expected, these liposomes were well-tolerated in humans and the systemic toxicity of highly potent drugs were significantly reduced. The liposomes were delivered passively through enhanced permeation and retention (EPR) effect to the target sites. This exploited the leaky vasculatures and poor lymphatic drainage of solid tumours for drug accumulation overtime, provided they stay in circulation for sufficiently longer time. Eventually, this mode of passive delivery of liposomes was limited by necrotic tumour cores or by absence of EPR at most target sites including certain cancer tissues.¹⁰ Although the first generation liposomes were successful in bioaccumulation and reduced systemic toxicity, these systems were far from

optimal in terms of bioavailability of the drugs and drug efficacy. This is achievable only when the drugs sufficiently accumulated at the target site and is effectively released by some mechanism. Alongside, an efficient monitoring of the drug carriers *in vivo* accounts for detailed understanding of the performance of the drug carriers in physiological conditions. Henceforth, a new class of second generation multimodal liposome based platforms with additional functions of active targeting, responsive release mechanisms and non-invasive imaging emerged to account for the shortcomings of the first generation liposomes.¹¹ While, adding targeting ligands adds up to the complexity of the system, stimuli responsive systems that used external stimuli such as light, magnetic field, ultrasound etc. offers great control over localized drug delivery.¹²

Liposomes with magnetic nanoparticles are controlled with magnetic field as remote trigger. Although this method is attractive for drug delivery to deeper tissues and organs, the use of magneto-liposomes for superficial tissues is cost consuming. Use of echogenic liposomes is often limited by its micron range size and dissolution of gas filled in it. While it is difficult to control the treatment area in a precise manner with most of the stimuli, light as an external trigger, provides great spatial and temporal control. Furthermore, the laser parameters offers great versatility such that the beam diameter, duration, wavelength and intensity of light exposure can be fine-tuned to meet the specific needs. Light controlled drug delivery methods are comparatively simpler, safer, and more economically viable than most other stimuli responsive systems. Such photo-responsive liposomes will find great potentials for topical and transdermal drug delivery and can be used for delivering drugs to superficial tissues like skin, eyes and mucous membranes.¹³

Although liposomes are made photo-sensitive by incorporation various molecules, gold nanoparticles stand out as unique choice due to their size-dependent optical tunability. Great advances have been made in the synthesis of gold nanoparticle that can now be prepared with well controlled morphologies and user-defined optical properties. Incorporation of gold nanoparticles also serve as an intrinsic and adaptive imaging modality, to monitor the spatio-temporal biodistribution of the liposomes, which are essential to understand to functioning of the carrier in physiological conditions. This allows rapid screening of potential nanotherapeutics.¹⁴ Comprehensively, the scope of this work is to synthesize a simple, photo-responsive liposomal platform that facilitates controlled, non-invasive drug delivery at the target site with simultaneous monitoring of the carrier.

1.2 Overarching Thesis Goals

Design of Photo-responsive Liposomes for Light Controlled Drug Release and Photoacoustic Imaging

1.2.1 Demonstrating light controlled release from Au NPs integrated liposomes through microbubble cavitation (Chapter 3)

Photo-responsive nanoparticles (NPs) have received considerable attention because of their potentials in providing spatial, temporal, and dosage control over the drug release. However, most of the relevant technologies are still in the process of development and are unprocurable by the clinics. In addition, reports on light controlled content release from photo-responsive liposomes using pulsed laser and

its underlying mechanism are limited. So in this first project, we demonstrated facile fabrication of these photo-responsive NPs by loading commercially available hydrophilic gold NPs within thermo-responsive liposomes. Calcein was used as a model drug to evaluate the encapsulation efficiency and the release kinetic profile upon heat/light stimulation. Furthermore, we characterized their size, morphology, phase transition temperature and stability. Finally, we demonstrated that this photo-triggered release might be due to the membrane disruption caused by microbubble cavitation.

1.2.2 Incorporation of Au NSs with liposomes for NIR responsive drug release and photoacoustic imaging (Chapter 4)

Liposomes encapsulating spherical Au NPs demonstrated laser controlled drug release through liposomal disruption due to microbubble cavitation generated by the Au NPs. However, use of visible light limits the penetration depth for clinical use. To synthesize liposomes responsive to NIR wavelength, branched or star shaped Au NPs, denoted as gold nanostars (AuNSs) were coated onto the liposomes' surface. The optical absorption and the photoacoustic spectrum of the AuNSs coated liposomes were in the NIR region, within the biological window. We evaluated the delivery of model hydrophobic and hydrophilic drugs and its potential as contrast agents for PAI.

1.2.3 *In-situ* synthesis of AuNSs within liposomes by a simple diffusion controlled reduction method for efficient encapsulation of AuNSs and Improved photo-responsiveness (Chapter 5)

We investigated a novel diffusion controlled reduction method for *in situ* synthesis of Au NSs to develop a one-step protocol for the synthesis of AuNS – liposome complex. This method provided improved encapsulation of AuNSs within liposomes resulting in better responsiveness to light trigger. Taking a step closer to potential clinical adaptability, a photoacoustic tomography (PAT) system that uses pulsed laser diode as light source was chosen for this study, so as to explore the use of the AuNS – liposome complex as both contrast agent as well as drug delivery platform. The PAT-PLD system allows high-speed PAT imaging, and its performance is comparable with the conventional, expensive OPO based PAT system. Being inexpensive and portable PAT-PLD system can be easily used in future translational biomedical applications. Instantaneous complete release (within 10 seconds of laser irradiation) of drug and significant PA contrast against blood was demonstrated.

1.2.4 Exploring NIR triggered release from liposomes for enhanced plasmid DNA transfection (Chapter 6)

Liposomes made of natural phospholipids from plant and animal sources are preferred for oral and topical delivery. Cholesterol is generally added to these liposomal formulations to improve the stability and membrane rigidity, resulting in higher phase transition temperature and slower drug release. To explore if the drug release from these systems, can be controlled using light, AuNSs were incorporated

within the aqueous core of the liposomes along with Calcein or GFP coding DNA plasmids. The liposomes with an optical absorbance at NIR region and a phase transition temperature of ~48 °C, exhibited improved calcein and plasmid release under light stimulation compared to the liposomes without AuNSs. To substantiate the finding, *invitro* studies were carried out by NIH-3T3 mouse fibroblast cells with liposomes containing AuNSs and GFP plasmid. Significantly enhanced gene transfection was achieved under NIR stimulation using a pulsed laser source, validating the role of photoacoustic mechanism of therapeutic release.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Liposomes

Liposomes are unilamellar or multilamellar, spherical nanocarriers comprised of self-assembled phospholipid bilayer, encompassing an aqueous core. Being an amphiphilic nanocarrier, liposomes can deliver both hydrophobic and hydrophilic drugs by capsulizing them in the lipid bilayer and aqueous core respectively. In addition, being artificial phospholipid vesicles, liposomes exhibit high biocompatibility and biodegradability. First reported in 1964 by A.D Bangham¹⁵, liposomes were initially used as mimicking models for biological bilayer membranes, to study physiological conditions such as membrane response to pharmacological molecules and diffusion kinetics of these molecules across the membrane.

Scheme 2-1: Schematic representation of liposomes composed of amphiphilic phospholipids; encapsulating lipophilic and hydrophilic drugs. The outer surface facilitates surface modifications for specific applications. Adapted from ¹⁶

Until recently, liposomes have been used to deliver a variety of molecules namely small hydrophilic molecules, genes (DNA, RNA, siRNA and mi-RNA), hydrophobic molecules, proteins and peptides. The ease of surface modifications that can be done on the liposomes facilitates them to be universal carriers for topical and systemic delivery of therapeutics. Liposomes have also been used as nanoreactors for controlled reactions to study reaction kinetics.

2.2 Liposome Preparation:

Preparation methods and the constituents of the lipid bilayer determine the surface charge, lamellar rigidity, size and shelf life of the liposomes. In addition, these liposomal membrane properties has a pronounced effect on the circulation time, pharmacokinetics, loading efficiency and drug release ability of the liposomes, thereby influencing the overall therapeutic efficacy of liposomes.

2.2.1 Constituents of Liposomes

The main constituent of the liposomes are the phospholipids that can be natural (Egg PC, Soy PC) or synthetic (DPPC, DOPE, DPPE). Glycolipids¹⁷, sphingolipids along with viral components¹⁸⁻²⁰ and membrane proteins²¹ may also be contained in certain preparations of liposomes for specific applications.

Liposomes can be Cationic (positively charged) or Neutral or Anionic (negatively charged) based on the charge of the lipids used for syntheses. Charged liposomes contains Dioleoylphosphoethanolamine (DOPE) as its major phospholipid component, along with cationic lipids such as DOTAP/DDAB or

anionic lipids such as DOPG-Sodium Salt, while neutral liposomes are usually composed of Dipalmitoylphosphatidylcholine (DPPC) along with a lysolipid, MPPC or DSPE. Cholesterol is also used for liposome synthesis to alter the membrane rigidity thereby facilitating drug release.

2.2.2 Methods of Synthesis

Liposomes can be prepared by various methods such as Reverse Phase Evaporation (REV)^{22, 23}, Thin Film Hydration (TFH)^{24,25}, Dehydration- Rehydration (DR)²⁶ and Freeze – Thaw (FT)²⁷ methods.

Scheme 2-2: Representative image of preparation of liposomes by thin film hydration method
Adapted from ²⁸

The most conventional method of preparation is the TFH²⁹, in which the lipid components are dissolved in an organic solvent (usually chloroform). The solvent is then evaporated using a rotary evaporator under reduced pressure to form a thin lipid bilayer which were then reconstituted by the aqueous phase. The

phospholipids self-assemble due to the hydrophilic interaction of the lipid head groups with water, to form spherical liposome vesicles.

Scheme 2-3: Representative image of Lamellarity of liposomes. Adapted from ²⁸

The liposomes can be unilamellar or multilamellar vesicles based on the number of lipid bilayers formed. For optimal drug encapsulation and tissue distribution, the multilamellar vesicles are usually made to form unilamellar vesicles namely small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and giant unilamellar vesicles (GUV) through mechanical methods such as ultrasonication³⁰ and size-extrusion.³¹ The therapeutic agent is usually encapsulated in the aqueous core or in the lipid bilayer of the liposomes based on their hydrophilicity. Post-preparation, the unencapsulated drug is removed by purification techniques such as ultra-centrifugation, ion-exchange chromatography, gel filtration chromatography and ultrafiltration.

2.2.3 Surface Modifications on Liposomes

As-synthesized, conventional liposomes are often functionalized with various molecules on the outer surface of the bilayer for additional benefits. For instance, polymers such as poly ethylene glycol (PEG) renders stealth properties to the liposomes. This increases the circulation time by preventing the adsorption of blood serum proteins, and enhances the probability of the liposomes permeation into tumour tissues.

Scheme 2-4: Schematic representation of different types of liposomes based on their surface modification/composition. Adapted from ³²

Some liposomes modified with polymers such as poly (N-isopropylacrylamide) (pNIPAAm) facilitate controlled drug release, as the polymers can cause conformational changes due to temperature variations.^{33, 34} Biomolecules such as monoclonal antibodies, peptides and targeting ligands on the exterior, act as recognition sequences that targets receptors or enzyme overexpressed by a particular target cell type. Liposomes modified with such biomolecules enhances

tissue uptake and bio-distribution at the target tissues. This technique of delivery is referred to as active targeting. These multifarious liposomes are being exploited for applications including imaging, diagnostics, bio-sensing, controlled drug release and photo-dynamic therapy.

2.3 Liposomes as Drug Delivery Agents in Market/ Clinical Trials

Liposomal compositions containing anticancer drugs have been successfully commercialized and have mainly been used for chemotherapeutic applications for cancer treatment. Commercialized liposomes, Doxil, Caelyx, and Myocet, encapsulating Doxorubicin are available for the treatment of Kaposi's sarcoma, ovarian cancer, and multiple myeloma. Various other formulations containing different anticancer agents such as Tretinoin, Vincristine, Cisplatin and Anamycin have been developed for clinical trials.³⁵ Different liposomal formulations containing the antifungal agent, Amphotericin-B has been commercialized viz AmBisome^{TM}, Abelcet^{TM} and Amphocil^{TM} for the treatment of systemic fungal infections.³⁶

Although the morphology and the composition of these formulations differ, a comparative study of these products indicate similar results in terms of their efficacy, biodistribution, systemic toxicity and cost effectiveness. The study suggests that lipid based therapeutics will soon replace the conventional treatments due to their improved bioactivity. However, these liposomal formulations that available in the market belong to the first-generation nanocarriers, wherein the drug molecule is encapsulated within the liposomes, without any complex modifications.

Although effective in reducing the systemic toxicity, these liposomes encounter few shortcomings. The commercially available Doxorubicin loaded liposomal formulations exhibit reduced cardio-toxicity effects, however did not demonstrate improved antitumor activity when compared to free Doxorubicin treatment. The poor efficacy of the product in terms of anticancer properties is often attributed to the inability of the liposomes to release sufficient drugs at the target site.³⁷ In order to improve the efficacy of the liposome system in drug delivery, it is important to develop systems that can specifically improve the bio-availability of the drugs at the target sites. This requires precise control over the location, time and dosage of the drug released which can be achieved through by liposome targeting or by initiating the drug release post liposome accumulation at the target site. The following section provides a brief overview on how the second generation liposomes will be helpful in improving the efficacy of nanomedicine.

2.4 Methods to Improve the Treatment Efficacy

As discussed earlier, controlled drug delivery systems with active drug targeting and triggered release are sought after to further improve the efficacy of the liposomal drug delivery. The surface of the liposomes contains numerous conjugation sites that can be modified with targeting moieties. This approach enhances the accumulation and cellular internalization of the targeted liposomes at the target site, whereas the accumulation in normal cells is limited.

Scheme 2-5: Schematic representation of liposomes conjugated with targeting ligands entering the cells by active targeting. The ligands facilitates the attachment of the liposomes to the cell surface receptors. Adapted from ³⁸

Certain types of cells such as cancer cells and stem cells overexpress certain enzymes or cell surface receptors compared to that of normal cells that serves as biomarkers to differentiate these cells from the surrounding cells. Liposomes coated with specific ligands targeting such biomarkers, exhibit high affinity to the target cells and therefore enhanced cell internalization. Monoclonal antibodies^{39, 40}, peptide sequences (RGD peptides)⁴¹⁻⁴³, aptamers⁴⁴, glycoproteins transferrin)^{45, 46} or small molecules targeting cell surface receptors (folate)^{47, 48} are conjugated to the liposomes' surface to achieve drug targeting. The targeting ligands on the surface of the liposomes, recognizes specific biomarkers on the target cells. More the amount of receptors/biomarkers expressed, higher is the affinity of the targeted liposomes towards the cells with overexpressed biomarkers. Hence they are easily taken up by the target cells and increases the bioavailability of the drug at target site. (Scheme 2-5). At the same time, healthy cells with lesser biomarkers do not internalize the liposomes resulting in reduced systemic toxicity. Although active targeting seems

promising, clinical advancement in this area has been quite slow and involves considerable challenges and risks. Complexity of synthesis and purification, conjugation process and choice of ligands might alter the liposomes property to some extent. Moreover, most of the ligands themselves are not FDA approved and novel conjugation processes that do not compromise the nanoparticle physiochemical properties need to be thoroughly investigated. These rises questions on scalability and translatability of such targeted liposomes. A novel alternative to this approach was the exploitation of the microenvironment of the target tissue or to manipulate the drug release externally with the use of stimuli responsive liposomes.

Liposomes that are responsive to internal or external stimuli such as pH, temperature, and magnetic fields aim to provide a spatial and temporal control over the drug release, in order to achieve a localized delivery of drugs to the target site, at regular intervals of time. This ensures the drug concentration to be maintained within the therapeutic window thereby improving the efficacy of the drug. Development of a novel stimuli-responsive liposomes initiates this type of controlled drug release from liposomes by means of a stimuli (either external or internal). The properties of stimuli responsive liposomes change to a large extent in response to even a small change in its microenvironment. Delivery vehicles which exhibits such non-linear response are preferred, as the physiological conditions differ at different target sites⁴⁹.

The availability of various types of phospholipids, allows us to choose the appropriate ones based on the application. In general, the phospholipids are dissolved in an organic solvent and once when the solvent is removed a uniform bilayer on the walls of the container. When hydrated with an aqueous solution, the

lipids in the bilayer self-assemble to form spherical liposome vesicles. The drug encapsulated might be embedded in the lipid bilayers or within the aqueous core of the liposomes based on the hydrophilicity of the drug. Usually, the drug molecules within the liposomes are well protected from other proteins and enzymes when in circulation. The bilayer structure is intact unless disrupted by a stimuli. External stimuli such as light, magnetic field, ultrasound and internal stimuli such as temperature and pH are used to control the drug release from the liposomes. Usually the applied stimuli destabilizes the bilayer structure of the liposomes to release the encapsulated drug. The mechanism by which the membrane structure is disrupted depends on the type of stimuli used, which in turn varies based on the intended application. The following sub-sections will briefly discuss about various stimuli responsive liposomes, their composition and factors influencing the drug release.

2.5 Liposomes Responsive to Internal Triggers

After many years being spent to develop targeted delivery and sustained release formulations, lately, studies that combine drugs with an externally applied “trigger” are gaining attention. The liposomes triggered using an internal stimuli, exploits the differences in the physiological conditions to achieve drug release. For instance, Compared to the physiological pH (pH =7), certain organelles like endosomes and lysosomes have lower pH (pH = 5). pH sensitive liposomes are designed by exploiting this property of the cells.⁵⁰ Also, some tissues like the tumour tissues have increased number of receptors or enhanced enzyme production than the normal tissues. As the stimuli responsive liposomes are designed to release

their drug only under conditions different from the physiological conditions, controlled drug release is achievable. However, more than one site can exhibit the conditions desirable for drug release from the liposomes.

2.5.1 pH Sensitive Liposomes

Most of the pH sensitive liposomes are comprised of an unsaturated phospholipid, diacylphosphatidylethanolamine (DOPE), which cannot form stable bilayered liposomes. These phospholipids are associated with different stabilizing moieties which are removed from DOPE under low pH conditions, thus destabilizing the membrane bilayers. This eventually leads to drug release at the target site.

Scheme 2-6: Representation of pH triggered release from pH sensitive liposomes. The liposomes are stable in the external low pH, whereas the change in the pH in the endosomes triggers the release of encapsulated drugs. Adapted from ³⁸

Based on the stabilizing groups, Drummond identified four types of pH sensitive liposomes, based on its composition.⁵¹

1. Liposomes containing diacylphosphatidylethanolamine (DOPE), an unsaturated phospholipid and oleic acid (OA), an acidic amphiphile. OA is protonated at acidic conditions.⁵²
2. Liposomes are made of engineered phospholipid components containing chemical bonds that are hydrolysed at low pH.⁵³
3. Liposomes incorporated with pH-sensitive peptides into the membrane bilayer. The peptides like GALA and influenza hemagglutinin-derived (HA2) are used, which initiate the fusion of liposomes with the endosomal/lysosomal membrane.⁵⁴ However, these peptides increase the content leakage from the liposomes.^{55, 56}
4. Liposomes in association with pH-titratable polymers. These polymers have large structure flexibility and low immunogenicity, with an ease of large-scale synthesis. Membrane destabilization is based on the polycationic or polyanionic properties of the polymers.^{57, 58}

2.5.2 Temperature Sensitive Liposomes

Hyperthermic conditions (39-42°C) are used to achieve drug release from temperature sensitive liposomes. A localized temperature elevation, causes vasodilation resulting in the extravasation of liposomes at the target site and also releases the encapsulated drug simultaneously.⁵⁹

Scheme 2-7: Representation of temperature triggered release from temperature sensitive liposomes. The liposomes, stable in physiological temperature, is stimulated at a higher temperature corresponding to the LCST of the liposomes and releases of encapsulated drugs. Adapted from ³⁸

The property of Lower Critical Solution Temperature (LCST), also known as the Phase Transition Temperature (T_c) is exploited to achieve drug release from the temperature sensitive liposomes. It is the temperature at which the lipids undergo a transition from a solid-like gel state to a liquid phase, leading to formation of pores in the liposomes, through the encapsulated drug will be released. DPPC, a saturated phospholipid, with a T_c of 41°C is the main component of temperature sensitive liposomes. DPPC along with the lysolipid MPPC and DSPE-PEG 2000 (for stealth properties), was synthesized to achieve drug release at $39\text{--}40^\circ\text{C}$.⁶⁰

2.5.3 Enzyme Sensitive Liposomes

Certain enzymes are upregulated at particular target sites like the tumour tissue. For instance, enzymes like secretory phospholipase A2 (sPLA2) that is involved in the phospholipid degradation and matrix metalloproteinases (MMP), are used to initiate liposomal drug release. While the sPLA2 causes drug release by hydrolysis of the liposomal phospholipids,⁶¹ the MMPs act on the lipopeptides within the lipid bilayer to cause membrane destabilization.^{62, 63} Other suggested

enzymes include as elastase,⁶⁴ alkaline phosphatase⁶⁵ and phospholipase C.⁶⁶ However, the efficacy of these enzyme sensitive liposomes is greatly dependant on the surface characteristics of the liposomes.^{67, 68}

2.5.4 Limitations

Although this type of responsive release avoids the challenges and complexity involved in targeted liposomes, internal stimuli responsive liposomes may sometimes lead to non-specific drug release. This is because certain physiological conditions such as tumour acidosis and temperature elevation are not always confined to one particular tissue type at a time. This might result in uncontrolled release of drugs at off-target sites. Relatively, liposomes responsive to external stimuli offer precise spatial control and the parameters controlling the release can be manipulated and optimized for improved release and efficacy.

2.6 Liposomes Responsive to External Triggers

The advent of metal nanoparticles has given new insights to controlled drug release, which led to the unprecedented synthesis of liposomes-metal nanoparticles hybrid systems.⁶⁹ In such hybrid systems, the liposomes is associated with the actuators such as gold, silver, iron oxide, silica nanoparticles etc. that act as absorbers of energy from the external source of triggers. Considering the safety and potential adaptation by pharmaceutical industry, temperature-sensitive liposomes are ideal for such hybrid systems. This approach controls deposition of the liposomes and/or the therapeutic in the targeted region by an external energy

field.⁷⁰⁻⁷² The controlled release of the encapsulated payload by an external trigger overcomes the major problem of unpredictable drug release associated with the internally triggered drug delivery systems.⁷³ Based on the properties of the sensitizing agent or the nanoparticles associated with the liposomes, these externally triggered liposomal systems can be sensitive to light, magnetic field, ultrasound and so on, which is discussed briefly in the following sub-sections.

2.6.1 Magnetic Liposomes

Liposomes contains magnetic nanoparticles and are responsive to a magnetic field. The most commonly used MNPs are magnetite NPs (Fe_3O_4) and maghemite (Fe_2O_3). Other metallic nanoparticles like Co, Ni, Zn, Gd, and Mn are also used in combination with Fe to form magnetic nanoparticles.

Scheme 2-8: Representation of triggered release from magnetic liposomes. The liposomes contains magnetic nanoparticles which under an external magnetic field, destabilizes the liposomes and initiates the release of encapsulated drugs. Adapted from ³⁸

The drug is released due to the heat generated by an external alternating magnetic field (AMF). The MNPs encapsulated within the liposomes, when triggered by an external AMF, causes a local heat generation, thereby creating thermally responsive openings in the temperature sensitive liposomes. The

encapsulated drug molecule escapes through the openings. This type of controlled release is also termed as drug delivery through enhanced permeation.⁷⁴ *In vivo* the magnetic liposomes injected intravascularly are guided to the target lesions by placing a small permanent magnet near the tumor site externally for superficial tumors or through non-invasive surgical procedures for deep-tissue tumors.⁷⁵

2.6.2 Ultrasound Triggered Liposomes

Liposomes sensitive to ultrasound contain air-pockets or nanoemulsions of liquid perfluorocarbon (PFC).⁷⁶ These liposomes are also termed as bubble liposomes or echogenic liposomes.

Scheme 2-9: Representation of triggered release from ultrasound sensitive liposomes. The liposomes contains air bubbles or liquid PFC that can trigger drug release upon ultrasound stimulation. Adapted from ³⁸

The ultrasound based drug delivery technique forms pores on the cell membranes and leads to drug delivery to the cytoplasmic space, thereby surpassing

the endocytic pathway.⁷⁷ Ultrasound based liposomal delivery are found to enhance the transfection efficiencies of gene deliveries.^{78, 79}

2.6.3 Light Sensitive Liposomes

Liposomes are made light-sensitive by adding a photosensitizer to the lipid bilayer or in the aqueous core. Lipid like photo-responsive moieties, photo-responsive polymers, molecular dyes, plasmon resonant and non-resonant gold nanostructures are used as photon absorbers. Drug release from light-sensitive liposomes broadly follows two mechanisms; photochemical and photophysical.

Scheme 2-10: Representation of triggered release from light sensitive liposomes. The liposomes contains photosensitizers that can trigger drug release upon irradiation with light. Adapted from ³⁸

Photochemical methods involves either of the three mechanisms namely photopolymerization, photoisomerization and photocleavage.⁸⁰ The light-responsive moiety (lipid or polymer based) is usually incorporated into the liposomal bilayer membrane and undergoes chemical changes upon light activation. This changes the conformation of the moieties, thus destabilizing the lipid membrane. Light induced conformational changes such as isomerization (photoisomerization), cleavage of amphiphilic moiety into polar and non-polar groups (photocleavage),⁸¹ and formation of polymerizable phospholipid domains (photopolymerization)⁸² of the

lipid or polymer based photo-responsive moieties eventually results in drug release. However, almost all the photo-responsive moieties used for photochemical release of the drugs have their optimal absorption for activation in the UV range of the spectrum. In addition, the use of UV light as a source of illumination results in the formation of reactive oxygen species leading to toxicity to the cells. Thus photochemical method of drug release from liposomes is associated with phototoxicity and limited penetration depth.

Photophysical mechanism of drug release do not cause any chemical changes to the lipid bilayer membrane. Rather, the fluidity of the membrane bilayer is altered by physical or mechanical changes such as thermal or vibrational forces, leading to drug release. Molecular dyes, non-resonant metallic particles and plasmon resonant gold nanoparticles are used for photophysical activation of drug release. The photosensitizers can be encapsulated within the aqueous core, or attached on the lipid surface or embedded within the lipid bilayer of the liposomes. Essentially, the molecular absorbers or metal NPs absorb the light when activated at an optimal wavelength and leads to generation of heat. This thermal effect increases the fluidity of the lipid bilayer membrane resulting in drug release.⁸³ Amongst the metal NPs, gold nanoparticles are usually preferred due to their tunable optical properties and surface Plasmon characteristics, which is discussed in the further sections.⁸⁴ In case of plasmon resonant activation of gold nanoparticles, the heat generated causes significant temperature gradient high enough to produce pressure waves. The generated pressure waves leads to mechanical disruption of the liposomal membrane and drug release. This phenomenon is discussed further in detail in the following sections.

2.7 Gold Nanoparticles

Colloidal gold has been in use for biomedical applications in humans over a century. Ever since, the properties of colloidal gold was studied by Michael Faraday, gold nanoparticles (Au NPs) have found tremendous applications in drug delivery, biosensing, imaging and photo-thermal therapy.⁸⁵ The physical confinement of Au NPs makes them different from the bulk gold, rendering them unique optical properties such as quantum size effect and surface plasmon resonance (SPR). Au NPs are superordinate in optical and biological applications due to the attributes such as strong absorption of light, ease of synthesis and surface modification and biocompatibility.

2.7.1 Optical Properties of Au NPs

The optical properties of the Au NPs are governed by the absorption and scattering properties of the Au NPs. The electrons in a particle get more confined as the particle size drops below the Bohr's radius which increases the band gap energy.⁸⁶ Due to this quantum size effect, the band gap emission shifts from red to blue as the size of the particle gradually decreases. Au NPs can absorb or scatter light whenever irradiate with laser of selective wavelength, depending on their size.⁸⁷ Moreover, optical absorption of Au NPs is quite strong due to a phenomenon known as SPR. The free electrons in the Au NPs oscillate when contacted by light. When the wavelength of the light corresponds to these EMF driven electron SPR is achieved, which enhances the absorption and scattering effects of the Au NPs.⁸⁸ When the light is absorbed, it is dissipated as heat to the microenvironment surrounding the Au NPs or scattered at same or shifted frequency known as

Rayleigh and Raman scattering respectively.⁸⁹ Based on the size of the particles and by exciting the Au NPs at SPR, it is desirable to fine-tune the thermal and optical properties of Au NPs for photothermal and imaging applications respectively.⁹⁰

2.7.2. Heating Au NPs using Laser

The absorption of the laser by the Au NPs is size-dependant. The interaction of photon (of light) with electron-phonon of Au NPs has been extensively studied.⁹¹⁻⁹³ The photons excite the electrons to a higher electronic state and by quickly redistributing the energy, the electrons reach the Fermi distribution state within 500 fs. The electrons at an elevated temperature than that of the lattice phonons, transfer the energy to phonons within few ps and this is known as electron-phonon relaxation. The heated up phonons, dissipate the heat to the microenvironment by phonon-phonon scattering or vibrational relaxation. This process takes place within several hundred ps depending on the size of Au NPs. This elevates the temperature of the Au NPs' microenvironment resulting in a temperature gradient. This gradient is sufficiently high in a colloidal solution causes vaporisation in the Au NPs-liquid interface, leading to the formation of mechanical waves that can travel as sound and heat. The heat generated and the generation of pressure waves can be used in photothermal therapy as well as for controlled release applications.

2.7.3 Monitoring Au NPs *in vivo*

In addition to drug release and therapy, Au NPs containing photo-responsive liposomes facilitates real-time monitoring of the *in vivo* biodistribution of the

carriers, through non-invasive imaging techniques. Various imaging techniques like optical microscopy, surface enhanced Raman spectroscopy, metallic surface enhanced fluorescence and photoacoustic imaging exploits the optical properties of the Au NPs.⁶

Optical microscopy is based on the unique scattering properties of the Au NPs and are effective for *invitro* imaging.⁹⁴ When samples containing Au NPs are illuminated with a white light, based on the scattering efficiency and the morphology of the particles⁹⁴, light frequencies corresponding to SPR wavelength is reflected. The scattered light is captured by a confocal microscope to generate a coloured image on a dark background.⁹⁵

Photoacoustic imaging (PAI) utilizes the combination of light and sound to acquire images. When excited by light, Au NPs undergo non-radiative vibrational relaxation and PAI utilizes the energy dissipated through this relaxation for image construction. The heat generated due to vibrational relaxation causes local heating effect as described in the previous section and a consequent pressure build up, releasing ultrasound waves. The acoustic waves are collected by transducers, amplified and converted to electrical signals which in turn are processed and reconstructed to form an image.^{96, 97} PAI has shown great potentials in *in vivo* imaging of vasculatures, animal brain and subcutaneous tumour models.⁹⁸⁻¹⁰⁰

Raman scattering is yet another phenomenon observed with the vibrational relaxation of the Au NPs.¹⁰¹ When the molecules to be detected are present close to the spatially confined Au NPs, Raman scattering is greatly enhanced. This might be due to the electric field provided by the metal surface or charge transfer in the

molecules.¹⁰² The enhancement in the signal intensity is detected by SERS. Greatly used for lab diagnostics on nanosubstrates,^{103, 104} Gradually, SERS is also finding applications in in vivo imaging of circulating cancer cells in blood and deep tissue and solid tumours.¹⁰⁵⁻¹⁰⁷

Similarly to the enhancement of Raman signal, EMF generated from Au NPs upon laser excitation also enhance the fluorescence yields of the fluorophores. This is known as surface enhanced fluorescence and can be detected by fluorescent microscopy techniques. However this effect is based on the size of the Au NPs and the distance between the Au NPs and the fluorophores.¹⁰⁸ This technique has been used for lab diagnostics and is in early stages of development.¹⁰⁹

Amongst the various imaging options available, photoacoustic imaging (PAI) is a new diagnostic and imaging modality which is advancing rapidly from preclinical phase to clinical phase. This hybrid imaging modality provides rich optical contrast and high ultrasonic resolution at depth inside the tissue. Moreover, the optical absorption of the Au NPs / molecule itself is directly detected rather than depending on the surface enhanced scattering of the Au NPs, making it much simpler technique to use. The basic principle of PAI is illustrated in Scheme 2-11. In PAI, a nanosecond laser beam illuminates the tissue surface. Due to absorption of incident light by the absorbers (tissue chromophores such as hemoglobin, melanin, water etc.), there is a local temperature rise, which in turn produces pressure waves emitted in the form of acoustics waves, also known as PA waves. A wideband ultrasound transducer (UST) receives the PA signals outside the tissue boundary. For PA imaging, PA waves are acquired at various positions around the sample and are used to reconstruct the initial pressure rise within the tissue sample.

Scheme 2-11: Basic principle of photoacoustic imaging: The incident pulsed light is absorbed and converted into acoustic waves by thermal expansion. The acoustic waves are detected by an ultrasound transducer (UST).

Being considerably cost-effective and enabling deeper penetration at NIR wavelengths for the imaging of superficial tissues, we have chosen PAI as our model imaging modality and have tested the Au NPs containing photo-responsive liposomes as contrast agents for PAI for deep tissue imaging and cell tracking.

2.8 Au NPs Containing Photo-responsive Liposomes

Owing to the unique nature of the liposomes that facilitates conjugation or encapsulation of various entities, liposome-AuNPs hybrid systems can be easily developed by various techniques. Colloidal gold containing liposomes were reported as early as 1983 by Straubinger *et al* and Hong *et al* to study the cellular uptake¹¹⁰ and as histochemical markers for tracking the liposomes in cells¹¹¹ respectively. However, not much was done in this area until the 2000s during when the applications of Au NPs containing photo-responsive liposomes in therapy and drug release were extensively studied. Au NPs can be associated with liposomes in three different ways – in the aqueous core, into the lipid bilayer and on the surface

of the liposomes. Different preparation methods and mechanism allows this association of Au NPs with the liposomes. Early works on Au NPs containing liposomes were focused on the studying the effect of Au NPs on the physical parameters of liposomes.^{112, 113} Stable complexes of spherical Au NPs associated liposomes were also formed by simply mixing the as-synthesized Au NPs and the liposomes in varying ratios. In this case, the Au NPs attached to liposomes' surface by physical adsorption.¹¹³ Au NPs containing liposomes were even used as delivery vehicles to deliver the Au NPs to cells for therapeutic applications and this platform was preferred to evade the exocytosis of small NPs.¹¹⁴

The first work on light induced content release from photo-responsive liposomes however, was demonstrated by Paasonen *et al* in 2007. Spherical Au NPs were associated with the liposomes in all three possible methods and UV light was used as the source of trigger. The LCST of liposomes prepared ranged between 40 °C and 48 °C. Liposomes intact at 37°C released calcein 30 minutes post laser irradiation and indicated that the release was due to local heating of the Au NPs microenvironment.¹¹⁵ Similarly, An *et al* demonstrated that the drug encapsulated in thermosensitive AuNPs-liposome can be released by low power UV light irradiation. They also stated that the AuNPs acts as a nano-switch for controlled drug release, meaning the photothermal effect is reversible and the drug release can be ceased by removing the laser irradiation source.¹¹⁶ Unlike previous reports, Wu *et al* focused on encapsulating hollow gold nanoshells (HGN) within the liposomes and used pulsed laser as the source of illumination instead of continuous laser. They reported that the transient cavitation that occurs as a result of temperature gradient that prevails around the microenvironment of HGN is the main mechanism of drug

release.¹¹⁷ Later, Troutman et al and Leung *et al* demonstrated the spectral selectivity of the photo-responsive liposomes by comparing gold coated liposomes resonating at different optical wavelengths. Their study demonstrated that laser illumination ensures 100% release from photo-responsive liposomes with a corresponding resonance, while causing minimal release from liposomes with an un-matching resonance. It was also found that faster drug release was achievable with the use of pulsed laser compared to that of continuous laser.^{118, 119} More recently, other anisotropic structures such as gold nanorods and gold nanostars have also been associated with pH and temperature sensitive liposomes. Light induced drug delivery in *invitro* mammalian cell models has been demonstrated from these liposomes with a synergistic effect of temperature, pH and NIR light activation influencing the drug release.¹²⁰ Simultaneous imaging has also been attempted with Au NPs containing liposomes due to the presence of Au NPs as intrinsic imaging modality using OCT imaging,⁸⁴ and X-ray CT imaging.¹²¹

Table 2-1 below, summarizes the studies on Au NPs containing photo-responsive liposomes specific to light controlled drug delivery.

Photo-responsive Liposomes for Light Controlled Drug Release				
<i>Mechanism of Release</i>	<i>Au NPs based Photo-actuators</i>	<i>Association of AuNPs with liposomes</i>	<i>Activation Wavelength</i>	<i>Ref.</i>
Photothermal local heating effect caused by non-resonant Au NPs	Hydrophobic spherical AuNPs (2.5 nm)	In the lipid bilayer	UV	115
	Hydrophilic spherical AuNPs (2.8 nm)	Within aqueous core	UV	
	Hydrophilic spherical AuNPs (1.4 nm)	Lipid-metal complex on the surface	UV	
Photoacoustic effect caused by plasmon resonant Au NPs	Hollow Gold nanoshells	Within aqueous core, in the lipid bilayer and suspended freely outside	820 nm	117
Photothermal local heating effect caused by non-resonant Au NPs	Hydrophobic spherical AuNPs (5 nm)	In the lipid bilayer	UV (250 nm)	116
Photothermal local heating by plasmon-resonant gold coating	Gold nanoshells	On the liposome surface by lipid vesicle metallization	655 nm / 1064 nm	119
Wavelength selective content release due to photothermal effect caused by plasmon resonant Au NPs	Gold nanoclusters forming a shell like assembly	On the liposome surface by lipid vesicle metallization	760 nm / 1210 nm	118
Photoacoustic effect caused by plasmon resonant Au NPs	Hydrophilic spherical AuNPs (5 nm)	Within aqueous core	532 nm	176
Photothermal local heating by plasmon-resonant gold coating, along with synergistic effect of pH sensitivity of liposomes	Hydrophilic gold nanorods (AuNRs:60 nm long and Dia 25 nm)	Within aqueous core	656 nm	120
	Hydrophilic gold nanostars (AuNSs: 50-60 nm)	Within aqueous core	850 nm	120

Table 2-1: Summary of studies on Au NPs containing photo-responsive liposomes for controlled drug delivery

From the above literature it is evident that limited work has been done in the field of Au NPs containing liposomes in terms of controlled drug release and in vivo studies are still in early stages of research. Although a long way to the clinics, they hold substantial potential as a therapeutic agent, delivery agent, and multimodal imaging contrast agent, all in one platform. Further investigation on use other anisotropic Au NPs to allow near infra-red activation and optimizing laser parameters suitable for clinics will allow for major breakthroughs for transdermal and topical drug delivery. Possibilities of exploring the use of biocompatible anisotropic Au NPs to develop Au NPs containing liposomes, use of translatable laser sources and imaging techniques is the motivation behind this work. Thus, we aim to design a photo-responsive system through facile synthesis and evaluate its potential as controlled drug delivery system along with imaging potentials. Keeping in mind the scalability and cost, simple thin film hydration or diffusion controlled synthesis are employed for the synthesis of liposomes in this work. The synthesis methods are also optimized for higher encapsulation of the Au NPs and the therapeutics for optimal efficiency of the system. A thorough understanding of photoacoustic mechanism involved in the drug release with pulsed lasers as light source has been employed to validate the instantaneous drug release. Finally, the optical properties of the liposomes are tuned to shorter and longer NIR wavelength for precise control and clinical adaptability of the technique.

CHAPTER 3

NON-INVASIVE CONTROLLED RELEASE FROM GOLD NANOPARTICLE INTEGRATED PHOTO- RESPONSIVE LIPOSOMES THROUGH PULSED LASER INDUCED MICROBUBBLE CAVITATION

Mathiyazhakan, M., Yang, Y., Liu, Y., Zhu, C., Liu, Q., Ohl, C.D., Tam, K.C., Gao, Y. and Xu, C., 2015. Non-invasive controlled release from gold nanoparticle integrated photo-responsive liposomes through pulse laser induced microbubble cavitation. *Colloids and Surfaces B: Biointerfaces*, 126, pp.569-574.

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3.1 Introduction

One of the active challenges in the field of drug delivery is to develop drug-carriers that are highly target-specific and biocompatible with minimal toxicity. Therapeutic efficacy of these carriers could be enhanced by their ability to induce controlled drug release in terms of time, location and dosage. However, release of drugs from the conventional nanocarriers such as liposomes and hydrogels, is uncontrolled and the passive drug release is slow.¹²² Recently, nanoparticle (NP) based drug delivery systems (DDS) responding to externally applied “triggers” are gaining considerable attention.^{77, 123} They are expected to deliver drugs in spatial-, temporal- and dosage-controlled fashions with maximized specificity and minimal adverse effects. Furthermore, complex engineering and unpredictable drug release in vivo associated with other targeted DDS are eluded.¹²⁴ Among a wide range of exogenous stimuli-responsive systems (light, magnetic field, ultrasound, microwave radiation), light-triggered platforms are appealing, owing to their non-invasiveness, simplicity and adaptability in the clinics. Extensive research in the past decade has provided a variety of platform technologies, such as near-infrared-light responsive gold (Au) nanocages coated with smart polymers,¹²⁵ polymeric NPs of photo-labile conjugate with drugs,¹²⁶ and self-assembled porphyrane nanovesicles¹²⁷. However, these technologies are still in the preclinical stages of development, and require a clear understanding and optimization of parameters involved in the process of initiating and controlling the drug release.

One of the simplest and easily accessible method for preparing such a system is to integrate Au NPs with thermal-responsive liposomes,^{128, 129} both of which are widely available in market and have been extensively investigated in preclinical and

even clinical trials. Despite the limitation of deep-tissue activation of Au NPs at their plasmonic wavelength, when compared to near-infrared-activated Au nanostructures (e.g. nanorods, nanoshells and nanocages), this system still holds great promise when being used in small animals or topical delivery for humans.¹³⁰ In this system, light absorbed by Au NPs dissipates to the surrounding microenvironment in the form of heat, resulting in an increase in the local temperature. When the ambient temperature proximal to liposomes rises above their phase transition temperature, the liposomes dissociate and release the encapsulated cargo. Besides, vapour bubbles will be generated during the laser heating if the local temperature gradient is high enough, and will cause mechanical disruption of liposomes due to bubble cavitation (Scheme 3-1).

Scheme 3-1: The release mechanism of photo-responsive liposomes: Au NPs (red dots) when being irradiated, generate microbubbles that disrupt the liposome membrane and thus trigger Calcein release. Calcein, self-quenched within liposomes (as shown), fluoresces as being released to the surrounding.

There are some early efforts in combining Au NPs with liposomes for light-triggered release.^{115, 117, 118, 131} While most of them focused on the novelty of materials, accessibility and scalability issues are still to be addressed. Moreover, reports on cellular studies as well as release mechanisms using these nanocarriers are still limited. Herein, we describe the fabrication and characterization of photo-

responsive liposomes, simultaneously loaded with drugs and hydrophilic Au NPs. Calcein is used as a model compound to evaluate the encapsulation efficiency and the release profile of the system. Their physical properties, possible release mechanism and cell viability are carefully studied.

3.2 Material and Methods

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (MPPC) and 1, 2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[carboxy (polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000) were purchased from Avanti Polar Lipids (Alabama, US). All chemicals unless otherwise mentioned were purchased from Sigma-Aldrich. Deionized (DI) water was purified by a Millipore Milli-DI water purification system.

3.2.1 Preparation of Liposomes Containing Calcein and Au NPs

Conventional thin film hydration method¹³² was used to prepare all four types of liposomes including plain liposomes (PL), liposomes encapsulating Au NPs (AuL), liposomes encapsulating calcein (CalL), and liposomes encapsulating both Au NPs and Calcein (AuCalL). Specifically, DPPC, MPPC and DSPE-PEG2000 were dissolved in chloroform in a molar ratio of 86:10:4 or 7.95:0.65:1.39 mg/mL to form the lipid bilayer. The organic phase was evaporated at 37°C under reduced pressure in a rotary evaporator (Buchi, Switzerland). For AuCalL, thin lipid film was hydrated at 45°C with 2 mL solution containing 60 mM calcein solution (pH adjusted to 7.4 using NaOH) and Au NPs (3.36×10^{16}

particles/mL). The control samples, PL and AuL were hydrated without Calcein. The liposomes were then subjected to size extrusion using 200 nm polycarbonate membrane filters (Avanti Polar Lipids). The unencapsulated Au NPs or Calcein were removed by gel filtration chromatography with PD-10 desalting columns (GE Healthcare). The samples were stored at 4°C for further use.

3.2.2. Quantification of Number of Au NPs per Liposome

Liposomes containing varying concentrations of Au NPs (50, 100, 200 $\mu\text{L/mL}$) were prepared. The approximate number of liposomes formed during the synthesis was determined as previously described¹³³ from the known concentration of the phospholipid, DPPC, which is about 10mM, the hydrodynamic diameter of the liposomes and the known headgroup surface area of DPPC. Au NPs were encapsulated within the aqueous core of the liposomes. In order to determine the gold concentration, a known concentration of liposomal solution was digested at 100°C with a mixture of HCl and HNO₃ (in a ratio 3:1). The sample was then diluted to 10 mL and the concentration of Au NPs was quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Aligent 7700) and lipid concentration. Using the values obtained from the ICP-MS results the concentration of the gold was calculated from which the number of Au NPs in 1mL of liposome solution. The number of Au NPs per liposome was determined by the formula L/A , where L is the number of liposomes and A is the number of Au NPs.

3.2.3 Determination of Loading Efficiency

The total lipid concentration by mass was estimated using the phospholipid assay. The standard curve was calibrated at an excitation max of 535 nm and the unknown concentration of the phospholipid in the purified sample was calculated

using the linear equation obtained from the curve. Similarly, a standard curve was calibrated for various concentrations of Calcein by measuring its absorbance intensity using a fluorescence microplate reader. The unknown concentration of Calcein encapsulated within the liposomes was determined using the standard curve. The loading efficiency was determined

$$LE = \frac{\text{Wt of Calcein encapsulated}}{\text{Wt of final total lipids}} \times 100$$

3.2.4. Differential Scanning Calorimetry (DSC)

The phase transition temperature was measured by a Differential Scanning Microcalorimeter (MicroCal VP-DSC). The liposomes samples were heated from 5°C to 95°C and cooled down to 5°C at the rate of 60 °C/hr. The results were average of 6 scans which was consistent after first heating – cooling cycle. Distilled water was used for sample dilution.

3.2.5. Cryo Transmission Electron Microscopy (cryo-TEM)

The sample for the cryo-TEM was prepared on freshly glow discharged Quanti foils (R 2/2, 200 mesh). 4 µL of the liposome sample was placed on the grids and was blotted for excess liquid. The grids were then plunge-frozen in liquid ethane using a Vitrobot (FEI Arctica) at 5°C and 100% RH conditions. The frozen grids were then transferred to a cryoholder and observed in FEI Technai field emission gun TEM at 200kV under low dose conditions and -180°. The images were captured using Gatan Ultrascan Camera.

Quantification of hydrodynamic diameter and zeta potential: The hydrodynamic diameter, polydispersity, and zeta potential of liposomes were collected by Malvern

Zetasizer Nano ZS90, after diluting the liposomes in distilled water. The average of 3 measurements was reported.

3.2.6 Stability of Liposomes Quantified with DLS

The stability of the liposomes encapsulating 1 μM and 30 μM Calcein solution was measured through quantifying the hydrodynamic diameter of liposomes for a period of 21 days. The results were the average of 3 independent scans.

3.2.7 Calcein Release from Liposomes by Thermal Treatment

Calcein release profiles from liposomes with or without Au NPs were studied using a reported method with slight modifications.¹³⁴ Specifically, liposome samples were diluted to 5mM lipid concentration and thermally heated using a circulating hot water bath. The samples were exposed to a temperature range of 25°C to 70°C and an aliquot of the sample was collected at different temperature points (27°C, 32°C, 37°C, 39°C, 41°C, 43°C, 45°C, 52°C, 57°C, 62°C, 67°C and 70°C). The fluorescence intensity of the collected samples was measured using a fluorescence microplate reader with excitation and emission wavelengths at 480 nm and 535 nm respectively. The samples were treated with 2% Triton X-100 to facilitate complete release of Calcein.

3.2.8 Calcein Release from Liposomes by Continuous Laser Irradiation

The liposome samples were exposed to a 514 nm laser for 10 mins at an approximate power density of 12mW/cm². An aliquot of the samples was collected at varying time points and diluted to a final lipid concentration of 5 mM. The

fluorescence intensity of the samples was measured using a fluorescence microplate reader with excitation and emission wavelengths at 480 nm and 535 nm respectively. The samples were treated with 2% Triton X-100 for the complete release of the encapsulated drug, which was considered as the 100 % release. The sample with no laser exposure was used as a control.

3.2.9 Calcein Release from Liposomes by Pulsed Laser Irradiation

The liposome samples were irradiated with pulsed laser of wavelength 532nm at varying pulse numbers (0, 25, 50, 75, 100, and 125). The collected aliquots of samples were diluted to a final lipid concentration of 5 mM and the fluorescence intensity of the samples was measured using a fluorescence microplate reader with excitation and emission wavelengths at 480 nm and 535 nm respectively. The sample with no laser exposure was used as a control. The percentage of the released Calcein was calculated using the formula:

$$\% \text{ Release} = 100 * (F_t - F_i) / (F_x - F_i)$$

F_t is the intensity at a given pulse number; F_i and F_x are the initial and final intensity values respectively. Calcein release triggered by pulsed laser was also conducted at a lower temperature (15°C) than the room temperature (25°C).

3.2.10. Measurement of Pressure Impulses

A hydrophone system was employed measure the pressure impulses inside the samples after the laser irradiation. The hydrophone was from ONDA (HNR-1000) with a 1000 micron diameter and 450 nV/Pa sensitivity. The acoustic signals were recorded using a digital oscilloscope from LECORY (WaveRunner 64Xi-A) with a 600MHz bandwidth and 10 GS/s sample rate. Ideally 100 μ L of the sample was placed on a microscopic slide and the hydrophone was immersed in the droplet

~ 2mm away from the laser beam to avoid the damages from the laser pulse. Then, the samples were irradiated with a pulsed Nd:YAG laser with a pulse duration of 6ns at 532 nm wavelength. The energy density of the laser waves were maintained below the safety threshold levels.

3.2.11 Cell Viability Assay

For the preliminary studies, B16-F10 mouse melanoma cells were treated with increasing concentrations of Au NPs loaded liposomes and plain Liposomes. Untreated cells were used as control. The cells were incubated with the liposomes for 24 hrs, after which the media was replaced with fresh media. The cells were left to recover for another 24 hrs and MTT assay was performed.

3.3 Results and Discussion

3.3.1 Synthesis and Characterization

The light-to-heat conversion efficiency of Au NPs is size-tunable, where a smaller particle provides higher transducing efficiency and hence 5nm AuNPs were chosen for encapsulation.¹³⁵ Both Au NPs (5 nm) and lipids were purchased directly from a commercial source, and the photo-responsive system was synthesized using the simplest lipid film hydration method.^{136, 137} Briefly, a mixture of dipalmitoylglycerophosphocholine (DPPC), palmitoylhydroxyglycerol-phosphocholine (MPPC), and distearoylglycerolphosphoethanolamine-[carboxy (polyethylene glycol)-2000] (DSPE-PEG-2000) was dissolved in chloroform and subsequently evaporated to yield a lipid film in a round-bottom flask. Hydrating medium containing Au NPs and Calcein was added to generate multi-lamellar vesicles,

which were then subjected to size extrusion and gel filtration chromatography to remove unencapsulated Au NPs and Calcein. According to phospholipid assay¹³⁸, there was 10-20% loss of lipids during the whole procedure, and liposomes without Au NPs and with Au NPs contained 24.1% and 8% calcein respectively.

Figure 3-1: Liposomes containing Au NPs and Calcein visualized by cryoTEM at (a) 0°, (b) 20°, (c) 40°, (d) 0° tilt. Scale bar is 50 nm.

Under Cryo-TEM, most of the Au NPs were either associated with the surface of liposomes or encapsulated within liposomes (Figure 3-1a). To identify whether Au NPs were inside the lipid bilayer or associated with the surface, the samples were examined with the tilting angle of 0°, 20°, 40°, and 60° (Figure 3-1a-d). The position of the Au NPs within (yellow arrows in Figure 3-1a) or associated with the surface (red arrows in Figure 3-1a) of the liposomes was almost the same, regardless of the tilting angles. Few free floating Au NPs (black arrows) were also seen outside the liposomes at tilting angles 40° and 60° tilting angles.

Figure 3-2: Characterization of thermal/photo-responsive Liposomes by DLS (a) Hydrodynamic diameter of liposomes (b) Stability of liposomes over a storage period of 3 weeks. (n=3)

The prepared liposomes possessed a size of 118–146 nm with a poly-dispersity index (PDI) of less than 0.2 (Figure 3-2a). The gold containing liposomes possessed a negative zeta potential of -9.6 ± 0.5 mV (Table 3-1) and were stable over a period of 21 days without any significant changes in the hydrodynamic diameter when stored at 4°C (Figure 3-2b).

Composition	Plain Liposomes	5Au- Liposomes	Calcein Liposomes	5Au-Calcein- Liposomes
T_c (°C)	40.8	40.6	41.8	41.5
D_h (nm)	118.5±51.28	116.7 ± 52.34	141.5 ± 51.63	124.8 ± 58.08
PDI	0.136	0.13	0.087	0.147
No. of Liposomes /mL	5.28×10 ¹³	5.45×10 ¹³	3.65×10 ¹³	4.74×10 ¹³

Table 3-1: Tabular representation of physical characteristics of the different liposomal compositions

The number of Au NPs per liposome was theoretically calculated and documented in Table 3-2. The number of Au NPs per liposome increased as the concentration of the Au NPs added to the aqueous core was increased.

Volume of AuNPs (μL) /mL of Liposomes	Hydrodynamic Diameter of Liposomes (nm)	No. of Liposomes/mL	No. of AuNPs/ mL of liposomes	No of AuNPs/ Liposome
50	142.7±68.01	3.58653×10^{13}	6.28387×10^{12}	0.175207
100	160.7±72.81	2.80602×10^{13}	6.37045×10^{13}	2.270282
200	144.7±73.93	3.48471×10^{13}	2.61345×10^{14}	7.499767

Table 3-2: Table representing the number of gold nanoparticles per liposome by varying concentrations of gold nanoparticles

One other critical parameter for thermally sensitive liposomes is its phase transition temperature (T_c), at which the lipid layers change from ordered gel phase to fluid phase. This parameter also reflects the membrane integrity of thermally sensitive liposomes. Ideally, nanoparticles associated with liposomes (i.e. Au NPs) should not interfere with the lipid membrane integrity in order to avoid premature release or chemical degradation.

Figure 3-3: Phase transition temperatures of Liposomes (40.8°C), Liposomes encapsulating Au NPs only (40.6°C), Liposomes encapsulating Calcein only (41.8°C), and Liposomes encapsulating both Calcein and Au NPs (41.5 °C).

Thus the T_c value of Au NPs associated liposomes should not change significantly comparing to plain liposomes. Using a differential scanning microcalorimeter (MicroCal VP-DSC), all samples were heated from 5°C to 95°C and cooled down to 5°C at the rate of 60 °C/hr. The heat capacity versus temperature curve was analysed to determine the T_c . As shown in Figure 3-3 and Table 3-1, T_c of all liposomes was within the mild hyperthermia temperature range of (39-42°C), indicating there was no significant effect on T_c of the liposomes when incorporated with Au NPs. It deserves to note that all liposome samples displayed sharp transition peaks, which confirmed the high purity of the lipid.^{139, 140}

3.3.2 Calcein release from Liposomes by Thermal Treatment

Figure 3-4: Heat triggered release of Calcein from liposomes: Percentage of released Calcein from liposomes with or without Au NPs, which were placed in water bath with various temperatures for 2 minutes.

Calcein self-quenches when being encapsulated at high concentrations within the aqueous core of the liposomes. The fluorescence intensity increases when Calcein is released from the vesicles to the surrounding medium.¹⁴¹ Relying on this property of Calcein, we first studied the response of our system to the temperature change. As indicated in Figure 3-4, regardless of the presence of Au NPs, the liposomes were almost intact at the physiological temperature (i.e. 37°C) with a leakage percentage of less than 10%. However, when temperature was raised to 41°C (slightly above T_c), 60-80% of the encapsulated Calcein was released from liposomes within 2 minutes. No significant difference in the release percentage was observed within the liposomes with and without Au NPs, when the release was triggered using heat. This quick and efficient release is due to the presence of 10%

MPPC, which enhances the permeability of liposome bilayer at the transition temperature.⁶⁰

3.3.3 Calcein Release from Liposomes by Continuous Laser Irradiation

Next, we explored the possibility of releasing Calcein from liposomes upon laser irradiation. The experimental setup of the laser experiments are shown in Scheme 2

Scheme 3-2: Schematic representation of the LASER experimental setup.

For the initial trials, we selected 514 nm continuous-wave (CW) laser (with a power density $\sim 12 \text{ mW/cm}^2$) as the radiation source to achieve triggered drug release (Scheme 3-2). The laser light of selective wavelength (514nm, power density is 12mW/cm^2) was focused onto the liposome solution contained in a cuvette inside a cuvette holder. The laser was turned „off“ after a given time point (0, 5, 10, 20, 30, 60, 120, 300, or 600 seconds) and the samples were collected in an Eppendorf tube and were immediately transferred to an ice water bath to prevent any further release of Calcein. However, the CW laser failed to trigger the Calcein release (Figure 3-5a). It is believed that the power density of the CW is insufficient to raise the local temperature above the transition temperature of liposomes.¹⁴²

Figure 3-5: Laser triggered release of Calcein from liposomes. (a) Fluorescence intensity of calcein released upon exposure to CW laser at (12mW/cm²) (“+” is the positive control, in which liposomes with Au NPs were treated with 2% Triton X to release all encapsulated Calcein and liposomes without Au NPs remained untreated), (b) Percentage of released Calcein from liposomes with Au NPs, which were treated with pulsed lasers (pulse duration = 6 ns; power density ~ 12mW/cm²)

3.3.4 Calcein release from Liposomes by Pulsed Laser Irradiation

One solution to address this problem is to use pulsed laser, which has much higher instantaneous power density in each pulse duration.¹¹⁷ By using Nd:YAG pulse laser with a pulse duration of 6 nano-second and an instantaneous power density approximately equal to 166.67 kW/cm² in each pulse duration, we exposed liposomes with a varying number of pulses in which the pulse frequency was fixed at 1Hz. Note that the average energy density was only 1 mJ/cm², which was much lower when compared to the CW laser we used earlier. As shown in Figure 3-5b, liposomes with Au NPs released the entire drug load within minutes, while less than 40% release was observed in the liposomes without Au NPs (data not shown) under the same conditions. Interestingly, we found that the percentage of Calcein released significantly increased as the time of laser exposure increased.

Figure 3-6: Liposome exposure to 532 nm pulsed laser at 2 different temperatures for a constant time (50s). (Final release indicates the liposomes with Au NPs were treated with 2% Triton X to release all encapsulated Calcein)

Also, no significant difference in the fluorescence intensity was observed when the experiment was conducted at lower temperature (Figure 3-6). This eliminates the possibility of the bulk temperature of the liposomal solution affecting the release from the Au NP loaded liposomes.

3.3.5 Mechanism of Light triggered Drug Release

One possible mechanism for the observed drug release at such a lower power density might be because of the microbubble cavitation close to lipid bilayers.¹⁴² Existing studies have shown that pulsed laser output power as low as 0.75 mW can generate microbubbles on the cell membrane, which break and disrupt the membrane.^{143, 144} Given that Au NPs were observed on the lipid bilayer of the liposomes (Figure 3-1a), the release of Calcein from liposomes might be due to the microbubble formation and cavitation effects. Wu *et al* have detected acoustic signal (~ 0.0005 V) in their near-infrared-activated hollow gold nanoshells system.

To verify if this hypothesis is also valid for Au NPs, we monitored the acoustic signals generated from liposomes during the laser irradiation with a hydrophone. Typical pressure impulse signals were observed for both Au NPs and Au NP-containing liposomes, but not for liposomes without Au NPs (Figure 3-7a). The pressure impulse showed both positive and negative phases (inset in Figure 3-7a). It suggests that microbubbles formed (positive phase) and disrupted (negative phase) in the solution. The maximum and the minimum values of the recorded peak in the Au NP-containing liposomes were 0.00093 and -0.00074 V respectively. Finally, we measured the average maxima and minima of the pressure impulses as a function of increasing laser energy. As expected, the acoustics signal amplitude gradually increased with the increasing laser energy indicating typical pressure fluctuations (Figure 3-7b). This might be due to the increased intensity of the oscillation of the particles attributing to the subsequent thermo-elastic expansion and contraction.^{145, 146} It also deserves noting that Au NPs are always near thermal equilibrium with surrounding medium under CW lasers. The lack of temperature gradients may prevent microbubble formation and disruption of liposomes.^{147, 148}

Figure 3-7: (a) Acoustic signals measured using a hydrophone system for Au NPs (red), liposomes with Au NPs (black) and liposomes without Au NPs (blue). While Au NPs and liposomes with Au NPs showed characteristic pressure wave signals, no signal was observed from liposomes without Au NPs. The inset shows an enlarged view of acoustic signals of photo-responsive liposomes. (b) Maximum and minimum peak value of the pressure impulses as a function of increasing laser power. The acoustics signal amplitude gradually increases with the increasing laser energy.

Figure 3-8: Cytotoxicity of liposomes with and without AuNPs on B16-F10 mouse melanoma cells

Finally, the photo-responsive liposomes were tested for cell-uptake and cytotoxicity. For this, melanoma cells were treated with Au NPs loaded liposomes

for 12 hours. While the plain liposomes were not cytotoxic to the cells, the Au NPs loaded liposomes were slightly cytotoxic when compared to that of the plain liposomes, which should be due to the presence of Au NPs (Figure 3-8).

3.4 Conclusion

A facile preparation method of photo-responsive liposomes by loading hydrophilic Au NPs within the thermally-responsive liposomes was demonstrated. Au NPs either located on the liposome surface or encapsulated inside the aqueous core, act as remote triggers to facilitate light controlled drug release from the liposomes. Hydrophilic fluorescent dye, Calcein, was encapsulated along with Au NPs in the aqueous core of liposomes to characterize the drug release profile. The loading capacity of Calcein was as high as 8% by weight and we achieved the controlled release of Calcein through both thermal treatment and pulsed laser irradiation. Finally, we observed the phenomenon of microbubble cavitation in the Au NP-containing samples, which suggests that Calcein release from Au NP-containing liposomes might be due to liposome membrane disruption caused by microbubble cavitation. The liposomes were found to be biocompatible.

3.5 Acknowledgements

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CHAPTER 4

NEAR INFRARED RESPONSIVE LIPOSOMES FOR CELL TRACKING USING PHOTOACOUSTIC IMAGING AND LIGHT TRIGGERED DRUG RELEASE

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4.1 Introduction

Amongst various nanocarriers, liposomes exhibit stable encapsulation of therapeutics and long circulation half-time¹⁴⁹⁻¹⁵¹ and are most successful clinically.¹⁵²⁻¹⁵⁵ The most conventional composition of photo-responsive liposomes contains dipalmitoylphosphatidyl choline (DPPC) as its main constituent, to enable content at mild hyperthermic temperature.^{60, 129} Attempts to modulate the temperature at physiological conditions have resulted in stimuli responsive liposomes that can be externally triggered by using light as the trigger.¹³ Liposomes associated with gold nanostructures facilitates deep tissue drug delivery due to the optical tunability of the gold nanostructures.^{115, 117, 118} Along with drug release applications, liposome-gold hybrid systems are being actively investigated for their applications in photothermal therapy and multimodal imaging.^{121, 156} PAT is a hybrid imaging modality which can provide rich optical contrast and high ultrasonic resolution. In PAT, short laser pulses are absorbed by the sample generating ultrasonic / PA waves that are collected by an ultrasonic detector. Emerging as a promising tool for imaging biological features from organelle to organs,⁹⁸ PAT applications includes, but not limited to, small animal brain imaging, breast cancer imaging, vascular imaging, tumour angiogenesis, blood oxygenation, total hemoglobin concentration etc.¹⁵⁷⁻¹⁶⁴ To date several exogenous contrast agents based on metal, inorganic, organic NPs and Quantum dots with strong absorption in the NIR region have been used to enhance the contrast for deep tissue PAT imaging.¹⁶⁵⁻¹⁶⁹ In this work, we will demonstrate liposome-AuNSs as a potential exogenous contrast agent for deep-tissue PAT imaging. Here we describe the design and synthesis of temperature sensitive liposome coated with gold nanostars

(AuNSs) with absorption peak in the NIR region. In general, coating the liposomes' surface with gold provides optimal encapsulation of the therapeutics within the liposomes. Moreover, the proximity of the AuNSs to the lipid bilayer is effective to cause the disruption of liposomes and subsequent drug release. In addition, the use of AuNSs coated liposomes with strong NIR absorption as a potential contrast agent in photoacoustic tomography (PAT) is also explored.

4.2 Materials and Methods

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (MPPC) and 1, 2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[carboxy (polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000) were purchased from Avanti Polar Lipids (Alabama, US). HAuCl₄ and Paclitaxel was purchased from ACROS Organics. Fresh mouse blood was collected from animal facility center, Nanyang Technological University. All other chemicals unless otherwise mentioned were purchased from Sigma-Aldrich. Deionized (DI) water was purified by a Millipore Milli-DI water purification system.

4.2.1 Synthesis of NIR Responsive Liposomes

Temperature sensitive liposomes were first prepared by the conventional thin film hydration method as reported previously.¹³² Briefly 7.95, 0.65 and 1.39 mg/mL of DPPC, MPPC and DSPE-PEG2000 respectively were dissolved in chloroform (in a molar ratio of 86:10:4). The organic phase was evaporated at 37°C under reduced

pressure in a rotary evaporator (Buchi, Switzerland) to form the lipid bilayer. For Paclitaxel loaded liposomes (PTX-L), 1mg of PTX was dissolved in the organic phase. For Calcein loaded liposomes (Cal-L), the thin lipid film was hydrated with 1 mL calcein solution (60 mM, 7.4 pH). The plain liposomes (PL) were hydrated with PBS. The liposomes were then subjected to size extrusion using 200 nm polycarbonate membrane filters (Avanti Polar Lipids). The unencapsulated Calcein solution was removed by gel filtration chromatography with PD-10 desalting columns (GE Healthcare). The liposomes were then centrifuged at 12000 rpm at 4°C for 60 minutes to form a concentrated pellet.

To coat the liposomes with AuNSs, liposomal pellet with a total lipid concentration of 2mg was reconstituted in 1 mL of 1M HEPES buffer (pH 7.4). 50 μ L of HAuCl₄ was added to the solution and was left at room temperature in dark under constant shaking at 400 rpm. After 1 hour, the liposomal solutions coated with AuNSs were centrifuged at 12000 rpm 4°C for 30 minutes to remove the free AuNSs and excess HEPES buffer. Plain liposomes coated with AuNSs (AuL), calcein loaded liposomes coated with AuNSs (Cal-AuL), PTX loaded liposomes coated with AuNSs (PTX-AuL) were thus formed by the above procedure. The liposomes were stored at 4°C and were used within 48 hours.

4.2.2 Characterization

The hydrodynamic diameter, polydispersity, and zeta potential of liposomes was measured using dynamic light scattering technique (DLS, ZetaPALS analyzer, BIC). Average of 6 measurements was reported for each sample. The concentration of gold in liposomes was quantified by Inductively Coupled Plasma Spectrometer

(ICP, Prodigy ICP Spectrometer). 100 μ L of liposome was digested overnight in a mixture of HCl and HNO₃ (in a ratio 3:1). The sample was then diluted to 10 mL and was quantified against the gold standards. The morphology of the liposomes and the presence of AuNSs coated on the lipid bilayer surface was determined by transmission electron microscopy (TEM). 10 μ L of sample solution was placed on 200 mesh formvar carbon coated copper grids (FCF-200-Cu, Electron Microscopy Sciences) and was air-dried. After negative staining with 3% uranyl acetate for 1.5 minutes, the grids were washed with DI water. Finally, the grid samples was air-dried overnight and were imaged using FEI Tecnai G2 Spirit Bio-TWIN electron microscope, operated at 120 kV. The morphology of the AuNSs were confirmed using high resolution TEM (HRTEM) imaging. For this, the liposomes were lysed with Triton X 100 and AuNSs were collected after centrifuging the samples at 15,000 g for 15 minutes at 4°C. The AuNSs were then re-dissolved in DI water and 5 μ L of the AuNSs solution was placed on 200 mesh formvar carbon coated copper grids (FCF-200-Cu, Electron Microscopy Sciences). The grids were completely dried before imaging with TEM (FEI G2 spirit, 120 kV, America). Phospholipid assay was performed to determine the total lipid concentration by mass. Loading efficiency was estimated from the on the standard curves constructed for known concentration of phospholipids, calcein and paclitaxel, at an excitation max of 535nm, 480 nm and 230 nm respectively. The loading efficiency was determined by

$$LE = \frac{\text{Wt of Calcein encapsulated}}{\text{Wt of final total lipids}} \times 100$$

4.2.3 Cell Culture

B16-F10 mouse melanoma cells (ATCC) were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM, high glucose 4.5 g/L and 4.0 mM L-Glutamine) supplemented with 10% FBS and penicillin/streptomycin (100 U/ml) at 37°C with 5% CO₂.

4.2.4 Acquisition of Photoacoustic Spectrum

An optical parametric oscillator, OPO (Continuum, Surelite), pumped by a Q-switched 532 nm Nd:YAG laser was used as an excitation source for photoacoustic measurements. It generates 5 ns duration pulses at 10 Hz repetition rate with wavelengths tunable from 660 nm to 2500 nm. The solution containing liposomes coated with AuNSs (AuL) was placed inside a low-density polyethylene (LDPE) tube with an inner diameter (ID) of 0.59 mm and outer diameter (OD) of 0.78 mm. The sample containing tube and the single-element UST (V323-SU/2.25 MHz, 13 mm active area, and 70% nominal bandwidth, Panametrics) were immersed in water for coupling of PA signals to UST. The tube was irradiated with wavelengths ranging from 660 - 900 nm with 10 nm increment. Respective PA signals were collected using the UST and these signals were subsequently amplified, and band pass filtered by an Amplifier/Filter unit (A/F) (Olympus-NDT, 5072PR). Finally, the output from A/F unit was digitized with a data acquisition card (GaGe, compuscope 4227) and stored in the computer. Peak-to-peak PA voltage was then normalized with the laser energy for each wavelength and was plotted against the wavelength to generate the PA spectrum.

4.2.5 Comparison of Photoacoustic Signal from Liposome/Animal Blood

The PA spectrum of liposomes coated with AuNSs (AuL) shows peak at 690nm. So, for further PA experiments, the laser was tuned to 690 nm and the incident laser energy density was maintained at 10mJ/cm². To measure PA signal, the AuNSs coated liposome sample/animal blood was placed inside a LDPE tube (0.59 mm ID, 0.78 mm OD). The sample containing tube was irradiated with 690 nm laser beam. Respective PA signals from liposome and blood samples were collected using the 2.25 MHz UST.

4.2.6 Deep-tissue PAT Imaging Experiment

The LDPE tubes (~1 cm long) filled with fresh mouse blood and AuNSs coated liposomes (2 mg/mL) were embedded in a chicken breast tissue. For PAT imaging, we have used the standard Nd:YAG/OPO based PAT system as reported previously.¹⁷⁰ The tissue surface was irradiated with an expanded 690 nm laser beam having energy density 5 mJ/cm² (< ANSI safety limit 20 mJ/cm²) and the PA signals were collected by continuously rotating the 2.25 MHz UST around the sample in a circular geometry.¹⁷⁰ The scan time was 1 minute. PA signals were collected when the tubes were at a depth of 1 and 2 cm beneath the laser-illuminated tissue surface. The depth-encoded PA singles were then reconstructed using a delay-and-sum algorithm to generate a two-dimensional PA cross-sectional image of the sample under investigation.

4.2.7 Photoacoustic B-mode Imaging of Cells Labelled with AuNSs Coated Liposomes

PA signals were also collected from B16F10 melanoma cells labelled with AuNSs coated liposomes. The cells were labelled with AuNSs coated liposomes with a total lipid concentration of 100 $\mu\text{g/mL}$ for 48 hrs and unlabelled cells were used as control. The labelled and the unlabelled cells were then trypsinized and centrifuged at 400 g for 5 mins to obtain the cell pellet. The experimental set up uses a clinical research ultrasound system (ECUBE 12R, Alpinion, South Korea) with photoacoustic imaging capability. In order to operate the system in the photoacoustic mode, laser excitation needs to be provided as well as a trigger which synchronizes the excitation of the laser and the signal acquisition by the ultrasound transducer.¹⁷¹ With a final concentration of 3×10^6 cells/mL in 1 mL transparent tubes at an imaging depth of 2 cm, the cells were irradiated at 690 nm and a framerate of 5 fps. The generated PA signals were acquired by a linear array transducer (L3-12 transducer), consisting of 128 array elements. Real time PA imaging was done

4.2.8 Drug Release Studies

1 mL Calcein encapsulated AuNSs coated liposome solution (Cal-AuL) was taken in a quartz cuvette and was irradiated for varying time points (0-60 seconds) with the OPO laser with an energy density of 10 mJ/cm^2 . 10 μL liposomal solution was collected after every 5 seconds and was replaced with fresh aliquots. Collected samples were transferred to an Eppendorf tube containing distilled water and were placed onto an ice box to avoid further release. The samples were centrifuged at

15000 rpm to remove AuNSs coated liposomes and the supernatant was transferred to 96 well plates. The percentage of Calcein released was quantified by measuring the fluorescence at an excitation and emission wavelengths of 480 nm and 520 nm using a fluorescence spectrophotometer (H4 Synergy). The experiment was repeated with Calcein encapsulated liposome without the AuNSs (Cal-L). Both Cal-L and Cal-AuL were treated at 37°C and the amount of Calcein released was measured as a control. In the same way, PTX release assay was also performed using PTX encapsulated AuNSs coated liposome solution (PTX-AuL) and PTX encapsulated liposome without the AuNSs (PTX-L). The percentage of PTX released was quantified by measuring the absorbance at 230 nm using a UV spectrophotometer (Spectramax). Both PTX-L and PTX-AuL were treated at 37°C and the amount of PTX released was measured as a control.

4.2.9 Qualitative Analysis of *Invitro* Calcein Release

For *invitro* experiments, B16F10 mouse melanoma cells were incubated with Cal-AuL (with a final concentration of 40 µg total lipids/mL) and were incubated for 6 hours at 37°C with 5% CO₂. The cells were these washed trice with PBS and then irradiated with an OPO laser at 690 nm for 30 seconds per group with an energy density of 10 mJ/cm². . Plain liposomes, free calcein and Cal-L treated groups were used as controls. To verify the Calcein release from the liposomes, the cells were imaged using LX71 inverted fluorescence microscope (Olympus) and Retiga-2000R CCD camera, one hour post laser treatment. It is to be noted that the settings (500 ms exposure time and 3x gain) for the all green fluorescent images, at 20x image magnification was kept constant throughout the imaging. Finally, the images were processed using the ImageJ software to normalize background signal.

The calcein release intensity was quantified using the fluorescence microplate reader with an excitation and emission wavelengths of 480nm and 515 nm respectively.

4.2.10 Cell Cytotoxicity Assay

The B16F10 mouse melanoma cells were incubated with PTX-AuL (with a final concentration of 40 μg total lipids/mL) and were incubated for 6 hours at 37°C with 5% CO₂. The cells were these washed trice with PBS and then irradiated with an OPO laser at 690 nm for 30 seconds per group with an energy density of 10 mJ/cm². Plain liposomes, free PTX and PTX-L treated groups were used as controls. To test the effectiveness of the release of Paclitaxel, cell viability assay was performed using Alamar Blue, 24 hours post laser treatment.

4.3 Results and Discussion

4.3.1 Synthesis and Characterization of AuNSs Coated Liposomes

Temperature sensitive liposomes were prepared using the conventional thin film hydration method and coated with AuNSs by reducing the gold precursor, HAuCl₄ using HEPES buffer. HEPES was found to reduce the Au³⁺ ions in HAuCl₄ to Au⁰ thus forming AuNSs^{172, 173} and this zerovalent state leads to the deposition of AuNSs on the surface of the liposomes.^{174, 175} The presence of AuNSs on the surface of the liposomes imparts characteristic optical properties to the AuNSs coated liposomes making it responsive to light.

Figure 4-1: Characterization profile of AuNSs coated liposomes represented by the (a) UV-Vis absorption spectrum of AuNSs coated liposomes (b) Hydrodynamic diameter of AuNSs coated liposomes and Plain liposomes.

The maximum absorbance of light occurred at 690 nm measured from the UV-Vis spectroscopy (Figure 4-1a). The plain liposomes and AuNSs coated liposomes were characterized for their size, surface potential and morphology. The hydrodynamic diameter measured by the dynamic light scattering method was found to be 141.4 ± 0.5 and 113.2 ± 0.7 nm (Figure 4-1b), while the surface charge was -28.38 ± 1.25 and -22.37 ± 0.23 mV for plain liposomes and AuNSs coated liposomes respectively.

Figure 4-2: Characterization profile of AuNSs coated liposomes represented by the (a) TEM images of AuNSs coated liposomes and (b) AuNSs. Scale bar: 50 nm

The TEM images indicated the presence of AuNSs on the surface of the liposomes and the size of the AuNSs liposomes from the TEM images was found to be 96.77 nm which is comparable to the hydrodynamic diameter measured (Figure 4-2a). The HR-TEM images clearly revealed the branched star shaped morphology of the AuNSs (Figure 4-2b). From the ICP results, the concentration of gold in AuNSs coated liposomes was found to be 160 μM . The loading efficiency of calcein and PTX was found to be 24% and 25% respectively.

4.3.2 Photoacoustic Imaging Studies

The photoacoustic spectrum revealed that the photoacoustic peak occurs at 690 nm, indicating high optical absorbance at this wavelength (Figure 4-3a). In general, the photoacoustic signal is proportional to the optical absorbance and a linear trend in photoacoustic signal intensity was observed for varying concentrations of AuNSs coated liposomes (Figure 4-3b).

Figure 4-3: Photoacoustic measurements with OPO/ Nd:YAG laser (a) PA spectrum of AuNSs coated liposomes (b) Photoacoustic signal intensity vs concentration of AuNSs coated Liposome (c) PA signal collected from blood and AuNSs coated liposomes

Based on this observation, the laser excitation was fixed at 690 nm for the further experiments. At 690 nm, it was found that the photoacoustic contrast of the 2mg/mL AuNSs coated liposomes was 2 times stronger than that from of the animal blood. (Figure 4-3c)

Figure 4-4: Deep tissue Photoacoustic Imaging (a) Photograph of the LDPE tubes filled with 2 mg/mL AuNSs coated liposomes and animal blood, (b-c) Reconstructed PA images of the tubes embedded inside a chicken tissue at (b) 1 cm, (c) 2 cm from the tissue illuminated surface.

Further the potential to use the AuNSs coated liposomes as a PAT contrast agent and their effective imaging depth at a wavelength of 690 nm, was tested. As shown in Figure 4-4a, two LDPE tubes - one filled with fresh mouse blood and the other with AuNSs coated liposomes (2 mg /mL) were sandwiched between chicken breast tissue of varying depths (1 and 2 cms). The SNR values of blood and AuNSs coated liposomes measured were at 1 cm depth was 13 and 25 respectively as shown in Figure 4-4b. Similarly, SNR values of blood and AuNSs coated liposomes measured were at 2 cms depth was 14 and 6 respectively as shown in Figure 4-4c. Our results indicate that AuNSs coated liposomes are promising contrast agents for PAT, providing good PA signal enhancement and image contrast in biological tissues.

Figure 4-5: Tracking of B16-F10 melanoma cells labelled with 2mg/mL AuNS coated liposomes using PAI (a) B-scan PA image unlabelled B16F10 mouse melanoma cells and (b) B-scan image of AuNSs coated liposomes labelled B16F10 mouse melanoma cells.

The B-scan PA image of the tubes containing the unlabelled and the labelled melanoma cells is shown in Figure 4-5. Melanin is an intrinsic photoacoustic contrast agent that has a good contrast against blood in a wide range of wavelength (600-800 nm). The bright red spots on the image show the melanoma cells. It can be seen that in both the labelled and the unlabelled samples, the cells can be imaged. However, the signal to noise ratio (SNR) of the unlabelled cells is 72.44 (Figure 4-5a), while that of the labelled cells is 225.28 (Figure 4-5b). This 3 time better signal intensity if the labelled cells further confirms that AuNSs coated liposomes can be used as a photoacoustic contrast agent for cell labelling and tracking.

4.3.3 Drug Release Studies

Later, Cal-AuL and PTX-AuL solutions were exposed to the NIR pulsed laser for varying time points (0-60 seconds) with an energy density of 10 mJ/cm². As shown in Figure 4-6, the presence of AuNS enabled 72.09 ± 0.88 % and 76.08 ± 3.28 % release of encapsulated Calcein (Figure 4-6a) and PTX (Figure 4-6b) respectively from the corresponding AuNSs coated liposomes within 60 seconds

under the laser exposure while the liposomes with AuNSs had only ~10% release under the same experimental condition.

Figure 4-6: Percentage of Drug Release (a) Release of Calcein from Cal-AuL and Cal-L and (b) Release of PTX from PTX-AuL and PTX-L upon laser irradiation at 690 nm with an energy density of 10 mJ/cm².

4.3.4 Qualitative Analysis of *Invitro* Calcein Release

B16F10 melanoma cells were incubated with free calcein solution (120 μ M), liposomes containing calcein and AuNSs coated liposomes containing calcein overnight. After washing the cells with PBS to remove the particles that were not taken up by the cells, the each of the treated groups were exposed to laser at 690 nm at 10mJ/cm² for 30 seconds. Laser untreated groups were compared against the laser treated groups. It was observed that free calcein stained the cells in both laser treated and untreated groups, no fluorescence was observed in both groups in case of cells incubated with liposomes containing calcein. However, the cells incubated with AuNSs coated liposomes containing calcein, significant increase in fluorescence was observed in laser treated groups while no fluorescence was

observed in groups unexposed to laser (Figure 4-7). When subject to a laser source at 690 nm, the absorbed light generates pressure waves from the microenvironment due to the temperature gradient caused by the thermal energy. These pressure waves' leads to the disruption of liposomes, releasing the encapsulated drug. This indicates that the photoacoustic effect and the local temperature increase in the nanoparticle microenvironment, releases the encapsulated calcein upon laser exposure.^{117, 176}

Figure 4-7: Release of Calcein from AuNSs coated liposomes containing calcein upon laser exposure. Scaling is same for all images and the Scale bar is 100 μ m Blue: DAPI Green: Calcein

It is also to be noted that the rise in temperature of the bulk solution containing the same concentration of AuNSs coated liposomes was found to be $\sim 1^{\circ}\text{C}$ upon laser exposure of about 10 mins at an energy density of 10 mJ/cm^2 . This

further validates that the encapsulated drug is released by the liposome disruption due to the generation of pressure waves. At a concentration of 60 mM, calcein self-quenches within the liposomes and the cells are non-fluorescent. As the laser triggers the AuNSs coated liposomes containing calcein, the released calcein stains the cells and can be detected as its fluorescence recovers.

4.3.5 Cell Cytotoxicity Studies

Figure 4-8: Cell viability of B16F10 melanoma cells treated with PTX Au-Liposomes with and without laser exposure.

To test the biocompatibility of the AuNSs coated liposomes, B16F10 mouse melanoma cells were incubated with AuNSs, free drug paclitaxel, liposomes loaded with paclitaxel, AuNSs coated liposomes loaded with paclitaxel for 24 hrs. The cells were then washed with PBS and the cell viability was assessed using Alamar blue assay against the untreated cells. Similarly, cells treated with laser at 690 nm for 30 sec were tested for viability 24 hours post the laser treatment to see the efficacy of the drug release. It was found that the cells that were not exposed to

laser did not show any significant changes in the viability. While the cells treated with PTX showed significant decrease in cell viability in both control and laser treated groups, about 20% cell death was observed from cells treated with AuNSs coated liposomes without PTX (Figure 4-8) This might be due to the microbubble cavitation that might have some adverse effect on the cell membrane leading to cell death.^{177, 178} However, the highest percentage of cell death was observed from cells treated with AuNSs coated liposomes containing paclitaxel upon laser exposure. Cells treated with liposomes with paclitaxel did not show significant changes in the cell viability ensure stable encapsulation of PTX within liposomes. This shows that the AuNSs causes significant drug release from the liposomes.

4.4 Conclusion

In summary we have described the design and synthesis of temperature sensitive liposome coated with gold nanostars (AuNSs) with absorption peak in the NIR region in order to improve the penetration depth of the light into the skin. The coating of the liposomes' surface with gold also facilitated optimal encapsulation of the therapeutics within the liposomes when compared to encapsulation of AuNPs in the aqueous core. The proximity of the AuNSs to the lipid bilayer was highly effective to cause the disruption of liposomes and subsequent drug release. In addition, it was observed that AuNSs coated liposomes can be used as contrast agents for photoacoustic imaging of superficial tissues and can be used to deliver both hydrophilic and hydrophobic drugs effectively. Thus we present AuNSs coated liposomes as a dual modal therapeutic and contrast agent responsive to NIR light.

Further use of these particles for *invivo* small animal models are under investigation for its potential use in the clinics.

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CHAPTER 5

***IN SITU* SYNTHESIS OF GOLD NANOSTARS WITHIN LIPOSOMES FOR CONTROLLED DRUG RELEASE AND PHOTOACOUSTIC IMAGING**

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5.1 Introduction

The most convenient and straightforward way to synthesize photo-responsive liposomes is to incorporate gold nanoparticles that absorb light and convert the photon energy into thermal energy.^{80, 179} Currently, the integration of gold nanoparticles and liposomes is mainly achieved through hydrating the lipid film with the aqueous solution containing pre-synthesized nanoparticles. For example, liposomes containing gold nanoparticles can be triggered with a 532 nm pulse laser to release the encapsulated fluorescent dyes and gold nanorods with a characteristic surface plasmon band at 792 nm (longitudinal) can be incorporated into lipid layers to provide a theranostic platform for siRNA delivery to the tumour.^{176, 180, 181} Although simple and convenient, it usually suffers from the low encapsulation of nanoparticles.¹⁸² As an attempt to improve the encapsulation of nanoparticles within the liposomes, this chapter reports a protocol for the *in-situ* synthesis of gold nanoparticles within liposome vehicles as an alternative strategy (Scheme 5-1). Specifically, it starts with the formulation of chloroauric acid (HAuCl₄) containing liposomes before reducing the encapsulated HAuCl₄ to gold nanoparticles using a mild reducing agent (i.e. 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)).^{172, 173} Compared with the conventional process that encapsulates the pre-synthesized gold nanoparticles into liposomes during the thin-film hydration step, this facile and convenient method allows the formation and simultaneous encapsulation of AuNSs within liposomes. The absorption spectrum of AuNSs can be tuned between visible and near infra-red (NIR) regions by controlling the size and morphology of AuNSs through varying the concentrations of HAuCl₄ and HEPES. Compared to other methods, the *in situ*

synthesis method provides a controlled environment¹⁸³ to achieve this, as a result of which the optical absorption of the particles can be shifted to longer wavelengths. As a proof-of-concept, gold nanostars (AuNSs) with the maximum absorbance in the near-infrared (NIR) wavelength range (i.e. 803 nm) were synthesized within the temperature-sensitive liposomes.

Scheme 5-1: Schematic of the synthesis of AuNS-liposome complex by diffusion controlled method and its use in light-controlled drug release

Alongside, we also tested this platform as contrast agents for photoacoustic imaging (PAI) using a pulsed laser diode photoacoustic tomography (PLD-PAT) system. While the performance of the PLD-PAT system is comparable to the conventional, bulky and expensive OPO-PAT systems, it is also portable, affordable and allows high-speed imaging. Thus PLD-PAT holds great promise for future translational biomedical applications.¹⁸⁴ The AuNSs containing liposomes showed stronger photoacoustic signals (1.5 fold) in the NIR region than fresh blood. Furthermore, when there were drugs (i.e. Doxorubicin (Dox)) within these liposomes, the irradiation with the NIR pulse laser would disrupt the liposomes and trigger the 100% release of pre-encapsulated Dox within 10 seconds. In contrast, there was negligible contrast enhancement and minor laser-triggered release (10%) for the liposomes without AuNSs. Finally, cancer cells were treated with these Dox-containing AuNS-Liposomes with or without the laser stimulation. The cell

viability assay showed that over 90% cells died only under the NIR laser treatment. Taking together, this article reports a convenient and efficient way to prepare the AuNS-liposome complex for both photoacoustic imaging and controlled drug delivery.

5.2 Materials & Methods

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-sn-glycero-3 phosphocholine (MPPC) and 1, 2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[carboxy (polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000) were purchased from Avanti Polar Lipids Alabama, US). HAuCl₄ was purchased from ACROS Organics. Fresh mouse blood was collected from animal facility canter, Nanyang Technological University. All other chemicals unless otherwise mentioned were purchased from Sigma-Aldrich. Deionized (DI) water was purified by a Millipore Milli-DI water purification system.

5.2.1 Synthesis of Liposomes Encapsulating HAuCl₄ or HAuCl₄/Dox

Liposomes encapsulating HAuCl₄ were synthesized by thin film hydration method.^{136, 137} Briefly, the constituent phospholipids (DPPC, MPPC and DSPE-PEG2000) were mixed and dissolved in chloroform in a molar ratio of 86:10:4 (15.9 mg : 1.3 mg : 2.8 mg). The mixture was evaporated in a rotary evaporator to form a thin lipid layer, which was then re-hydrated by solution containing HAuCl₄ (20 mM) for AuNS-liposome complex. Then the synthesized liposomes were extruded using a hand-held mini-extruder. Liposomes were purified from unencapsulated HAuCl₄ by centrifuging the liposomes at 10,000 g at 4°C for 60 minutes. To

prepare liposomes containing HAuCl₄/Dox, 2 mg of Dox and 1 μ L of triethylamine (TEA) were added to the chloroform along with the lipids as above.¹⁸⁵ The rest procedure was the same as the above.

5.2.2 Diffusion Controlled Reduction of HAuCl₄ in Liposomes with HEPES Buffered Solution

After the removal of unencapsulated HAuCl₄, the liposomes were reconstituted with 5 mL solution containing 1 M HEPES. The mixture was left in dark for one hour to allow the reduction of HAuCl₄ by HEPES.¹⁸⁶ Later liposomes were purified by gel filtration chromatography using PD-10 desalting column (GE Healthcare) to remove the excess HEPES. Purified samples were then stored at 4 °C until further use.

5.2.3 Characterization

Hydrodynamic diameter and zeta potential of the prepared liposomes were measured with dynamic light scattering (DLS, ZetaPALS analyser, BIC). Each sample was measured at least 6 times. The UV-vis absorbance was measured with UV-Visible spectrophotometer (UV-2450, Shimadzu).

For transmission electron microscopy (TEM), 10 μ l of sample solution was placed on 200 mesh formvar carbon coated copper grids (FCF-200-Cu, Electron Microscopy Sciences) and air-dried. The sample was then negatively stained using 3% uranyl acetate for 1.5 minutes followed by washing with DI water. Finally, the grid samples was air-dried overnight before imaging with FEI Tecnai G2 Spirit Bio-TWIN electron microscope, operated at 120 kV.

For high resolution TEM (HRTEM) imaging, the liposomes were lysed with Triton X 100 and AuNSs were collected after centrifuging the samples at 10,000 g

for 15 minutes at 4°C. AuNSs were then re-suspended in DI water and 5 μ L of this suspension was placed on 200 mesh formvar carbon coated copper grids (FCF-200-Cu, Electron Microscopy Sciences). The grids were completely dried in a dry cabinet and were imaged using TEM (FEI G2 spirit, 120 kV, America).

The concentration of AuNSs in the liposomal solution was determined using Inductively Coupled Plasma Spectrometer (ICP, Prodigy ICP Spectrometer). Briefly, 100 μ L AuNS-Liposome complex or Dox-AuNS-Liposome complex solutions after purification were digested using 250 μ L aqua-regia overnight. The digested samples were then diluted to 10 mL using DI water and the gold content was measured by ICP Spectrometer. Gold standard solution was used to construct the standard curve.

5.2.4 Photoacoustic Measurement

The setup for the photoacoustic system is depicted in Scheme 5-2. Pulsed laser diode (QD-Q1910-SA-TEC, Quantel, France) operating at 803 nm was the light source with a pulse width of ~136 ns and a pulse energy of 1.4 mJ at 7 kHz repetition rate. A ground glass was used in front of the laser window to make the laser beam more uniform. The sample was placed inside a water bath. The liposome solution and/or tissue sample was irradiated with the homogeneous laser beam and the photoacoustic signals were collected by the ultrasound transducer. A single element ultrasound transducer (V323-SU/2.25 MHz, Olympus NDT) with 2.25 MHz central frequency, 13 mm active area, and 70% nominal bandwidth was used for all the photoacoustic experiments.

Scheme 5-2: Schematic representation of the setup of photoacoustic imaging system. The sample kept immersed in a water bath is irradiated with a pulsed laser and the photoacoustic signals are collected by an ultrasound transducer (UST).

The photoacoustic signals were subsequently amplified, and band pass was filtered by an Amplifier/Filter unit (A/F) (Olympus-NDT, 5072PR). Finally, the output from A/F unit was digitized with a data acquisition card (GaGe, compuscope 4227) and stored in the computer. For photoacoustic spectrum measurement, an optical parametric oscillator (Continuum, Surelite OPO) system pumped by a 532 nm Nd:YAG laser (Continuum, Surelite Ex) was used. The laser wavelength was varied from 660 to 900 nm to obtain the photoacoustic spectrum.

5.2.5: Laser Triggered Dox Release from Dox-AuNS-Liposome Complex

50 μ L of Dox-AuNS-Liposome complex was placed on a microscopic slide, 2 centimetres away from the laser window. The sample was irradiated with the pulsed laser at 803 nm for varying time points (0-10 seconds) with 5 mm laser spot and the output energy of 1.4 mJ/cm². The solution could be collected after each cycle and was replaced with fresh aliquots. Collected samples were diluted in DI water and were centrifuged at 10,000 g to remove Dox-AuNS-Liposome complex. The supernatant containing released Dox was quantified by measuring the absorbance of Dox at 480 nm using a UV-vis spectrophotometer (Spectramax). The same protocol was carried for Dox-liposome complex without Au NSs.

For *invitro* drug release experiments, B16 F10 melanoma cells (ATCC) were seeded on a 48-well plate overnight before being incubated with DMEM medium containing 10% FBS and the liposome samples. Free Dox and AuNSs acted as controls. After 24 hours of labelling, the liposome-containing medium was replaced with fresh one. The cells were immediately treated with 803 nm pulse laser for 120 s with the energy of 1.4 mJ/cm². Subsequently, the viability of cells was quantified using Alamar Blue and the results were expressed as the percentage of viable cells as against the untreated cells. Alongside, live/dead staining was performed using Calcein Acetomethoxy (CAM) and Propidium Iodide (PI) for qualitative analysis of the effect of laser treatment. Upon exposure to laser, cells were labelled with CAM (2 μ M) and PI (4 μ M) in DMEM without phenol red solution. Following 30 minute incubation, cells were washed and rinsed with PBS before being placed in DMEM without phenol red. The cells were then imaged

using LX71 inverted fluorescence microscope (Olympus) equipped with a Retiga-2000R CCD camera. The images were captured at 20X image magnification with an exposure time of 50 ms and a gain number of 7. Finally, the images were processed to normalize the background colour using ImageJ software.

5.3 Results:

5.3.1 Synthesis and Characterization of AuNS-liposome Complex

HAuCl₄ containing liposomes were prepared by hydrating the lipid film with 20 mM HAuCl₄ aqueous solution (Scheme 5-1). After removing the free HAuCl₄, the liposomes were reconstituted with 1 M HEPES solution for one hour in the dark. Later, the HEPES buffer was replaced with DI water by using gel filtration chromatography.

Figure 5-1. Characterization of AuNS-liposomes: **a)** UV spectrum of plain liposomes (dotted line) and AuNS-liposome complex (solid line). **b)** TEM image of AuNS-liposomes. Scale bar: 50 nm; **c)** HRTEM image of AuNSs collected from AuNS-liposomes. Scale bar: 50 nm

The as-synthesized liposomes had a hydrodynamic diameter of 422.6 ± 0.9 nm and a zeta potential of -35.33 ± 1.53 mV. The concentration of gold nanoparticles was found to be 1.22 mM from the ICP analysis. Their maximum UV-Vis absorption was 803 nm (Figure 5-1a), which falls within the ‘optical window’ in biological tissues.^{187, 188} When the liposomes were examined under TEM, AuNSs were seen on the lipid membrane of liposomes (Figure 5-1b). After being extracted from the liposomes and examined under HRTEM, these AuNSs with branches had the similar irregular shape with the diameter of approximately 44 nm (Figure 5-1c).

5.3.2 AuNS-liposome Complex as Contrast Agents for Photoacoustic Imaging

The photoacoustic spectrum of AuNS-liposome complex was collected as the laser was continuously tuned from 660 nm to 900 nm in a 10 nm increment.

Figure 5-2: a) Photoacoustic spectra of AuNS-Liposome complex; b) Photoacoustic signal intensity vs concentration of AuNS-Liposome complex. The excitation laser pulse was at 803 nm for (b).

As shown in Figure 5-2a, the complex showed the strongest photoacoustic contrast between 750 nm and 870 nm. Note: this laser scanning didn’t change the absorption of AuNS-liposomes. Taking 803 nm as the preferred wavelength, we

further studied the relation between the liposome concentration and photoacoustic signal. As shown in Figure 5-2b, there was a linear relationship between the photoacoustic signal strength and the concentration of liposomes.

The photoacoustic signals were further recorded from both liposome samples and fresh blood (from mice). Blood being an intrinsic absorber of light produces photoacoustic signals and therefore, any potential contrast agent is usually compared against the blood for their potency.^{99, 165, 189}

Figure 5-3: AuNS-liposome complex as contrast agents for photoacoustic imaging **a)** Comparison of photoacoustic signals of 2 mg/mL AuNS-Liposome complex and fresh mouse blood; **b)** Comparison of photoacoustic signals of 2 mg/mL AuNS-Liposome complex and fresh mouse blood under 1 cm fresh chicken breast tissue. The excitation laser pulse was at 803 nm.

Figure 5-3a shows the photoacoustic signal comparison between the liposomes and blood. The peak-to-peak photoacoustic signal from AuNS-liposome complex was 675.9 mV while that of blood was 456.8 mV. AuNS-liposome complex showed almost 1.5 fold increase in the photoacoustic signals compared to that of blood.

More excitingly, this signal difference was not significantly influenced when they were placed within 1-cm deep inside chicken breast tissue. As shown in Figure 5-3b, AuNS-liposome complex showed a peak-to-peak photoacoustic signal of 108.6 mV when being placed under 1 cm fresh chicken breast tissue while blood produced 66 mV. In this case, peak-to-peak photoacoustic signal intensity of AuNS-liposome complex was almost 1.6 times more than that of blood. Similarly, at a depth of 2 cm, AuNS-liposome complex and animal blood recorded a peak-to-peak photoacoustic signal of 30.64 mV and 17.69 mV respectively (data not shown). In this case, peak-to-peak photoacoustic signal intensity of AuNS-liposome complex was almost 1.7 times more than that of blood. It is evident that the AuNS-liposome complex can produce significantly higher photoacoustic signals in comparison to blood under all conditions suggesting that it can be used as a contrast agent for the photoacoustic imaging applications.

5.3.3 Controlled Drug Release from AuNS-liposome Complex

To explore the utilization of AuNS-liposome complex for drug delivery, Dox was encapsulated within the lipid bilayer of the AuNS-liposome complex to form Dox-AuNS-liposome complex. In order to facilitate the encapsulation of Dox in lipid bilayer, TEA was used to deprotonate Dox-HCl to the hydrophobic Dox.^{185,}
¹⁹⁰ For Dox-liposome complex, DI water instead of HAuCl₄ was used during the synthesis.

The concentration of Dox within liposomes was ~10% by weight, which was calculated by digesting the Dox-loaded liposomes using Triton X-100 and measuring its absorbance at 480 nm. Formation of AuNSs in the Dox-AuNS-liposome was firstly confirmed through the change in the maximal peak absorbance

in the UV-vis spectrum (Figure 5-5a). Different from Dox-liposomes that showed the highest absorbance at 480 nm, the maximal absorbance of Dox-AuNS-liposomes showed up at 650 nm. Clearly this NIR absorption was due to the appearance of AuNSs.

Figure 5-4. Characterization of Dox-AuNS-Liposomes: **a)** TEM image of AuNSs extracted from Dox-AuNS-Liposomes. Scale bar: 100 nm; **b)** Hydrodynamic diameter of Dox-AuNS-liposomes; **c)** Photoacoustic spectra of AuNS-Liposomes and Dox-AuNS-Liposomes; **d)** UV-vis spectra of AuNS-Liposomes and Dox-AuNS-Liposomes.

The formation of AuNSs were further confirmed by lysing the liposomes and examining the extracted AuNSs through TEM (Figure 5-4a). Dynamic light

scattering study also revealed that the presence of AuNSs didn't significantly change the size of liposomes (129.3 ± 1.6 nm vs. 115.9 ± 2.5 nm, Figure 5-4b). We further examined the photoacoustic spectra of Dox-AuNS-liposomes that presented their strongest signal between 660 nm and 750 nm (Figure 5-4d).

Later, Dox-AuNS-liposome solution was exposed to the NIR pulsed laser for varying time points (0-10 seconds) with an energy density of 1.4 mJ/cm^2 . As shown in Figure 5-5b, the presence of AuNS enabled 100% release of encapsulated Dox from the liposomes within only 10 seconds under the laser exposure while the Dox-liposomes had only ~10% release under the same experimental condition.

Figure 5-5. AuNS-liposome complex as the drug carrier for controlled release: **a)** UV-vis spectra of Dox-liposomes and Dox-AuNS-liposome complex; **b)** Release of Dox from Dox-liposomes and Dox-AuNS-liposome complex under the irradiation of NIR pulse laser.

5.3.4 Cell Cytotoxicity Assay with Dox-AuNS-liposomes

To examine the potential therapeutic effectiveness of Dox-AuNS-Liposome complex, melanoma cells were incubated with AuNS-liposomes, Dox-liposomes, or Dox-AuNS-Liposomes with or without the treatment of pulsed laser. Later the cell viability was quantified using Alamar Blue assay and was normalized against the viability of the untreated cells. Dox was maintained at $\sim 30 \mu\text{g/mL}$ for the Dox-

containing groups and Au concentration was kept at 70 μM for AuNSs containing samples.

Figure 5-6. Viability of B16-F10 melanoma cells after the treatment of Dox, AuNS-Liposomes, Dox-Liposomes, and Dox-AuNS-Liposomes with or without pulsed laser by Alamar Blue assay

As shown in Figure 5-6, there was negligent cell death for all samples without the treatment of pulsed laser, except the Dox treated groups. However, if the pulsed laser was applied, AuNS-Liposome complex and Dox-AuNS-Liposome complex resulted in approximately $82.42 \pm 5.23\%$ and $93.75 \pm 5.37\%$ of cell death.

Besides Alamar blue assay, live/dead assay was also performed with CAM and PI (Figure 5-7), in which the results echoed the Alamar Blue viability assay.

Figure 5-7. Viability of B16-F10 melanoma cells after the treatment of Dox, AuNS-Liposomes, Dox-Liposomes, and Dox-AuNS-Liposomes with or without pulsed laser Live/dead staining assay with Calcein Acetomethoxy and Propidium Iodide. Scale bar: 100 μm .

5.4 Discussion

HEPES is a moderate reducing agent for the synthesis of gold nanoparticles with different morphologies.^{172, 173} The piperazine ring in the HEPES is responsible for the reduction of HAuCl_4 from Au^{3+} to Au^0 . In this study, we explored the use of HEPES to reduce HAuCl_4 that was pre-encapsulated within liposomes. The key is whether HEPES could diffuse through the lipid bilayer of liposomes and whether the HAuCl_4 concentration encapsulated in liposomes was sufficient for the formation of AuNSs. The concentrations of HAuCl_4 (20 mM) and HEPES (1M) during the synthesis were selected based on the early work by Xie and Maiorano.^{172, 191} Although the diffusion process could not be directly observed, the successful formation of AuNSs on the lipid membrane of liposomes suggested both conditions were met (Figure 5-1b & 5-1c).

Surface-plasmon resonance active nanoparticles such as Au nanoparticles are able to generate photoacoustic signals when being irradiated with a pulsed laser and thus can provide contrast in photoacoustic imaging.¹⁹² The incident pulsed laser

is absorbed by Au nanoparticles and converted to heat. Then the thermal energy causes a thermoelastic expansion resulting in pressure wave. The pressure wave is recorded as photoacoustic signal by an ultrasound transducer. We first examined the photoacoustic spectrum of AuNS-liposomes (Figure 5-2a). Between 660 nm to 900 nm, the complex showed the strongest photoacoustic contrast between 750 nm and 870 nm. Figure 5-1a showed that AuNS-liposomes had a maximal absorbance at 803 nm in UV-vis spectrum. Therefore, pulsed diode laser at 803 nm was chosen as the laser source for further experiments.

As expected, the photoacoustic signal intensity has a linear relation with the Au concentration (Figure 5-2b). Moreover, the peak-to-peak photoacoustic signal amplitude was higher than that of blood even under 1-cm of chicken breast tissue. This confirms that for *in vivo* photoacoustic imaging applications AuNS-liposome complex can be used as a potential photoacoustic contrast agent.

Recently, we demonstrated that pulsed laser could disrupt liposomes containing Au nanoparticles through microbubble cavitation, which resulted in the release of encapsulated drugs.¹⁷⁶ We believe that a similar phenomenon would be observed with the AuNS-Liposome complex. As a proof-of-concept, Dox was incorporated within the lipid bilayer of the liposomes. Although the formation of AuNSs in the Dox-AuNS-Liposome complex was evident from the UV-vis absorption spectra (Figure 5-3a) and the TEM examination (Figure 5-4a), there was a blue-shift of the absorption spectrum of the Dox-AuNS-Liposomes (Figure 5-4c), when compared to that of AuNS-Liposomes. This could be attributed to the morphological change of the AuNSs and further studies are required to understand this phenomenon with more details. In addition, the characteristic peak of Dox at

480 nm was not observed in the Dox-AuNS-Liposomes (Figure 5-3a). This effect may be due to the negligible or very weak absorption of the Dox or dominant effect of the peak of the AuNSs present in the complex.¹⁹³

The synthesized Dox-AuNS-Liposomes were then irradiated with the pulsed laser at 803 nm at the wavelength where Dox-AuNS-Liposomes showed good absorption (Figure 5-3b). Complete release of Dox was achieved within 10 seconds from Dox-AuNS-Liposome complex. However, there were only ~10% Dox released from the Dox-Liposomes under the same condition. This clearly indicates that the Dox release from Dox-AuNS-Liposome complex was due to the laser stimulation that might execute through heat or microbubble formation.

Finally, the therapeutic effect of Dox-AuNS-Liposome complex was explored with melanoma cells (Figure 5-6 and 5-7). Compared with AuNS-Liposomes, Dox-Liposomes, and Dox-AuNS-Liposomes, Dox showed consistent cytotoxicity under both conditions. In contrast, Dox-liposomes did not have much effect on the cell viability. This is because the liposomes had a phase transition temperature at 40.8 °C¹⁷⁶ and it has been reported that the cytotoxicity of the drug encapsulated in temperature-sensitive liposomes was highly reduced when maintained at 37 °C.^{194, 195} AuNS-Liposomes were non-toxic without laser treatment. However, the exposure to NIR laser dramatically changed their cytotoxicity, which should be due to the cavitation induced by the microbubble formation due to the transient heating of AuNS-Liposomes.¹⁹⁶⁻¹⁹⁸ As a combination of Dox-liposomes and AuNS-Liposomes, Dox-AuNS-Liposome complex showed similar non-toxic effects without laser treatment. However, with the treatment of

laser, it also showed the highest cytotoxicity with due to the synergetic effect of local heating, microbubble cavitation and Dox.

5.5 Conclusion:

In summary, this report introduces the synthesis of AuNSs containing liposomes by the *in situ* reduction of the gold precursor HAuCl_4 through HEPES diffusion and reduction. It allows the formation and simultaneous encapsulation of AuNSs within liposomes. Due to the presence of AuNSs, these liposomes can produce strong photoacoustic signals in the NIR region even at 1 cm deep inside biological tissue. Furthermore, when there are drugs (i.e. Dox) within these liposomes, the irradiation with the NIR pulse laser would disrupt the liposomes and trigger the release of these pre-encapsulated drugs to perform the local treatment. We believe this platform might have the potential as a dual-modal theranostic agents since it can be used for both imaging as well as therapy with controlled drug delivery and targeting. In the future studies, we will be testing the capability further with an *in vivo* small animal model.

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CHAPTER 6

NEAR-INFRARED LIGHT-SENSITIVE LIPOSOMES FOR ENHANCED PLASMID DNA TRANSFECTION

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6.1 Introduction

In our previous works, the temperature sensitive liposomes were primarily constituted of a synthetic phospholipid, DPPC, with a phase transition temperature in the mild hyperthermic range of 41 °C. Liposomes made from synthetic phospholipids are mostly used as a drug carrier for intravenous administration. In most other drug delivery routes such as oral, pulmonary and dermal, usage of natural phospholipids from plant and animal sources like soybeans, egg yolk, bovine heart and spinal column to synthesize liposomes is generally preferred.¹⁹⁹ However, the membrane rigidity of such liposomes are often compromised due to the permeability of the phospholipids used, resulting in stability and drug leakage issues. Hence to improve the membrane rigidity, cholesterol (Cho) is incorporated in such liposomes compositions. Addition of Cho increases the T_c of the membrane and decreases the permeability of the bilayer. This enhances the stability of the liposomes and minimizes drug leakage.²⁰⁰ Release of drugs from such liposomes with hyperthermic T_c requires modulating the physiological temperature above 45 °C, which may result in considerable thermal damage to tissues. Since AuNSs based light triggered release is mainly based on photoacoustic effect, light triggered drug release will be a great platform to control the delivery of therapeutics from such liposomes.

In this light, we incorporated AuNSs with liposomes composed of Phospholipon 90G (P90G), cholesterol (cho) and skin-penetrating-and-cell-entering (SPACE) peptides. P90G is a natural phospholipid lecithin which is produced from soybean. Due to its natural presence in the human skin, it is highly

biocompatibility.²⁰¹ SPACE peptides enhances the potential transdermal penetration and cellular internalization.^{202, 203} Calcein molecules or GFP-encoding DNA plasmids were used as model therapeutics which were encapsulated in the core of the liposomes to demonstrate light triggered therapeutic release. Release of Calcein or the GFP plasmid was induced by microbubble cavitation and subsequent liposomal disruption due to the presence of AuNSs. The elevated cellular fluorescence due to the release of Calcein or GFP plasmid following NIR treatment, served as an indicator of successful controlled delivery & gene transfection.

6.2 Materials and Methods

All materials except specifically mentioned were obtained from Sigma-Aldrich (Singapore). P90G (composed of Phosphatidylcholine 94.4%, lysophosphatidylcholine 2.5%, non-polar lipids 1.5%, water 0.4%, tocopherol 0.2%, and ethanol 0.1%) was a gift from Lipoid (Lipoid, Switzerland). Cholesterol was purchased from Avanti (Avanti polar lipids Inc., USA). SPACE peptide (ACTGSTQHQ-CG, Disulfide Bridge 2–10) was purchased from RS Synthesis (USA). POPE-NHS was obtained from NOF (USA). pEAK12-GFP plasmid as described previously was amplified by transforming into DH5 α -competent cells and purified by gel electrophoresis.^{204, 205} PD10 column was obtained from GE Healthcare (Singapore). SnakeSkin™ Dialysis Tubing and BSA protein assay kit were supplied by ThermoFisher Scientific (Singapore). Hoechst 33342 was purchased from Life Technologies (Singapore).

6.2.1 Synthesis of Liposomes

Liposomes were synthesized according to the previously published protocol.²⁰³ Briefly, P90G and Cho were dissolved in 2 ml ethanol at different mole ratios (7:0, 7:2, 7:7 and 7:14). For SPACE peptide conjugation, POPE-NHS (5 mg/ml in ethanol) and SPACE peptide (5 mg/ml in phosphate-buffered saline/PBS, pH 8) were added into the mixture following a 2 hrs pre-incubation at room temperature. Ethanol was evaporated using a rotary evaporator to form a thin film in a round-bottom flask. The film was then hydrated with 1 ml calcein solution (200 μ M) or DNA plasmid solution (150 ng/ml) pre-mixed with 50 μ l CaCl_2 (50 mM) with magnetic stirring. pH of the solution was adjusted to 7.4 with 1 M NaOH solution. Un-encapsulated dyes or plasmid were removed through PD10 column.

6.2.2 Synthesis of AuNSs and AuNS-liposomes

AuNSs were synthesized by adding 50 μ l gold precursor (HAuCl_4 , 20 mM) to 1 ml HEPES buffer (1 M, pH 7.4) under constant shaking (600 rpm) at room temperature.¹⁷⁶ One hour later, AuNSs were purified through centrifugation (15k rpm for 30 min, 4°C) and rinsed with deionized water. The process was repeated thrice. AuNS-liposomes were synthesized through hydrating the lipid film with a mixture of AuNSs and calcein or DNA plasmid solution. The subsequent steps were similar to the synthesis of liposomes.

6.2.3 Characterization of AuNS-liposomes

The encapsulation efficiency of calcein or DNA plasmid was evaluated by disrupting the liposomes with 10% Triton X-100 solution and quantifying the concentration of encapsulated molecules. Calcein was quantified by measuring the fluorescence signal of the disrupted liposome solution at an excitation and emission

wavelength of 480 and 520 nm respectively and the signal intensity was plotted against a calcein fluorescence standard curve. DNA was quantified through measuring its absorbance at 260 nm (NanoDrop spectrophotometer) and plotting against the DNA absorbance standard curve.

The hydrodynamic size and polydispersity index (PDI) of liposomes and AuNSs were characterized with Zetasizer nano-Z (Malvern). To examine their stability of liposomes, both factors were tracked for one month at 4°C. Phase transition temperature for unloaded and AuNS-loaded liposomes were analysed by a Differential scanning calorimetry (DSC) technique using Pyris™ Diamond DSC (PerkinElmer). Briefly, ~4-8 mg of liposome samples was loaded into aluminium pan for measurement. Systems were heated from 35°C to 70°C at a rate of 5°C/min, and recorded heat flow was plotted after normalizing the sample weight.

The morphology of synthesized AuNSs were examined with high-resolution TEM (FEI G2 spirit, 120 kV). 10 µl of aqueous solution containing AuNSs was placed on 200-mesh formvar carbon-coated copper grids (FCF-200-Cu) and dried in the dry cabinet for 72 hrs before imaging. To quantify the concentration of Au, 100 µl of AuNS-liposomes (5 mg/ml) were treated with 250 µl aqua-regia overnight to dissolve the AuNSs. The solution was then diluted with 10 ml DI H₂O. Inductively Coupled Plasma Spectrometer (ICP, Prodigy ICP spectrometer) analysis was used to determine the concentration of Au.

6.2.4 Photoacoustic (PA) Spectrum Acquisition

The PA excitation laser consists of an optical parametric oscillator (OPO) (Continuum, Surelite OPO) system pumped by a 532 nm Nd:YAG laser (Continuum, Surelite Ex). The OPO generates 5 ns duration pulses at 10 Hz

repetition rate with tunable wavelength. AuNSs were irradiated with 680–920 nm lasers with 10 nm increments, and corresponding PA signals were collected using a 2.25 MHz UST (V323-SU/2.25 MHz, Panametrics). Peak-to-peak PA signal was then normalized with the laser energy for each wavelength.

6.2.5 Cell Culture and Liposome Labelling

NIH-3T3 cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose 4.5 g/L and 4.0 mM L-Glutamine) supplemented with 10% FBS and penicillin/streptomycin (100 U/ml) and kept at 37°C with 5% CO₂. Cell labelling was performed by dispersing liposomes in DMEM with 1% FBS to a final liposome concentration of 5 mg/ml. Cells were incubated with liposome-containing medium for 4 hrs before being rinsed with PBS for further studies.

6.2.6 Fluorescence Imaging

Fluorescence imaging was performed using a LX71 inverted fluorescence microscope (Olympus) and Retiga-2000R CCD camera. The same settings (500 ms exposure time and 5x gain) were used to capture all green fluorescent images, at 100x image magnification. Finally, image processing with ImageJ was done to normalize background signal and quantify cellular intensity.

6.2.6 NIR Triggered Therapeutic Release

3T3 cells were seeded on a 48-well plate and labelled with AuNS-liposomes for 4 hrs in culture medium containing 1% FBS. Stimulation with pulsed OPO laser at 690 nm was performed for a total of 20 s per sample (10 pulse/s, with energy density of 10 or 40 mJ/cm²).

6.2.7 Alamar Blue Viability Assay

Alamar Blue viability assay was used to evaluate cytotoxicity of laser-treated 3T3 (~30k cells/well in a 48 well-plate). Alamar Blue reagent was added into cell culture medium in 1 to 10 volume ratio for cell incubation. 24 hrs later, fluorescence measurement (Ex: 570 nm/Em: 585 nm) was taken and normalized against untreated samples to measure the cell viability.

6.2.8 Statistical Analysis

One-way ANOVA was utilized to determine the significance in differences (p-values), accompanied with Tukey post-hoc analysis using the SPSS Statistics 20 software (IBM). Each experiment was performed by involving at least three replicates ($n \geq 3$).

6.3 Results and Discussion:

6.3.1 Synthesis and Characterization of AuNS-liposomes

The AuNSs were synthesized by reducing 50 μ L of 20 mM HAuCl₄ with 1M HEPES at room temperature. The as-synthesized Au NSs were washed and encapsulated in the aqueous core of the liposomes using conventional thin film hydration technique gel filtration chromatography using PD10 desalting columns.

The hydrodynamic diameter of the liposomes with SPACE peptides (LipS) was ~170 nm while the liposomes without space peptides (Lip) was ~ 130nm. The zeta potential of Lip and LipS was -25 mV to -70 mV respectively. It is to be noted the conjugation of the SPACE peptides did not alter the stability and the drug

loading efficiency of the liposomes since the conjugation was done on the surface of the liposomes. As expected, conjugation of SPACE peptides enhanced the cellular delivery of loaded molecules and plasmid to the cells which were confirmed by fluorescent imaging and flow cytometry. The molar ratio of P90G: Cho was fixed at 7:14 to avoid premature release of encapsulated drugs.

Figure 6-1: Characterization of AuNS and AuNS-loaded liposomes. A) TEM images of synthesized AuNS for liposomes incorporation. B) Photoacoustic spectra of AuNS showing signal peak at 690 nm. C) DSC analysis showing glass transition temperature of Lip and AuNS-incorporated Lip. Scale bar=100 nm.

The hydrodynamic diameter of AuNSs was ~45-50 nm (Figure 6-1a) and radiation-to-acoustic conversion rate for light occurred at 690 nm (Figure 6-1b). . ICP analysis revealed that $40.05 \pm 0.532 \mu\text{mol Au}$ was incorporated in 5 mg of total lipids per mL of liposomes. In addition, the phase transition temperature of the LipS without and with AuNSs was found to be 48.75°C and 48.34°C respectively, indicating that the incorporation of AuNSs did not have any significant effect on the membrane fluidity of the LipS (Figure 6-1c).

6.3.2 NIR Triggered Calcein Release

Based on this observation, pulsed OPO laser tuned to 690 nm was chosen as the light source for NIR irradiation. 3T3 cells seeded on a 48-well plate were labelled with liposomes for 4 hours in culture medium containing 1% FBS. Post incubation, the cells were washed and were irradiated at 690 nm with energy density of 10 or 40 mJ/cm² for a total of 20 s per well.

Figure 6-2: Alamar viability assay of 3T3 labeled with liposome carrying both calcein and AuNS after laser treatment.

Laser settings were first optimized to minimize the phototoxicity to the cells, by incubating the cells with Calcein loaded Lip and LipS containing/not containing AuNS. Although minimal cytotoxic effects were seen from all the groups tested, irrespective of the laser energy, ~5% and ~8-10% cell death was observed with 10 mJ/cm² or 40 mJ/cm² respectively. Thus for subsequent experiments, the laser energy was set to 10 mJ.

Figure 6-3: Fluorescence signal quantification showing laser-induced delivery of calcein in LipS Au. *** represents $P < 0.001$.

Next, NIR-triggered release of calcein and GFP plasmid from liposomes was demonstrated. As quantified in Figure 6-3 and represented in Figure 6-4 for calcein loading, cell labelling with LipS and AuNS-containing LipS (LipS Au) led to similar fluorescence intensity, unlike calcein only group which is not well internalized by the cells. However, after laser illumination, LipS Au-labelled cells exhibited strong fluorescence which is not observed from both calcein only and LipS group. Specifically, 1.94-fold stronger signal intensity was observed following laser stimulation. Meanwhile, laser activation does not significantly change the fluorescence in calcein only and LipS group (1.19 and 1.01-fold, respectively). This demonstrates the effective laser-triggered unloading of the proposed carrier. To substantiate this finding, we also compared calcein delivery from Lip and Lip Au (without SPACE peptide).

Figure 6-4: Representative images showing NIR laser-stimulated calcein delivery with AuNS-containing Lip and LipS. Scale bar = 100 μ m.

6.3.3 NIR Triggered DNA Plasmid Release

Figure 6-5: Fluorescence signal quantification showing laser-induced GFP plasmid transfection using AuNS-containing Lip/LipS. *** represents $P < 0.001$.

Similar observation was found for GFP plasmid delivery (Figure.6-5). Without laser treatment, minimal GFP fluorescence can be observed from all the groups (plasmid only, LipS and LipS Au). Following laser activation however, significant signal enhancement was seen on LipS Au group (1.80-fold). On the other hand, plasmid only and LipS group show insignificant GFP increase 24hrs post-laser treatment (1.1 and 1.02-fold, respectively). As expected, only cells labelled with AuNS-containing liposomes exhibited significantly stronger fluorescence after laser treatment (Figure. 6-6). It is to be noted that, AuNSs encapsulated liposomes group exhibited enhanced release of both Calcein and GFP plasmid upon laser irradiation, confirming the role of AuNSs as triggers for drug release.

Figure 6-6: Representative fluorescence images NIR laser-stimulated GFP plasmid transfection using AuNS-containing Lip/LipS reveals laser-induced plasmid delivery. Green: GFP Blue: Nucblue (B). Scale bar=100 μ m

6.4 Conclusion:

Here we report the construction of NIR-responsive liposomes with AuNSs. Considering biodegradability and deformability, natural phospholipids (P90G) was used in conjunction with Cho as the basic components of the liposomes. To ensure minimal loss of molecules prior to cellular internalization, a composition of 7:14 mol ratio between P90G and Cho was identified to provide the longest sustained release for both small (calcein) and big molecules (GFP plasmid). SPACE peptides were then conjugated to improve the cellular uptake of the liposomes. Incorporation of AuNSs within the liposomes permits the conversion of NIR-radiation energy to heat energy, which induces microcavitation and disrupts the liposome integrity.¹⁷⁶ Although the liposomes exhibited higher stability and had a phase transition temperature in the hyperthermic region, instantaneous release of drugs within the cell cytoplasm after the NIR-laser trigger was observed. This indicates that the drug release can be precisely controlled in terms of location, temperature and dosage using photoacoustic cavitation. Therefore, the proposed light-responsive, SPACE-modified liposome can be applied to animal models for the treatment of genetically-related skin diseases.

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CHAPTER 7

SUMMARY AND FUTURE DIRECTIONS

7.1 Summary

Liposomes has been established as clinically safe and successful nanocarriers. By protecting the encapsulated drugs against degradation and leakage they have shown to reduce non-specific systemic toxicity. Moreover, being biomimics of the cell membrane cellular internalization is achieved through endocytosis accounting for enhanced bioaccumulation. By exploiting the versatile surface properties of liposomes, gold nanoparticles can be associated with the liposomes either by encapsulation or deposition coating or through in situ synthesis. In either case, the gold nanoparticles impart their optical properties to the gold-liposome complex. This makes gold-liposome complex responsive to light ranging from UV-NIR depending on the spectral absorption of the gold nanoparticles. This additional property of the complex will allow us to achieve controlled delivery of encapsulated drug through external manipulation using light as stimulus. Additionally, presence of gold nanoparticles will facilitate non-invasive, multimodal imaging of the complex in vivo in order to track its biodistribution.

First, using a simple thin film hydration technique, we encapsulated spherical gold nanoparticles into temperature sensitive liposomes through passive encapsulation. The gold nanoparticles were within the aqueous core of the liposomes. The gold nanoparticles being excellent absorbers of photons, act as photosensitizers and generate heat which is dissipated to the surrounding causing a local heating effect. However, if this heat generated is sufficiently high to form a temperature gradient in the interface of the gold nanoparticle and the surrounding medium, vaporisation occurs, resulting in microbubble cavitation. This cavitation

eventually collapses leading to pressure wave generation that disrupts the liposomal membrane and releases the encapsulated drug. However, it was observed that this temperature gradient can be achieved only by pulsed lasers and not by continuous laser. This was because the instantaneous power density in each pulse duration in pulsed laser is much higher than the power density of continuous laser. Based on this mechanism, release of a model drug, calcein was demonstrated using Nd: YAG laser operating at 532 nm as external light source. By increasing the exposure time, the amount of drug released was also increased. The occurrence of microbubble cavitation was confirmed by measuring the photoacoustic signals generated, using a hydrophone. As the power of the laser was increased, the photoacoustic signal intensity was also increased. Thus these results indicated that by manipulating and controlling the laser parameters such as time of exposure and laser power, the dosage of the drug delivered can be controlled precisely.

One shortcoming of this system is its sensitivity to visible light that results in penetration depth limitation. To improve the effectiveness of the system, liposomes responsive to NIR wavelengths that fall within the biological window are required. For this, we synthesized photo-responsive liposomes coated with gold nanostars using a reduction-deposition method. Here, HEPES buffer solution used as a reducing agent of HAuCl_4 , forms branched gold nanostars that deposits on the surface of the liposomes as they are formed. The anisotropic nature of the gold nanostars increased the optical absorption of the AuNSs coated liposomes to 690 nm. Moreover, coating of AuNSs on the surface had the benefits of increased aqueous volume for drug encapsulation and the proximity of the AuNSs to the liposome membrane that improves the responsiveness of the system to light. Using

an optical parametric oscillator pumped by a Q-switched 532 nm Nd: YAG laser tuned at 690 nm as the light source we delivered the model drugs, Calcein and Paclitaxel to B16 F10 melanoma cells. Light controlled Calcein delivery was confirmed by the green staining of the cytoplasm of the cells treated with AuNSs coated liposomes encapsulating Calcein. To verify the functioning of Paclitaxel, a simple cell viability assay using Alamar blue was performed. It was found that the cells treated with AuNSs coated liposomes encapsulating Paclitaxel exhibited the highest cell toxicity when exposed to laser. At the same time, no staining or cell death was observed in cells not treated with laser, indicating very minimal drug release. Additionally, the AuNSs coated liposomes were tested as contrast agents for non-invasive photoacoustic imaging. The photoacoustic signal from AuNSs coated liposomes with a concentration of 2mg total lipids and 21.94mM AuNSs was measured and compared with that of animal blood. A higher contrast with 2 times increase in signal intensity was observed from AuNSs coated liposomes. Deep-tissue imaging upto a depth of 2 cms and tracking of melanoma cells labelled with AuNSs coated liposomes were demonstrated and similar trend in the signal to noise ratio was found. Thus these particles served as effective carriers for light controlled drug release and as potential contrast agents for non-invasive PAI.

Various methods to synthesize photo responsive liposomes are known. Amongst them, diffusion controlled in situ synthesis of nanoparticles within liposomes provides a controlled environment for particle formation. Moreover, this method is convenient one-step synthesis protocol for simultaneous synthesis of liposomes and gold nanostars. It also allows for a higher concentration of gold nanostars associated with the liposomes. HAuCl₄ encapsulated liposomes were

reconstituted with HEPES buffer solution. HEPES diffuses through the bilayer and reduces the HAuCl_4 to form AuNSs associated with the liposomes. In the diffusion controlled reduction process, AuNS-liposome complex with an optical absorption at 803 nm was obtained using the same initial concentrations of HAuCl_4 and HEPES used in the previous work. Presence of AuNSs in the liposomes produced strong photoacoustic signals in the NIR region even at a depth of 2 cms in biological tissue with a PLD-PAT system. Irradiation of drug encapsulated AuNS – liposome complex with the NIR pulsed laser, disrupted the liposomes and trigger the release of the pre-encapsulated drug within seconds. In addition to this, AuNS – liposome complex due to the higher encapsulation of AuNSs, AuNS – liposome complex alone could induce cell death upon laser irradiation, making it as a photothermal therapeutic agent. Thus this platform have the potential as a dual-modal theranostic agent, since it can be used for both imaging as well as therapy with controlled drug delivery and targeting.

Finally, the efficacy of the system was tested for construction of NIR-responsive liposomes with AuNSs made from natural phospholipids (P90G) in conjunction with Cho. The formulation was optimized to ensure minimal drug loss leading to an increase in the T_c to $\sim 48^\circ\text{C}$, resulting in the longest sustained release of both small (calcein) and big molecules (GFP plasmid). SPACE peptides were then conjugated to improve the cellular uptake of the liposomes. Incorporation of AuNSs within the liposomes facilitated the conversion of NIR-radiation energy to transient thermal energy, which induces microcavitation and disrupts the liposome integrity. Despite the liposomes' improved stability and increased T_c , faster release of drugs within the cell cytoplasm after the NIR-laser trigger was observed. This indicates that the

drug release can be precisely controlled in terms of location, temperature and dosage using photoacoustic cavitation. The proposed light-responsive, SPACE-modified liposome can be applied to animal models for the treatment of genetically-related skin diseases.

So far, we have demonstrated laser induced content release from photo-responsive liposomes synthesized by various methods using mammalian model cells for *invitro* studies. With the use of biocompatible components such as liposomes, gold nanostars and lasers at safe operating protocols, we believe that this technology will be a significant contribution to topical and transdermal drug delivery as well as non-invasive imaging and diagnosis at superficial tissues. Despite its potential, the photosensitive liposomes have to be tested on *invivo* models for deeper understanding on the interaction and functioning of the liposomes in physiological conditions. Also, it is important to understand the clearance of the nanoparticles from the body to affirm its biocompatibility. On account of this, the future work on the photo-responsive liposomes is elaborated in the following section

7.2. Future Directions and Scope

7.2.1. Photo-responsive Liposomes as Topical Delivery Agent

Skin is the largest organ of the body and forms a natural physical barrier to protect the internal organs. Although this protective layer is rarely invaded due to its innate defence mechanisms, the skin is quite susceptible to various diseases due to environmental and genetic factors. Some of most common skin disorders ranges

from temporary conditions such as acne, contact dermatitis, rashes from infections to chronic illness such psoriasis, vitiligo, skin cancer. Some conditions like keloids are also recurrent even after surgical removal, while treatment of temporary conditions can result in permanent scar formation and pigmentation at times. Topical treatment of skin disorders using nanoparticles is advantageous as it prevents the systemic toxicity due to localized delivery of the therapeutic.

Liposomes encapsulating active pharmaceutical ingredients have been developed to treat conditions such as atopic eczema, acne, psoriasis and melanoma. Liposome encapsulated therapeutics ensured low systemic availability of the drugs and enhanced bioavailability. The structural and physiochemical properties of liposomes and the vantage of light penetrations makes photo-responsive liposomes good candidates for topical drug delivery. We will be testing the delivery and drug penetration of photo-responsive liposomes into rat and pig skin model. One way to deliver the liposomes to skin is through a topical hyaluronic acid based gel formulation or through penetration enhancing peptides. However, we will be using the microneedle technology which is widely studied in our group to deliver the liposomes to the skin. Once the liposomal delivery is established, we will study the drug release and penetration using light stimuli. The temperature and morphology of the laser treated and the adjacent tissues will be constantly monitored to understand the effect of laser interaction with the skin.

To test the efficacy, biodistribution and clearance of photo-responsive liposomes in a more detailed manner, *in vivo* studies with small animal models are required. Further investigations on this are currently being done to use subcutaneous melanoma models for *in vivo* studies.

7.2.2 Multimodal Imaging and Therapy

The performance of photo-responsive liposomes in other imaging modalities and therapy can be explored. The unique capability of photo-responsive liposomes for photoacoustic (PA) imaging in addition to controlled drug release had been explored. The functioning of photo-responsive liposomes in other imaging modalities like SERS and Optical Coherence Tomography (OCT) can also be studied. Additionally, portable Raman microscopy is emerging as a rapid analysis method to detect and quantify metal based nanoparticles on skin. This will be an inexpensive and quick analysis method to quantify photo-responsive liposomes in topical formulations. Besides controlled drug release and imaging, photo-responsive liposomes can also be used as a therapeutic agent for photothermal, photoacoustic therapy and laser therapy.

7.2.3 Exploring the Use of AuNSs as Photo-actuators for Polymeric Nanoparticles

We wanted to explore if other biocompatible materials such as polymer based hydrogels can be made photo-responsive by incorporation of AuNSs, as an attempt to extend the light controlled release mechanism to polymer based drug delivery systems.

For this, PLGA nanoparticles were synthesized by a double emulsion method. Briefly, 1 mg 5FU dissolved in phosphate buffer saline (pH 7.2) was mixed with 500 μ L chloroform containing 10 mg PLGA (50:50, with Mw: 30000–60000) and 1% span 80 (v/v). The mixture was homogenized for 30 s at 30k rpm (Scilogex D160) to form the first emulsion, which was then mixed with 5 ml of 3% poly-vinyl

alcohol (PVA) solution to form the double emulsion particles under further homogenization. Subsequently, chloroform was evaporated under magnetic stirring for 4 hours in a fume hood, and the excess PVA was removed by washing the particles with PBS. Finally, the PLGA microparticles were freeze-dried and stored at $-20\text{ }^{\circ}\text{C}$.

To coat the PLGA particles with AuNSs, 2mg PLGA microparticles encapsulating 5FU (PLGA-5FU) was reconstituted in 1M HEPES buffer solution. To this, 50 μL of 20 mM HAuCl_4 was added and the solution was kept at room temperature for 1 hour to form PLGA microparticles encapsulating 5FU coated with AuNSs (PLGA-5FU-AuNS). The particles were then washed with distilled water thrice to remove the uncoated AuNSs was freeze-dried and stored at $-20\text{ }^{\circ}\text{C}$. Similarly plain PLGA microparticles and PLGA microparticles coated with AuNSs (PLGA-AuNS) were also synthesized.

Figure 7-1: FESEM images of (a) PLGA microparticles and (b) PLGA-AuNS microparticles. Scale bar: 1 μm

As a preliminary proof of concept, light triggered release of 5FU in B16F10 melanoma cells was demonstrated through cell viability assay. The cells were incubated with PLGA-5FU-AuNS and were irradiated with a pulsed laser diode at 803 nm for 60 seconds. Cells were also incubated PLGA, PLGA-AuNS, PLGA-5FU and 5FU for comparative analysis. Cells without any particles were treated as

control and was taken as 100% viable cells. While the laser untreated groups exhibited little or no cytotoxicity, PLGA-AuNS, PLGA-5FU-AuNS and 5FU treated groups exhibited 70%, 88% and 39% cell cytotoxicity respectively.

Figure 7-2: Cell viability of B16F10 melanoma cells treated with PLGA-5FU-AuNSs microparticles with and without laser exposure at 803 nm for 60 seconds with and energy density of 1.4 mJ/cm².

Cells incubated PLGA particles did not show any cytotoxicity with laser exposure, while 16% cytotoxicity was observed in cells incubated PLGA-5FU. This might be due to the effect of 5FU diffused out of PLGA. The results indicate that presence of AuNSs initiates the light triggered drug release and is responsible for the increased cytotoxicity. This system needs to be characterized and optimized further for enhanced control on the drug delivery, which is still ongoing. However, the SEM images and the *invitro* studies indicate that with the help of AuNSs, novel and simple responsive drug delivery systems can be achieved.

7.3 Conclusion

To conclude, light triggered controlled drug delivery using photo-responsive liposomes was evaluated in this thesis, alongside photoacoustic imaging. The photo-responsive liposomes were not only effective as drug carriers for light controlled drug delivery but also served as potential contrast agents for photoacoustic imaging. The light triggered drug release was achieved by liposomal disruption through a photoacoustic mechanism, when the irradiation source matches the SPR wavelength of the gold nanoparticles. Hence, by optimizing the parameters of the laser; the location, time and dosage of the therapeutic can be efficiently controlled paving way for personalized treatment. Furthermore, facile syntheses of this photo-responsive liposomes has inspired the development of many other light sensitive nanoparticles with varying optical and therapeutic properties. Owing to the organic and biodegradable nature of phospholipids, lack of complexity in the formulation and the intrinsic multifunctionality of Au NPs, photo-responsive liposomes will have broad implications for clinical translation for topical and ocular drug delivery.

7.4 Outlook

The photo-responsive liposomal platform has a great potential as drug carriers for superficial tissues such as skin and eyes. Guided laser therapies may be effective in reaching certain tissues such as mucous membranes. Although inexpensive and efficient for topical or ocular therapies and for small animal models, photo-responsive systems are held back by the penetration depth limits of

the light. In order to target deep tissue organs such as liver, kidneys etc. other external stimuli such as magnetic field and ultrasound are greatly sought after. Thus 'one-system-fits-all' is difficult to be applied for personalized and precision medicine. The ultimatum of developing stimuli responsive systems is to achieve localized release of at target sites thus delivering tailored treatment to each individual. Hence, the demand for DDS that allows for disease state monitoring, triggered release or real-time tracking of stimuli responsive release are constantly increasing. Effectuating such optimized treatment systems will eventually lead to higher patient survival and better quality of life. The great progress in nanotechnology has paved way for the development of DDS that can be controlled efficiently. However, for these systems to be translatable, certain bottlenecks need to be overcome. This requires a thorough understanding of physio-chemical parameters of the nanoparticles which in turn affects their *in vivo* efficacy. First and foremost, the clearance of the NPs from the human body and long-term effects of chronic usage of these NPs based systems has to be studied. Also, optimizing a specific NPs based systems also calls for analysis of the physical properties of the NPs and the target area. Such a rationale when applied to nanomedicine, will expedite NP drug delivery systems to future clinical translation.

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