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Nanoemulsions Within Liposomes for Cytosolic Drug Delivery to Multidrug-Resistant Cancer Cells

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Doctor of Philosophy

Cancer cells that survive chemotherapy treatment often develop resistance to the administered chemotherapeutics, as well as to many other types of drugs, because the cancer cells increase their production of efflux pumps in the cell. This undesired phenomenon of resistance to cancer drugs is known as multidrug resistance. This work uses a novel drug carrier, called an eLiposome, to achieve cytosolic drug delivery to kill multidrug-resistant cancer cells.

An eLiposome consists of a perfluoropentane (PFC5) emulsion droplet inside of a liposome. Folate attached to the eLiposome facilitates uptake into the cell. The PFC5 droplet is metastable at body temperature, but will rupture the liposome as the droplet expands during vaporization, and will release any drugs encapsulated inside of the liposome directly to the cell cytosol.

Laser and ultrasound were examined as triggers to initiate the vaporization of the PFC5 droplet and actuate the release of doxorubicin (Dox) from folated eLiposomes containing Dox (feLD). Gold nanorods (GNRs) were synthesized and transferred to PFC5 droplets. Although GNRs are efficient at converting irradiated laser light to heat, no vaporization of the PFC5 droplets was observed when irradiated with laser light. Further investigation into the energy required for vaporization of PFC5 droplets revealed that there are currently no portable and wearable lasers available to provide enough energy to vaporize PFC5 droplets.

Two seconds of ultrasound can release 78% of encapsulated Dox from feLD. Dox-sensitive KB-3-1 cells and Dox-resistant KB-V1 cells treated with feLD (without ultrasound) had cell viabilities of 33% and 60%, respectively. Ultrasound had negligible additional effect on the cell viability of KB-3-1 and KB-V1 cells treated with feLD (33% and 53%, respectively). We hypothesized that the Dox fiber formed during the loading of Dox into the eLiposome is a site for heterogeneous nucleation once the feLD is endocytosed by the cell, and vaporization and drug release occurs with or without ultrasound.

Blocking the efflux pumps with verapamil decreases the rate at which Dox is exported from multidrug-resistant cells. When verapamil is co-delivered with feLD, the cell viability of KB-3-1 and KB-V1 cells decreases to 29% and 25%, respectively; thereby reversing the multidrug resistance possessed by KB-V1 cells. The delivery of doxorubicin inside of folated eLiposomes with an efflux pump blocker is a novel way to kill multidrug-resistant cancer cells as effectively as non-resistant cancer cells independent of lasers or ultrasound.

Keywords: multidrug resistance, liposome, drug delivery, doxorubicin, verapamil, co-delivery, vaporization, emulsion, ultrasound, gold nanorod, laser, eLiposome
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1 INTRODUCTION

Cancer is the 2\textsuperscript{nd} leading cause of death in the United States, close behind heart disease; however, it is the leading cause of death in the United States for individuals between ages 40-79 [1]. It is estimated that there will be 595,690 cancer related deaths and 1,685,210 new cases of cancer in the United States in 2016 [1]. Treating cancer is a national priority.

Cancer cells are characterized by rapid division and uncontrolled growth that eventually inhibits other functions of the body. Current methods for treating cancer include surgery, radiation, or chemotherapy, as well as combinations of these treatments [2]. Chemotherapy is the delivery of cancer drugs at a high enough dosage to kill the cancer cells while not killing the patient. Unfortunately, no cancer drug in current use perfectly distinguishes between normal and healthy cells, so significant side effects, such as hair loss, nausea, weight loss, and fatigue, are associated with chemotherapy treatments [3].

In 2000, Hanahan and Weinberg proposed six hallmarks of cancer: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [4]. In 2011, they noted four more emerging hallmarks of cancer: deregulating cellular energetics, avoiding immune destruction, genome instability and mutation, and tumor promoting inflammation [5]. Drugs can target cancer cells by preferentially targeting cells with one or more of the hallmarks described by Hanahan and Weinberg.
Targeted drug delivery helps cancer drugs preferentially target tumor cells over healthy cells and decreases the negative side effects associated with chemotherapy. It allows for a higher concentration of drug to accumulate at the tumor site (an abnormal growth of cancerous tissue), and a lower concentration of drug to accumulate around healthy cells. One common method to target cancer cells is to attach a ligand to the drug or drug carrier that corresponds to membrane receptors that are upregulated in cancer cells due to the hallmarks explained above. For example, the folate receptor is upregulated in over 1/3 of cancer cells [6] and is associated with sustaining proliferative signaling.

Another method to minimize the negative side effects of chemotherapy, as well as increase drug efficacy, is to have the drug remain inactive until it reaches the tumor. This can be done by either developing a prodrug (an inactive compound that becomes active after it is chemically altered) or by encapsulating the drug inside of or attached to a drug delivery vehicle and only activating or releasing the drug at the tumor site. Drugs are released, or prodrugs are chemically changed from inactive to active, when exposed to specific stimuli; thus the drug actions are localized to specific sites in the body. Triggered drug release from a drug delivery vehicle can be actuated by internal mechanisms (i.e., changes in pH [7] or temperature [8]) or by external stimuli (i.e., ultrasound [9], near-infrared light [10], or magnetic fields [11]).

Delivering drugs directly to the tumor site will decrease the negative side effects of chemotherapy, but there is another problem associated with chemotherapy. It is the acquired resistance to chemotherapeutics. More specifically, over time the cancer can often become resistant to both the initially administered cancer drug as well as to unrelated cancer drugs—a phenomenon known as multidrug resistance. Resistance to the chemotherapeutics limits the
treatment methods available to the patient because a higher or more frequent dosage is required for efficacy, often resulting in a required dosage that exceeds the lethal level.

One of the established mechanisms for multidrug resistance is an increased number of efflux pumps [12-15] which increase the rate at which undesired compounds inside of the cell (such as cancer drugs) are pumped out of the cell. In conventional drug delivery, cancer drugs accumulate at the tumor site and diffuse into the cancer cells. The rate of accumulation inside of resistant cancer cells is much lower than the rate of accumulation inside of drug-sensitive cancer cells using conventional drug delivery; however, accumulation of the drug inside of resistant cells can be increased by inhibiting the function of the efflux pumps [16].

The objective of my research was to develop drug delivery methods to overcome the resistance of multidrug-resistant cancer (MDR) cells. My hypothesis is that blocking the efflux pumps while delivering cancer drugs directly to the cytosol of MDR cells will kill MDR cells as effectively as sensitive cancer cells. My method for achieving this result used a targeted drug delivery vehicle known as a folated eLiposome, which is a liposome containing an emulsion droplet. I attempted two different controlled release mechanisms to vaporize the emulsion droplet and release drugs directly to the cell cytosol: gold nanorod heating via laser, and ultrasound.
2 LITERATURE REVIEW

2.1 Liposomes as Drug Delivery Vehicles

Liposomes are one of the most common drug carriers. They were first discovered in 1961 [17] when A.D. Bangham and colleagues observed that phospholipids spontaneously formed lipid bilayers when dispersed in water. Liposomes range in size from 0.02 to 10 μm and are characterized as vesicles that have an aqueous core enveloped by a hydrophobic lipid bilayer [18]. This allows for the possibility of transporting both hydrophilic drugs within the aqueous core and/or hydrophobic drugs within the lipid bilayer (Fig. 1) [19].

![Figure 1. Schematic representation of a drug loaded liposome (modified from [19]).](image)

Encapsulating drugs inside of a carrier is commonly used to avoid systemic delivery of chemotherapeutics and to reduce the negative side effects of chemotherapy. Additional advantages of encapsulating drugs inside of liposomes include biocompatibility, prevention of
premature degradation of encapsulated cargo, entrapment of both hydrophilic and hydrophobic drugs, targeted delivery, site avoidance (entrapped drugs prevented from interacting with healthy tissue) and regulation of biodistribution (locations where the liposomes travel inside the body) by size or lipid component variation [19]. For example, in vivo studies by Gabizon et al. [20] show that <2% of doxorubicin loaded inside of PEGylated liposomes (liposomes with polyethylene glycol on the surface) is released during circulation, allowing for most of the loaded drug to be released at the tissues where the liposomes accumulate. Encapsulation prevents the drugs from interacting with any cells until they are released or escape from the drug carrier.

Liposomes can still interact with serum proteins and be cleared quickly from circulation when opsonins bind to the liposome. When bound to liposomes, opsonins interact with receptors on the surface of macrophages or hepatocytes, thereby enhancing uptake by these cells and clearance from blood circulation [21]. In 1990, two different groups proposed to incorporate polyethylene glycol (PEG) into the lipid bilayer in order to improve the circulation time of liposomes [22, 23]. PEGylated liposomes, now often referred to as stealth liposomes, have increased half-life, decreased plasma clearance, and decreased distribution volume compared to non-PEGylated liposomes [24]. The PEG coating on liposomes reduces the adsorption of opsonins on the vesicles and thus decreases the clearance of PEGylated liposomes by the immune system [21, 24] and increases the probability that PEGylated liposomes will accumulate at the tumor site.

2.1.1 Targeting of Liposomes

Passive targeting of drugs to cancer cells occurs when drug delivery vehicles are able to collect at tumor sites without any external assistance. In some cases, liposomes passively target
tumors because of their small size and via a mechanism known as the enhanced permeation and retention (EPR) effect. Specifically, the EPR effect is that small particles, such as liposomes, can escape from blood vessels into the tissue of tumors due to the leaky vasculature of the tumor [18]. Conversely, these same liposomes are too large to enter healthy tissues, which have less permeable vasculature. However, within a single tumor the vascular permeability can vary, such that particles as large as 200 nm can leak through at certain locations in the tumor while at other locations particles as small as 4 nm may not be able to leak through [25]. Furthermore, once extravasated the liposomes might flow right past the cancer cell without stopping to release its drug. Therefore, while it is beneficial that the liposomes can passively target tumors, it would be more effective if they were also able to actively target the cancer cells.

One way liposomes can actively target tumor cells is to attach a ligand to the outer surface of the liposomes that has a corresponding receptor on the surface of the cancer cell. Researchers are still looking for new molecular features that are uniquely specific to cancer cells, such as an over expression of an uncommon receptor molecule on the cell surface. While one specific receptor truly unique to cancer cells has yet to be discovered, researchers have found receptors that are expressed at much higher levels in cancer cells than in normal cells. Ligands commonly used to exploit this difference include folate, transferrin, and galactosamine [25] because the corresponding receptors for these molecules are often overexpressed in rapidly growing cells.

Folate is one of the oldest and most well-known ligands attached to liposomes, as many cancer cells overexpress folate receptors [18]. The binding of the attached folate to the folate receptor also induces endocytosis [26-28], which is the process of a cell invaginating a section of its cell membrane to engulf a volume of external fluid. Thus, if folate is attached to the liposome,
the cancer cells have a greater uptake of the liposomes into the cell compared to non-targeted liposomes [9, 29], which may be only randomly and rarely endocytosed.

Targeted delivery allows more of the drug from PEGylated liposomes to accumulate on the surface or within the cancer cells due to an increase in liposomes collecting at the tumor site instead of circulating in the blood. However, without a quick release mechanism the drug is only able to escape the liposome through diffusion across its lipid bilayer. Rapid release of the drug is desirable over slow release so that the cells do not have time to respond by upregulating expression of defensive proteins that block, inactivate, or pump out the drug. Quick release of the drug from the liposome leads to increased efficacy.

2.1.2 Triggered Release

2.1.2.1 Passive Triggers

In order to provide faster and/or more controlled release, many different types of release mechanisms have been studied, both through passive and remote triggers. Passive triggers rely on the environment of the tumor to trigger the release of the payload at the tumor site. The most common passive trigger is a response to a change in pH [30]. The extracellular pH of normal tissues and blood is regulated at a constant pH of 7.4; the intracellular pH is about 7.2. The extracellular pH of various solid tumors, however, can be as low as 5.7 due to rapid metabolism and poor circulation [31]. Drug delivery vehicles that are endocytosed will also experience a drop in pH, with a pH of 6.5 inside the early endosome, pH of 5.0-6.0 inside the late endosome, and a pH of 4.0-5.0 inside the lysosome [32]. Therefore, drugs can be released inside or outside of the cell depending on the pH sensitivity of the drug carrier. The drugs released inside of the endosome/lysosome must still escape this compartment to the cytosol in order to have their
desired effect. Drugs released outside of the tumor cells in response to the lower pH result in a higher than average local extracellular concentration of the drug. However, the externally released drug must still diffuse into the cell or be endocytosed by the tumor cells in order to have a cytotoxic effect. Unfortunately, despite a higher extracellular concentration of the drug, cancers that have developed multidrug resistance might still be able to pump out the drug before the drug can induce a cytotoxic response.

Many pH-sensitive liposomes contain specially synthesized pH-sensitive lipids that are incorporated into the liposomal bilayer [33]. These lipids will change in size or shape depending on the acidity of the surrounding environment [34]. In an alternative approach, Nahire et al. have developed a pH-sensitive liposome that does not require lipids that are structurally modified at a reduced pH [35]. They encapsulate ammonium bicarbonate inside of their liposomes, which will generate carbon dioxide gas bubbles and burst the liposome in response to hydronium ions diffusing into the liposomes when in an acidic environment. The release of encapsulated cargo from the burst liposomes by Nahire et al. is enhanced through the application of ultrasound, so such release can be a combination of passive and active mechanisms.

Another common passive trigger is a response to a change in temperature. Thermo-sensitive liposomes [19, 36] are liposomes that incorporate a thermo-sensitive polymer which undergoes a coil-to-globule transition when the polymer is heated above its lower critical transition temperature. Therefore, thermo-sensitive liposomes are used in combination with mild hyperthermia (sometimes found in large tumors or induced through external heating between 41 and 42°C) [19] in order to control the release of the liposomal payload. Methods for localized hyperthermia in conjunction with thermo-sensitive liposomes are also being explored with the various external triggers (magnetic [37], laser heating [38, 39], ultrasound [40]).
2.1.2.2 Remote Triggers

Magnetic fields, light, electric fields, and ultrasound are externally applied stimuli commonly used in triggered drug delivery. Each of these stimuli has advantages as well as disadvantages. Magnetically responsive systems have the ability to guide the drug delivery vehicle to a target location and have the possibility to perform diagnostics and therapy simultaneously. One major disadvantage is the complexity involved in the setup of external magnetic fields in order to achieve adequate focusing and penetration depth [41].

Light-triggered delivery systems are non-invasive and have the possibility of spatiotemporal control. Another advantage of light-triggered release is the possibility of having a one-time release or a repeatable on-off release. UV light has a disadvantage of low penetration depth (~10 mm); however, near-infrared (NIR) light (650-900 nm) is able to penetrate deeper because tissues do not absorb or scatter the light as much as the shorter wavelength light. A current disadvantage of NIR-responsive systems is finding biocompatible photosensitive materials that will be continue to be non-toxic after use (i.e. safe biodegradability and clearance of nanoparticles) [41, 42].

Electroresponsive systems can have pulsed or sustained drug release using weak electric fields (typically about 1 V). They offer significant freedom in the design of the device that controls the electric signal, but they have low penetration depth and lack the ability to avoid undesired tissue damage [41].

Ultrasound is an effective method to achieve spatiotemporal control, is noninvasive, lacks ionizing radiations, and has the ability to control penetration depth by tuning frequency, duty cycles and time of exposure. Ultrasound can also increase drug transport across cell membranes and tissues. One potential drawback of ultrasound is the possibility of metastatic dissemination
[41], or the spread of cancer from its primary site to other distant organs in the body. The following two sections will review the use of lasers and ultrasound to trigger the release of therapeutics, as they are the two methods presented in this research to trigger the release of doxorubicin from eLiposomes.

2.2 Lasers

Lasers can be designed to emit light at different wavelengths and intensities. One of the challenges with light-activated biomedical applications is the ability of light to reach its target location without being absorbed or scattered by other molecules in its path. Hemoglobin and water have high absorption of visible and infrared light, respectively; however, they both have minimal absorption of light between 650 and 900 nm, what has been termed the near-infrared (NIR) window [43]. For this reason, NIR lasers have greater penetration depth in tissues than UV lasers.

Light can be delivered from lasers continuously or in short (nano-femtoseconds) high-energy pulses. Continuous wave lasers are often used for laser-induced mild hyperthermia [44, 45]. Pulsed lasers are often used for imaging applications [46-48] and/or quick heating for release of therapeutics [46, 49]. Pulsed lasers are not typically used for prolonged heating with gold nanorods because the short, high-energy pulses can lead to particle structural changes and ablation due to the excessive heating of the gold nanorods without sufficient cooling between pulses [50].

Leung and Romanowski [51] review light-activated release from liposomes, which includes the release by photochemical activation and photophysical activation. Content release from liposomes through photochemical activation utilizes the destabilization of the lipid
membrane by light induced (typically UV) isomerization, cleavage, or polymerization of its components [51]. Photophysical activation does not involve any chemical changes in the liposome bilayer or any components within the liposome. Rather, it involves thermal or mechanical changes in the liposome or surrounding medium. The following section of the literature review will focus on the heating of gold nanorods to induce a thermal change to trigger release from liposomes.

2.2.1 Gold Nanorod Heating

Gold nanorods (GNRs) are often used for photothermal therapy because they can be synthesized to absorb light over a wide range of optical frequencies, including the NIR window. Gold nanorods are most effective at absorbing light and converting it to thermal energy when the irradiating light wavelength corresponds to the oscillations of the electrons around the long axis of the gold nanorods, a phenomenon known as localized surface plasmon resonance (LSPR) [52]. Gold nanorod heating is commonly used for mild hyperthermia treatments (40-45°C) or diagnostic imaging via vaporization of contrast agents [47, 53]. GNRs are also used for drug delivery with temperature sensitive nanoparticles [54] and even DNA/siRNA delivery [55].

Gold nanorods are not used as frequently for drug release from liposomes, but there are some investigators researching this and related types of light-activated release. Viitala et al. [56] loaded GNRs inside of DPPC liposomes along with calcein (a model drug). They observed 60% release of calcein after 15 min of illumination (3 W). Drug release from these liposomes was attributed to heating the DPPC above its phase transition temperature (41°C) and thus increasing the rate of diffusion of calcein out of the liposomes. Instead of encapsulating GNRs inside of liposomes, Yu et al. [54] investigated the release of doxorubicin when Dox-loaded liposomes
were co-delivered with a solution of GNRs. They found the greatest amount of release when irradiating GNRs in the presence of Dox-loaded liposomes that also contained ammonium bicarbonate (ABC-Lip). Han et al. [57] showed that CO₂ bubbles were not generated at 37°C (physiological temperature) using ABC-Lip; however, bubbles were generated at temperatures as low as 42°C. Therefore, when GNRs were used to induce mild hyperthermia (approximately 45°C in the experiments by Yu et al.), CO₂ bubbles could generate in ABC-Lip and facilitate quicker release of Dox from the liposomes. Yu et al. [54] observed approximately 80% release of Dox from ABC-Lip after 5 min of irradiation (810 nm, 1.6 W/cm²), whereas they observed only 30% Dox release from liposomes with ammonium sulfate instead of ammonium bicarbonate.

Gold nanorod heating can also be used to vaporize perfluorocarbon droplets [46, 47, 58, 59]. Liu et al. vaporized GNR-loaded perfluoropentane droplets and applied ultrasound to facilitate the perforation of cell membranes using ultrasound and cavitation [58]. Zhong et al. vaporized perfluorohexane to release paclitaxel from nanoparticles while concurrently using it as a contrast agent for real-time imaging [46]. Using different loading procedures, Hannah et al. [47] and Wilson et al. [59] loaded GNRs in perfluoropentane droplets to use as contrast agents for imaging.

2.3 Ultrasound

Ultrasound (US) consists of pressure waves having frequencies of 20 kHz or greater [60]. The pressure waves from US are longitudinal compressional waves, in which the particles are displaced in the same direction as the travelling pressure wave. If one were to “take a picture” of the ultrasonic wave as it travels through a medium, one would see areas of rarefaction (regions of low pressure) and areas of compression (regions of high pressure) [61]. As the ultrasonic wave
propagates through the medium, it causes cyclic displacement of the particles without net movement of the particles in any one direction. Ultrasonic waves can also be focused, reflected and refracted through a medium [61, 62]. Under the right conditions it is possible to vaporize liquid droplets [63, 64] and to cause acoustic cavitation [62, 65].

Important considerations for the phase change of liquid droplets (emulsions) and the cavitation of the resulting gas bubbles include the peak negative pressure, frequency and exposure time of the incoming ultrasound, as well as the size of the droplet or bubble [61]. Larger droplets are easier to vaporize in part because of the lower Laplace pressure of the droplet. The Laplace pressure is the increase in pressure inside a suspended droplet (or bubble), and is given by

\[ \Delta P_{Lp} = \frac{2 \gamma_g}{R_{em}} \]  

(2-1)

where \( \Delta P_{Lp} \) is the Laplace pressure, \( \gamma_g \) is the interfacial energy of the droplet, and \( R_{em} \) is the radius of the droplet. The interfacial energy of the droplet is a function of the surfactant used to stabilize the droplet. The Laplace equation shows that as the size of the droplet decreases, the Laplace pressure increases, and the peak negative pressure needed to allow the droplet to vaporize also increases in magnitude. These relationships are captured in the following equation [66]

\[ P_{US} \leq P_{vap}(T) - P_{atm} - \Delta P_{Lp} - P_{hyd} \]  

(2-2)

where \( P_{US} \) is the ultrasonic peak rarefractional pressure, \( P_{vap}(T) \) is the vapor pressure of the liquid of the droplet at temperature \( T \), \( P_{atm} \) is the atmospheric pressure, and \( P_{hyd} \) is the hydrostatic pressure (usually negligible compared to the other terms in Eq. 2-2).
A nucleation event must occur to initiate the liquid-to-gas phase change of a droplet. Mountford and Borden suggest that the stability of phospholipid stabilized PFC droplets is more than simply stabilization through the Laplace pressure. Stability is also attributed with the high energy barrier associated with homogeneous nucleation [67]. At a given peak pressure and pulse length, lower frequencies of the incoming US increase the probability of a nucleation event occurring within a droplet [63]. One explanation for this is that there is a longer time window in which stochastic events may satisfy the inequality of Eq. 2-2 or exceed the activation energy barrier for nucleation. The lower the US frequency and the more negative the rarefractional pressure (higher amplitude in pressure wave), the more probable it is to vaporize droplets [63].

There are limitations to the peak negative pressure and frequency allowed for biological applications. In 1991, Apfel and Holland observed that the ratio of the acoustic pressure and the square root of the frequency can approximately predict the onset of collapse cavitation for a single acoustic cycle [68]. This observation has led to the establishment of the Mechanical Index (MI), which is a measure of the likelihood of collapse cavitation occurring (possibility to damage healthy tissue), and is defined as

\[
MI = \frac{P^-/\text{MPa}}{f/\text{MHz}}
\]

(2-3)

where \(P^-\) is the peak negative pressure amplitude and \(f\) is the applied frequency. The onset of collapse cavitation begins between MI values of 0.3-0.4 and detrimental biological effects begin when MI > 1 [69]. This definition of MI assumes that there are stabilized gas pockets or free bubbles present as nuclei for cavitation [62]. Modes for ultrasound delivery approved by the Food and Drug Administration include frequencies between 0.02 – 7.5 MHz [70].
2.4 Acoustic Droplet Vaporization

The use of sound waves to induce a phase change in a liquid emulsion droplet is known as acoustic droplet vaporization (ADV). The liquids used in this work were perfluorocarbons (PFCs); therefore, they will be the only liquids of interest in this literature review. PFCs are good choices for biomedical application because of their low solubility in aqueous formulations and very low toxicity [63]. Perfluoropentane (PFC5) and perfluorohexane (PFC6) have normal boiling points of 29°C and 59°C respectively. However, when coated with a surfactant, both PFC droplets (1 µm) can have a spontaneous phase transition temperature (to gas) above 70°C [65]. Mountford and Borden [67] report that the spontaneous vaporization of PFC droplets occurs at temperatures around 90% of the critical temperature of the PFC ($T_c = 423.1K$ for PFC5 [71], 90% $T_c = 107°C$).

Ultrasound pressure waves change the local pressure inside and outside the PFC droplet, thus providing a driving force for vaporization. The probability of nucleation within the PFC droplet is proportional to the amount of time the droplet spends with its local pressure below its vapor pressure (see Figure 2). Therefore, it can be proposed that acoustic droplet vaporization is more likely as the frequency decreases, the number of cycles per pulse increases, the peak negative pressure increases in magnitude, and the Laplace pressure decreases (lower interfacial energy and/or increased droplet radius) [63].

Some recent research on ADV has centered on capturing the vaporization dynamics and lifetime evaluation of PFC droplets using ultra-high speed optical imaging [72-81]. A recent study by Shpak et al. [76] looked at the physical mechanism responsible for ADV and concluded that it is initiated by the focusing of a nonlinear acoustic wave on a specific spot inside the
droplet. The authors propose that the phenomenon of ADV is facilitated by the nonlinear propagation of ultrasound, which builds up superharmonics necessary to induce a focusing effect within the droplet. They observed that, depending on the diameter of the droplet and frequency of the incoming ultrasound wave, the initial site of nucleation went from a random site in the droplet, to one of two specific sites, and finally to one specific site. This site in the droplet is the location where the focused pressure amplitude is maximal. This focusing effect (using the droplet itself as a lens) could be obtained by either increasing the droplet diameter or by increasing the frequency of the incoming ultrasound. Thus, higher frequency US can increase ADV through a focusing of superharmonics, while lower frequency US (20 kHz) can increase ADV through a greater time for stochastic events to occur.

Figure 2. Schematic of an ultrasonic acoustic wave in a PFC emulsion. The upper figure illustrates an US acoustic wave whose peak rarefactional pressure stays above the vapor pressure of the PFC minus the Laplace pressure. The lower figure illustrates a gas phase forming around the emulsion droplet when the peak rarefaction pressure drops below the vapor pressure of the PFC minus the Laplace pressure. Figure used by permission from [82].
Miles et al. [83] investigated the nucleation pressure threshold in ADV and predicted that the local pressure needed to nucleate a 10 µm PFC5 droplet at 25°C is \(-9.33 \pm 0.30\) MPa (7.5MHz, 5 cycles/pulse). Their model combined classical homogeneous nucleation theory with superharmonic focusing, and their model predictions agreed with approximately two-thirds of their experimental data. This pressure threshold predicted by Miles et al. is for droplets much larger than the ones used in experiments presented in this work (10 µm v. < 0.2 µm) and Miles et al. also used a much higher frequency (7.5 MHz v 0.02 MHz). The size of the droplet is a factor in deciding whether high or low frequency US would be better for inducing ADV. The focusing effect for ADV is applicable for larger droplets, as used by Shpak et al. [76] and Miles et al. [83], but not for small droplets as used in this work. Shpak et al. observed that a frequency of 5.0 MHz was necessary for a focusing effect to induce ADV at one site for a 6-10 µm droplet [76], whereas at 3.5 MHz there were two spots of nucleation. The size of the droplets proposed in this research is on the order of 100-200 nm. The significantly smaller size of the droplet would require frequencies outside of the FDA approved frequencies for US if focusing were the only trigger. Therefore, lower frequencies of US were used in the research presented in this dissertation and focusing is probably not involved in ADV. As noted above there is also a greater probability for nucleation at lower frequencies due to a greater time window at a pressure below the vapor pressure of the droplet.

2.5 Multidrug Resistance

Chemotherapy is a common treatment method for cancer and involves delivering cancer drugs to a patient in an effort to kill the cancer cells without killing the patient. One obstacle of successful chemotherapy is the development of multidrug resistance in which the cancer cells develop resistance not only to the administered cancer drug but also to multiple unrelated drugs.
that can be structurally and/or functionally different [15]. Developing resistance to cancer drugs will eventually require such a high dose of cancer drugs to kill the cells that is not manageable or safe for the rest of the body. Multidrug resistance is manifest in patients whose tumor growth initially responded to chemotherapy, but then shows a loss of response after the initial treatments [84].

Several cellular factors have been attributed to causing multidrug resistance including a decreased influx of drugs, an increased efflux of drugs (ATP dependent efflux pumps), activation of DNA repair, activation of detoxifying systems (cytochrome P450), and blocked apoptosis (e.g. decreased ceramide levels) [12]. While more than one resistance mechanism can be present in any cancer cell population exposed to chemotherapeutics, one of the most studied and most common mechanisms of multidrug resistance is an elevated expression of drug efflux pumps [12-15]. The drug efflux pumps are energy dependent transmembrane proteins that are capable of transporting a broad spectrum of unwanted compounds across the cell membrane [12]. Increased numbers of drug efflux pumps lower the intracellular drug concentration quickly enough so that the drug is unable to have a cytotoxic effect. Consequently, the sensitive cancer cells will die after a chemotherapy treatment, while the multidrug-resistant (MDR) cancer cells will survive, increasing the fraction of cells with MDR characteristics. Cancers that develop resistance in response to treatment are said to have acquired resistance. Some cancers, however, have an intrinsic overexpression of efflux pumps and are said to have primary or natural drug resistance [85].
2.5.1 Current Treatments to Overcome MDR

There have been multiple investigations to develop drugs that can inhibit, circumvent, or overwhelm the efflux transporters of MDR cancer cells. The most common transporter studied is the permeability glycoprotein (P-gp) [86]. Cancers with low initial levels of P-gp expression show elevated levels of expression after commencement of chemotherapy. Furthermore, some cancers that are intrinsically resistant to chemotherapy overexpress P-gp without any prior exposure (i.e., renal, adrenocorticoid, hepatocellular, pancreatic, and colorectal carcinomas) [85].

Reversing multidrug resistance by inhibiting P-gp has been studied for more than two decades [87]. There are currently three different generations of inhibitor compounds for the P-gp pump with the classification based on their affinity for the transporters and the relative side effects [15]. The first generation of P-gp inhibitors needed a high concentration of the inhibitor that also resulted in an unacceptably high systemic toxicity. These included verapamil, quinine, quinidine, cyclosporin A, nifedipine, tamoxifen and others, all of which were drugs that were approved for other medical purposes but were found to inhibit P-gp as well [88]. However, the clinical trials did show enough promise and encouraged the second generation of less toxic inhibitors, most being analogues of first generation inhibitors [15].

Second generation inhibitors include Valspodar (cyclosporin D analogue), dexverapamil (R-enantiomer of verapamil), dexnigulpidine (structural analog for nifedipine), cinchonine (structural analog for quinidine) and toremifene (structural analog for tamoxifen). The second generation inhibitors are more potent and selective, but clinical trials were generally disappointing. These include the clinical trials that were halted due to elevated intrinsic toxicity (dexverapamil) and poor efficacy (dexnigulpidine) [88].
The third generation inhibitors are the most specific and only require nanomolar concentrations. They are compounds designed with specific characteristics—such as lipophilicity, positive charge at neutral pH, and/or the presence of aromatic rings—that are able to overcome problems seen with the first and second generation compounds [88]. These inhibitors include biricodar, laniquidar, zosuquidar, elacridar, and tariquidar [87]. As of 2016, these inhibitors still show unwanted inherent toxicities and pharmacokinetic interactions in clinical trials, and none of them have yet found general clinical use [89].

In short, despite decades of research and multiple clinical trials, no P-gp inhibitors are currently in clinical use. The problems associated with poor performance of P-gp inhibitors include poor specificity, low affinity for the binding site, interference with the physiological role of P-gp, and interference with the pharmacokinetics of the associated chemotherapy [87]. One major problem with systemic delivery of these inhibitors is that they will affect not only the cancer cells, but normal cells as well, resulting in a large number of negative side effects.

Since shutting down P-gp pumps has yet to be proven “safe and effective” in killing MDR cancer cells, another approach is to deliver the drug into the cell faster than it can be pumped out. In this research, my goal was to very quickly load the cell cytosol with drug by triggering the release of the drug from a liposomal carrier once the carrier was endocytosed by a cancer cell. Slowing the removal of drug by inhibiting the P-gp pumps would help accomplish this goal.

### 2.5.2 GNR Heating to Overcome MDR

No currently published articles were found that used GNRs to treat multidrug-resistant cancer cells by releasing drug from a liposome or vaporizing an emulsion droplet. There are a
few articles, though, that use laser heating via GNRs to facilitate the release or uptake of Dox into MDR cells [90-93]. Zhang et al. [92] and Qiu et al. [90] developed different drug delivery vehicles, but both trapped Dox inside of DNA and attached the complex directly to GNRs. The Dox was released when the DNA denatured after GNR heating. Wang et al. [91] used the heating of silica coated GNRs to increase the uptake of Dox in a Dox-resistant breast cancer cell line. Zhong et al. [93] developed a Dox-loaded GNR-cored micelle to treat MDR cells. GNR heating increased the local temperature of the micelles above the phase transition temperature of the poly(e-caprolactone) portion of the block copolymer making up the micelle. My research attempted to use GNR heating to vaporize emulsion droplets located inside of a Dox-loaded liposome in order to trigger cytosolic delivery. Results are presented in Chapter 6.

### 2.5.3 Ultrasound Used to Overcome MDR

Using ultrasound to induce a phase change in emulsions for drug delivery is a current area of research [94-98], as are using microbubbles and US to deliver drugs to MDR cells [99-104], using liposomes in an effort to overcome MDR [105-110], and using US to overcome MDR [111-118]. However, there is currently only one published article (Deng et al. [119]) which uses US, liposomes, and microbubbles to deliver drugs in an effort to overcome MDR. Deng et al. [119] used a construct developed by Kheirolomoom et al. [120] of liposomes attached to a PFC microbubble by an avidin-biotin linker (Figure 3). Doxorubicin was loaded into the liposomes and their drug delivery vehicle was administered in vitro to a breast cancer cell line resistant to doxorubicin (MCF7/ADR). The average size of their construct was about 2 µm, which is too large to passively accumulate at tumor sites, and without any targeting moiety attached to the construct there is no means of either actively or passively targeting cancer cells. Nevertheless, the authors found that there was a reduction in viability of the MDR cells in vitro.
when US was applied in conjunction with their liposome-microbubble construct. Their construct depends on the ability of the ultrasound and cavitation of the microbubble to cause pores in the cell membrane of the cancer cells, making it easier for doxorubicin to enter the cell. Their study hinted that rapid delivery to the cytosol could overcome or bypass MDR mechanisms. The method presented in this dissertation is novel in that it will deliver the drug directly to the cytosol of MDR cells using a different design than Deng et al. used but similar concepts of ultrasound-triggered release. We hypothesize that rapid delivery directly to the cytosol of the cell will be more effective at killing the MDR cancer cells.

![Figure 3. Schematic illustration of the liposome–microbubble construct developed by Kheirolomoom et al. Deng et al. loaded Dox in the liposomes and used US to trigger the release of Dox to MDR cells. Figure used by permission from ref [120].](image)

### 2.6 eLiposomes (folated)

Javadi et al. developed a method to encapsulate PFC nanoemulsions inside of liposomes [66]. Briefly, lipids were dried onto the bottom of a round bottom flask and then rehydrated in the desired buffer. As mentioned in Section 2.1, liposomes spontaneously form in aqueous solutions with phospholipids. Ultrasound was used to reduce the size of the liposomes. The liposomes were then extruded through a 200-nm filter to achieve a uniform distribution of unilamellar liposomes. In a separate container, US was used to form emulsions of
perfluorocarbons which were extruded to 100 nm. The emulsions and liposomes were mixed together in an equivolume ratio and sonicated on ice for 3 cycles of 30 s with 1 min pauses. The lipid bilayer of the liposomes continually breaks and reforms around the emulsions, thus forming eLiposomes (liposomes with emulsion droplets inside).

The eLiposomes were separated from the empty liposomes and free emulsions using a pillow density separation technique. The eLiposome mixture was put in an Eppendorf tube, and then NaCl, glucose, and sucrose (in that order) were carefully pipetted to the bottom of the tube. After centrifugation (3000 rpm for 10 min) the eLiposomes collected between the glucose/sucrose interface due to differences in density. Folate was attached by incubating eLiposomes with PEG-folate micelles (synthesis described in [66]) for 1 hour (20 µL of micelles for 1.5 mL of eLiposomes).

Lattin et al. investigated the release of calcein (a model drug) from eLiposomes [121, 122] and found that increased exposure time, intensity of ultrasound, and droplet size all increased the release of calcein from eLiposomes. Javadi et al. also showed that cytosolic delivery of calcein to HeLa cells (not a MDR cell line) via folated eLiposomes can be controlled through the application of ultrasound [9, 66]. The research presented in this dissertation attempted to use ultrasound to control the cytosolic delivery of doxorubicin to multidrug-resistant cancer cells.

### 2.7 Loading of Drugs into Liposomes

Liposomes are versatile drug carriers in that they can entrap hydrophilic drugs in their aqueous core and/or hydrophobic drugs in their bilayer. For liposomes made via the film hydration technique, lipids are dried in a thin film on a flask and then rehydrated with another solution. Drugs can be loaded during the synthesis of the liposomes (passive loading) by simply
having the drug in the solution used to rehydrate the liposomes [123]. Javadi et al. [9] and Lattin et al. [122] used passive loading to encapsulate calcein inside of eLiposomes. Passive loading of drugs is an easy method to encapsulate drugs in liposomes; however, drugs loaded this way have a relatively low encapsulation efficiency (<30%), resulting in a significant loss of unused drugs [123]. Haran et al. developed a method to load amphipathic weak bases in liposomes using a pH gradient [124]. Liposomes are formed by rehydration in an acidic buffer. Then the external fluid is replaced by a solution with a neutral pH via a size-exclusion column. Drug in neutral solution is then added to the neutral suspension of liposomes, and the neutral form (uncharged) of the drug can diffuse into the liposome across its bilayer. Once inside, the acidic environment protonates the drug and the charged drug can no longer cross the membrane. Often, the drug precipitates and becomes trapped inside of the liposome. This is the most common method used to encapsulate Doxorubicin inside of liposomes.

2.7.1 Doxorubicin

Anthracyclicines are among the most effective cancer drugs developed. Doxorubicin (Dox) (see Figure 4) was one of the first anthracyclicines developed and is one of the anthracyclicines currently approved for clinical use [125]. There are currently three commercialized liposomal drug formulations containing Dox (Doxil, Lipo-Dox, and Mycoet) [19]. Dox is used to treat multiple types of cancer including breast cancer, Kaposi’s sarcoma, and ovarian cancer [125]. However, problems with its use include severe cardiotoxicity [125], multidrug resistance [126], and typical side effects characteristic of all cancer drugs including hair loss, nausea, fatigue, etc. Dox induces apoptosis through its intercalation of DNA in the nucleus of cells [127].
Doxorubicin is loaded into liposomes using remote loading via a transmembrane pH gradient demonstrated by Haran et al. [124]. The method used in this dissertation is based on the work from Lin et al. [98], which was modified from Haran et al. [124]. Briefly, the internal compartment of the eLiposomes contains ammonium sulfate buffer with a pH of 4.5, while there is a buffer containing free Dox (doxorubicin hydrochloride, Dox-NH$_3$Cl) at a neutral pH (7.4) external to the liposome. The Dox-NH$_3$Cl is incubated with the eLiposomes. Although the Dox-NH$_3^+$ cationic form cannot cross the liposomal bilayer, at neutral pH, the proton is removed and the neutral doxorubicin (Dox-NH$_2$) can diffuse across the liposomal bilayer. Once inside of the liposome, the neutral doxorubicin (Dox-NH$_2$) will become protonated, react with the sulfate, precipitate [(Dox-NH$_3$)$_2$SO$_4$], and become trapped inside of the liposome (Figure 5). Li et al. [129] and Johnston et al. [130] show via cryo-microscopy that Dox will form fiber bundles as the concentration of Dox inside of the liposome increases. The concentration of Dox can be determined using a spectrophotometer and measuring the absorbance at 488 nm [98].
2.8 Cancer Cell Lines

2.8.1 MCF-7 Cells

Michigan Cancer Foundation (MCF)-7 cells are a line of human breast cancer cells. Doxorubicin is commonly used to treat MCF-7 cells [119, 131, 132]; however, MCF-7 cells can develop MDR after exposure to doxorubicin [119]. Folate is also used to target MCF-7 cells via folate-mediated endocytosis [133].

2.8.2 KB Cells

KB cells are a derivate of HeLa cells [134, 135] and have a high expression of folate receptors [136], many times higher than MCF-7 cells. Doxorubicin is also used to treat KB cells [137]. Dr. Gottesman and colleagues at the National Institute of Health (NIH) have developed a MDR strain of KB cells (KB-V1) through culturing a sensitive KB strain (KB-3-1) in increasing concentrations of vinblastine [138]. KB-3-1 cells were derived from KB epidermal carcinoma cells (ATCC, Manassas, VA) after two subclonings [134]. Shen et al. showed that KB-V1 cells are resistant to vinblastine, cochinine, and Dox [138].

Figure 5. Schematic representation of Dox encapsulation process in eLiposomes using a transmembrane pH gradient.
3 OBJECTIVES

The overall objective of this research was to develop a drug delivery method to overcome the acquired resistance of multidrug-resistant cancer cells. As mentioned in Section 2.5, the development or inherent nature of multidrug resistance in cancer cells is a significant obstacle in treating cancer. This resistance often drives the required effective dosage of chemotherapy drugs to a toxic level. The systemic administration of inhibitors to reverse multidrug resistance is still problematic due to many unwanted side effects. The use of localized and controlled drug delivery will significantly decrease unwanted side effects, and increase the effectiveness in cancer treatment. The specific goals of this research were to:

I. Investigate the effectiveness of triggered drug release in the cytosol of MDR cells
   a. Confirm the stability of eLiposomes at temperatures above the normal boiling point of perfluoropentane and investigate the mechanisms of calcein release from eLiposomes at the higher temperatures
   b. Determine the feasibility of controlled release of drugs from targeted eLiposomes using gold nanorod heating via lasers
      i. Synthesize gold nanorods and load them into targeted eLiposomes
      ii. Use a low-powered continuous wave laser to vaporize emulsion droplets
      iii. Deliver model drugs to the cytosol of MDR cells
   c. Determine the cytotoxicity of drug-loaded targeted eLiposomes using
i. Gold nanorod heating via lasers

ii. Ultrasound

II. Investigate the enhanced killing of MDR cells by co-delivering an inhibitor with the drug-loaded folated eLiposomes

It was our hypothesis that drugs delivered directly to the cytosol of MDR cells will be able to kill the resistant cells as effectively as, or better than, the action of the free drug against the non-resistant cells. The delivery of cancer drugs directly to the cytosol of cells via vaporization of emulsion droplets inside of targeted liposomes provides a novel way to treat multidrug-resistant cancer cells. We reasoned that localizing the drug to the cytosol would minimize the unwanted side effects as well as decrease the effective systemic dose of current cancer treatments. This delivery method may then provide the ideal location for any therapeutic that needs to be delivered to the cytosol or that can be easily transported from the cytosol to the nucleus.
4 MATERIAL AND METHODS

4.1 Materials and Equipment

4.1.1 Materials

The phospholipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphate, sodium salt (DPPA) were purchased from Echelon Biosciences, Inc (Salt Lake City, UT). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000], ammonium salt (DSPE-PEG-NH₂) was obtained from Laysan Bio, Inc. (Arab, AL). Dodecafluoro-n-pentane (PFC5) was purchased from SynQuest Laboratories (Alachua, FL). Calcein was obtained from MP Biomedicals, LLC (Solon, OH). Phosphate buffered saline, 10x solution (PBS), sodium chloride (NaCl), sodium hydroxide (NaOH), tetrahydrofuran (THF), pyridine, ammonium sulfate ((NH₄)₂SO₄), sodium lauryl sulfate (SDS), and Whatman® Nuclepore Track-Etch Membrane filters (19 mm diameter) were purchased from Fisher Scientific (Hampton, NH). Sodium borohydride, powder 98+% (NaBH₄) was purchased from Acros Organics (New Jersey, USA). Dulbecco’s modified eagle medium (1X) (DMEM)(+ 4.5 g/L D-glucose, + L-glutamine, - sodium pyruvate), RPMI 1640 (1X) (+ L-glutamine, + phenol red, - folic acid), penicillin streptomycin (Pen Strep), and fetal bovine serum (FBS) were purchased from Gibco® by Life Technologies (Grand Island, NY). 1,1'-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Molecular Probes™ by Life
Technologies (Eugene, OR). Trypsin-EDTA was purchased from Invitrogen (Carlsbad, CA). Sucrose, chloroform, sulfuric acid (H₂SO₄), L-ascorbic acid, and silver nitrate (AgNO₃) were purchased from Avantor Performance Materials, Inc (Phillipsburg, NJ). Glycerol was purchased from MilliporeSigma (Billerica, MA). Nitrogen gas was purchased from Airgas (Salt Lake City, UT). Doxorubicin HCl injection, USP (10mg/mL) was purchased from Pfizer (New York, NY). Vinblastine sulfate injection (1 mg/mL) was purchased from APP Pharmaceuticals, LLC (Schaumburg, IL). Cholesterol (powder, BioReagent, suitable for cell culture, ≥99.0%), α-D-glucose, gold(III) chloride trihydrate (HAuCl₄), hexadecyltrimethylammonium bromide (CTAB) (BioUltra, for molecular biology, ≥99.0%), 1H,1H,2H,2H-perfluorodecanethiol (PFD-SH), folic acid (≥97%), ninhydrin, (±)-verapamil hydrochloride (≥99% (titration), powder), dimethyl sulfoxide, ≥99.9% (DMSO), methanol, N,N'-dicyclohexylcarbodiimide, 99% (DCC), dichloromethane (CH₂Cl₂), L-glutamic acid potassium salt monohydrate (≥99%, (HPLC), powder), and poly(ethylene glycol) methyl ether thiol (Average Mn, 2000) (mPEG-SH) were purchased from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid SVS was purchased from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ). Dimethyl sulfoxide-D6 (D, 99.9%) +0.05% V/V TMS DLM-10TB-10 was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Poly-L-lysine (MW 30-70kD) 12 mm round, No. 1 German glass coverslips were purchased from Corning Inc. – Life Sciences (Oneonta, NY). Sephadex G-25 columns were purchased from GE Healthcare Life Sciences (Pittsburgh, PA). KB-3-1 and KB-V1 cells were kind gifts from Dr. Michael Gottesman (NIH, Bethesda, MA). Greiner Bio-One CELLSTAR® 24-well cell culture plates were purchased from BioExpress (Kaysville, UT). Cell culture flasks were purchased from Sarstedt AG & Co (Nümbrecht, Germany). Borosilicate micro glass cells (1 mm I.D., 50 mm long) were purchased from Friedrich and Dimmock, Inc. (Millville, NJ). All
water used was deionized water distilled through a Corning Mega-Pure™ MP-1 Glass Still (ddH\(_2\)O).

### 4.2 Methods

The experiments done in this work require the creation of emulsions, liposomes, eLiposomes and gold nanorods. I will first explain the methods to create eLiposomes loaded with the fluorescent dye calcein (Section 4.2.1) and the experiments to measure the release of calcein. The calcein-loaded eLiposomes were used observe the stability of eLiposomes at body temperature (37°C) over time and the results are presented in Chapter 5. I will then explain the methods used to synthesize gold nanorods and to transfer them to perfluoropentane (Section 4.2.2). The results of the gold nanorod experiments are presented in Chapter 6. In Section 4.2.3, I will explain the methods used to created doxorubicin-loaded folated eLiposomes (folated eLipoDox) and the experiments used to observe cellular uptake and cytotoxicity of folated eLipoDox. The results of these experiments are presented in Chapter 7. Finally, I will describe the methods used to measure the enhanced cytotoxicity of co-delivering folated eLipoDox with verapamil. The results of the co-delivery experiments are presented in Chapter 8.

#### 4.2.1 Calcein Release Experiments (Ch. 5)

##### 4.2.1.1 PFC5 Emulsion Droplet Formation

Calcein loaded eLiposomes were synthesized as described in [139]. Briefly, DPPA in chloroform was dried on a flask under vacuum using a rotary evaporation and was subsequently rehydrated in PBS (5 mg/mL). Once the flask was cooled in an ice-water bath, PFC5 (0.1g/mg DPPA) was added to the flask. The solution was then sonicated on ice with a 20 kHz probe (Sonics and Materials, CVX400, Newton, CT) at 1 W/cm\(^2\) for 1 min. The DPPA coated PFC5
emulsion droplets were filtered (LiposoFast™, Avestin, Ottawa, ON, Canada) through a 100-nm filter.

4.2.1.2 Liposome Formation

Liposomes were synthesized by drying DPPC, cholesterol, and DSPE-PEG(2000) amine onto a glass round bottom flask in a 3:1:1 molar ratio. The lipids were then rehydrated in PBS (50 mg/mL) and filtered through a 200-nm filter to make a uniform distribution of unilamellar liposomes.

4.2.1.3 Calcein eLiposomes

Calcein-loaded eLiposomes were made by mixing 2 mL of 30 mM calcein with 1 mL of 100-nm DPPA-coated PFC5 emulsion droplets and 1 mL of 200-nm liposomes. This solution was sonicated on ice for three 30-s intervals with 60-s pauses between each sonication. External calcein was removed by centrifugation (2500 rpm, Eppendorf 5414C, Hamburg, Germany). The supernatant was discarded and the eLiposome pellet was resuspended in 1.5 mL of PBS. This washing procedure was repeated twice more to remove nearly all of the external calcein.

Calcein-loaded eLiposomes were separated from external emulsion droplets and empty liposomes using a pillow density separation technique. 0.3 mL eLiposome mixture was pipetted into a 1.5 mL centrifuge tube. NaCl, glucose, and sucrose (~0.3 mL each) were then slowly added to the bottom of the centrifuge tube using a Pasteur pipet in order of increasing density, and centrifuged for at 3000 rpm for 10 min. Calcein-loaded eLiposomes accumulate between the sucrose and glucose layers, whereas empty liposomes remain between the NaCl and glucose layers. The external emulsion droplets amass at the bottom (below the sucrose layer).
4.2.1.4 *Calcein Release from eLiposomes*

Calcein concentration inside of the eLiposomes was estimated to be 15 mM, which is in the self-quenched region and thus has minimal fluorescence. The fluorescence of calcein increases as calcein is released from eLiposomes, and the concentration of calcein is proportional to fluorescence. For these experiments 30 µL of calcein-loaded eLiposomes were mixed with 2 mL of PBS and placed in a cuvette in a QuantaMaster fluorometer (Photon Technology International, Birmingham, NJ, USA). Temperature was kept constant and the fluorescence was continuously recorded with the excitation and emission wavelengths set at 488 and 520 nm, respectively.

4.2.2  **Gold Nanorod Experiments (Ch. 6)**

4.2.2.1  **GNR Synthesis**

Gold nanorods were synthesized using a slightly modified procedure to the synthesis presented by Nikoobakht et al. [140]. Briefly 0.28 mL of AgNO₃ (0.004 M) was added to 5 mL of aqueous CTAB (0.2 M) and placed in a 28°C water bath (growth solution). In another vial, 5.0 mL of HAuCl₄ (0.5 mM) was added to 5 mL of CTAB (0.2 M) (seed solution). 600 µL of ice cold NaBH₄ (0.01 M) was added to the seed solution and stirred for 2 min. The seed solution was then placed in the water bath and 5 mL of HAuCl₄ (1 mM) was added to the growth solution and stirred for 2 min. 70 µL of ascorbic acid (0.0788 M) was then added to the growth solution. 13.35 µL of the seed solution was then added to the growth solution and stirred for 2 min before being placed in the water bath. The growth solution remained in the water bath overnight before taking an absorption spectrum (Beckman Coulter DU-640 UV, Fullerton, CA).
4.2.2.2 GNR Phase Transfer

Once the GNRs were synthesized, we needed to transfer them to PFC5. Gold nanorods were transferred from an aqueous phase to PFC5 using a modified procedure of Wilson et al. [59]. Briefly, 5 mL mPEG-SH (2.0 mg/mL) was added to 5 mL of the GNR growth solution. This solution was sonicated (1.25 W/cm², 20 kHz, Sonics and Materials, CVX400, Newton, CT) for 2 min at room temperature (~23°C). The solution was left at room temperature for 2 hours in the dark before being centrifuged at 11,000 rpm for 10 min (Eppendorf 5414C, Hamburg, Germany). The GNRs were resuspended in 5 mL of ddH₂O and the solution was again sonicated for 2 min and allowed to sit for 1 hour at room temperature in the dark. The GNR solution was then centrifuged again (11,000 rpm, 10 min) and resuspended in 5 mL of THF. The previous sonication and washing steps were designed to replace the CTAB on the surface of the GNRs with mPEG-SH. Five mL of PFD-SH in THF (3 µL/5 mL) was added to the GNR solution. This solution was then sonicated in an ultrasonic bath (120V, 50/60Hz, Sonicor Instrument Corporation, Copiague, NY) for 30 min at room temperature, and 60 min at 50°C. The vials were then left overnight in the dark. This part of the procedure was done to replace some of the mPEG-SH or remaining CTAB with the PFD-SH. The GNR solution was then centrifuged for 10 min at 11,000 rpm, and the GNRs were resuspended in 5 mL of CH₂Cl₂. One mL of the GNRs was then placed in a separate vial and the CH₂Cl₂ was evaporated with a flowing stream of nitrogen gas. A pink residue was left on the bottom edges of the vial. Then 0.25 mL of PFC5 was added to the vial, and the solution was sonicated in the ultrabath sonicator for 3 min at room temperature. Two mL of DPPC in ddH₂O (0.2 mg/mL) was then added to the GNR-PFC5 solution and the solution was probe sonicated for 20 s at 1 W/cm² at room temperature.
4.2.2.3 PFC5 Vaporization Using GNR Heating

The PFC5-GNR emulsion droplet solution was placed inside of a borosilicate micro glass cell and irradiated with either a 450 mW continuous wave (cw) laser (785 nm, BWF1 series, B&W TEK Inc., Newark, DE) in a chamber at 37°C, or a pulsed laser (mode-locked ti:sapphire laser capable of producing pulses of about 30 femtoseconds, Dr. John Colton’s lab, BYU) at room temperature. No observable vapor bubbles were observed after laser irradiation.

4.2.3 Folated eLipoDox Experiments (Ch. 7)

4.2.3.1 DSPE-PEG-Folate Synthesis

Folate was conjugated to DSPE-PEG-NH₂ using a previously described method [141] with slight modifications. Briefly, 16.7 mg of folic acid was dissolved in 0.667 mL of anhydrous DMSO. DSPE-PEG-NH₂ (66.7 mg) was dissolved in 0.333 mL of pyridine and added to the folic acid solution. N,N’-dicyclohexylcarbodiimide (DCC) (21.7 mg) was then added to the reaction mixture. Nitrogen gas was added to the round bottom flask and the round bottom flask was capped. The reaction proceeded under mild stirring for 4 hours at room temperature (~23°C) in the dark. Every hour the reaction progress was checked using thin layer chromatography on silica gel plates using a 75:36:6 chloroform/methanol/water mobile phase. Ninhydrin spray (0.2 g ninhydrin/100 mL ethanol) was used to confirm the disappearance of the amine on the DSPE-PEG-NH₂. The pyridine was then removed by rotary evaporation and 6 mL of ddH₂O was added to the mixture. The solution was centrifuged to remove trace insolubles and the supernatant was dialyzed in 3500 molecular weight Spectra/Por® (Spectrum Laboratories, Inc., Rancho Dominguez, CA) tubing against NaCl (50 mM, 2 x 700 mL) and then ddH₂O (3 x 700 mL). An equal amount of chloroform was added to the dialysate to extract the product (DSPE-PEG-folate). Hydrochloric acid was added to the aqueous phase to make the product more soluble in
chloroform, as was seen by the shift of the characteristic yellow of folate from the aqueous to chloroform phase. This chloroform phase was collected and stored at 4°C. H-NMR was used to confirm the synthesis of DSPE-PEG-folate (in DMSO-d6).

4.2.3.2 PFC5 Emulsion Droplet Formation

eLiposomes were synthesized using a modified procedure of Javadi et al. [66] in order to make larger sized eLiposomes. Briefly, DPPA in chloroform was dried on a flask using rotary evaporation and was subsequently rehydrated (5 mg/mL) in 129 mM ammonium sulfate (pH 4.5). Once cooled, PFC5 (0.02g/mg DPPA) was added to the DPPA solution. The mixture was then sonicated on ice with a 20 kHz probe (Sonics and Materials, CVX400, Newton, CT) at 1.25 W/cm² (30% amplitude setting) for five 1-min intervals with a 1-min pause between each sonication. The DPPA coated PFC5 emulsion droplets were extruded (LiposoFast™, Avestin, Ottowa, ON, Canada) through a 100-nm polycarbonate track-etched filter (Whatman® Nuclepore).

4.2.3.3 Liposome Formation

Liposomes were synthesized by drying 37.5 mg DPPC and 12.5 mg cholesterol onto a glass round bottom flask. For folated liposomes, 0.2 mL of the DSPE-PEG-folate in chloroform (see section 4.2.3.1) was added to the lipids before drying. The lipids were then rehydrated (50 mg/mL) in 129 mM ammonium sulfate (pH 4.5) and extruded through a 400-nm polycarbonate filter to make a uniform distribution of unilamellar liposomes.
4.2.3.4 **eLiposome Synthesis**

eLiposomes were made by mixing 1 mL of DPPA-coated PFC5 emulsion droplets and 1 mL of liposomes. This solution was sonicated (1 W/cm²) on ice for three 15-s intervals with 60-s pauses between each sonication. eLiposomes are separated from external emulsion droplets and empty liposomes using a pillow density separation technique as described in section 4.2.1.3. Briefly, eLiposomes followed by NaCl and then sucrose (~0.4 mL each) were added to a centrifuge tube and centrifuged at 3000 rpm for 10 min. The eLiposomes were collected and passed through a Sephadex G25 column to replace the external media with PBS (pH 7.4), establishing a transmembrane pH gradient.

4.2.3.5 **Doxorubicin Loading**

Doxorubicin was loaded into eLiposomes using a transmembrane pH gradient. An equal volume of Dox in PBS (0.1 mg/mL) was added to the eLiposomes and left for 18 hrs in the fridge (4°C). The solution was then pipetted and centrifuged for 10 min at 3000 rpm. The Dox loaded eLiposomes (eLipoDox) were resuspended in PBS and diluted to an absorbance value of 0.5 (Beckman Coulter DU-640 UV, Fullerton, CA) measured at 480 nm. This corresponds to a Dox concentration of 0.024 mg/mL.

4.2.3.6 **Dynamic Light Scattering and Zeta Potential**

Folated eLipoDox and non-targeted eLipoDox were sized using dynamic light scattering (NanoBrook Omni, Brookhaven Instruments Corporation, Holtsville, NY). The zeta potential of folated Liposomes and non-targeted liposomes were measured using phase analysis light scattering (NanoBrook Omni, Brookhaven Instruments Corporation, Holtsville, NY).
4.2.3.7 Doxorubicin Release Experiments

Experiments investigating the release of doxorubicin from eLiposomes using ultrasound (Sonics and Materials, CVX400, Newton, CT) were conducted using a QuantaMaster fluorometer (Photon Technology International, Birmingham, NJ, USA). Folated eLipoDox or non-targeted eLipoDox (20 µL) was added to PBS (2 mL) in a cuvette and gently mixed by re-pipetting. Fluorescence was measured using excitation and emission wavelengths of 475 nm and 588 nm, respectively. After 50 s, the reading was stopped, the cuvette was removed, and ultrasound was applied (1 W/cm², 20 kHz, 2 s total of US applied in 20 sec of 0.1-s pulses, 1:10 duty cycle). The cuvette was then replaced and the reading continued. This procedure was repeated one more time. SDS (30 µL) was then added to the solution and gently re-pipetted to release all of the Dox, and the fluorescence was then measured.

4.2.3.8 Cell Culture

KB-3-1 and KB-V1 cells were cultured in DMEM containing 10% FBS and 1% Strep Pen and incubated at 37°C, 5% CO₂. Vinblastine (3.5 µg/5 mL DMEM) was added to the KB-V1 cells growth media to maintain its multidrug resistance. Before experiments, cells were washed and grown for 48 hours in folate free RPMI media (10% FBS, 1% Step Pen), and no vinblastine was added to KB-V1 cells. Cells were seeded in a 24-well plate 24 hours before the addition of drugs at an approximate cell density of 1 x 10⁴ and 2 x 10⁴ for KB-3-1 and KB-V1 cells, respectively.

4.2.3.9 Confocal Experiments

KB-V1 cells were seeded at approximately 6 x 10⁴ cells on poly-L-lysine (MW 30-70kD) 12 mm round, No. 1 German glass coverslips in a 24-well plate. Cells had been growing for 48
hours in RPMI folate free media before the addition of folated or non-targeted eLipDox (0.2 mL, 0.5 Abs at 480 nm). Drugs were allowed to incubate (37°C, 5% CO₂) for 2 hours before the media was removed. Cells were then rinsed with 0.5 mL of ice-cold PBS. The PBS was removed and 1.0 mL of ice-cold methanol was added to the cells and the 24-well plate was kept on ice for 10 minutes. The methanol was removed and the cells were rinsed twice with ice-cold PBS. The cell-covered cover slips were then placed on plain selected precleaned VWR micro slides (25 x 75 mm, 1.0 mm thick, VWR International, LLC, Radnor, PA) using 5 µL of a 50% glycerol/ddH₂O solution. Confocal images were obtained using an Olympus FluoView FV1000 (Tokyo, Japan) confocal microscope.

4.2.3.10 MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to measure the metabolic activity of living cells [142]. It has a yellow color; however metabolic enzymes in living cells can reduce MTT to an insoluble formazan, which is purple. For cell experiments, 0.1 mL of MTT in PBS (5 mg/mL) was added to the cells (0.9 mL growth media) and incubated at 37°C, 5% CO₂ for 2 hours. The growth media containing MTT was removed and the formazan was solubilized using 1.0 mL of DMSO. After 15 min, the absorbance at 570 nm and 700 nm was measured using a Synergy™ MX multimode microplate reader (BioTek® Instruments, Inc., Winooski, VT). Seeding density for cells was optimized to obtain an absorbance value (570 nm) of approximately 1.0 for the PBS control.

4.2.3.11 IC₅₀ Experiments

Doxorubicin (0.2 mL) was added to the cells (1.0 mL growth media) in a 24-well plate to obtain Dox concentrations between 0.001 and 500 µM. PBS was used as a positive control. The
media was removed after 2 hours incubation (37°C, 5% CO₂) and the cells were rinsed with 0.5 mL of PBS. The PBS was then replaced with fresh DMEM (10% FBS, 1% Pen Strep). An MTT assay was performed 48 hours later.

**4.2.3.12 Ultrasound Experiments with Folated eLipoDox**

KB-3-1 and KB-V1 cells were seeded in a 24-well plate, and 0.2 mL of PBS, Dox, eLipoDox, or folated eLipoDox was added to cells (1.0 mL of RPMI). Dox, eLipoDox, and folated eLipoDox had an absorbance of 0.5 measured at 480 nm (Beckman Coulter DU-640 UV, Fullerton, CA), which corresponds to a final Dox concentration of 7 µM. Cells were incubated (37°C, 5% CO₂) for 2 hours with the drugs. To minimize the cellular damage or detachment of the cells from the bottom of the well during insonation, 1.8 mL of DMEM was added to the cells just before ultrasound was applied to some of the cells (1 W/cm², 20 kHz, 2 s total of US applied in 20 sec of 0.1-s pulses, 1:10 duty cycle). The probe was placed in the growth media so that the tip was just below the liquid surface. Following ultrasound, the media was removed and the cells were rinsed with 0.5 mL PBS to remove any drug not internalized by the cells. After removing the PBS, 0.9 mL of fresh DMEM (10% FBS, 1% Pen Strep) was added to the cells. An MTT assay was performed 48 hours after US.

**4.2.3.13 Dox Loading with Glutamate vs Sulfate**

Folated liposomes were synthesized as described in section 4.2.3.3 using either ammonium sulfate or potassium glutamate to rehydrate the liposomes and emulsion droplets (4.2.3.2). Otherwise, eLiposome synthesis proceeded as described in section 4.2.3.4. Doxorubicin was loaded into folated eLiposomes using a pH gradient as described in 4.2.3.5, except that the Dox solution added to folated eLiposomes had a concentration of 2 mg/mL.
instead of 0.1 mg/mL. The loading process was at 4°C for 17 hours, and then the folated eLipoDox solutions sat at room temperature (23°C) for 90 min.

4.2.4 Co-delivery of Folated eLipoDox and Verapamil Experiments (Ch. 8)

Folated and non-targeted eLipoDox were synthesized as described in section 4.2.3. Cell culture (4.2.3.8) and MTT assays (4.2.3.10) were also performed as described in section 4.2.3.

4.2.4.1 Optimal Verapamil Concentration

For determining the cytotoxicity of verapamil, KB-V1 and KB-3-1 cells were seeded in 24-well plates. After 24 hours, 0.1 mL of verapamil (0.003–3 mg/mL) was added to the cells (in 1.0 mL growth media) followed by 0.2 mL of PBS. The PBS was added as a substitute for the 0.2 mL of Dox that was added in the co-delivery experiments with Dox and verapamil. For the co-delivery experiments with Dox and verapamil, 0.2 mL of 50 µM Dox was added following the addition of verpamil. The drugs were allowed to incubate (37°C, 5% CO₂) for 2 hours before the media was removed. The cells were then washed with 0.5 mL of PBS. The PBS was removed and 0.9 mL of fresh DMEM (10% FBS, 1% Pen Strep) was added. The cells were incubated for 48 hours, and then the MTT assay was performed.

4.2.4.2 Co-delivery of Folated eLipoDox and Verapamil

Folated eLipoDox was synthesized and doxorubicin and folated eLipoDox were diluted to absorbance (480 nm) values of 0.5 (Beckman Coulter DU-640 UV, Fullerton, CA). A solution of verapamil in PBS (3 mg/mL) was also prepared. KB-V1 and KB-3-1 cells were cultured in folate free RPMI (10% FBS, 1% Pen Strep) and seeded in a 24-well plate. Verapamil solution or PBS (0.1 mL of either) was added to the cells (in 1.0 mL RPMI) followed by the addition of 0.2
mL of PBS, Dox or folated eLipoDox. The cells were incubated (37°C, 5% CO2) with the drugs for 2 hours. Then 1.8 mL of DMEM was added to the cells just before ultrasound was applied to some of the cells (1 W/cm², 20 kHz, 2 s total of US applied in 20 sec of 0.1-s pulses, 1:10 duty cycle). The probe was placed in the growth media so that the tip was just below the surface. Following insonation, the media was removed and the cells were rinsed with 0.5 mL PBS to remove any drug not internalized by the cells. After removing the PBS, 0.9 mL of fresh DMEM (10% FBS, 1% Pen Strep) was added to the cells. An MTT assay was performed 48 hours after insonation and washing.
5 RELEASE MECHANISM OF CALCEIN AT HIGHER TEMPERATURES

5.1 Introduction

Previous studies [9, 66] showed that eLiposomes were capable of delivering a model drug (calcein) to cells via ultrasound; however, the question remained as to the stability of the eLiposomes at temperatures above the normal boiling point of perfluoropentane (PFC5) (29°C). eLiposomes need to be stable at body temperature (37°C) in order to reach the target tumor without premature release of the therapeutic. Premature release can happen from the therapeutic diffusing through the liposomal bilayer or from vaporization of the PFC5 emulsion before ultrasound (US) is applied. Because the PFC5 emulsion droplet increases 125-fold in volume as it changes from a liquid to a vapor phase, and the bilayer can only sustain 3% expansion by area and maintain integrity[143, 144], vaporization of only a fraction of the emulsion droplet would be enough to rupture the liposomal bilayer and release the drug.

Perfluorocarbon (PFC) emulsion droplets have been shown to be stable at physiological temperatures [65, 145-148], although the temperature is above its normal boiling point. Many studies have attributed this phenomenon to the added Laplace pressure on the droplet, which will increase the temperature at which vaporization occurs and make the droplet stable at physiological temperatures. A recent study by Mountford and Borden [67], however, suggests that the added Laplace pressure on nanodroplets is not sufficient to explain the high temperatures
needed to vaporize PFC emulsion droplets. They contribute the stability of PFC droplets to the high energy barrier associated with homogeneous nucleation.

Experiments with Dr. Ghaleb Husseini were conducted to determine the thermal stability of eLiposomes by measuring the kinetics of calcein release at various temperatures. Calcein was loaded in eLiposomes at a concentration of 15 mM, which quenches calcein fluorescence. Calcein release at temperatures above (37°C, 49°C, 59°C) and below (25°C) the natural boiling point of PFC5 was measured using a QuantaMaster fluorometer (Photon Technology International, Birmingham, NJ, USA). No ultrasound was applied in these experiments. The purpose of these experiments was to investigate the thermal stability of eLiposomes at temperatures above the normal boiling point of PFC5. Results from these experiments were published in the Journal of Colloid Sciences and Biotechnology [139] and are presented below.

5.2 Results

Figure 6 shows the results of the experiments done to determine the stability of eLiposomes as a function of temperature. The release of calcein from the eLiposomes is depicted as a function of time for four different temperatures – 59, 49, 37, and 25°C. A rapid release of calcein from eLiposomes was observed at 59°C and 49°C (89% and 57 % after 1 hour, respectively), but minimal release was observed at body temperature (37°C) or room temperature (25°C) (8% and 5% after 1 hour, respectively). The latter temperature is below the normal boiling point of PFC5 (29°C), but the former temperatures are significantly above the normal boiling point. The stability of the PFC5 emulsions above its normal boiling point, specifically at 37°C, was previously attributed to the Laplace pressure, which increases the internal pressure inside the droplet such that the vapor pressure remains below the local (internal) pressure and no
gas forms [149]. However, Mountford and Borden suggest that their data are more consistent
with the high energy barrier associated with homogeneous nucleation [67]. We postulate and
discuss three possible causes for the rapid release observed at 49°C and 59°C, namely: 1)
increasing temperature increases the passive diffusion of calcein across the liposomal bilayer 2)
the phospholipids in the bilayer experience a membrane transition at its transition temperature 3)
the liposome is ruptured by the formation of a gas bubble. Below, we test these postulates against
the experimental evidence to reject or accept possible physical mechanisms at play in this drug
delivery system.

![Graph showing fraction of calcein release over time at different temperatures](image.png)

Figure 6. Average fraction of calcein release from eLiposomes incubated at various

5.3 Discussion

5.3.1 Temperature Dependent Diffusivity

For the first postulate, we assume that the temperature dependence of the diffusivity of
calcein through the lipid bilayer membrane can be modeled by an Arrhenius-type equation,

\[ D = D_0 \exp \left( \frac{-E_d}{RT} \right) \] (5-1)
where $D$ is the diffusivity coefficient, $D_o$ is the pre-exponential constant, $E_d$ is the activation energy for diffusion, $R$ is the universal gas constant, and $T$ is the absolute temperature. Initial release rates, which are proportional to $D/D_o$, were calculated and the negative of the natural logarithms of the release rates were plotted versus the inverse of absolute temperature. The data were then fit using a linear equation (see Figure 7). Using the slope of the fitted line, the activation energy for diffusion was estimated to be 110 kJ/mol.

![Figure 7](image-url)

Figure 7. Negative of the natural logarithms of the initial fractional calcein release rate versus inverse temperature. The line is a linear regression of the data points.

Work done by Maherani et al. [150] who investigated the temperature dependency of the permeability of DPPC-liposomes to calcein provides data from which the activation energy for diffusion was calculated to be 51 kJ/mol. Although this activation energy is approximately 50% lower than what we deduced in our experiments, it is quite significant and thus casts doubt upon the first above-mentioned hypothesis. To illustrate the significance of this difference, at 15 min of incubation, Maherani et al. reported just over 1% release of calcein at 50°C, whereas in this work just over 20% release was observed at 49°C after 15 min. This is 20-fold faster release than
published data of liposomes with no internal PFC droplets. Therefore, although the data apparently fits Eq. 5-1, the value of the activation energy is so high that it is unlikely that this is the sole or even primary mechanism of calcein release.

5.3.2 Membrane Transition Temperature

As for the second postulate, several studies have shown that liposome permeability has a local maximum at the lipid melting transition temperature, so an increase in calcein release as a result of membrane transition would show a maximum at the transition temperature on a plot of rate vs. temperature [151, 152]. The membrane melting transition temperature for DPPC is 41°C [150]. As seen in Figure 8, the data do not have a maximum release at the transition temperature that would be expected if release was solely based on membrane transition and if there were

![Figure 8. Plot of initial fractional calcein release rates versus temperature. The dotted vertical line represents the literature value of the transition temperature for DPPC (41°C). The solid line is a representation of expected changes in membrane permeability [151] for passive transport across phospholipid bilayers. The points are the experimental data.](image-url)
changes in membrane diffusivity as it changed from gel to liquid crystal states of the bilayer. The rate actually appears to increase exponentially as a function of temperature (See Figure 7). Therefore, it is also unlikely that the membrane transition of the phospholipids is the sole mechanism of calcein release.

5.3.3 Homogeneous Nucleation

The third postulate consists of gas bubble nucleation above the normal boiling point and involves fairly complex theory. Firstly, the apparent boiling point of the PFC5 droplet increases due to the additional pressure imposed by the Laplace pressure ($\Delta P_{lp}$). The Laplace pressure is an additional pressure imposed on the interior of a sphere due to the curved interface, and is a function of the interfacial energy ($\gamma$) and the radius ($R$) of the emulsion droplet: $\Delta P_{lp} = \frac{2\gamma}{R}$. The total pressure of the emulsion droplet is the sum of atmospheric pressure ($P_{atm}$), the hydrostatic pressure ($P_{hyd}$), and the Laplace pressure. If gas nucleation sites are present, a gas bubble may form when the total pressure inside the nanoemulsion (left side of Eq. 5-2) drops below the local vapor pressure:

$$P_{atm} + P_{hyd} + \Delta P_{lp} \leq P_{vap}(T)$$  \hspace{1cm} (5-2)

Ignoring any hydrostatic pressure ($\rho g \Delta h$), if one takes the interfacial energy of the phospholipid-coated PFC nanoemulsion to be 3.8 dyn/cm [153], and a nanoemulsion with a diameter of 100 nm, the boiling point will increase from 29°C to approximately 57°C. If the droplet were 200 nm, the apparent boiling point would only be 45 °C. Emulsions for this study may range from 100 nm or lower to about 200 nm in diameter; therefore, the first bubble could form once the temperature reached somewhere between 45°C to 57°C, with more bubbles likely to form as the temperature increases.
However, nucleation must also occur in order for a gas phase to be formed. According to homogeneous nucleation theory, the number of nucleation events occurring at a given temperature for a given unit volume per unit time can be approximated with the following equation:

\[ J(T) = J_o \exp \left( \frac{-E_c}{k_b T} \right) \]  

where \( J_o \) is a proportionality constant that has a weak dependence on temperature, \( E_c \) is the activation energy barrier for the formation of a nucleating cavity of a critical radius \( (r^*) \), and \( k_b \) is Boltzmann’s constant [154]. A derivation of \( J(T) \) is given in Section 5.3.3.1. If we assume that the release rate of calcein is proportional to the number of nucleation events occurring, then the two events (nucleation and release) should follow the same trend. As seen in Figure 9, this is somewhat observed. The nucleation of gas bubbles begins about 10°C above the apparent boiling point. The nucleation rates increase exponentially at first and then continue to increase linearly. While the trends of homogeneous nucleation theory and the experimental data somewhat match, the experimental data begin to increase at an earlier temperature than homogeneous nucleation theory indicates. This suggests that there are emulsion droplets much larger than 200 nm, or that the theory is not sufficient.

Mountford and Borden investigated the spontaneous vaporization of 800 nm perfluoropropane (PFC3) and perfluorobutane (PFC4) droplets. Their results indicate that although the release of calcein appears to coincide with the boiling point of 100-200 nm PFC5 droplets adjusted for local Laplace pressure (45-57°C), spontaneous vaporization of PFC droplets occur at approximately 90% of the critical temperature \( (T_c) \) [67]. According to Mountford and Borden, spontaneous vaporization of PFC5 should occur at approximately 107°C \( (T_c=423.1K, [71]) \). In our experiments, calcein release is seen at a much lower temperature than
can be explained by homogeneous nucleation, therefore we turn to heterogeneous nucleation theory in Section 5.3.4.

Figure 9. Plot of initial fractional calcein release rates (left axis) and rate of nucleation events (right axis) versus temperature. The points indicate the experimental data and correspond to values on the left axis. The dotted line (d = 200 nm) and solid line (d = 100 nm) are theoretical homogeneous nucleation data and correspond to values on the right axis.

5.3.3.1 Derivation for Homogeneous Nucleation

The following derivation was used to provide the theoretical homogeneous nucleation data shown in Figure 9. Blander [155] provides a derivation for obtaining the nucleation rate, \( J \), for homogeneous nucleation in the form shown in Eq. 5-3. \( J_0 \) is a proportionality constant assumed to be the number density of the liquid and is given by the following equation

\[
J_0 = N \left( \frac{2\gamma_g}{\pi m} \right)^{1/2}
\]

(5-4)

where \( \gamma_g \) is the interfacial energy of the gas bubble with radius \( r^* \), \( N \) is the number density (molecules/m\(^3\)) of liquid PFC5, and \( m \) is the molecular mass of PFC5 [154].
The interfacial energy is a function of temperature and was taken from the DIPPR database [71]. The dependence on temperature for $J_o$ is weak compared to the terms inside of the exponent in Eq. 5-3.

There are two factors that contribute to the energy required ($E$) to form a gas cavity within a liquid droplet: 1) surface energy of the cavity formed, and 2) free energy change during vaporization. The surface energy of the nucleus formed is calculated as the interfacial energy of the gas bubble, $\gamma_g$, multiplied by its surface area, $4\pi r^2$, where $r$ is the radius of the gas bubble. The free energy change during vaporization can be accounted for by the free energy of vaporization, $\Delta G_v$, of the entire vapor bubble: $\frac{4}{3}\pi r^3 \Delta G_v$. The energy required to form a gas cavity with radius, $r$, is

$$E = \frac{4}{3}\pi r^3 \Delta G_v + 4\pi r^2 \gamma_g. \quad (5-5)$$

The first term on the right hand side of Eq. 5-5 is negative at temperatures above the apparent boiling point of the PFC5 nanoemulsion, and is the driving force for nucleation. The second term on the right hand of the equation is always positive. The critical radius, $r^*$, is the radius of the bubble when $E$ is at a maximum. Differentiating Eq. 5-5 with respect to $r$ and setting it equal to zero provides a value for $r^*$ (after rearranging):

$$r^* = \frac{-2\gamma_g}{\Delta G_v}. \quad (5-6)$$

$\Delta G_v$ can be obtained from the following equation

$$\Delta G_v = \Delta H_v - T\Delta S_v \quad (5-7)$$

where $\Delta H_v$ is the latent heat of vaporization, and $\Delta S_v$ is the change in entropy during vaporization. For a pure liquid in equilibrium with its vapor phase at constant temperature and
pressure, the free energy of the vapor phase is equal to the free energy of the liquid phase. Therefore, at equilibrium and the apparent boiling point, $T_v$,

$$\Delta S_v = \frac{\Delta H_v}{T_v}. \quad (5-8)$$

Assuming that $\Delta S_v$ does not change much with temperature above the apparent boiling point,

Eqs. 5-7 and 5-8 can be combined to yield

$$\Delta G_v = \left(\frac{T_v - T}{T_v}\right) \Delta H_v. \quad (5-9)$$

The latent heat of vaporization can be obtained from the Clapeyron Equation,

$$\frac{dP_v}{dT} = \frac{\Delta H_v}{RT_v(V_v - V_L)} \quad (5-10)$$

where $P_v$ is the corresponding pressure of the vapor phase in equilibrium with the liquid nanoemulsion droplet, and $(V_v - V_L)$ is the change in volume accompanying the change from liquid $(V_L)$ to vapor phase $(V_v)$. $V_L$ was assumed to be constant and was obtained from the DIPPR database [71]. Assuming the vapor phase is an ideal gas, $V_v$ can be written as

$$V_v = \left(\frac{R_g T}{P_v}\right) \quad (5-11)$$

where $R_g$ is the universal gas constant. Assuming the $P_v$ in Eq.5-11 is constant and equivalent to the pressure inside the emulsion droplet, it can be defined as

$$P_v = P_{atm} + P_{hyd} + \Delta P_{Lp} \quad (5-12)$$

where $P_{atm}$ is the atmospheric pressure, $P_{hyd}$ is the hydrostatic pressure, and $\Delta P_{Lp}$ is the Laplace pressure. $P_{hyd}$ was neglected for this analysis, and $\Delta P_{Lp}$ is defined as

$$\Delta P_{Lp} = \frac{-2\gamma_{Lp}}{R_{em}} \quad (5-13)$$
where $\gamma_{LP}$ is the interfacial energy of the nanoemulsion liquid and the phospholipids, and $R_{em}$ is the radius of the PFC5 nanoemulsion droplet. The value of $\gamma_{LP}$ used in this analysis was 3.8 dyne/cm² [153].

The apparent boiling point, $T_v$, was determined by finding the temperature that corresponds to the $P_v$ calculated in Eq.5-12 and using the DIPPR [71] vapor pressure correlation. This function was differentiated with respect to $T$ and combined with Eqs. 5-10-13 to yield the following equation:

$$\Delta G_v = \left(\frac{T_v-T}{T_v}\right) P \left(\frac{R \beta T}{2\gamma_{LP}} - V_L\right) \frac{dP_v}{dT}$$  \hspace{1cm} (5-14)

$\Delta G_v$ is a function of both the local temperature and the size of the nanoemulsion droplet. The critical radius, as a function of temperature, can now be calculated by inserting Eq.5-14 into Eq.5-6. $E_c$ is defined as the activation energy at $r^*$, obtained by inserting $r^*$ in Eq.5-5. Now $J(T)$ (see Eq. 5-3) can be plotted as a function of temperature using the derivation above, and is plotted in Figure 9 as the theoretical homogeneous nucleation data. Apparently homogeneous nucleation does not fit the data since release of calcein appears to occur at temperatures lower than homogeneous nucleation predicts. So we turn to heterogeneous nucleation to look for a better theory to fit the data.

5.3.4 Heterogeneous Nucleation

It is impossible to have homogeneous nucleation of a liquid at its equilibrium boiling point because the gas bubbles are unstable and will collapse spontaneously [155]. Therefore, nearly all experimentally formed gas bubbles are nucleated on surfaces of impurities in the system. Brennen [154] points out that the homogeneous nucleation is exceedingly difficult to
obtain in the laboratory because even carefully distilled solvent still contains solid or dissolved chemical contaminants. The presence of contaminating particulates in the PFC5 emulsion would present sites for nucleation leading to heterogeneous nucleation. The extent to which a particulate can lower the activation energy for heterogeneous nucleation depends upon the contact angle of the nucleating agent on the contaminant surface [156]. Lower contact angles will result in a lower energy barrier. The activation energy for heterogeneous nucleation is lower than the activation energy for homogeneous nucleation; therefore, nucleation will happen at lower temperatures in heterogeneous nucleation. For example, homogeneous nucleation (see Section 5.3.3 and 5.3.3.1 and Figure 9) predicts 0 (or at least less than $10^{-15}$) nucleation events per nm$^3$ per second at 49°C for a 200-nm PFC5 emulsion droplet; whereas heterogeneous nucleation (see Figure 10 and Section 5.3.4.1) predicts about $10^{11}$ nucleation events per nm$^3$ per second at 49°C for a 200-nm PFC5 emulsion droplet. Using the same derivation for homogeneous nucleation (see 5.3.3.1), but using only a fraction of the interfacial energy of the nanoemulsion droplet, it is possible to fit the trend in the data to the theory for nucleation at temperatures above the adjusted boiling point (Figure 10).

5.3.4.1 Derivation for Heterogeneous Nucleation

For heterogeneous nucleation, the possible contaminants in the liquid phase provide a nucleation site and therefore reduce the energy required for nucleation. Depending on the mechanism, the energy required to create a nucleating gas phase is lowered. This is often attributed to a lower energy associated with forming the gas-liquid interface. To account for this reduction in surface energy required, we simply employed a scale factor, $f$, to the surface energy
Figure 10. Plot of initial fractional calcein release rates (left axis) and rate of nucleation events (right axis) versus temperature. Experimental data (points) correspond to values on the left axis. Theoretical heterogeneous nucleation of 200 nm droplets (line) corresponds to values on the right axis.

term to determine the $E_c$ for heterogeneous nucleation. At the critical radius, $r^*$, Eq. 5-5 then becomes

$$E_c = \frac{4}{3} \pi r^* \Delta G_v + 4 \pi r^* \gamma_f f$$  

(5-15)

Using Eq. 5-15 in Eq. 5-3, the value of $f$ to fit the experimental data shown in Figure 10 was found to be $f = 0.6678$. It should be noted that this equation is only valid for temperatures above the apparent boiling point or the boiling point adjusted for the local Laplace pressure. Heterogeneous nucleation theory (Figure 10) predicts that nucleation events happen at the lower temperatures where calcein release was observed (i.e. 49°C), whereas homogeneous nucleation theory ($f = 1$, dotted line in Figure 9) predicts that nucleation will not occur.

5.4 Conclusions

The analysis presented above suggests that, of the several possible causes of calcein release from the liposomes containing nanosized PFC5 droplets, we can rule out some possibilities, and
perhaps accept others. The calcein release data do not fit what would be expected for
temperature-induced changes in DPPC membrane permeability as heating caused a change from
the solid-gel phase of the bilayer membrane to the liquid crystal phase. Others have observed a
maximum in permeability right at the transition temperature [151], which would be 41°C in this
study. Thus, we will dismiss this possibility as a major contributor, although this could possibly
be happening in a small degree while other mechanisms dominate.

Likewise, we can dismiss a simple thermal increase in diffusion of calcein through the
DPPC bilayer. According to Eq. 5-1, higher temperatures should result in a faster diffusion
through the liposomal bilayer. While greater release rates of calcein at higher temperatures is
seen (Figure 7), the very high activation energy predicted for diffusion casts doubt on diffusion as
a major mechanism for calcein release.

Homogeneous nucleation theory predicts that the release of calcein temperature would
occur at higher temperatures than observed; and this rarely occurs except in highly purified and
controlled experiments. Mountford and Borden point out that the temperature must approach
\(~0.9\) (107°C for PFC5 [71]) of the critical temperature for spontaneous vaporization of PFC
droplets. Calcein release occurs at temperatures much lower than suggested by the vaporization
of PFC5 droplets as a result of homogeneous nucleation.

Heterogeneous nucleation theory was able to capture the general shape and trends observed
experimentally, but definitive proof is lacking. Heterogeneous nucleation could be caused by a
variety of sources, such as micelles of DPPC in the PFC5 droplets, solid contaminants in the
PFC5 droplets, or even dissolved oxygen or nitrogen in the PFC5.

As will be shown in Chapter 7, we have strong evidence for heterogeneous nucleation of gas
bubbles when doxorubicin sulfate crystals are formed inside the liposomes; but there is no
doxorubicin in these experiments employing calcein. The most important finding from these experiments is that the DPPC liposomes containing PFC5 emulsions and calcein are stable at body temperature (37°C), even though this temperature is above the normal boiling point of PFC5 (29°C).
6 GOLD NANORODS

6.1 Introduction

Gold nanoparticles are a promising new nanotechnology with a wide variety of biomedical applications including hyperthermia [44, 45], imaging [46-48], and controlled release of therapeutics [46, 49]. In all of these biomedical applications, lasers are used to heat the nanoparticles, which in turn heat their surroundings to produce a therapeutic or diagnostic response. The lasers typically operate at a wavelength in the near-infrared (NIR) window (650-900 nm) [43] because hemoglobin and water have their lowest absorption coefficients in this region and the laser can therefore penetrate deeper in the tissues. One of the major benefits of gold nanoparticles, particularly gold nanorods (GNRs), is that they can be synthesized to absorb laser light in the NIR window. Other optimal properties of gold nanoparticles in biomedical applications include gold being non-toxic, the relative ease of attaching some ligands to the gold surface, and the efficiency of gold emitting heat from irradiated laser-lights.

Gold nanorods were chosen in the current research because of the ability to tune the wavelength of maximum absorbance to the wavelength of our laser by adjusting the length and radius of the gold nanorod. GNRs have 2 maximums in their absorbance corresponding to the plasmon resonance of the electrons around the length and width of the nanorod. The aspect ratio of the nanorod (length/width) can be changed by slight variations in the synthesis process. The larger the aspect ratio is, the larger the wavelength is for the maximum absorbance peak. The
desired aspect ratio to obtain a maximum absorbance wavelength around 785 nm is about 3.8 [157]. Gold nanorods also demonstrate the highest efficiency of converting laser irradiation to heat with an absorption cross section over 13 times larger than its geometrical cross section [158]. The geometrical cross section is the physical area of the nanorod that intercepts irradiated light from the laser, whereas the absorption cross section is the area corresponding to the effective area of light absorbed due to the plasmon surface resonance.

The goal of this research was to place gold nanorods inside of perfluoropentane (PFC5) emulsion droplets, which were then encapsulated inside of liposomes. These eLiposomes would then be loaded with a therapeutic and when the laser heated the gold nanorods, the emulsion droplet would vaporize and form a large gas bubble, which would break open the liposome and release the therapeutic. The desired application would be to release the therapeutic using a wearable and portable laser device. This type of application would provide an optimal opportunity for an individual undergoing chemotherapy to have controlled release of a therapeutic, without the negative side effects of a systemic release, from the comfort of one’s own home, or even while at work.

6.2 Surface Modification

The first step in obtaining GNR loaded PFC5 emulsion droplets was to synthesize the nanorods and modify their surface chemistry. GNRs were synthesized with a coating of cetyl trimethyl ammonium bromide (CTAB) which is toxic to the body in µM amounts when freely administered. Since CTAB is not covalently bound to the GNR it would eventually desorb from the gold nanorod into the body in toxic amounts [159]. For this reason CTAB is commonly replaced with a PEGylated thiol [52], in which the stronger covalent bond (Au-S) replaces the
ionic bonding of CTAB to gold without changing the GNR shape. Another method of reducing cytotoxicity from desorbing CTAB is to encapsulate the CTAB-coated GNR in a shell (often a silica shell) without actually removing the CTAB [160].

The surface of GNRs were also modified in order to transfer GNRs from water into PFC5. GNRs were synthesized to be hydrophilic and freely suspended in an aqueous solution and would not transfer to PFC5 without first modifying the surface of the GNRs. There are investigators who are currently researching how to transfer GNRs to an organic media [161-163]. As mentioned, some investigators simply encase the GNRs (CTAB included) with a silica shell, as that process is easier than replacing the CTAB. The silica shell can then be derivatized to make the surface hydrophobic. While this method is effective in transferring the GNRs to an organic media, the silica shell will act as a thermal insulator, thus requiring more energy to heat the silica surface sufficiently to vaporize the surrounding perfluorocarbon droplet. One group reports a successful transfer of GNRs to perfluoropentane using both a thiol replacement [59] and encapsulation of the GNRs in silica [47], but there are no reports of drug delivery using this construct.

In our lab, gold nanorods were synthesized (Figure 11) according to slightly modified published procedures [140]. The established procedures were modified to obtain a maximum absorbance wavelength around 785 nm, the operating wavelength of our laser. Briefly, a seed solution was made by mixing 5 mL of HAuCl4 (0.5 mM) with 5 mL of CTAB, followed by the addition of 600 µL of ice cold NaBH4 (0.01 M). A growth solution was prepared by mixing 0.28 mL of AgNO3 (0.004 M) with 5 mL of CTAB and 5 mL of HAuCl4 (1 mM). 70 µL of ascorbic acid (0.0788 M) was then added to the growth solution which resulted in a clear, colorless
solution. 13.35 μL of the seed solution was then added to the growth solution and stirred for 2 min before being left in a 28°C water bath overnight.

![TEM image of GNRs synthesized in our lab.](image)

Figure 11. TEM image of GNRs synthesized in our lab.

After GNRs were synthesized, a slightly modified procedure from Wilson et al. [59] (see Section 4.2.2.2) was used to replace the CTAB with thiolated polyethylene glycol (mPEG-SH) and pefluorodecanethiol (PFD-SH). This procedure was difficult to reproduce in that it was not always successful in resuspending the GNRs in PFC5. Communications with Katherine Wilson confirmed that the transfer of GNRs to PFC5 did not always work well, but it was good enough for what she was attempting to do. My purpose in transferring GNRs to PFC5 was to vaporize the PFC5 through laser-induced heating of the GNRs. A change from clear to a pink opaque PFC5 phase indicated a resuspension of GNRs in the PFC5. The PFC5 emulsions with GNRs were suspended in thin capillary tubes and irradiated with a 450 mW continuous wave (cw) laser, as well as a pulsed laser. Samples were incubated at 37 °C for the irradiation with the cw laser,
while samples were kept at 37 °C until immediately before irradiation with the pulsed laser. No detectable vaporization of the emulsion droplets was observed with irradiation from either laser.

### 6.3 Laser-induced Heating

Heating of gold nanorods occurs when photons from the laser excite the electrons of the gold nanorods, and the electrons then heat the gold lattice, which dissipates the heat to its surrounding medium. All of this heating occurs on the order of femtoseconds to picoseconds. When heating at the nanoscale, one important concept to understand is thermal confinement, which occurs when the laser pulse duration is less than the thermal relaxation time for the irradiated volume. This relaxation time ($\tau$) can be estimated using the following equation

$$\tau = \frac{r^2}{\alpha}$$  \hspace{1cm} (6-1)

where $r$ is the particle radius, and $\alpha$ is the thermal diffusivity of the surrounding medium. An effective radius ($r_{eff}$) is used for a GNR, which is equal to the radius of a sphere of equivalent volume. For a 10 nm x 40 nm rod ($r_{eff} = 9.1$ nm) in water ($\alpha = 1.47 \times 10^{-7}$ m$^2$/s), the corresponding relaxation time is approximately 0.56 ns. This suggests that the rod will heat up to a high temperature and cool down to the initial temperature of its surroundings after approximately $3 \times \tau$, or 1.7 ns. This allows for quick and significant heating of GNRs with a high powered fs pulsed laser.

The steady state temperature ($T_{ss}$) of a single gold nanoparticle irradiated by a laser in which the laser pulse duration is significantly longer than the thermal relaxation time is given by

$$T_{ss} - T_\infty = \frac{I_{abs}}{4\pi kr_{np}}$$  \hspace{1cm} (6-2)
where $T_\infty$ is the temperature of the liquid surrounding the gold nanoparticle, $I$ is the laser fluence (W/m²), $C_{abs}$ is the absorption cross section of the nanoparticle, $k$ is the thermal conductivity of the liquid, and $r_{np}$ is the characteristic radius of the nanoparticle. While this equation is developed for a sphere, the effective radius ($r_{eff}$) can be used to estimate heating from gold nanorods. In order for a nucleation event to occur, the liquid interface of the gold nanorod surface must reach a sufficiently high temperature, perhaps as high as 0.9 x $T_c$ (critical temperature). The governing equation for the gold nanoparticle-medium interface is given by

$$I C_{abs} = G A (T_{ss} - T_{med})$$  \hspace{1cm} (6-3)

where $G$ is a thermal conductance at nanoparticle-liquid interface, $A$ is the geometrical cross section, and $T_{med}$ is the temperature of the fluid or solid material at the nanoparticle interface.

Rearranging Eq. 6-3 to solve for $T_{ss}$ and substituting the equality into Eq. 6-2 (noting the direction of heat flow), the required fluence necessary to increase the temperature of the liquid at the nanoparticle interface can be determined. Since the value of $G$ (105.0 x 10⁶ W/m² K) has been reported for GNRs in water [164] the results of this calculation is given in Figure 12 for the heating of single GNR in water using the property values given in Table 1. It should be noted that to raise the temperature at the surface of the water just 1 K requires a laser fluence that is 3 orders of magnitude higher than the laser fluence used in current in vivo medical therapy [158].

This high energy requirement to heat a single GNR occurs because of the rapid heat dissipation from the GNR due to the large surface to volume ratio of this nanoparticle, and the very small value of $r$.

<table>
<thead>
<tr>
<th>Size</th>
<th>$\lambda_{spr}$ (nm)</th>
<th>$C_{abs}$ (nm²)</th>
<th>$A$ (nm²)</th>
<th>$k$ (W/m K)</th>
<th>$G$ (W/m² K)</th>
</tr>
</thead>
</table>

Table 1. Gold nanorod properties and gold nanorod-water interface properties used in calculating fluences in Figure 12.
Besides increasing the laser fluence of a cw laser, pulsed lasers (ns and fs) can be used to achieve significant temperature increases within the GNR. There is a caution, though, with delivering too much energy to a GNR via a pulsed laser. The GNR can melt if its temperature rises above its melting point (1336 K); and when it solidifies again it will no longer be in the shape of a rod, drastically changing the maximum absorbance wavelength. This caution does not apply to typical cw lasers, but for pulsed lasers (where the pulse length is closer to or shorter than the relaxation time) it can present a problem. Attia et al. [166] observed no physiological changes or changes in absorption when GNRs were irradiated with a cw laser; however, they observed melting of the GNRs and a significant change in absorption when irradiated with a pulsed laser. Although cw lasers used in in vivo applications do not have enough power to significantly raise the temperature of a single GNR, cw lasers can cause a significant heating
effect when there is collective heating due to a large number ($10^9$/mL) of GNRs in a relatively large region. To illustrate, Maltzahn et al. [167] administered 14 x 47 nm PEGylated GNRs (20 mg/kg) to tumor-bearing mice; and with ~7% ID/g (injected dose/gram of tissue, $10^{11}$ GNRs/mL in an assumed 21-gm mouse) accumulation of GNRs at the tumor site, they observed a 40K increase in temperature at the irradiated tumor site (810 nm, 2 W/cm², 5 mm laser beam size, 5 min). Although the collective heating of gold nanorods using a cw laser can significantly heat the media surrounding GNRs, we did not observe vaporization of our GNR-loaded PFC5 emulsion droplets using a cw laser or a pulsed laser.

### 6.4 Discussion

Possible reasons for failure to vaporize PFC5 emulsion droplets in our experiments include an inadequate transfer of GNRs to the PFC emulsion droplets and not enough laser energy delivered to vaporize the emulsion droplets. A recent article [47] by the same group as Wilson et al. (see Section 6.2) used a different, more efficient, method for transferring GNRs to PFC5. In this method they fluorinated the rods with 1H,1H,2H,2H-perfluorodecyl-triethoxysilane, which forms a silica shell around the surface of the rods. The CTAB is not replaced before the addition of the fluorinated silane. While this method proved effective at transferring GNRs to PFC5 and even in imaging with lasers, the energy required for vaporization is not feasible with a portable and wearable device.

Pulsed lasers can easily deliver enough laser energy to vaporize a GNR loaded PFC5 droplet. The study by Wilson et al. [59] used PFC5 droplets (200 nm diameter) loaded with 2-15 GNRs, and they were able to see vaporization of the droplets using a pulsed laser (5.0 mJ/cm² pulse energy, 10 Hz pulse repetition rate and 5-7 ns pulse length for 60 s; 5.0mJ/cm² delivered in
6-ns pulses is equivalent to $8.3 \times 10^5$ W/cm² per pulse). Hannah et al. [47] never mentioned the number of GNRs/droplet or showed a cryoTEM image of their GNR loaded droplets. The droplets used in their experiments were 200-800 nm in diameter, and they used a pulsed laser delivering laser light at fluences of up to 90 mJ/cm² (Nd:YAG, 1064 nm, 27 mm² spot size, unknown pulse length). They noticed the photoacoustic signal as a result of vaporization immediately after pulsed laser irradiation ($t = 0.5$s). A signal was observed at fluences as low as 4 mJ/cm², but only with the largest droplets. Our work presented in Section 6.2 used smaller droplets and lower fluences than those used by Hannah et al. Their results highlight a few of the factors required for vaporization of the droplets including the size of the droplets and the required fluence. While pulsed lasers can deliver enough laser energy to heat GNRs sufficiently to vaporize relatively small PFC5 nanodroplets, pulsed lasers are too large to wear as a therapeutic device.

The calculated results shown in Figure 12 suggest that portable cw lasers, which might have at most 1 W cw power, do not have sufficient power to heat a single GNR to induce a phase change in a PFC5 droplet. The question remains as to whether multiple gold nanorods in PFC5 emulsion droplets can produce enough of a collective heating to trigger the nucleation of a PFC5 vapor bubble with a wearable cw laser. Liu et al. [58] showed a cryoTEM image of their GNR loaded PFC droplets which show 2 GNRs in the droplet. Their droplets were formed by mixing HSA with ice-cold PFC5 droplets and sonicating the solution. Liu et al. used a 2.6 W cw laser to vaporize 220-340 nm PFC5 droplets containing GNRs. It should be noted that a 2.6 W cw laser is in the most dangerous class of lasers and will leave scars and damage skin when administered. As such, it would not be approved to be a portable device for a patient to wear as they obtain
treatment. However, Liu et al. did notice the vaporization of the PFC5 droplets and significant heating of the tumor sites after 5 min of laser irradiation using a cw laser.

The majority of studies investigating GNR heating to cause vaporization of a droplet use a pulsed laser to concentrate enough power to heat the GNR sufficiently to vaporize the droplet. These studies are interested in the acoustics of vaporization for diagnostic purposes, and the large amount of power delivered in nanosecond to femtosecond pulses is enough to vaporize the droplets. Most studies using a cw laser and GNRs are using the GNR heating to produce a local hyperthermia, not to vaporize droplets. Liu et al. [58] mentioned above did use a cw laser to initially vaporize PFC droplets; however, they also used the GNRs to heat the tumor site. For local hyperthermia, the goal is to have a sustained temperature between 40-45 °C [168]. Continuous wave lasers are better for the longer time associated with local hyperthermia because the lower amount of energy delivered will do less damage to healthy tissue compared to high power pulses for a sustained period of time.

6.5 Conclusion

Our objective in attempting laser-induced GNR heating was to have a portable and wearable laser to vaporize nanodroplets. The pulsed lasers used for nanodroplet vaporization are not portable or wearable. The cw lasers that are safely portable do not provide enough energy to vaporize the nanodroplets. While the future may still hold the possibility of a wearable device, we concluded investigating GNR heating via lasers and moved on to investigating ultrasound as a means to actuate the release of therapeutics from liposomes.
7 CYTOSOLIC DELIVERY OF DOX USING ELIPSOmES

7.1 Introduction

Cancer drugs that are not delivered inside of a carrier (free drug) often have a relatively low bioavailability in tumors unless administered in very high doses [169]. One reason for the low bioavailability of free drugs is that they are rapidly cleared from the blood. For example, free doxorubicin (Dox) has a distribution half-life of 3.6 min, whereas Doxil (a PEGylated liposome loaded with Dox) has a distribution half-life of over 50 hours [170]. PEGylation of the liposomes avoids clearance by the reticuloendothelial system (RES). Therefore, PEGylated liposomes are often used as drug carriers in order to increase the circulation time of cancer drugs.

Liposomes will only be a benefit in cancer drug delivery if the drug is released at or inside the tumor cells, thus increasing the drug concentration at the tumor site and decreasing the drug concentration around healthy tissue. Gabizon et al. [20] reported that there is minimal release of Dox (<2%) during circulation of Doxil, and therefore Dox is initially delivered to the tissues while still inside of a liposome. This work does not study how the liposomes get to the tumor site, but how the drug is released once the liposomes arrive at the targeted location. Liposomes can be “targeted” to cancer cells by attaching a ligand to the surface of the liposomes, which corresponds to a receptor found on the surfaces of the cancer cells. The ligand on the liposome does not act like a homing missile targeted at cancer cells (which implies swimming against the current and crossing barriers), but more like one half of a piece of Velcro with the other half
being found more frequently on the surface of cancer cells compared to healthy cells. The targeting of cancer cells, therefore, is only as good as the specificity of the ligand for receptors on the surface of cancer cells and does not occur instantly when the liposomes are injected. The liposomes build up in the tissues over time.

Folate is the targeting ligand of choice in this work because it is overexpressed on over 1/3 of cancer cells [6], and it is relatively inexpensive to use as a model ligand. Furthermore, folate will induce endocytosis once the ligand is bound by a receptor on the surface of a cell. Once taken in by the cell, the drug will still need to escape from the liposome and the endosome (cellular vesicle surrounding the liposome after endocytosis). A higher concentration of drugs can be achieved inside the cell cytosol if there is a release mechanism that can be activated after endocytosis rather than traditional release outside the cell followed by diffusion into the cell. The objective of study in this chapter is to determine the efficacy of using ultrasound (US) to control the release of Dox from eLiposomes in order to enhance the killing of multidrug-resistant (MDR) cancer cells.

Cancer cells that survive exposure to cancer drugs can develop an acquired resistance to the administered drugs and to many other types of cancer drugs as well. This undesired phenomenon is known as multidrug resistance. One of the established mechanisms for multidrug resistance is the production of an increased number of export pumps [12-15], which will increase the rate at which undesired compounds inside of the cell (such as cancer drugs) are pumped out of the cell. We hypothesize that delivery of a significant amount of Dox directly to the cytosol of a MDR cancer cell will increase both the concentration and the residence time of Dox in the cell cytosol, and therefore increase the probability that Dox will initiate apoptosis at the nucleus and kill the MDR cancer cell.
Previous work in Dr. Pitt’s lab has shown the successful synthesis of eLiposomes [66] and the controlled delivery of calcein (a model drug) [9, 122] to the cytosol of non-MDR HeLa cells. Ultrasound-induced vaporization of the emulsion droplet was the method of controlled release from the liposome, and in the previous work [9] calcein was only observed in the cell cytosol when US was applied to folated (targeted) eLiposomes loaded with calcein. It is our desire to use ultrasound as a remote trigger that can induce the expansion of the emulsion droplet to gas through acoustic droplet vaporization, and thus trigger the release of Dox to the cytosol of multidrug-resistant cancer cells only where US is focused. The aim of this work, therefore, was to determine if cytosolic delivery of Dox from folated eLiposomes, triggered by US, will enhance the killing of MDR cells.

7.2 Results

7.2.1 MCF 7 Cells

Our lab had access to a Dox-resistant breast cancer cell line (MCF7/ADR, kindly donated from Dr. You Han Bae, University of Utah) as well as a Dox-sensitive breast cancer cell line (MCF7, kindly donated from Dr. O’Niell, MMBio, BYU). Preliminary results from our lab indicated no large, significant differences in the killing of these MCF7 cells (sensitive or resistant) treated with folate-targeted or non-targeted Dox-loaded liposomes. While some researchers use folate to induce folate-mediated endocytosis in MFC 7 cells [133, 171, 172], many researchers classify MCF 7 cells as having a low expression of folate receptors [173-175]. We suspected that the lack of response to folation of liposomes was due to the low intrinsic concentration of folate receptors. Therefore, we obtained a different cell line (KB) which has significantly higher expression of folate receptors compared to MCF7 cells [136, 174].
7.2.2 KB Cells

KB cells are a derivative of HeLa cells, a cervical cancer harvested from Henrietta Lacks in 1951 [134, 135]. These cells were chosen to investigate the cytotoxicity of Dox-loaded folated eLiposomes (folated eLipoDox) because the KB cells have a higher expression of folate receptors [136]. Furthermore, both a resistant and sensitive strain of these cells are available. KB-V1 (Dox resistant) and KB-3-1 (Dox sensitive) cells were a kind gift from Dr. Gottesman (NIH, Bethesda, MD). The KB-V1 cells have an acquired multi-drug resistance produced from culturing the cells in an increasing concentration of vinblastine [138]. They are also resistant to Dox and colchicine while never being exposed to these drugs [138].

Various concentrations of free (soluble) Dox were administered to both KB-V1 and KB-3-1 cells to confirm the resistance of the KB-V1 cells to Dox and to determine an optimal concentration for subsequent experiments with folated eLipoDox. Cell viability was determined via an MTT assay (see Section 4.2.3.10), a colorimetric assay which measures the cell metabolic activity. Figure 13 shows that the dose response curve for the MDR KB-V1 cells is shifted to the right compared to KB-3-1 cells, indicating that the KB-V1 cell line is indeed more resistant to treatment with free Dox compared to the KB-3-1 cell line. The shift is about 2 log units, indicating that 100-fold more Dox (external to the cell) is required to produce a similar toxicity in the MDR KB-V1 cell line even though this cell line had been developed by vinblastine exposure, thus proving its multidrug-resistant behavior.
Figure 13. Dose response curve for KB-3-1 and KB-V1 cells treated with Dox. Predicted values were fit to a four parameter logistic equation using a least square regression analysis. Error bars represent the standard deviation of experimental data points (n=3).

The Dox IC$_{50}$ values of KB-3-1 and KB-V1 cells were determined using the following four parameter logistic equation

$$Y = D + \frac{A-D}{1+10^{(\log_{10} \{A\}-\log_{10} \{B\})C}}$$

(7-1)

where $Y$ is the % cell viability, $D$ is a minimum value for the cell viability, $A$ is a maximum value for the cell viability, $B$ is the IC$_{50}$ concentration (µM), $C$ is the slope at the midpoint, and $x$ is the Dox concentration (µM). The four parameters $A, B, C,$ and $D$ were optimized using a least squares regression, and the values are given in Table 2. KB-3-1 and KB-V1 cells have a Dox IC$_{50}$ of 1.3 µM and 184.0 µM, respectively, which shows that the KB-V1 cells are 147 times more resistant (ratio of B-parameters) to a 2-hour treatment of free Dox treatment than are KB-3-1 cells.

Table 2. Parameters for the four parameter logistic equation to determine the IC$_{50}$ of KB cells.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B (IC$_{50}$ µM)</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB-V1 cell line</td>
<td>0.9164</td>
<td>184.0</td>
<td>1.009</td>
<td>0.0008</td>
</tr>
<tr>
<td>KB-3-1 cell line</td>
<td>0.9867</td>
<td>1.250</td>
<td>0.9888</td>
<td>0.0000</td>
</tr>
</tbody>
</table>
As seen in Figure 13, there is a sharp decrease in cell viability for KB-3-1 cells between 1 (59%) and 10 µM (9%), whereas there is no statistical difference for the KB-V1 cells between 1 (81%) and 10 µM (86%). Therefore, Dox concentrations in the range of 5-10 µM were chosen for further experiments to provide an optimal sensitivity to examine if folated eLipoDox can reduce the viability of resistant KB-V1 cells to match that of sensitive KB-3-1 cells, thus effectively reversing (or overcoming) the multidrug resistance of KB-V1 cells.

7.2.3 Characterization of Folated eLipoDox

eLiposomes were synthesized using the method described in Section 4.2.3. The technique was slightly modified from published procedures [66] to formulate eLiposomes containing larger emulsion droplets. Larger droplets have a lower Laplace pressure and may require less peak negative pressure in order for ultrasound to provide optimal conditions for vaporization [121]. eLiposomes were made with or without targeting folate ligands on the surface. The zeta potential of folate-targeted liposomes was -21.41 ± 1.85 mV (mean ± s.d.), whereas the zeta potential of non-targeted liposomes was -9.48 ± 0.60 mV. The more negative zeta potential of folated liposomes confirms the successful incorporation of DSPE-PEG-folate in the surface of the targeted liposomes, as at neutral pH, the folic acid groups are ionized and negatively charged. A pH gradient between the inside and outside of the eLiposome was established in order to facilitate the remote loading of Dox inside of the eLiposomes. Synthesized eLipoDox had an average diameter of 448 ± 16 nm (mean ± 95% c.i.) as determined by dynamic light scattering, and was not statistically different (p=0.28) in size compared to folated eLipoDox, which had an average diameter of 469 ± 36 nm.
The concentration of Dox and eLipoDox was determined using UV/VIS spectrophotometry. The peak absorbance at 480 nm was used with an extinction coefficient of 12,043 M\(^{-1}\) cm\(^{-1}\) as determined via a calibration curve of free Dox (Figure 14). For experiments with eLipoDox, the target absorbance of Dox and eLipoDox was 0.50, which corresponds to a Dox concentration of approximately 7 µM delivered to the cells.

![Figure 14. Calibration curve for the absorbance (480 nm) of free Dox in PBS (pH 7.4) at room temperature (23°C).](image)

\[
y = 0.01204x \\
R^2 = 0.9955
\]

### 7.2.4 Uptake of Folated and Non-folated eLipodox

The first set of experiments with eLipoDox was designed to see if eLipoDox particles had been taken inside of KB-V1 cells by the time ultrasound would be applied. The liposomes were tagged with a fluorescent label (DiI), and folate-targeted (Figure 15A,C) and non-targeted (without folate, Figure 15B,D) eLipoDox were delivered to KB-V1 cells. After 2 hours, the cells were rinsed with PBS and imaged using a confocal microscope (no US was applied). Any fluorescence appearing in Figure 15C,D is the result of eLipoDox either attached to the surface of the cell or inside of the cell. A sample slice from the stack of confocal images taken is shown
in Figure 15A-D, corresponding to the slice with the highest fluorescence. The mean fluorescence (Figure 15E) of KB-V1 cells exposed to folated eLipoDox was 300 times more than
the mean fluorescence of cells exposed to eLipoDox (without folate). For the cells exposed to folated eLipoDox, every cell appeared to emit fluorescence (Figure 15C); whereas when the cells were exposed to eLipoDox without a targeting ligand, little to no fluorescence was observed (Figure 15D). This suggests that folate promotes cytosolic uptake of liposomal doxorubicin to KB-V1 cells.

7.2.5 Dox Release from eLipoDox

Previous studies [9, 121, 122] showed that calcein (a model drug) could be released from eLiposomes upon insonation. Lattin et al. [122] synthesized 800-nm liposomes with PFC emulsion droplets of two sizes (100 nm and 400 nm). They observed approximately 20% release of calcein from PFC5 eLiposomes after 0.1s of 20-kHz US (1 W/cm²), and the release increased with time (approx. 78% release after 5 s for 400 nm droplets, 40% release for 100-nm droplets). They also looked at smaller (200 nm) liposomes with 100-nm droplets and observed less release (approx. 12% release after 1s and 21% after 5s, 20 kHz, 1 W/cm²) compared to the larger liposomes [121].

A similar study was performed in order to determine the release of doxorubicin from eLiposomes. Dox is self-quenched at high concentrations [176]; therefore, the fluorescence of Dox will increase as it is released from the liposome and diluted. The increase in the fluorescence of Dox is proportional to the amount released. A QuantaMaster fluorometer (Photon Technology International, Birmingham, NJ, USA) measured the fluorescence of Dox released from folated eLipoDox and folated LipoDox (no emulsion droplet) after insonation using excitation and emission wavelengths of 475 nm and 588 nm, respectively. The same ultrasound conditions applied to cells in viability experiments (Section 7.2.6) were used in the
release experiments (1 W/cm², 20 kHz, 2 s total of US applied in 20 sec of 0.1-s pulses, 1:10 duty cycle). Figure 16 shows the raw data collected. A baseline fluorescence was first measured and the fluorometer was paused, the cuvette was removed from the fluorometer, US was applied to the cuvette, the cuvette was then replaced, and the fluorometer reading was continued. The reading was paused after 50 seconds of measuring the fluorescence for another similar application of US. After 2 rounds of insonation, SDS was added and gently mixed by repipetting to release all of the encapsulated Dox from the liposomes and produce the fluorescence corresponding to 100% release. The % Release was calculated using the following equation:

\[
\% \text{ Release} = \frac{f_{US} - f_i}{f_{SDS} - f_i} \times 100\%
\]  

(7-2)

where \(f_i\) is the initial (baseline) fluorescence, \(f_{US}\) is the fluorescence after sonication, and \(f_{SDS}\) is the fluorescence after Dox release using SDS. Results shown in Figure 16 are very noisy because of the low signal to noise ratio. Average release data are summarized in Table 3 and Figure 17. Folated eLipoDox released an average of 78% of encapsulated Dox after 2 s of pulsed US, whereas folated LipoDox only released 29% (Figure 17, Table 3, \(p=0.0002\)).

The presence of an emulsion droplet inside of the liposome caused a significant increase in the release of Dox from liposomes. The majority of Dox was released from folated eLipoDox after only 2 s of pulsed US. An additional 2 s of US did not produce any further significant increase in Dox released from folated eLipoDox compared to the first 2 s of US (82% total release to 78%, \(p=0.25\)); however, another 2 s of US did show significant increase in the release of Dox from folated LipoDox (no emulsion droplet) (29% to 51%, \(p=0.02\)).
Figure 16. Dox release from folated eLipoDox (black) and folated LipoDox (red) measured by fluorescence. Pulsed US (1 W/cm², 20 kHz, 2 s total of US applied in 20 sec of 0.1-s pulses, 1:10 duty cycle) was applied at 50 s and 100 s. SDS was added at 150 s.

Figure 17. Average Dox release from folated eLipoDox (feLD, black/solid) and folated LipoDox (fLD, red/hatched) measured by fluorescence. A baseline fluorescence (No US) was first measured. US was then applied twice (1 W/cm², 20 kHz, 2 s total of US applied in 20 sec of 0.1-s pulses, 1:10 duty cycle) with the fluorescence measured after each insonation. Finally, SDS was added to achieve 100% release. Error bars represent the 95% confidence interval. (n=3).
Table 3. Average release of Dox from folated eLipoDox (feLD) and eLipoDox (eLD) after insonation (1 W/cm², 20 kHz, 2 s total of US applied in 20 sec of 0.1-s pulses, 1:10 duty cycle).

<table>
<thead>
<tr>
<th>US</th>
<th>feLD 1</th>
<th>feLD 2</th>
<th>feLD 3</th>
<th>fLD 1</th>
<th>fLD 2</th>
<th>fLD 3</th>
<th>feLD Avg</th>
<th>fLD Avg</th>
<th>p-value (feLD v fLD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>US 1</td>
<td>76.3%</td>
<td>75.7%</td>
<td>80.9%</td>
<td>29.0%</td>
<td>35.1%</td>
<td>24.0%</td>
<td>77.5%</td>
<td>29.4%</td>
<td>0.0002</td>
</tr>
<tr>
<td>US 2</td>
<td>77.0%</td>
<td>82.1%</td>
<td>86.6%</td>
<td>44.7%</td>
<td>59.7%</td>
<td>49.6%</td>
<td>81.9%</td>
<td>51.3%</td>
<td>0.0042</td>
</tr>
</tbody>
</table>

Previous data by Javadi et al. [9] showed that the presence of both a targeting folate ligand on the surface of the eLiposome and an emulsion droplet inside the liposome increased the calcein fluorescence inside of HeLa cells after insonation. Our results show that folated eLipoDox is taken inside of the cells after 2 hours (Figure 15C), and that, at this time, a 2-sec pulse of US can cause the release of the bulk of encapsulated Dox from folated eLipoDox (Figure 17). These results suggest that US can be used to trigger an instantaneous release of Dox from folated eLipoDox directly to the cell cytosol of MDR cells.

### 7.2.6 Cell Viability Experiments

We investigated the viability of KB cells after the delivery of folated eLipoDox and the application of US. Folate-targeted and non-targeted eLipoDox, as well as folate-targeted LipoDox (no emulsion droplet) were synthesized and diluted to approximately 7 µM in the cell suspension. Both KB-V1 and KB-3-1 cells were incubated with either PBS, free Dox (7 µM), folated eLiposomes (No Dox), folated LipoDox (no emulsion droplet), eLipoDox, or folated eLipoDox for 2 hours before the samples were insonated in selected wells in a 24-well plate (1 W/cm², 20 kHz, 2 s total of US applied in 20 sec of 0.1-s pulses, 1:10 duty cycle). After US was applied to the selected wells, all of the cells were rinsed with 0.5 mL of PBS. The PBS was removed and 0.9 mL of fresh growth media was added to the wells. An MTT cell viability assay was performed 48 hours after insonation and the cell viability results are shown in Figure 18.
Figure 18. Mean cell viability of KB-V1 (blue) and KB-3-1 (orange) cells measured by an MTT assay 48 hours after drug delivery. Concentration of Dox is approximately 7 uM. Cells were either treated with US (hatched bars, 1 W/cm², 20 kHz, 2 s total of US applied in 20 sec of 0.1-s pulses, 1:10 duty cycle) or without US (solid bars). Error bars represent the 95% confidence interval. (n ≥ 3).

Absorbance at 560 nm was measured using a plate reader with the absorbance at 700 nm subtracted. The percent viability for each condition is referenced to cells exposed to PBS but without any insonation (PBS No US) for the given cell line (resistant or sensitive). At the concentration of Dox used in these experiments (7 µM), the viability of MDR KB-V1 cells exposed to free Dox is above 90% with or without ultrasound, suggesting that the resistant cancer cells can quickly export any Dox that diffuses into the cell. KB-3-1 cells are unable to export Dox as quickly due to the lack of P-gp pumps, compared to KB-V1 cells [177], as can be seen from the lower viability (47%) in response to treatment with free Dox.

Figure 18 contains all data from many experiments. For clarity, I will discuss various aspects, comparisons, and analyses of sub-sections of this data set in the subsequent sections.
7.2.6.1 Cytotoxicity of folated eLiposomes

KB-V1 and KB-3-1 cells were both treated with folated eLiposomes without Dox to determine the cytotoxicity of the drug delivery vehicle. During the synthesis of folated eLipoDox, the folated eLiposomes were split into two separate vials before loading Dox into the eLiposomes. Dox in PBS was added to one vial (folated eLipoDox), and to the other vial an equal amount of PBS was added (folated eLiposomes without Dox). Folated eLiposomes were then treated the same way as the folated eLipoDox. KB-V1 cells had a cell viability of 85% (Figure 19) and KB-3-1 cells had a cell viability of 83% when treated with folated eLiposomes (no Dox) without US (not statistically different). Cell viability slightly increased for both KB-V1 cells (90%) and KB-3-1 cells (94%) when US was applied, but the change was not statistically significant. The viability of cells exposed to folated eLiposomes without Dox averaged less than 100%, but was not statistically different for any unloaded formulation.

Figure 19. Cell viability of KB-V1 and KB-3-1 cells treated with folated eLiposomes. Error bars represent 95% confidence interval (n=10).
Other studies [119, 178] investigating the cytotoxicity of different cell lines treated with Dox liposomes attached to PFC microbubbles similarly report 10-20% killing of cells treated with just their constructs and US (no Dox). In this study, the killing of KB cells treated with folated eLiposomes with or without US was not statistically significant compared to the PBS only control (Figure 18). Thus, folated eLiposomes without drug have minimal effect on the cell viability of KB cells.

### 7.2.6.2 Effect of ultrasound

Some cells were treated with PBS and US to verify that the US conditions did not have a significant effect on the cell growth. The results show that there is no significant difference due to the application of only US to KB-V1 (99%) or KB-3-1 (97%) cells ($p>0.05$ in both cases, see Figure 20A).

Ultrasound did significantly increase the killing of KB-V1 cells treated with free Dox, decreasing the viability from 97% to 90% ($p=0.029$, Figure 20B). This slight decrease in viability could be attributed to the cell membrane becoming more permeable for a short time after insonation, which is not unusual [179], thus increasing the influx of Dox. The cells were washed within 20 minutes of US being applied, which would allow a short time for a small amount of free Dox to more easily diffuse into the cell than normally would enter without insonation.
Figure 20. Cell viability of KB-V1 and KB-3-1 cells treated with PBS (A), free Dox (B), folated LipoDox (C) or folated eLipoDox (D). Error bars represent 95% confidence interval (n≥3).

Figure 17 showed that ultrasonic insonation can release approximately 29% of encapsulated Dox from folated LipoDox (no emulsion droplet) and 78% from folated eLipoDox. Although insonation produces 29% release of Dox from folated LipoDox with US in the absence of cells, there is no significant difference due to insonation when folated LipoDox was used against KB-V1 or KB-3-1 cells (Figure 20C). There is, however, a significant \( p=0.047 \) decrease in the cell viability observed after insonation of KB-V1 cells treated with folated eLipoDox (Figure 20D). Yet, while statistically significant, the decrease in viability is not substantial (60% to 53%). The reason for this relatively small decrease in viability is attributed to the instability of
the folated eLipoDox once it has been endocytosed by the cell, and therefore produces low viability of cells exposed to folated eLipoDox even when not insonated (60% for KB-V1 cells, 33% for KB-3-1 cells). This will be discussed later in Section 7.2.7. Surprisingly, ultrasound has a negligible effect on the viability of KB cells for any of the conditions shown in Figure 18 and Figure 20 which includes folated eLiposomes with and without Dox in the liposomes. While ultrasound does not produce a pronounced difference, Dox produces a significant difference, as does folate, which will be discussed next.

7.2.6.3 Effect of folate

As mentioned previously, folate induces much more uptake of eLipoDox. Figure 15 shows the uptake of folated eLipoDox compared to non-folated eLipoDox. The cells shown in Figure 15 were rinsed with PBS prior to preparation for confocal microscopy, so only free Dox inside of the cell or eLipoDox inside of or bound to the cell was present to create fluorescence in the image. This result suggests that folated eLipoDox can release a significant amount of Dox to the cytosol of the cell, whereas eLipoDox does not induce uptake by the cell, so there is a negligible amount of Dox released directly to the cell cytosol. This is supported by the cell viability data shown in Figure 21. KB-V1 cells have a viability of 84% when treated with eLipoDox (Figure 21A), which is statistically higher when compared to cells treated with folated eLipoDox (60%, \(p=0.003\)), but not statistically different when compared to cells treated with free Dox (97%, \(p=0.076\)).
The importance of a folate ligand for cytosolic delivery of Dox is also observed by comparing the viability of KB-3-1 (sensitive) cells treated with Dox, eLipoDox, or folated eLipoDox (Figure 21B). Non-folated eLipoDox kills significantly less KB-3-1 cells (62%) than either free Dox (47%, \( p<0.01 \)) or folated eLipoDox (33%, \( p<0.0001 \)). It is unlikely that large quantities of eLipoDox (without folate) are taken into the cell during the 2 hours of incubation (see Figure 15D). Gabizon et al. report that PEGylated liposomal doxorubicin (no emulsion droplet) passively accumulates in the tumor interstitial fluid and gradually releases doxorubicin without any significant interaction with tumor cells [180]. Thus, Dox released from eLipoDox (either by diffusional escape or by PFC5 droplet vaporization) will remain outside of the cell, and the concentration of free Dox outside of the cell will be less than that of cells treated with free Dox. Since, in our experiments, the media containing any drug is removed after 2 hours of incubation with eLipoDox, and the cells are rinsed with PBS to reduce any residual drug, there is minimal time for any Dox released from eLipoDox to diffuse into the cell. Attaching folate to eLipoDox facilitates endocytosis by the cell (see Figure 15C); thus, Dox can be released directly
from folated eLipoDox to the cell cytosol. Cell viability of KB-3-1 cells treated with folated eLipoDox (33%, Figure 21B) is significantly less than the cell viability of Dox-treated KB-3-1 cells (47%, \( p<0.0001 \)). These results support the hypothesis that folate greatly enhances the cytosolic delivery of doxorubicin from eLipoDox.

### 7.2.6.4 Effect of emulsion droplet

As mentioned, KB cells were treated with folated LipoDox (no emulsion droplet) to confirm that an emulsion droplet is necessary to get adequate release to the cytosol of the cells. There is little to no killing of KB-V1 or KB-3-1 cells treated with folated LipoDox (see Figure 22); however, cell viability is significantly reduced (\( p<0.0001 \), No US) for both KB-V1 and KB-3-1 cells when there is an emulsion droplet encapsulated inside the liposome (folated eLipoDox). The presence of an emulsion droplet makes a statistically significant difference, with or without ultrasound.

Figure 22. Cell viability of resistant KB-V1(A) and sensitive KB-3-1 (B) cells treated with folated LipoDox or folated eLipoDox. Error bars represent 95% confidence interval (\( n \geq 3 \)).
The presence of an emulsion droplet inside of folated eLipoDox apparently provides a possible mechanism for the release of Dox from the liposome directly to the cell cytosol. The PFC5 droplet (100-200 nm) will expand 5 times in diameter (500-1000 nm) when it vaporizes, thus rupturing the liposome and most likely the endosome as well, as endosomes are less than 1 \( \mu \)m in size [181]. This will provide a greater opportunity for Dox molecules to reach the nucleus and initiate apoptosis. Interestingly, ultrasound does not appear to be necessary to provide a significant amount of killing of KB-V1 or KB-3-1 cells with folated eLipoDox, and insonation provides only a marginal increase in killing KB-V1 cells (Figure 22).

7.2.7 Instability of Endocytosed Folated eLipoDox

As mentioned in Section 7.2.6.2 one possibility for the significant amount of killing when cells were incubated with folated eLipoDox (in the absence of US) is that the folated eLipoDox is not stable once it is endocytosed by the cell. In order for US to trigger the release of doxorubicin from eLipoDox to deliver it to the cell cytosol, the eLipoDox construct must be endocytosed and the PFC5 droplet must vaporize when, and only when, US is applied. One hypothesis as to why there is significant killing when KB cells are treated with folated eLipoDox (but without insonation) follows: the folated eLipoDox is endocytosed by the cell; however, the PFC5 droplet vaporizes whether or not US is applied. This hypothesis is consistent with all the data, but is difficult to prove.

Our proposed mechanism underlying this hypothesis is that the Dox precipitates with sulfate to form fibrous crystals during the process of loading doxorubicin. These solid fibers provide nucleation sites for the PFC5 molecules inside of the liposome to nucleate to a gas bubble, independent of the application of US. When folated eLipoDox enters the cell through
folate-mediated endocytosis, the PFC gas bubble forms spontaneously, releasing Dox to the cell cytosol without application of ultrasound. Confocal images (see Figure 15) show that folated eLipoDox is taken into the cell, whereas eLipoDox (no folate) has minimal uptake into the cell. This observation along with the increased killing of KB-V1 and KB-3-1 cells (see Figure 18) treated with folated eLipoDox compared to either folated eLiposomes (no Dox) or eLipoDox (no folate, Dox released external to the cell) suggests that the Dox is released from the folated eLipoDox after endocytosis, and not before. The environment within the endosome is significantly different from the environment surrounding the cell. The endosome has proton pumps to lower the pH and regulate the osmotic pressure [182]. It also has various enzymes to degrade encapsulated cargo [6]. Early endosomes can also have a smaller diameter (~250 nm) than the liposome (~450 nm) [181], which would compress the liposomal bilayer and increase the contact between the Dox fiber and PFC5 droplet. While we do not postulate what it is about the new environment within the endosome that causes the release of Dox from eLiposomes, we do postulate that the release is caused from the vaporization of the PFC5 droplet. The observation that an emulsion droplet within the liposome is necessary to achieve significant killing of KB-V1 or KB-3-1 cells (see Figure 22) suggests that vaporization is likely the cause of Dox release from the eLiposome once the construct has been endocytosed. The hypothesis that the Dox fibers provide nucleation sites for the PFC5 droplet inside of the folated eLipoDox was explored and tested by gleaning results from literature and from some additional quantitative and qualitative experiments.

The formation of fibrous crystals of Dox sulfate when using an ammonium sulfate pH gradient has been reported by many groups [183]. But there are also other reports that Dox fibers form when liposomes are loaded using a citrate pH gradient [129, 184] and a magnesium sulfate
pH gradient [130]. These studies show that the precipitated Dox salts will form bundles of fibers as the drug to lipid (D/L) mass ratio increases, with simple rod-like fibers forming at least by a D/L ratio of 0.05 (see Figure 23). As the D/L ratio increases further, the fibers become thicker and appear to take up more volume inside the liposomes, with some shapes of the Dox crystals appearing to be circular or triangular and even globular at higher (D/L) ratios [129, 130]. As can be seen in Figure 23, the Dox fibers can distort the spherical shape of the liposomes, which would introduce significant stress on the liposomal membrane.

Both Johnston et al. and Li et al. noted that the percentage of encapsulated Dox eventually released from liposomes decreased as the D/L ratio increased [129, 130]. Additionally, Li et al. showed that the significant increase in retention of Dox inside of the liposomes corresponds to the Dox fibers forming bundles of fibers [129]. We postulated that these Dox fibers (Figure 23) provide heterogeneous nucleation sites for the initiation of the PFC5 gas phase, and that the liquid droplet will provide PFC5 molecules to form a gas phase, whether or not US is applied. For our experiments, the initial D/L ratio was 0.005, assuming no lipids were lost during the synthesis procedure. It should be noted that some lipids were lost during the pillow density separation (process that separates empty liposomes from eLiposomes and emulsion droplets), although the exact amount of loss was not quantified.

To test this nucleation hypothesis, experiments were performed with folated eLipoDox to determine the effect of the initial drug to lipid ratio. Folated eLiposomes were synthesized and split into multiple vials, with each vial having the same lipid concentration. Various concentrations of Dox in PBS, but equal volumes, were then added to the multiple vials. At the highest concentration of Dox (2 mg/mL) used in these experiments the initial D/L ratio was 0.16,
assuming no lipids were lost. But even in the most dubious case of losing 90% of lipids, the ratio would be even higher, sufficiently high to form fibrous crystals.

Figure 23. Cryo-transmission electron microscopy of DSPC/Chol (55/45 mol%) liposomes containing doxorubicin at different drug-to-lipid ratios. Panels represent empty liposomes (A) and D/L ratios (wt/wt) of 0.05 (B), 0.18 (C), 0.37 (D), and 0.46 (E). The bar in panels A to E represents 200 nm in all micrographs and all micrographs (A–E) are shown at the same magnification. Each panel is a representative image from at least 20 images per D/L ratio. Figure used by permission from [130].
We expected that due to the presence of a PFC5 emulsion droplet in the liposome, there would be a maximum amount of loading possible before the emulsion droplet would be crowded into touching the Dox fibers and form vapor even before delivering eLipoDox to the cells. Thus, eLipoDox loaded at high D/L ratios were expected to be the most unstable and therefore the most likely for the PFC5 emulsion droplet to vaporize inside of the liposome (due to less space inside of the liposome for the emulsion droplet). We expected eLipoDox at lower D/L ratios to be more stable and have higher cell viability when not insonated. When the D/L ratio is too low, few if any Dox crystals would form and molecular Dox would be more likely to permeate out [129] before the folated eLipoDox could be endocytosed; thus we expected the cell viability to also be higher (especially for KB-V1 cells) because the Dox would escape before endocytosis and would need to diffuse across the cell membrane into the cell, which is less likely to reduce cell viability.

Several experiments were performed with Dox loading concentrations between 0.05-2 mg/mL, corresponding to D/L ratios between 0.004-0.16 (assuming no lipids were lost during synthesis). The D/L ratios would increase if lipids were lost during synthesis (i.e. if 50% lipids are lost, then D/L ratios would be between 0.008-0.32). We were not able to make and measure a D/L ratio lower than 0.001 due to limitations in detector sensitivity.

Figure 24 shows that except for the peak at the Dox loading of 0.25 mg/mL (-0.6 on a log basis), there was no significant and meaningful difference due to the D/L ratio in cell viability of KB-3-1 or KB-V1 cells. Furthermore, there was no significant difference in viability between cells with and without insonation (data not shown) for the D/L ratios investigated. These results suggest that even a low concentration of Dox loading is sufficient to provide a possible nucleation site for PFC5 emulsions also loaded inside the liposome.
Figure 24. Cell viability of KB cells treated with folated eLipoDox with different initial drug to lipid ratios. The x-axis refers to the logarithm of the initial Dox concentration (mg/mL) added to an equal volume of folated eLiposomes. If no lipids were lost during the synthesis of folated eLiposomes, the largest D/L ratio would be 0.16. Error bars refer to the 95% confidence interval (n≥4).

Further support of the heterogeneous nucleation of gas was provided by doing experiments in which Dox fibers were not formed. Li et al. [129] showed that Dox fibers are not formed when Dox is loaded using a monoanionic buffer (such as glutamate). We performed qualitative experiments comparing the presence of gas bubbles when Dox was loaded into folated Liposomes containing a PFC5 emulsion droplet using glutamate pH gradients versus ammonium sulfate pH gradients. Our hypothesis suggests that there would be the nucleation of bubbles when ammonium sulfate is used as the pH gradient due to the presence of a Dox fiber inside the eLiposome. Minimal bubbles will be observed, if any, when glutamate is used because no Dox fibers are formed during the loading process and no Dox fibers would be present to nucleate the PFC5 liquid droplets to gas.
Dox was loaded into folated eLiposomes using an ammonium sulfate pH gradient or a potassium glutamate pH gradient. An equal volume of Dox in PBS (2 mg/mL) and folated eLiposomes were added together and allowed to incubate in the refrigerator (4°C) overnight for 17 hours. The next morning (at 17 hours) bubbles were observed in the vial containing folated eLipoDox formed using an ammonium sulfate pH gradient, but no bubbles were observed in the vial containing folated eLipoDox formed using a potassium glutamate pH gradient (Figure 25A). The vials were then kept at room temperature (23°C) for 90 additional minutes (Figure 25B) after which we observed more (and larger) bubbles for the ammonium sulfate loaded eLipoDox sample. There were also a few small bubbles that did appear in the vial with folated eLipoDox loaded using a glutamate pH gradient. Loading of Dox in folated eLiposomes for experiments in Section 7.2.6 used an ammonium sulfate pH gradient at 4°C and 18 hrs, and a Dox concentration of 0.1 mg/mL; yet no bubbles were observed. The presence of a significant amount of bubbles when loading 2 mg/mL in the ammonium sulfate folated eLipoDox but not in the glutamate folated eLipoDox (Figure 25) supports our postulate that the presence of a Dox fiber provides a nucleation site to form perfluoropentane gas bubbles.

Figure 25. Folated eLipoDox after synthesis and incubation with Dox (2mg/mL). Right vial in (A) and (B) is Dox loading with potassium glutamate. Left vial in (A) and (B) is Dox loading with ammonium sulfate. (A) Immediately after loading Dox for 17 hours at 4°C. (B) After 90 additional minutes sitting at room temperature (23°C).
Li et al. [129] report that when glutamate is used as the pH gradient, Dox is could not be loaded to as high of a Dox concentration inside of the liposome because Dox does not form fibrous crystals. They also report that at the maximum loading of Dox inside glutamate liposomes, 60% of the Dox is released within 30 minutes in 50% human plasma. In comparison, Dox that formed fibrous bundles with citrate released less than 10% of Dox after 30 minutes in 50% human plasma. Thus while loading folated eLipoDox using a glutamate gradient is a possibility, we did not perform any cell viability experiments with glutamate eLipoDox because the resulting construct would not be useful in a clinical application.

7.3 Summary and Conclusion

Dox-sensitive KB-3-1 cells and Dox-resistant KB-V1 cells were used to investigate the effectiveness of cytosolic delivery of Dox to overcome multidrug resistance. The folated eLiposomes (no Dox) used in this study had minimal effect on the growth of KB cells (10-20% killing) and viability was not statistically different from the negative control of PBS without insonation. Folated eLipoDox was shown to be inside of the cell after 2 hours of incubation, whereas there was no observable uptake of non-folated (non-targeted) eLipoDox after 2 hours. Release of the drug inside the cell, as is the case with folated eLipoDox, significantly increased the killing of sensitive KB-3-1 (47% to 33%) cells and resistant KB-V1 (97% to 60%) cells compared to cells treated with free Dox. Non-targeted eLipoDox actually killed KB-3-1 cells less than free Dox (62% v 47%) presumably because the construct is still outside of the cells when the Dox is released, and the lower concentration of external free Dox resulted in a lower driving force for Dox to diffuse into the cell.
Ultrasound at mild conditions (1 W/cm², 20 kHz, 2 s total of US applied in 20 sec of 0.1-s pulses, 1:10 duty cycle) can release approximately 78% of encapsulated Dox inside of folated eLipoDox in vitro. Despite folated eLipoDox being endocytosed by cells and US being able to release Dox, there was no substantial difference in cell viability of KB-3-1 or KB-V1 cells due to the application of US on cells treated with folated eLipoDox.

Our current hypothesis as to why insonation produces no significant difference in KB cell viability when treated with folated eLipoDox is that the emulsion droplet vaporizes once endocytosed by the cells, even without US. This hypothesis is difficult to prove, but is supported by the presence of bubbles in vials containing folated eLipoDox loaded using an ammonium sulfate buffer, but not with a potassium glutamate buffer. The Dox purportedly forms a heterogeneous phase of fibers inside of the liposomes only when loaded using a multianionic buffer. It is our hypothesis that the Dox fiber provides a nucleation site for PFC5 to form a gas phase that ruptures the liposome and causes the release of Dox without the application of US.

While US did not make a substantial difference in killing cells treated with folated eLipoDox, a higher percentage of KB cells were killed when treated with folated eLipoDox compared to an equivalent concentration of free Dox. Cell viability assays also show that the most cytotoxicity is produced by a combination of folate attached to the liposome, an emulsion droplet inside of the liposome, and Dox loaded in the liposome. Cytosolic delivery of Dox via folated eLipoDox did enhance the killing of MDR cells, reducing the viability to 60%, compared to 97% for MDR cells treated with free Dox. However, it did not fully overcome the multidrug resistance of MDR KB-V1 cells at the administered Dox concentration (7 µM).
8 CO-DELIVERY OF FOLATED ELIPODOX AND VERAPAMIL

8.1 Introduction

In the previous chapter, we investigated the effectiveness of folate-targeted doxorubicin-loaded eLiposomes (folated eLipoDox) at overcoming the resistance of KB-V1 cells to doxorubicin (Dox). Experiments were conducted at concentrations of Dox that killed a negligible amount of Dox-resistant KB-V1 cells and more than 50% of Dox-sensitive KB-3-1 cells when treated with free Dox (not encapsulated inside of a liposome). Results showed that folated eLipoDox reduced the viability of KB-V1 and KB-3-1 cells; however, the viability of KB-V1 cells was still higher than the viability of KB-3-1 cells treated with either free Dox or folated eLipoDox. We concluded that the cytosolic delivery of folated eLipoDox at this concentration is not sufficient to overcome the resistance of KB-V1 cells to Dox.

These KB-V1 cells acquired a resistance to Dox through exposure to increasing concentrations of vinblastine [138]. This phenomenon of acquiring resistance to drugs other than the sensitizing drug is known as multidrug resistance. KB-V1 cells are multidrug resistant because they express an increased number of active efflux pumps in the cell membrane of the cancer cells and can therefore export Dox at a much faster rate than cancer cells or other cells with normal expression levels of efflux pumps. It is our hypothesis that inhibiting the function of the efflux pumps would increase the effectiveness of cytosolic delivery of Dox via folated eLipoDox and thus overcome the multidrug resistance of KB-V1 cells.
Co-delivery of Dox with an inhibitor is not a new concept and many researchers have observed a synergistic enhancement of killing multidrug-resistant (MDR) cells through the co-delivery of verapamil and Dox [185-188]. Verapamil (Vrp) is a first generation inhibitor that was used in the experiments presented in this chapter. As a calcium channel blocker, it inhibits the function of the efflux pumps and reduces the ability of the cells to export unwanted materials from the cytosol, thereby giving Dox a longer opportunity to reach the nucleus and initiate apoptosis. The objective of the studies in this chapter is to determine if co-delivering verapamil with folated eLipoDox will enhance the killing of KB-V1 cells compared to folated eLipoDox alone and be able to kill KB-V1 cells with the same effectiveness as the non-resistant KB-3-1 cells.

8.2 Results

8.2.1 Optimal Concentration of Free Verapamil to Deliver with Free Doxorubicin

Doxorubicin and verapamil have a synergistic relationship in regards to killing resistant cancer cells, depending on the concentration of Dox and verapamil delivered. The first sets of experiments were conducted to determine the toxicity of free verapamil toward KB cells and to find an optimal concentration of verapamil to use in combination with Dox. Various concentrations (0.003-3 mg/mL) of 0.1 mL of free (soluble) verapamil were delivered to KB-V1 and KB-3-1 cells in 24-well plates. The final concentration of verapamil was between 0.5 and 500 µM. Verapamil was incubated (37°C, 5% CO2) with the cells for 2 hours before the media was removed, the cells were rinsed with 0.5 mL of PBS, and 0.9 mL of fresh media was added to the cells. An MTT assay was performed 48 hours later to determine cell viability. As seen in Figure 26, KB-V1 and KB-3-1 cells had a viability of 87% and 93%, respectively, when treated with the maximum concentration (500 µM) of verapamil. These viabilities were not statistically
different ($p=0.62$). By itself, verapamil did not produce a significant amount of toxicity at the concentrations and the conditions for these experiments.

![Figure 26](image-url)

Figure 26. Mean cell viability of resistant KB-V1 and sensitive KB-3-1 cells measured by an MTT assay 48 hours after drug delivery. Cells were treated with 0.1 mL of free verapamil (0.003-3 mg/mL). Final concentration of verapamil was between 0.5-500 µM. Error bars represent the 95% confidence interval (n=3).

The optimal concentration of Dox needed to observe the synergistic effects of the co-delivery of Dox and verapamil (Dox+Vrp) would be one in which Dox-treated KB-V1 cells are very viable while the Dox-treated KB-3-1 cells are not. This would allow the KB-V1 cells to have a range of viability that would show sensitivity. As seen in Figure 27 the optimal Dox concentration for this experiment is about 50 µM (1.7 on a log scale, red circles) as Dox killed the majority of KB-3-1 cells, but KB-V1 cells were still very viable (76%).

Various concentrations of verapamil (0.5-500 µM) were co-delivered with free Dox (50 µM) to KB-V1 cells (Figure 28). The objective of these experiments was to determine the optimal verapamil concentration that produces a synergistic enhancement of killing KB-V1 cells when co-delivered with Dox. As seen from Figure 28, the synergistic toxicity of the co-delivery
of Dox+Vrp increased as the concentration of verapamil increased, and the effect did not appear to taper off at the maximum delivered concentration of verapamil (500 µM). KB-V1 cells had a viability of 77% (Figure 28) when treated with 50 µM free Dox and a viability of 87% (Figure 26) when treated with 500 µM free verapamil. The viability of KB-V1 cells dropped to 39% (Figure 28) when Dox (50 µM) + Vrp (500 µM) were delivered together. If the toxicity of co-delivered Dox+Vrp were only additive functions of the toxicity of free Dox and free verapamil, the viability of Dox+Vrp-treated KB-V1 cells should be about 64%; however, the viability is much lower (39%), showing that Dox and verapamil have a synergistic toxicity towards KB-V1 cells at certain drug concentrations. While the viability of Dox+Verap-treated KB-V1 cells (39%, Figure 28) was significantly ($p=0.005$) less than Dox-treated KB-V1 cells (77%), Dox-treated KB-3-1 cells still had a significantly lower viability (6.4%, $p<0.0001$). The experiments reported in the following section (8.2.2) investigated the enhanced toxicity of Dox+Vrp when Dox was delivered directly to the cytosol via folated eLipoDox.
There is a minimum concentration of verapamil needed to produce a synergistic effect in the co-delivery of Dox+Vrp. The cell viability of Dox+Vrp treated KB-V1 cells at verapamil concentrations \( \leq 5 \, \mu M \) were not significantly different from Dox-treated KB-V1 cells. However, at some verapamil concentration between 5 and 50 \( \mu M \) the co-delivery of Dox+Vrp produced the desired synergistic effect. Since the viability of KB-V1 and KB-3-1 cells treated with 500 \( \mu M \) free verapamil (Figure 26) was still relatively high and this concentration had the greatest synergistic effect when co-delivered with Dox (Figure 28), all subsequent co-delivery experiments used 500 \( \mu M \) verapamil.

### 8.2.2 Cell Viability for Co-delivery Experiments

KB-V1 and KB-3-1 cells were treated with free Dox, free verapamil, free Dox+Vrp, folated eLipoDox, and folated eLipoDox plus verapamil (feLD+Vrp). Dox and synthesized folated eLipoDox were diluted to absorbance values of approximately 0.5 (which corresponded
to a final concentration of approximately 6.5 µM delivered to the cells). Cells were incubated with the drug(s) or control for 2 hours before US was applied. After US, the cells were rinsed with 0.5 mL of PBS, the PBS was removed, and 0.9 mL of fresh media was added to the cells. An MTT assay was performed 48 hours after administering the drugs to the cells. Absorbance at 560 nm was measured using a plate reader with the absorbance at 700 nm subtracted. The percent viability for each condition has a reference to cells exposed to PBS but not US (PBS No US) for the given cell line, and the cell viability results are shown in Figure 29. Various aspects, comparisons, and analyses of subsections of these data are discussed in the subsequent sections.

Figure 29. Mean cell viability of resistant KB-V1 (blue) and sensitive KB-3-1 (orange) cells measured by an MTT assay 48 hours after drug delivery. Concentration of Dox and verapamil was approximately 6.5 µM and 500 µM, respectively. Cells were either treated with US (hatched bars, 1 W/cm², 20 kHz, 2 s total of US applied in 20 sec of 0.1-s pulses, 1:10 duty cycle) or without US (solid bars). Error bars represent the 95% confidence interval. (n ≥ 4).

8.2.2.1 Effects of Ultrasound

Chapter 7 presented and discussed reasons why ultrasound was not effective at controlling the release of doxorubicin from folated eLipoDox. It was our hypothesis, considering the results in Chapter 7, that US would not have a significant additional effect on the killing of
KB cells treated with a co-delivery of folated eLipoDox and verapamil (feLD+Vrp). As can be seen in Figure 30A, there was no significant difference due to US for KB cells treated with feLD+Vrp (25 v 23%, \( p = 0.62 \); 29 v 31%, \( p = 0.59 \)).

![Graph A](image)

Figure 30. Cell viability of KB-V1 and KB-3-1 cells treated with folated eLipoDox plus verapamil (A) or free Dox plus verapamil (B). Error bars represent the 95% confidence interval (n≥4).

The cell viability of Dox+Vrp-treated KB-3-1 cells did decrease from 47% to 29% when US was applied (Figure 30B); however, the change was not significant (\( p = 0.07 \)). There were no conditions for any of the experiments presented in Figure 29 where US significantly enhanced the killing of KB-cells. Section 7.2.6.2 discussed reasons why US had a negligible effect on the viability of KB cells treated with free drug or folated eLipoDox, and it is believed these same reasons apply when free Vrp is present.

8.2.2.2 *Co-delivery of Free Dox and Free Verapamil*

KB-V1 and KB-3-1 cells treated with 500 µM of free verapamil (Figure 31) had a cell viability of 81% and 85%, respectively. The concentration of Dox delivered to the KB cells was 6.5 µM, slightly less than the concentration of Dox (7.0 µM) delivered to KB cells in Chapter 7.
As shown in Figure 31, KB-V1 cells treated with 6.5 \(\mu M\) Dox have a viability of 99% whereas KB-3-1 cells have a cell viability of 46%. If the toxicity of Dox+Vrp were only additive functions of the toxicity of free Dox and free verapamil, KB-V1 and KB-3-1 cells would have an approximate cell viability of 80% and 31%, respectively. We observed (Figure 31), however, that compared to Dox-treated KB cells, the co-delivery of Dox+Vrp had a synergistic enhancement in killing resistant KB-V1 cells (50% v 99%, \(p=0.004\)) while making no difference in the killing of sensitive KB-3-1 cells (47% v 46%, \(p=0.89\)). More importantly, however, there was no statistical difference between the viability of KB-V1 and KB-3-1 cells treated with free Dox+Vrp (50 v 47%, \(p=0.74\)). At this concentration of Dox and Vrp, the multidrug resistance of KB-V1 cells to the treatment of Dox was overcome with the co-delivery of verapamil (Dox+Vrp, Figure 31).

Verapamil is a calcium channel blocker, and will inhibit the function of the export pumps of KB-V1 cells, thus decreasing the rate at which Dox is removed from the cytosol of the cells. Perez-Soler et al. [16] measured the accumulation of Dox in KB (Dox-sensitive) and KB-V1 cells after 1 hour incubation with Dox (10 \(\mu g/mL\)) or Dox +Vrp (10 \(\mu g/mL\) each). Compared to
cells treated with Dox alone, KB-V1 cells treated with Dox+Vrp increased the accumulation of Dox by a factor of 2, but Dox+Vrp had no effect on the accumulation of Dox in sensitive KB cells. Perez-Soler et al. [16] also looked at the retention of Dox in KB-V1 and KB cells. After a 1-hour incubation with Dox or Dox+Vrp, the cells were washed and cellular Dox retention was measured 1 hour thereafter. Verapamil had no effect on the retention of Dox in sensitive KB cells, but increased the retention of Dox by a factor of 2 for KB-V1 cells. The decreased cell viability of Dox+Vrp-treated KB-V1 cells compared to Dox alone (Figure 31) can be attributed to the increased accumulation and retention of Dox in the cytosol of the cell, which would grant Dox molecules a longer time to accumulate in the nucleus and initiate apoptosis.

In sensitive cells, we observed no decrease in the viability of Dox+Vrp-treated KB-3-1 cells compared to KB-3-1 cells treated with Dox alone (Figure 31). Perez-Soler et al. [16] observed no increase in the accumulation or retention of Dox in Dox-sensitive KB cells. Therefore, verapamil had a negligible effect on the efflux of Dox molecules from the cytosol of Dox-sensitive KB cells. Furthermore, we can infer that the accumulation, and therefore cytotoxicity, of Dox in sensitive KB-3-1 cells was limited by the influx of Dox molecules to the cytosol and not the export of Dox molecules from the cell. The viability of KB-3-1 cells can still be reduced with a higher concentration of external Dox (Figure 27). Higher concentrations outside of the cell would increase the concentration gradient between the inside and outside of the cell, thus providing a greater driving force for diffusion of Dox into the cell and a higher accumulation of Dox in the cytosol. While Dox+Vrp does not increase the killing of Dox-sensitive KB cells, co-delivery of Dox+Vrp is as cytotoxic to Dox-resistant KB-V1 cells as to Dox-sensitive KB cells.
8.2.2.3  Co-delivery of Folated eLipoDox and Verapamil

While the co-delivery of free Dox+Vrp was shown to overcome the resistance of KB-V1 cells to Dox (Figure 31), it is not an optimal form of drug delivery because of the negative side effects associated with the systemic delivery of non-encapsulated drugs. Encapsulation of Dox in liposomes will help to reduce the cardiotoxicity and other negative side effects associated with the systemic delivery of free Dox. As seen in Figure 29, folated eLipoDox was better at killing the MDR KB-V1 cells than free Dox; however, it did not overcome the Dox resistance and kill the KB-V1 cells as well as Dox alone killed the KB-3-1 cells. It was our hypothesis that the co-delivery of folated eLipoDox and Verapamil would overcome the resistance of KB-V1 cells to Dox and be more cytotoxic to KB cells than free Dox or free Dox+Vrp.

Figure 32 shows the cell viability of KB-V1 and KB-3-1 cells when treated with free Dox+Vrp, folated eLipoDox, and feLD+Vrp. The viability of KB-V1 cells treated with free Dox+Vrp was lower, but not statistically different, than the cell viability of KB-V1 cells treated with folated eLipoDox (50 v 65%, \( p = 0.10 \)). Folated eLipoDox-treated KB-3-1 cells, interestingly, had a lower cell viability than Dox+Vrp-treated KB-3-1 cells (47 v 35%); however the difference was also not significant (\( p = 0.08 \), Figure 32). We can therefore infer that delivering Dox directly to the cell cytosol was as effective at killing KB cells as delivering free Dox and inhibiting the export pumps with verapamil.
The co-delivery of feLD+Vrp significantly reduced the viability of KB-V1 cells compared to both folated eLipoDox alone (25 v 65%, *p*<0.0001) and Dox+Vrp (25 v 50%, *p*=0.047; Figure 32). While the cell viability of feLD+Vrp-treated KB-3-1 did not significantly decrease compared to feLD-treated KB-3-1 cells (29 v 35%, *p*=0.08), the cell viability did significantly decrease compared to Dox+Vrp treated KB-3-1 cells (29 v 47%, *p*=0.0007, Figure 32). KB-V1 and KB-3-1 cells treated with feLD+Vrp had the lowest viability of any of the experimental conditions (Figure 29). More importantly, KB-V1 cells were killed as effectively as KB-3-1 cells when treated with feLD+Vrp (25 v 29%, *p*=0.38), supporting our hypothesis that multidrug resistance can be overcome through inhibiting the efflux pumps as cancer drugs are delivered directly to the cell cytosol.

### 8.3 Summary

The objective of the research presented in this chapter was to determine if the co-delivery of verapamil with folated eLipoDox would be able to overcome the resistance of KB-V1 cells to
Dox and kill KB-V1 cells as effectively as the sensitive KB-3-1 cells. Verapamil was shown to have minimal toxicity to KB cells at verapamil concentrations up to 500 µM, and the co-delivery of Dox (50 µM) with verapamil (500 µM) showed a significant synergistic toxicity to KB-V1 cells.

Further experiments showed that the co-delivery of Dox+Vrp was able to overcome the resistance of KB-V1 cells to Dox. However, there was no enhanced killing of KB-3-1 cells treated with Dox+Vrp. KB-V1 and KB-3-1 cells both saw a decrease in viability when treated with fLipD+Vrp, and, most importantly, there was no significant difference in their viability. As observed in the results from Chapter 7, there was no significant difference in cell viability due to the application of US for any of the experimental conditions presented in this chapter. We conclude that the cytosolic delivery of Dox via folated eLipoDox combined with the blocking of export pumps via verapamil can overcome the multidrug resistance of KB-V1 cells and even significantly reduce the viability of Dox-sensitive KB-3-1 cells.
9 CONCLUSIONS AND RECOMMENDATIONS

9.1 Summary and Conclusions

The overall objective of the presented research was to investigate the controlled release of therapeutics directly to the cytosol of cancer cells in order to overcome multidrug resistance. Cytosolic delivery of cancer drugs was hypothesized to be more effective at killing resistant cancer cells than delivery of the drug outside of the cell. Producing drug release to the cytosol would not only increase the amount of drug inside the cell, but also minimize the negative side effects associated with a systemic (whole body) delivery of unencapsulated drugs. The results from this research suggest that cytosolic delivery via a folated eLiposomes was more effective at killing MDR cells and can be enhanced when co-delivered with an inhibitor to block the functions of the export pumps.

The first set of experiments investigated whether eLiposomes were stable at body temperature (37 °C) Results showed that a model drug (calcein) had minimal release at body temperature; however, the release significantly increased as the temperature increased above body temperature. The stability of the PFC5 emulsion droplets at body temperature (above PFC5’s normal boiling point of 29 °C) is presumed to be the result of a high energy barrier for nucleation after the formation of phospholipid stabilized emulsions. Release at higher temperature was most likely the result of heterogeneous nucleation.
Vaporization of PFC5 emulsion droplets at body temperature was first attempted with laser-induced GNR heating. Problems with consistency in transferring GNRs from an aqueous solution to PFC5, and the further investigation of the required power to vaporize the emulsion droplets prompted a change from temperature induced vaporization of PFC5 droplets to a pressure-induced vaporization. While studies do show that it is possible to vaporize PFC5 droplets with laser-induced GNR heating, the power required is not feasible in a safe, portable device.

A Dox-sensitive and Dox-resistant strain of KB cells were used to investigate the cytotoxicity of folate-targeted dox loaded eLiposomes (folated eLipoDox). Confocal images showed that folate was required to accumulate a high concentration of eLipoDox inside the cell. Fluorescence spectroscopy showed that an emulsion droplet significantly increased the release of Dox from liposomes using ultrasound. Cell viability assays showed that folated eLipoDox was significantly better at killing MDR KB cells than freely administered Dox. Folate attached to the liposome, PFC5 emulsions inside of the liposome, and Dox loaded in the liposome were all required to get the best killing of MDR KB cells. When folated eLipoDox was co-delivered with verapamil, an export pump inhibitor, resistant and sensitive KB cell viability was reduced to under 30%, which was significantly better than the killing of sensitive KB cells treated with free Dox.

One significant, but unexpected, result was that ultrasound had a minimal effect on the killing of KB cells treated with folated eLipoDox. We surmise, with some supporting evidence, that the reason why so much killing occurred with folated eLipoDox without US was that the dox crystals inside of the liposome provided nucleation sites for the PFC5, and vaporization caused Dox release from the liposome at some time after the folated eLipoDox was endocytosed.
Despite ultrasound being unable to provide a means to externally trigger the release of Dox from eLiposomes, folated eLiposomes were able to overcome the multidrug resistance of KB-V1 cells. The effectiveness of eLiposomes to control the delivery of Dox to cancer cells is dependent on the specificity of the targeting ligand. Folated eLipoDox could be endocytosed by any cell that has a folate receptor. Many cancer cells overexpress folate receptors and therefore folated eLipoDox has a greater probability of being endocytosed by such a cancer cell. Folated eLipoDox provides an optimal drug delivery vehicle for delivering drugs directly to the cytosol, which is more cytotoxic than conventional drug delivery.

**9.2 Recommendations**

In order to confirm that folated eLipoDox can preferentially target cancer cells that overexpress folate receptors, folated eLipoDox can be delivered to a co-culture of cells in vitro with one cell line that has a high expression of folated receptors and another cell line that has a low expression of folate receptors. Folated eLipoDox is effective at releasing Dox to the cell cytosol without ultrasound. The effectiveness of using folated eLipoDox in vivo will depend on its ability to accumulate at the site of the tumor. Optimizing the size of the liposomes will help them escape capture by the RES and allow them to escape any leaky vasculature at the tumor site. A passive release mechanism is also beneficial in targeting metastatic sites, which are located at unknown sites in the body.

Another possibility in future research is to make folated eLipoDox stable inside of the cell until US is applied. Avenues to pursue include using different nucleating agents, such as PFCs with higher normal BPs (e.g. perfluorooctane), and optimizing the drug to lipid ratio and the size of the emulsion droplets and liposomes. Different drugs that do not form a fiber when loaded in
liposomes might also be able to control the release using US. Previous experiments [9, 122] showed calcein released from eLiposomes only when US was applied. Calcein was loaded in liposomes passively when the liposomes were formed. This was not as efficient in loading as the remote loading of Dox (pH gradient loading), meaning that more of the drug was wasted during the loading process; but a different loading process might be able to allow for controlled release using ultrasound.

Co-delivery of folated eLipoDox and verapamil was the most effective drug delivery method for killing the MDR KB-V1 cells. However, the verapamil was not delivered inside of an eLiposome. The stability and encapsulation efficiency of verapamil inside of eLiposomes should be investigated. If verapamil cannot be delivered to the cytosol in a sufficient concentration, other efflux pump inhibitors should be investigated to find an inhibitor that can be loaded in an eLiposome and co-delivered with folated eLipoDox at sufficient concentrations to overcome multidrug resistance.
10 REFERENCES


