Surface Modification of Liposomes Containing Nanoemulsions

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ABSTRACT

Surface Modification of Liposomes Containing Nanoemulsions

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Many attempts have been made to make cancer therapy more selective and less detrimental to the health of the patients. Nanoparticles have emerged as a way to solve some of the problems of traditional chemotherapy. Nanoparticles can provide protection for the therapeutic from degradation or clearance, as well as protection to healthy tissue from the damaging effects of chemotherapy drugs. Researchers are pursuing different strategies but all have the same goals of improving the outcomes of cancer patients.

The field of controlled release of drugs has increased significantly in hopes of better treating diseases like cancer. Improved control of drug release has great potential for improving patient outcomes. Still there exist certain barriers such as circulation time, cell specificity, and endosomal escape.

In this study a novel drug delivery vehicle was studied in vitro. The novel construct consisted of a liposome containing perfluorocarbon emulsions—an eLiposome—that was activated by ultrasound to break open on demand. Two targeting moieties were attached to the eLiposome to increase cell specificity and induce endocytosis. These studies determined the localization of eLiposomes in vitro using flow cytometry and confocal microscopy.

Results indicated that eLiposomes modified with a targeting moiety attached to HeLa cells to a greater extent than non-targeting eLiposomes. Confocal images indicated localization of eLiposomes around the membrane of cells. Flow cytometer results indicated that ultrasound does in fact disrupt the eLiposomes but evidence of significant delivery to the cytoplasm was not obtained. However cells that were incubated with eLiposomes for 24 hours showed over 60% of the cells had green color association indicating eLiposome uptake.

Keywords: liposomes, nanoemulsions, drug delivery, ultrasound, targeted drug delivery, vesosomes, eLiposome
ACKNOWLEDGMENTS

I would not have been able to accomplish this work without the unwavering patience and love of my wife, Sara. She motivated me when experiments did not go as planned and celebrated with me when things went well. Her writing expertise is so very much appreciated as her had the patience to review this work and provide critique. I greatly appreciate the financial support and encouragement of my parents, who taught me the importance of education. I am grateful for Sara’s family who never hesitated to help our family.

I feel deep gratitude to Dr. William Pitt for giving me a chance to work in his lab four years ago. It has been the highlight of my education here at Brigham Young University. Dr. Pitt’s help has been invaluable during my time here in my research, class work, and career plans. I am especially grateful for his good example and friendship. I wish to thank friends and coworkers including Tara Pandy, Chris Tracy, Marjan Javadi, James Lattin, Melissa Tovar who helped with eLiposome preparation, emulsion preparation, cell culture, and experimental expertise.

I wish to thank my committee, Dr. William Pitt, Dr. Kenneth Solen, and Dr. Morris Argyle. Their critique of my thesis and patience in scheduling the annual reviews and the defense is greatly appreciated.

Finally, I would like to thank Brigham Young University for this marvelous opportunity to study chemical engineering and attempt to solve important problems facing the world today.
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1 INTRODUCTION

Cancer is among the leading causes of death. Approximately 400 people out of 100,000 were diagnosed with cancer in 2008 [1]. Significant progress has been made in understanding cancer and combating the disease with current treatments including surgery or chemotherapy. Chemotherapy is a somewhat effective treatment for those with cancer; however it also damages healthy cells in the body. Many attempts have been made to make chemotherapy more selective and less detrimental to the health of the patients.

Nanoparticles have emerged as a way to solve some of the problems of traditional chemotherapy. Nanoparticles can provide protection for the therapeutic from degradation or clearance, as well as provide protection to healthy tissue from the damaging effects of chemotherapeutic drugs. Researchers are pursuing different strategies, but all have the same goals of improving the outcomes of cancer patients.

The field of controlled release of drugs has increased significantly in hopes of better treating diseases like cancer. Improved control of drug release has great potential for improving patient outcomes. Still there exist certain barriers such as circulation time, cell specificity, and endosomal escape.

Other therapies are emerging to treat cancer, such as gene therapy using plasmid DNA, mRNA, or siRNA. These therapeutics show promise but suffer from poor circulation kinetics
and degradation in the body. Delivery vehicles for these therapeutics are also required, which would overcome some of the current barriers.

This thesis research uses surface-modified liposomes containing nanoemulsions to deliver fluorescent molecules and siRNA to cancer cells. These eLiposomes are incubated with cells then later sonicated to burst the eLiposomes, providing a means for therapeutics to escape from the endosome or bypass the endosomal pathway all together. The conjugation of ligands to eLiposomes and the localization of the eLiposomes in vitro is specifically investigated in this research.

This thesis begins with an overview of relevant literature pertaining to drug delivery and ultrasound. The specific objectives are then set forth followed by the experimental approach to the project. Given the nature of the data, the results and discussion are sometimes combined for the benefit of the reader. This thesis document concludes with recommendations on how questions and problems encountered in this work can be answered and solved.
2 LITERATURE REVIEW

2.1 Drug Delivery

As stated above controlled drug delivery can solve many problems in treating diseases. A variety of drug carriers are being investigated including polymer conjugates, liposomes, and polymer vesicles. Each system has advantages and disadvantages, but given the diversity of delivery needs they all may play important roles in future therapeutics. Specific goals of drug delivery include 1) maintenance of drug levels within therapeutic ranges, 2) reduced non-specific harmful effects due to targeting specific tissues, 3) usage of less drug, 4) reduced dosing frequency and invasiveness, 5) easier administration of therapeutics with short half-lives [2].

2.2 siRNA

One particular therapeutic that holds great potential is short interfering RNA (siRNA). From its discovery in 1998 by Fire, Mello and colleagues [3], billions of dollars have been invested in the related technologies. Short interfering RNA is able to bind to complementary mRNA and induce cleavage. Once the mRNA is broken up, the associated protein is not produced. Therefore delivering siRNA to the cytoplasm of cells can be used to “knock down” a specific protein. Already siRNA has shown ability to decrease protein expression in various diseases in vivo, including hypercholesterolaemia, liver cirrhosis, hepatitis B virus (HBV), human papillomavirus, ovarian cancer and bone cancer [4]. However these therapeutics still
require additional design to make them more clinically suitable. Free siRNA undergoes rapid degradation in the body and requires a protective delivery vehicle in order to reach the targeted tissues.

2.3 Liposomes

There are two broad categories of delivery systems: polymer-based and liposome-based systems. Liposomes have an advantage over other carriers in their facile preparation and relative biocompatibility. Complete biocompatibility was previously accepted until recently where PEGylated liposomes were shown to activate the complement system in some cases [5].

Significant progress has been made in using liposomes for drug delivery since their discovery by Alec D. Bangham in 1961 [6]. He discovered that phospholipids self-assemble into bilayers. The phospholipid head group is hydrophilic while the acyl chains are hydrophobic. Thousands of lipids will self-assemble in an aqueous solution into spheres with an aqueous interior. Large amounts of water soluble drugs can be encapsulated inside of liposomes.

Many new clinical applications of liposomal carriers have emerged in the last 20 years. Liposomal doxorubicin, commercially known as Doxil®, has been approved for the treatment of recurring breast cancer [6]. There are more than a dozen liposomal drug formulations that have been approved for clinical use.

Liposomal carriers have been modified over the years into more complex, multifunctional carriers. Instead of just plain liposomes, they have been modified with polymers, stimuli responsive lipids, stimuli responsive polymers, diagnostic labels, and positively charged lipids. These modifications provide means to overcome barriers to more specific and smart delivery of therapeutics. Adding functionalities is relatively easy. Ligands
can easily be attached to lipids then inserted into the liposome bilayer. In addition to facile functionalization, liposomes can be easily extruded to the desired size.

Many researchers desire to deliver genes using liposomes. Researchers discovered that positively charged lipids could complex DNA and be used for delivery. Now many cationic liposomal formulations are commercially available for use in vitro [6]. Use in vivo has been unsuccessful due to the positive charges on the liposomes, which render them toxic to cells. While liposomal applications are promising, using simple liposomes is not enough. Liposomes are rapidly cleared from the circulation system through capture by the reticulo-endothelial system (RES). Thus, liposomes must be designed to be evasive as well as targeting.

### 2.4 Targeted Drug Delivery

Targeted drug delivery has the potential to significantly improve the efficacy of current therapeutics and provide the means to deliver new types of drugs such as genes or proteins. In order for new nanocarriers to be effective, they need to contain multiple functionalities to overcome biological barriers in the body. Nanocarriers that combine longevity in the blood and specific target recognition have the potential to significantly increase therapeutic efficacy [7]. The surfaces of nanocarriers have been modified to interact differently in a biological environment to provide needed properties such as prolonged circulation and specific localization [7]. Nanocarriers comprising long-chain polyethylene glycol on their surface exhibit increased blood circulation time because of reduced recognition by the RES [8]. Increased circulation time leads to increased extravasation through leaky capillaries into tumors [9]. Extravasation (the escape from blood vessels) of drugs is referred to as passive targeting, which requires that nanocarriers be a certain size to leak into the tumor tissue [9]. Active targeting can be
accomplished by attaching ligands to the surface of nanocarriers that target specific tissues in the body. Maruyam et al. attached transferrin to the distal end of a polyethylene glycol chain on a liposome and observed liposome binding to tumor cells in vitro [9]. The ligands that are selected preferably only interact with the target tissue. This approach has the potential to minimize adverse side effects of certain drugs because the drug will only be delivered to the target cell at the target site [10].

2.4.1 Polyethylene Glycol (PEG)

In order to achieve longer blood circulation times, the reticular endothelial system (RES) must be avoided [9]. Liposomes in the blood become opsonized (coated by proteins) then rapidly cleared from circulation, thereby limiting drug therapeutic efficacy. One way to “hide” the nanocarriers from the RES is to attach polyethylene glycol (PEG) chains to the surface of the nanocarrier. PEG has been shown to increase blood circulation time and increase drug accumulation at the tumor site [7]. Any drug delivery vehicle administered via the blood stream should incorporate PEG if prolonged circulation is desired [7].

Even though PEG has solved an important problem of circulation time, it creates another problem once the liposome reaches target cells. The long PEG chains increase the hydrodynamic radius and thus reduce extravasation; more importantly PEG chains reduce endocytosis of the liposomes. Research groups have overcome this problem by attaching ligands to the distal end of PEG that bind to receptors on the cell surface that actively induce endocytosis [9].
2.4.2 Ligands and Endosomal Pathways

Fortunately some cancer cells overexpress receptors that can be targeted using ligands. Attaching ligands to the end of PEG increases receptor-mediated endocytosis (RME), but it also reverses the stealth endowed, increasing detection by the RES [9]. This is especially true when large molecules such as antibodies are attached to PEG. The large size increases opsonization and subsequent clearance from the circulation. Only small amounts of ligand conjugated to PEG (~1%) should be included in the liposomal membrane.

Researchers have used many different kinds of ligands for targeted drug delivery and have reported increased specificity [11]. Ligands that target the endosomal pathway have significant potential for use in targeted drug delivery [11]. Ligands that target the cell surface are usually taken into the cell through RME. Depending on the ligand, the intracellular tracking path could be clathrin-dependent or clathrin-independent. Clathrin-dependent RME traffics contents to the lysosomes for degradation while clathrin independent RME leads to endosomal accumulation and sorting [11].

2.4.3 Folate

Folate receptors are overexpressed in many cancers, which make them an ideal target receptor. Furthermore, folate receptors, upon activation, are taken up by clathrin-independent RME, so trafficking is not to the digestive lysosome. Liposomes containing doxorubicin and decorated with folate have shown increased in vitro cell toxicity, which suggests increased endocytotic uptake [12]. Lee et al. decorated liposomal doxorubicin with folate on the distal end of PEG and observed that uptake into KB cells was 45 times higher than non-targeted liposomes [13]. They also observed that only those cells that overexpressed the folate receptor internalized
folate-PEG-liposomal calcein. Folate is an attractive possible ligand due to its small size, tumor specificity, and ease of conjugation to PEG.

Recently Gabizon et al. compared folate-targeted liposomal doxorubicin with non-targeted liposomal doxorubicin in several different tumor models. They found folate-targeted liposomal doxorubicin to be more effective than non-targeted liposomal doxorubicin [14].

Folate can be attached via an amide bond to PEG that is functionalized with a terminal primary amine [15]. The PEG spacer provides a greater ability of the ligand to associate with receptors on the cell surface. Ligands attached directly to the surface of liposomes are not internalized as extensively as ligands with a spacer. Optimal targeting was observed with a PEG spacer of molecular weight 3350 [16].

Folate has been used for targeted delivery of genes. Bruckheimer et al. constructed DNA lipoplex complexes (DNA-lipid-PEG-Folate). They observed a 1.6 fold increase in binding and internalization of the folate-targeted complexes over the non-targeted. They used this formulation to treat tumors in mice and found a significant reduction in tumor volume compared to mice treated with non-targeted liposomes [17].

2.4.4 Thymidine Kinase 1 (TK1)

The protein thymidine kinase 1 (TK1) was found to be a biological marker for cancer [18]. Thymidine kinase levels in the serum of breast cancer patients indicated a statistically significant positive correlation with cancer stage. Researchers at BYU developed the use of an antibody to the TK1 protein that binds preferentially to many cancers. A TK1 antibody has not yet been reported as a targeting moiety to delivery drugs but shows promising application.
2.4.5 Conjugation Chemistry

The type of chemistry that is required to attach a ligand to a nanocarrier will depend on the available reactive groups on the ligand and the assembly needs of the nanocarrier. Attaching ligands to a liposome can be accomplished in different ways and at different times in the formation of the nanocarrier. Ligands can be attached by covalent or non-covalent coupling [19]. Covalent coupling provides increased stability in the blood stream and is the preferred method of attachment. A ligand can be attached to a lipid before incorporation into a liposome, or the ligand can be attached after the liposome is formed in an aqueous environment. Common covalent bonds used to link ligands with nanocarriers include thioether, disulfide, carboxamide, amide, and hydrazone [19]. Thioether bonds have been used extensively because of the ease of reacting a thiol with a maleimide group. Thioethers can also be formed in an aqueous environment. Antibodies are commonly attached using this method due to available thiol groups. Ligands bearing a carboxylic acid group can be attached to a lipid terminating in an amine group or vice versa using carbodiimide conjugation [15]. Disulfide bonds are formed by reacting two thiol groups together and are relatively stable in the blood, but the bond is cleaved in the cytosol or endosome of a cell.

High efficiency chemistries that can be performed at low temperature in an aqueous environment are very desirable. Certain therapeutics and targeting ligands degrade or denature at higher temperatures or in organic solvents. Preservation of therapeutic efficacy targeting specificity is essential. For example an antibody fragment may denature at temperatures above 60°C, rendering the fragment useless. Thiol-maleimide reactions and carbodiimide chemistry can often be performed at room temperature.
2.5 Endosomal Escape

Escaping from the endosome remains a significant challenge for efficient drug delivery. Contents in the endosome are sent to the lysosome where enzymes degrade the cargo. Therefore escape from the endosome or bypassing the endosomal pathway altogether is essential. Varkouhi et al. reviewed four different methods used for endosomal escape: pore formation in the endosomal membrane, pH-buffering effect (the proton sponge effect), fusion in the endosomal membrane, and photochemical disruption of the endosomal membrane [20].

Pore formation in the endosomal membrane can be accomplished by using certain peptides that have high affinity for the inside of the endosomal membrane. Many of these peptides respond to the low pH, which induces a conformation change and insertion into the endosomal membrane. The insertion causes a pore to form, and the contents of the endosome leak out to the cytosol.

The proton sponge effect occurs when an agent with many basic chemical groups can be protonated at lower pH (5-6 range). When inside an endosome the amine groups will become protonated, causing an increased influx of protons and other ions into the endosome. The influx creates an osmotic imbalance and causes the endosome to swell and burst. However, these proton sponge molecules can be toxic to cells if not properly designed.

Fusogenic peptides have been used to fuse the membranes of liposomes carrying the peptide and the membrane of the endosome. Upon fusion, the contents of the liposomes spill into the cytoplasm. Using peptides risks the development of an immune response. For example, viruses use peptides to breach membrane barriers.
Photochemical disruption of the endosome occurs when light is applied to drug carriers that include photosensitive chemical agents. These agents can, upon activation, produce reactive oxygen species that break up the endosomal membrane.

2.6 Ultrasound and Drug Delivery

Ultrasound (US) consists of pressure waves with frequencies of 20 kHz or greater and can be produced by transforming a voltage into mechanical movement [21]. Ultrasound waves can be manipulated like audio waves and even be focused on a specific location, thus making them very useful in improving site-specific targeting for drug delivery. Ultrasound can also enhance transport of drugs in tissue. It can heat up tissue in a targeted location to trigger release of drugs that are in thermo-sensitive carriers.

An important effect of ultrasound is cavitation. Cavitation is the “formation and/or activity of gas or vapor filled cavities (bubbles) in a medium exposed to an ultrasonic field” [22]. Barnett describes two types of cavitation. First, stable cavitation, where a bubble oscillates continuously in an ultrasonic field around some equilibrium value. Second is inertial or collapse cavitation. Collapse cavitation occurs when the bubble diameter exceeds a limiting diameter and collapses violently [22]. Large amounts of energy are deposited during collapse cavitation, but it is a highly localized and nearly instantaneous event. Extremely high temperatures and pressures are reached during these cavitation events. Shock waves are produced, and liquid jets traveling at high velocities can be formed when the bubble collapses next to a rigid boundary [23].

Both stable and collapse cavitation can cause damage to cells [21]. The high shears produced by cavitation can puncture cell membranes and allow external molecules to diffuse into the cytosol of the cell.
2.6.1 Microbubbles and Nanoemulsions

Microbubbles are spherical gas bubbles stabilized by a surfactant or polymer. These microbubbles have useful applications in combination with ultrasound. Since sound travels at different speeds through liquid and water, the microbubbles provide a contrast that can be exploited for imaging purposes. In addition to imaging, bubbles can be used to generate cavitation events. Cavitation can be more easily achieved using low frequency ultrasound [24].

Perfluorocarbon (PFC) nanoemulsions have received attention for use in imaging and drug delivery [25]. Unlike microbubbles, which have a mean diameter between 1 to 5 microns, PFC nanoemulsions can be formed to have a mean diameter as small as 100 nm. Ultrasound can be used to stimulate the PFC liquid to transition to a gas. The transition occurs due to local mechanical agitation and through subpressurization. Once the PFC emulsion has formed a gas bubble it becomes a cavitation center [24]. Smaller particles can perfuse hard-to-reach tissue for imaging or drug delivery. The size of nanoparticles is especially important for delivery to tumors. The tumors have leaky capillaries, and particles ~200 nm will accumulate passively in the tumor. Unger suggests that PFC nanoemulsions are easier to prepare than microbubbles and have the advantage of being smaller, which is essential for extravasation into tumor tissue [24].

The Laplace pressure increases the boiling point of PFC inside droplets [25]. Stability of PFC nanoemulsions at temperatures higher than the PFC’s normal boiling temperature is a result of the Laplace pressure inside of spherical droplets:

\[ \Delta P = P_{\text{inside}} - P_{\text{outside}} = \frac{2 \gamma}{R_p} \]  

where \( P_{\text{inside}} \) and \( P_{\text{outside}} \) are the pressures inside and outside the droplet respectively, \( \gamma \) is the surface tension at the interface and \( R_p \) is the radius of the droplet [25]. The boiling temperature
of the PFC inside the droplet is the temperature at which the vapor pressure matches the pressure inside the droplet.

Rapoport’s group observed a droplet-to-bubble transition when they sonicated PFC nanoemulsions. Following the transition the bubbles experience collapse cavitation, which then enhances drug delivery [25].

2.7 Vesosomes

Researchers have developed a method to achieve higher encapsulation volumes inside liposomes. The higher encapsulation volumes can be achieved by refolding large sheets of lipid into spherical liposomes. Liposomes made in this manner are called vesosomes [26]. The lipid sheets are formed when the acyl chains of the lipids interdigitate or stack together uniformly in the presence of alcohol. The alcohol molecules associate with the heads of the phospholipids, which causes the liposome to form sheets of bilayers. The alcohol can be removed and the lipids will remain in their interdigitated form until heated above their melting temperature (T_m). Sheets are more easily formed from liposomes if there is already a high curvature in the liposome. So smaller liposomes, on the order of 50 nm readily form sheets with the addition of alcohol to the concentration of 3 M. When the sheets are heated above their T_m they refold into unilamellar spherical structures with a size of about one micron. This technique provides a higher capture volume than other liposome formation techniques. Large particles on the order of a few hundred nanometers can be encapsulated inside the vesosomes [26].
3 OBJECTIVE

The objective of my research is to attach targeting moieties to a liposome containing perfluorocarbon emulsions and evaluate their \textit{in vitro} efficacy. The targeting moiety is designed to cause the liposome to attach to certain cells, such as cancer cells, and induce endocytotic uptake by the cell. Upon ultrasound activation, the perfluorocarbon emulsion is expected to vaporize and cause the emulsion droplet to expand to a gas, thus causing the liposome to burst. If the liposome happens to be inside an endosome, the endosome may also burst. Since delivery via the endosome is desired, a ligand that induces receptor mediated endocytosis (RME) is required. Folic acid and TK1 antibody fragments have been selected as targeting ligands. Folic acid is a vitamin that is essential in certain biological processes. The receptor for folic acid is overexpressed on the surface of some cancer cells. Thymidine kinase 1 is a protein that is overexpressed on many cancers. A TK1 antibody has been developed that attaches to many different cancers. Folic acid can be attached to 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG(2000)-amine) via carbodiimide chemistry. The TK1 antibody fragment can be attached to DSPE-PEG-maleimide through a reaction between a free thiol on the fragment and the maleimide group creating a thioether bond.

Drug delivery to the cytosol of cells requires overcoming several barriers. Encapsulating emulsions in targeting liposomes (eLiposomes) provides a means to overcome one of the
barriers—the endosome. A concept drawing of an eLiposome is picture in . In order to efficiently deliver eLiposomes to the endosomes of cells, targeting ligands can be used so that the eLiposomes are taken up by receptor-mediated endocytosis, after which the emulsions can be activated by ultrasound. Upon rupture of the liposome and the endosome, the contents would be spilt into the cytosol. The specific goals of this study include:

1. Attaching folate and TK1 antibody fragments to polyethylene glycol (PEG)
2. Insert the ligand-modified DSPE-PEG into the bilayer of an eLiposome
3. Validate localization of eLiposomes in cells
4. Evaluate effect of ultrasound on cells treated with eLiposomes in vitro

Figure 1. An eLiposome which consists of a lipid bilayer surrounding perfluorocarbon emulsions. The spheres on the outer membrane represent targeting molecules connected to the lipids in the bilayer via a polyethylene glycol spacer.
4 EXPERIMENTAL APPROACH

4.1 Synthesis of DSPE-PEG-Folate

Folate was conjugated to DSPE-PEG-amine as previously described [15]. Briefly, a molar excess of folic acid (16.7 mg) was added to a round bottom flask and dissolved 667 µL of anhydrous dimethyl sulfoxide (DMSO). Anhydrous DMSO was further dried by addition of molecular sieve. DSPE-PEG-amine (66.7 mg) was dissolved in 333 µL of pyridine and added to the round bottom flask. N,N'-dicyclohexylcarbodiimide (DCC) (21.7 mg) was then added to the reaction mixture. The reaction ran for 4 hours under nitrogen at room temperature in the dark. The pyridine was removed by evaporation. Distilled deionized water (ddH₂O) was added to make a mixture of 90% water by volume. The unreacted folic acid, DCC, byproducts, and DMSO were removed by dialysis with a 3500 molecular weight cut-off. Other larger byproducts were removed by centrifugation. After removal of byproducts and reactants, an equal volume of chloroform was added to extract the product (DSPE-PEG-folate). A drop of hydrochloric acid was added to the aqueous phase to protonate the product to make it more soluble in chloroform. The characteristic yellow of folate shifted from the aqueous phase to the chloroform phase. NMR was used to confirm attachment of the folic acid to DSPE-PEG-amine.
4.2 Synthesis of DSPE-PEG-TK1 F(ab’)

TK1 monoclonal antibody was kindly donated by Dr. Kim O’Neill of the Microbiology and Molecular Biology department at BYU. TK1 F(ab’)_2 fragments were isolated using Invitrogen’s F(ab’) digestion kit as directed. Fc fragments were removed by dialysis against ddH₂O with a 50,000 molecular weight cut-off. Further reduction of the F(ab’)₂ was performed by incubating the protein in a reduction buffer of 2-mercaptoethanolamine for 90 minutes at 37°C under constant stirring. Dialysis against ddH₂O in a 3,500 molecular weight cut-off membrane removed the reduction buffer while retaining F(ab’) fragments.

Micelles were prepared by adding 2 mg of powder DSPE-PEG-MA lipid to 6 mL of PBS and heating the solution to 60°C for one hour with periodic stirring. The micelles and F(ab’) fragments were incubated together overnight under nitrogen. Unreacted F(ab’) was removed when external calcein was removed as described in section 4.3.1.

4.3 eLiposome Preparation

Chloroform was removed from 60 mg of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) lipids under vacuum with a rotary vacuum evaporator, leaving a thin dry lipid film on the flask. The lipid film was hydrated by adding an aqueous solution (water or PBS) to the flask to a total lipid concentration of 30 mg/mL and heating the mixture to 50°C while rotating for 30 minutes or until the lipid was suspended to form liposomes. When necessary, a sonicating bath was used to remove any remaining lipid residue from the flask. To form small unilamellar vesicles (SUVs), the liposomes were sonicated with a 20 kHz sonicating probe for 15 minutes at 30% intensity at room temperature. The resulting solution was clear and had a blue tint. At this
point vesicles were typically 30 to 80 nm in diameter as measured by dynamic light scattering (DLS).

Interdigitated DPPC sheets were formed by adding ethanol drop wise while stirring to a total concentration of 3 M (approx. 0.2 mL of ethanol per 1 mL of original vesicle solution). As the ethanol was added, the solution became more opaque white and showed an increase in viscosity. Then the ethanol was removed by centrifugation as follows. The lipid sheets were diluted in ~45 mL of ddH2O and placed in a 50 mL centrifuge tube, and spin for 3 minutes at 2500 rpm. The ethanol rich supernatant was discarded, and the lipid pellet resuspended in ddH2O and centrifuged again. The resulting sheets were then resuspended to a final volume of 2mL in a 150mM Hepes/50mM NaCl buffer (pH 7.4).

Nanoemulsions were prepared with DPPC as a surfactant to stabilize the liquid perfluorohexane (PFC6). First small unilamellar vesicles (SUVs) of DPPC were prepared using a probe sonicator as described previously. Perfluorohexane was then added to the SUVs in a 10:1 mass ratio DPPC:PFC6. The PFC6/liposome mix was then placed on ice and sonicated with a 20 kHz sonicating probe for 30 seconds at 20% intensity twice with a minute rest in between. Emulsions were then extruded through a 100 nm polycarbonate filter 15 times. The resulting emulsion was placed on ice and sized using the DLS.

eLiposomes were formed by adding 0.1-0.2 mL of emulsion solution (10 mg lipid, 0.1 g PFC6, in 1 mL volume) to the interdigitated lipid sheets. Calcein at a concentration of 30 mM was added to the sheets/emulsion mix to a final concentration of 0.5 mM in some experiments and 10 mM for others. The emulsion concentration chosen results in encapsulating approximately 4 emulsion droplets inside each eLiposome. The solution was then heated to 50°C and stirred for 30 minutes, allowing the sheets to fold around the adjacent aqueous
solution, including emulsion droplets. The eLiposomes were extruded through a 200 nm polycarbonate membrane filter using a hand extruder (LiposoFast-Basic, Avestin, Ottawa Ontario).

4.3.1 Surface Modified eLiposomes

DSPE-PEG-folate micelles were prepared by dissolving 2-6 mg of DSPE-PEG-folate in 0.6 mL of DMSO and then adding 5.4 mL of ddH₂O. The DMSO was removed by dialysis in a 3500 molecular weight cut off (MWCO) bag at room temperature. The first dialysate was removed after 4 hours and the second was left overnight. DSPE-PEG-folate micelles were then mixed with the eLiposome formulation in an eppendorf tube to a concentration of approximately 1.2 mol%. The mix was incubated at 50°C for one hour.

Micelles of DSPE-PEG were prepared in a similar way as the DSPE-PEG-folate micelles. Micelles of DSPE-PEG-maleimide were prepared by simply adding 2-6 mg of lipid to ddH₂O and heating to 60°C. DSPE-PEG and DSPE-PEG-F(ab’) were inserted into eLiposomes as described above.

When eLiposomes containing calcein are formed, the calcein solution is found inside and outside the eLiposome. External calcein was removed using centrifugation. Hepes/NaCl buffer was added to the eLiposome samples to a volume of 1 mL in an eppendorf tube. eLiposomes were centrifuged at 3000 rpm for 10 minutes using a microfuge; then the supernatant was removed. The pellet was re-suspended in a Hepes/NaCl buffer to a final volume of 1mL. Each sample contained between 3 to 7 mg of DPPC lipid. Samples of 0.5 mM calcein were centrifuged once and samples of 10 mM calcein were centrifuged 3 times.
4.3.2 eLiposome Characterization

The labeled eLiposomes were characterized using dynamic light scattering (DLS). Size distributions of the eLiposomes were obtained after extrusion. Approximately 20 µL of eLiposomes were added to 3 mL of PBS for analysis using DLS.

4.4 In vitro Experiments

HeLa (CCL-2™) cells were grown in DMEM (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS). Experiments were conducted on cells passaged between one and 20 times after receiving them from ATCC. HeLa cells were starved of folate for 24 or 48 hours. Approximately 660,000 cells were seeded per well in a 12-well plate and cultured in RPMI 1640 folate free media for 24 hours. Approximately 300,000 cells were seeded per well in a 12 well plate for cells culture in folate from media for 48 hours. eLiposome preparations were added to the wells in 100 µL aliquots. eLiposomes and cells were allowed to incubate for 1 to 24 hours.

Free folate was used to verify the mechanism of attachment. A folate solution was prepared with a concentration of 6-8 mg/mL. An aliquot of 100 µL pipetted into a well containing 1mL of media resulted in a final folate concentration of 100 mM. The folate was incubated with the cells for 2 minutes to occupy folate receptors on the HeLa cells.

4.4.1 Sonication

The 20 kHz probe was suspended with the tip approximately 1.5 cm above the cell monolayer. The power generator was set to 20% duty cycle which is an intensity of about 1 W/cm². Ultrasound was applied for 0.2 seconds. After sonication, the media was removed from
all the wells, and the cells were washed with PBS four times to remove any un-incorporated eLiposomes and calcein. The cells were harvested and suspended in 0.5 mL RPMI 1640 folate free media for flow cytometry and confocal slide preparation. The cell suspension was placed in 12x75 cell culture tubes used for flow cytometry.

4.4.2 Flow Cytometry

Samples were run through a BD Facscanto™ flow cytometer equipped with a 488 nm wavelength laser to excite fluorescence of calcein. Calcein fluorescence in/on the cells was quantified using flow cytometry. 10,000 events were collected for each sample. Flow results were analyzed using FACSDiva software (Becton Dickinson, New Jersey) along with Summit v4.3 software (Dako, Denmark).

4.4.3 Confocal Microscopy

Slides were prepared using a Cytospin™ (Thermo Scientific, Asheville, NC) to concentrate suspended cells onto the glass slide. The slides were spun at 800 rpm for 3 minutes. A drop of PBS was placed on the cells, and a 22x22 mm glass coverslip was gently placed over the spot. Slides were placed in the dark until viewing under the confocal microscope.

Intracellular uptake was observed using calcein and fluorescein dextran 10,000 MW (Invitrogen, Carlsbad, California). HeLa cells were imaged using an Olympus FluoView FV 300 confocal laser scanning microscope. The confocal is equipped with an argon laser providing excitation light at wavelengths of 458 nm, 488 nm, and 515 nm, as well as green Helium-Neon and red Helium-Neon laser sources with respective excitation wavelengths of 543 nm and 633
nm. The argon laser with excitation wavelength 488 was used to visualize the localization of eLiposomes containing calcein.

4.5 **siRNA Delivery Experiment**

The effectiveness of eLiposome delivery and ultrasound treatment was evaluated by “knocking down” the phosducin-like protein (PhLP) protein. The PhLP protein, which is a chaperone for G-protein, is expressed in cells including HeLa cells. In this experiment the HeLa cells were plated in the wells of a 12-well plate. Cells were exposed to eLiposomes containing PhLP siRNA or control (non-specific) siRNA +/- folate or knocked down with 20 nM siRNA against PhLP or control, and then harvested 90-96 hours later. Additionally siRNA against PhLP or control siRNA was complexed with oligofectamine, which is known to transfect cells and was used as a positive control in most experiments.

Cells were treated with 100 µL of eLiposomes or oligofectamine. The DPPC concentration in the eLiposome suspension was ~1 mg/mL and the size was ~200 nm, resulting in an estimated concentration of ~2 billion eLiposomes/mL. The cells receiving ultrasound were sonicated two hours after eLiposomes were added. Cells treated with siRNA complexed with oligofectamine were washed after four hours, and fresh media was added. After 90-96 hours, the cells were washed with ice-cold buffer and treated with a lysis buffer to solubilize the cells. The cells were centrifuged to separate membranes and other debris from the protein in the cell. A DC-Lowery protein assay was conducted to determine the relative protein concentration in each sample before loading the samples in the gel.

The protein supernatant in each sample was placed in reduction buffer and heated to denature the proteins and prepare them to be placed on a polyacrylamide gel. The samples were
placed on the gel and separated according to molecular weight. The protein on the gels was transferred to a nitrocellulose membrane and blotted for PhLP. Blots were scanned on the Licor Odyssey imager and quantified.
5 RESULTS AND DISCUSSION

5.1 NMR of DSPE-PEG-folate

NMR results for the synthesis product of the reaction between DSPE-PEG-amine and folic acid show the appearance of peaks corresponding to folic acid. Peaks at 8.643 and 7.643 ppm indicated the presence of folate, which suggests successful conjugation. Not all of the peaks appeared for folic acid, which may be a result of having a much lower fraction of the hydrogens than the DSPE-Peg amine. Also this could indicate a less efficient coupling.

5.2 Attaching TK1 F(ab) Fragment

Usually radioactive antibodies are used to quantify attachment of the antibodies to PEG. Resources were not available to verify attachment directly. The supposedly TK1-F(ab’)-modified-PEG-lipids were inserted into eLiposomes containing calcein. These TK1-modified eLiposomes were incubated with HeLa cells, which are known to express TK1 proteins. Flow cytometer results showed that 7.9% of cells treated with TK1 eLiposomes displayed the green fluorescence of calcein compared to 0% of cells treated with non-labelled eLiposomes. This result, however, has not yet been repeated with the same success. These results are supportive but not conclusive that TK1-F(ab’) was attached to the eLiposome. Additional results obtained using TK1-F(ab’) fragments are disclosed later.
5.3 **Dynamic Light Scattering Results**

Dynamic light scattering was used to investigate the size distribution of emulsions and liposomes. The sizes are listed in Table 1. The size distribution of eLiposomes before extrusion show two peaks, a peak at 100 nm and another at ~500 nm. The 500 nm peak represents liposomes formed from the refolding of the DPPC lipid sheets. When the eLiposomes were extruded through a 200 nm polycarbonate membrane, the bi-modal distribution disappeared and the two peaks were not distinguishable. It is probable that the two peaks are present, but the curve fitting algorithm in the DLS software merged them into one peak.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak Type</th>
<th>Size Peak 1</th>
<th>Size Peak 2</th>
</tr>
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<tbody>
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<td>90 to 150 nm</td>
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<tr>
<td>Vesosome</td>
<td>single</td>
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<td>Extruded eLiposome</td>
<td>single</td>
<td>130 to 210 nm</td>
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5.4 **eLiposome Localization**

Localization of the folated eLiposomes was examined using confocal imaging and flow cytometry. Many experimental parameters were unknown at the beginning of the experiments such as what calcein concentration to use inside the eLiposomes, how many eLiposomes to add to the cells, incubation time, and sonication time. The results listed below do not repeat all the same parameters but rather show an evolving experimental protocol in order to achieve the best delivery possible.

The results shown in Figure 2 show the percentage of HeLa cells with calcein fluorescence. These cells were treated with 100 µL of eLiposomes at a concentration of ~2 mg/mL DPPC. The calcein concentration inside the eLiposomes was 0.5 mM. External calcein
was removed using centrifugation as described earlier in the experimental approach section. The green bars (bars 3, 7, 11, 14) labeled Lipo-Folate were liposomes recovered from the supernatant of the centrifuged eLiposomes. The supernatant contains micelles of DSPE-PEG-folate and any liposomes that do not contain emulsions. This experiment investigated the effectiveness of the centrifugation and the inhibitory effect of free folate. Two minutes before adding folated eLiposomes to the cells, free folate was added to the cells. Free folate and folated eLiposomes compete for binding sites on the folate receptors of cells. Free folate inhibited binding of folated eLiposomes by occupying folate receptors, thereby out-competing folated eLiposomes for binding sites, which manifests itself as a decrease in calcein fluorescence of the eLip-Folate and Lipo-Folate (compare bars 1, 9 and 3,11).

eLiposomes modified with three different PEG-lipids were compared. Other wells of the 12-well plate were used to assess the inhibitory effect of free folate. Folated and TK1-modified eLiposomes were compared against non-targeting eLiposomes. Cells treated with folated eLiposomes showed a 4-fold increase over non-targeting PEGylated eLiposomes (see bars 1 and 2). Cells treated with free folate and folated eLiposomes showed green association at the same level as non-targeting eLiposomes (see bars 2, 6, 9, 10, 11, 13). These results suggest that the folate-lipid inserted into the eLiposomes is binding to the folate receptors on the HeLa cells. TK1-modified-liposomes showed an increase in fluorescence over non-targeting eLiposomes (compare bars 2 and 4), suggesting that the TK1-F(ab’) may be present and attaching to the cells.
Figure 2. The percent of cell populations with significant fluorescent shift. eLip-Folate indicates the cells treated with folated eLiposomes, eLip-PEG indicates the cells treated with pegylated eLiposomes (non-targeting), Lipo-Folate indicates cells treated with liposomes without nanoemulsions, and eLip-TK1 indicates cells treated with TK1 F(ab’) fragments attached to eLiposomes. eLip/Lipo + US indicates those cells that were sonicated after being incubated with folated eLiposomes. eLip/Lipo + Free Folate indicates the cells treated with free folate prior to exposure to eLiposomes or liposomes. N/A indicates that these experiments were not done.

Upon sonication, the cell-associated fluorescence decreased in both the case of the folated eLiposomes and TK1-modified-eLiposomes (see bars 1, 5 and 4, 8). Unexpectedly the percent of fluorescent cells increased when the cells were treated with folated eLiposomes, free folate, and ultrasound (see bar 12).

The calcein used in these experiments displays the phenomenon of fluorescence self-quenching, in which fluorescent molecules interact and suppress photon release at high
concentrations. As the calcein is diluted the fluorescence intensity increases to a maximum then decreases to zero at infinite dilution. At high concentrations of calcein > 0.07 mM the fluorescence will decrease to near zero, but upon dilution the fluorescence will increase to a maximum and then decrease again. High concentrations inside the eLiposomes > 0.07 mM were chosen with the goal of showing calcein release with ultrasound. It was hypothesized that the fluorescence would increase upon sonication as self-quenched calcein was released and diluted, and that this increase would be reflected in the flow cytometer and confocal results. Unexpectedly the fluorescence decreased when ultrasound was applied. It was expected that the fluorescence would increase since the concentration inside the eLiposomes was in the self-quenched range, so upon release would dilute and show increased fluorescence. There are two possible explanations for this result. First, some or all of the eLiposomes could be attached to the surface of the cells so when they are sonicated they release the entrapped calcein, which is washed away in the washing steps after sonication. Another explanation is that the eLiposomes could be inside the cells and upon sonication could be releasing all their contents but the calcein gets so diluted beyond the maximum fluorescence that it does not produce enough of a signal to be picked up by the detector on the flow cytometer.

Curiously, when eLiposomes modified with folate were sonicated in the presence of excess free folate the percent of green cells increased. It was hypothesized that this could be a result of increased uptake of the surrounding solution by the cells caused by the presence of free folate, which stimulates endocytosis activity. When the external eLiposomes are ruptured by the ultrasound and release calcein, the cells could be taking in the free calcein in solution.

These data suggest that the eLiposomes labeled with folate are sticking to cells via folate receptors since free folate lowered the fluorescence to the levels of non-targeted eLiposomes.
Free folate inhibited uptake of calcein in eLiposomes and also inhibited uptake of the folated liposomes (without emulsions) collected in the supernatant (green bars, 3, 7, 11, 14). This observation suggests that the separation techniques seems to separate eLiposomes from empty liposomes. The Lipo-Folate + US sample did not show the same decrease in fluorescence as the folated eLiposome sample (compare bars 1, 5 to 3, 7). This is consistent with the hypothesis and previous data in the Pitt group that eLiposomes are more sensitive to rupture by ultrasound than liposomes not containing emulsions.

A similar experiment was conducted in order to test the hypothesis that the eLiposomes were sticking to the outside of the cells and that sonication was breaking open those eLiposomes and causing the observed decrease in fluorescence. Instead of calcein, fluorescein dextran (MW = 10,000) was used which did not self-quench at the concentrations used.

Figure 3 shows the same pattern observed in the above experiment with concentrated calcein. Upon sonication the cell-associated fluorescence decreased to the level of controls. However when ultrasound and free folate were combined the cell-associated fluorescence remained high when a decrease in calcein fluorescence was expected. These results further support the hypothesis that the eLiposomes were attaching to the outside of cells and upon sonication were releasing their contents. Again the inhibitory effect of the free folate was observed. The free folate out-competes the folated eLiposomes for binding sites on the cells.
Figure 3. The percent of cell populations with significant fluorescent shift using fluorescein dextran. eLip-Folate indicates the cells treated with folated eLiposomes. eLip + US indicates those cells that were sonicated and treated with folated eLiposomes. eLip + Free folate indicates the cells treated with free folate before incubation with folated eLiposomes.

Figure 4 shows the images of the cells described above in Figure 3. The flow cytometry results showed that free folate decreased cell-associated fluorescence. The cells treated with free folate show a noticeable decrease in calcein fluorescence when comparing the middle images of rows A and B. In the overlay pictures very, few cells have green inside the membrane. Most of the fluorescence is localized on the outside around the membrane. This indicates that the eLiposomes are associating with the cells via the folate receptor and the folate attached to the eLiposomes. In row B the calcein fluorescence decreased suggesting that the free folate out-competed the folated eLiposomes for folate receptors.
Figure 4. Confocal images of HeLa cells. Row A contains images of cells treated with folated eLiposomes: left: light image, center: fluorescent image, and right: overlay image. Row B shows images of cells treated with folated eLiposomes and free folate.

The results above showed very little if any intracellular uptake. Most of the green fluorescence is concentrated near the membrane. In addition to low uptake, only ~15% of the cells had any green associated with them. For eLiposomes to be a more viable option for drug or gene delivery, higher delivery rates are required along with endocytotic uptake.

There are a few possible explanations for the low uptake and also the low cell-associated fluorescence. First, low uptake could result from particles that are too large to be quickly endocytosed or there was insufficient time for the cells to endocytose the eLiposomes. Folate receptors are associated with clathrin-independent endocytosis, and particles endocytosed in this pathway can be as large as 500 nm [27]. These eLiposomes were 150 nm to 1 μm due to eLiposome agglomeration during centrifugation to remove calcein. Larger sized particles are associated with longer incubation times to increase uptake. Furthermore, the cell-associated fluorescence could be a result of low folate receptor expression. In other words the HeLa cells
may not have been starved of folate long enough. In previous experiments, zero calcein fluorescence was detected with cells that were cultured in media containing folate (data not shown).

To test these two hypotheses another experiment was designed. Two 12-well plates were prepared containing HeLa cells cultured in RPMI 1640 folate free media. After 24 hours one of the plates was treated with eLiposomes (targeted and non-targeted) containing 10 mM calcein. The eLiposomes and cells were allowed to incubate together for 24 hours.

After ~45 hours the other twelve-well plate was treated with eLiposomes (targeting and non-targeting) containing 0.5 mM calcein. These eLiposomes were allowed to incubate with the cells for 2.5 hours at which time the cells in selected wells were sonicated. All the cells were harvested and prepared for flow cytometry and confocal microscopy.

Figure 5 shows the flow cytometer results of the cells incubated with eLiposomes for 24 hours. Cell-associated fluorescence increased dramatically over the previous experiments (65% vs. 10%). The cells incubated with non-targeting eLiposomes maintained a calcein percentage of about ~2% as seen in previous experiments. However, the same pattern is seen in this experiment as seen in the previous with a decrease in percent calcein fluorescence upon sonication, a decrease with excess folate, and an increase in calcein fluorescence when combining free folate and sonication. It is still not known whether these differences are statistically significant or what the error ranges are on these results.
Figure 5. The percentage of cells with calcein fluorescence after incubation with eLiposomes for 24 hours. eLip-Folate are cells treated with folated eLiposomes and eLip-PEG are cells treated with pegylated eLiposomes (non-targeting). Each eLiposome formulation was sonicated (US). Percentage of green cells treated with Free folate alone and in combination with US appear in the figure. N/A indicates that these experiments were not done.

Figure 6 is an overlay of the fluorescence shift on the flow cytometer. The red histogram population (vertical hatch) is the cell population treated with folated eLiposomes. The green histogram (left-slanting hatch) is the cell population treated with free folate just before adding folated eLiposomes. The mean of the green histogram is 133.83 arbitrary fluorescence counts (afc) while that of the blue histogram is 101.16 afc. A downward shift in the fluorescence mean was also observed in the sample that was exposed to folated eLiposomes and sonicated as shown in Figure 7. In this case the mean shifted from 133.83 to 62.97 afc.
Figure 6. Histogram overlays of the fluorescent shift observed in cells treated with folate eLiposomes (red vertical hatch) and those cells treated with folate eLiposomes and free folate (green left-slanting hatch). The x-axis is on a log scale and the tic marks indicate 10, 100, and 1000 respectively.

Figure 7. Histogram overlays of the fluorescent shift observed in cells treated with folated eLiposomes (red vertical hatch) and those cells treated with folated eLiposomes and ultrasound (green left-slanting hatch). The x-axis is on a log scale and the tic marks indicate 10, 100, and 1000 respectively.

Statistical analysis was performed on the raw data collected in Figure 6 and Figure 7 to determine if the change in the populations was significant. Methods described by Zhou et al. for comparing log normal distributions were used [28]. Briefly the log of the arbitrary fluorescence
units for each event within a specified region was taken and averaged. The standard deviation of the log of the fluorescence values was also calculated. Figure 8 shows the results obtained from this statistical analysis. What this analysis reveals (that is not immediately apparent in the histogram data) is the increase in the mean fluorescence of the cells exposed to folated eLiposomes and ultrasound. These results suggest that release of entrapped calcein inside of eLiposomes was released into the cells. If the eLiposomes were on the outer membrane of the cell the calcein would have been washed away as the cells were washed four times with PBS after sonication.

Figure 8. The means of the log(arbitrary fluorescence units) taken from the raw data in Figure 6 and Figure 7. Only cells treated with folated eLiposomes are compared at various conditions: US, free folate, and US + free folate.

Figure 9 shows the confocal images collected on the cells that were starved of folate for ~45 hours and incubated with folated eLiposomes for 2.5 hours. In row A (cells treated with folated eLiposomes) the green is associated with cell membranes. Very little green is seen inside
of cells. The effect of ultrasound can be seen in comparing pictures in rows A and C (row C images are of cells that were sonicated and treated with folated eLiposomes). Row B contains images of cells treated with free folate and folated eLiposomes. These results support the hypothesis that the ultrasound is breaking up the eLiposomes or shearing them off of the surface of the HeLa cells. The calcein is then washed away and does not show up in flow or confocal data. Row D contains images of cells treated with free folate, folated eLiposomes, and then later sonicated. Row E contains images of cells treated with non-targeting eLiposomes. Row F contains images of cells treated with non-targeting eLiposomes and then sonicated.

Figure 10 through Figure 15 show the flow cytometer data of the cells pictured in Figure 9. The y-axis is arbitrary light units collected by the side-scatter detector. The x-axis is arbitrary light units collected by the forward scatter detector. Figure 10A is what these scatter plots normally look like for normal HeLa cells. In Figure 10B the cells were starved of folate for 48 hours, and a new population appeared within this experiment that was not observed previously. The population shows up lower on the forward scatter, which is correlated to size, and higher on the side scatter plot, which is correlated to cell cytoplasm complexity, which means these particles are small and complex. The population is completely green (signal is picked up by green fluorescence detector on flow cytometer) and does not resemble fragments of dead cells, which emit fluorescence on all channels not just green.
Figure 9. Confocal images taken of cells exposed to folated eLiposomes after being starved of folate for 48 hours. Cells in rows A-D were exposed to folated eLiposomes, A folated eLiposomes, B folated eLiposomes + free folate, C folated eLiposomes + US, D folated eLiposomes + US + free folate, E pegylated eLiposomes (non-targeting), and F pegylated eLiposomes + US.
Figure 10. Plots of the side scatter versus the forward scatter measured by the flow cytometer. Panel A shows negative control HeLa cells (no treatment). Panel B shows the new population that appeared with those cells that were exposed to folated eLiposomes after being starved of folate for 48 hours.

Each population was separated from the other and viewed as a histogram to show where these populations show up on fluorescence shift. The new population is completely green. There are a couple of hypotheses that might explain this observation. Since the cells were starved for longer they could have taken in eLiposomes and died and/or shriveled, which would account for the location on the side-scatter-versus-forward-scatter plot. Another explanation is that the population is agglomerated eLiposomes attached to cells. The confocal images above undermine the shriveled cell hypothesis since all of the green is visualized around the membrane of the cells. More random location of the green fluorescence would be expected if they were shriveled cells.
Figure 11. The gated population of the data from Figure 10 (B).

Figure 12. The histogram plot of the gated data (red, peak below 10) from Figure 11. The other grey data is the non-gated cell population. The dotted line is the histogram of the un-gated population.
Figure 13. The gated population from the data shown in Figure 10.

Figure 14. The fluorescent shift of the gated population in Figure 13. The other data is the non-gated event population. The dotted line indicates non-gated events with a similar fluorescent shift.
Figure 15 shows the histogram of events collected from the cell sample that was treated with folated eLiposomes then sonicated 2.5 hours later. A noticeable decrease in the peak between 10 and 100 is consistent with earlier results and supports the hypothesis that the eLiposomes are first attaching but being subsequently removed by ultrasound.

Another experiment was conducted to investigate time dependent uptake. In this experiment a calcein concentration of 0.5 mM was used to be consistent with other experiments. The cells were starved of folate for 24 hours when the first eLiposomes were added at the 24 hour time point. In other wells eLiposomes were added at 10 and 6 hours before being harvested. After the cells incubated with the eLiposomes, selected wells were sonicated. The cell-associated fluorescence is reported in Figure 16. Folated eLiposomes were compared with non-targeting eLiposomes, and the effect of ultrasound and free folate were tested. Free folate inhibited cell-associated fluorescence as seen in early experiments.
Ultrasound caused a decrease in fluorescence as seen in previous experiments already mentioned. However, when ultrasound and free folate were combined the cell-associated fluorescence did not increase as it did in earlier experiments (Figure 2 and Figure 3). For the earlier experiments it was hypothesized that the cells endocytosed calcein in the surrounding media after the calcein was released from the eLiposomes. It was believed that the free folate increased endocytosis events, which increased uptake of surrounding calcein. It makes sense that after a certain period of time the amount of endocytosis events for free folate would decrease. The decrease in endocytosis events would explain the decrease in fluorescence seen in Figure 16. By the time the cells were sonicated, the endocytosis events likely decreased so that any calcein in the surrounding media was not taken into the cells.

Figure 17 shows confocal images of cells from the experiment explained in the previous paragraph. Row A contains images of cells incubated with folated eLiposomes for 24 hours. Row
B contains images of cells incubated with free folate and folated eLiposomes for 24 hours. The fluorescent image in row B is noticeable less green than the image in row A indicating that the eLiposomes are binding to cells via folate. More fluorescence is observed inside the membrane of the cells in row A (overlay image) than in Figure 9 (A overlay) and Figure 4 (A overlay).

![Figure 17. Confocal images of cells treated with folated eLiposomes for 24 hours (A). Cells were also treated with free folate before adding folated eLiposomes and allowed to incubate for 24 hours (B).](image)

5.5 siRNA Delivery

The use of eLiposomes coupled with low frequency ultrasound is an attractive delivery system for therapeutics targeting the cytoplasm of cells. It may be possible to effectively deliver siRNA to the cytosol of cells using this technique. SiRNA targeting the phosducin-like protein
(PhLP) was used to evaluate the efficacy of the eLiposome as a delivery vehicle (Figure 18). Control siRNA was used, which is a non-specific siRNA. The cells were sonicated two hours after exposure to eLiposomes.

![Figure 18. Scans taken of the immunoblots conducted for PhLP. The top two images (A and B) were the non-sonicated replicates. The bottom two images (C and D) were the sonicated replicates. From left to right the lanes are: folated eLiposomes containing siRNA targeting PhLP, folated eLiposomes containing control siRNA, non-targeting eLiposomes containing siRNA targeting PhLP, non-targeting eLiposomes containing control siRNA, oligofectamine + siRNA targeting PhLP, and oligofectamine + control siRNA. Panels A and B were the non-sonicated samples and served as controls. Panels C and D were the sonicated samples. All the samples were replicated once so there are two panels for non-sonicated (A and B) and two for the sonicated (C and D) samples. The lysate from the cells was added to one of the six lanes. From left to right the lanes contain protein collected from cells treated with the following: folated eLiposomes containing PhLP siRNA, folated eLiposomes...](image-url)
containing control siRNA, non-folated eLiposomes containing PhLP siRNA, non-folated
eLiposomes containing control siRNA, oligofectamine and PhLP siRNA, and oligofectamine and
control siRNA. Panels C and D contain lysates of cells that were sonicated while panels A and B
contain lysates that were not sonicated. In panel C there is approximately a 50% difference
between the sonicated folated eLiposomes carrying PhLP siRNA and the sonicated folated
eLiposomes carrying control siRNA, which is evidence of significant protein knockdown by the
siRNA. However, PhLP protein did not decrease in the replicate (Panel D). Figure 19 shows the
quantified results from Figure 18. SiRNA delivered using oligofectamine knocked down the
PhLP protein to 1.27 and 2.87, about a 90% decrease in protein levels.

![Graph showing relative protein content](image)

**Figure 19.** The quantified values obtained from Figure 18. The y-axis are arbitrary fluorescence units obtained from the image quantifying the PhLP protein.

Obviously more experiments are needed to determine if the differences achieved are statistically significant. Although these results are not an immediate answer to the challenges of
effectively delivering drugs, they are still encouraging enough to continue investigating the delivery of siRNA.

5.6 Discussion of the Feasibility of Using Ultrasound

The original hypothesis underlying this work is that as the local pressure in an ultrasonic field decreased below the vapor pressure of the PFC, the PFC would start to boil to a gas. It was recognized that some under-pressurization would be required to nucleate the gas phase to commence boiling, but no literature or theory was found on how much under-pressurization and for how long under-pressurization would be required. Of course higher acoustic intensity and lower frequency ultrasound would increase the under-pressurization and the dwell time (length of time of under-pressurization). Theoretically, the transition to gas phase would happen spontaneously and instantly if the pressure could be reduced to the spinodal decomposition pressure, and bubble formation would be guaranteed instantly. The microbubble would then undergo collapse cavitation in an ultrasonic field; however, according to data presented by Brennen [29], subpressurizations of about 10 atm negative pressure would be required for the spinodal gas formation of perfluorohexane and perfluoropentane. This exceeds available instrument capacity and would probably damage cells. For lighter perfluorocarbons, the spinodal decomposition could theoretically be reached such as for perfluoropropane and perfluoromethane. However, at room temperature these chemicals are gases and would require additional processing adjustments to condense to liquid nanoemulsions. Furthermore, such emulsions may not be stable long enough to be practical. Perfluoropentane, with a boiling point of 29°C, will vaporize readily in emulsion preparations unless the emulsions are prepared on ice.
Therefore in these experiments the stochastic nucleation of a PFC6 gas phase during the millions of cycles of ultrasound exposure was relied on, but such nucleation was not guaranteed for PFC6 with a boiling point of 56°C.

Although the spinodal decomposition pressure cannot be reached with available equipment, ultrasound can be useful in sub-pressurizing the volume around an emulsion, thereby increasing the probability of nucleation. The mechanism by which nucleation occurs will most likely be heterogeneous.

Is it possible to stabilize emulsions of PFC5 (boiling point of 29°C) for use in the body at 37°C? The Laplace pressure equation calculates the pressure gradients across spherical interfaces of liquid or gas bubbles. The lipid bilayer of a liposome has two interfaces, water to hydrocarbon and hydrocarbon to water, so the pressure increase occurs twice in traversing the liposome membrane. The reported surface tension of a phospholipid bilayer is between $2 \times 10^{-8}$ N/m and $10^{-6}$ N/m, so low that the pressure gradient across the membrane is nearly zero even for a highly curved membrane [30]. The Laplace pressure across the interface of the perfluorohexane nanoemulsions can be estimated from the interfacial tension reported for perfluorocarbons [31]. As longer chain surfactants are introduced into the interface, the surface tension goes down. Values for surfactants similar to DPPC were not completely obtained due to the long equilibrium times needed. Therefore it was estimated that the surface tension for DPPC is about $3.5 \times 10^{-3}$ N/m. Thus for a PFC6 emulsion of 100 nm diameter stabilized by DPPC, the Laplace pressure is ~1.4 atm, and the absolute pressure is about 2.4 atm inside the emulsion. Using a vapor pressure correlation for bulk perfluorohexane this pressure corresponds to a boiling point of 85°C. Alternatively, a sub-pressurization of -194 kPa would induce equilibrium boiling at 37°C as shown in Figure 20. In contrast a PFC5 nanoemulsion would only need -0.04 atm of sub-
pressurization to reach the boiling point of PFC5. The PFC5 emulsion would be stabilized by the same lipid so the surface tension in a PFC5 and PFC6 emulsion was assumed to be the same, which results in the same Laplace pressure in each emulsion. The pressure in a PFC5 emulsion (2.4 atm) is still higher than the vapor pressure of PFC5 at 37°C (1.36 atm). This analysis shows that it may be possible to make very small PFC5 emulsions that are stable at 37°C because of their high Laplace pressure. It would be easier to nucleate these PFC5 emulsions than PFC6 emulsions.

Figure 20. Reduced pressure required to boil PFC6 and PFC5 in a 100 nm emulsion stabilized by DPPC.
6 SUMMARY AND RECOMMENDATIONS

6.1 Summary

In this work eLiposomes with targeting ligands were investigated as a potential drug delivery vehicle. The eLiposome surface was successfully modified with folate and the TK1-F(ab’) fragment. NMR of DSPE-PEG-folate showed characteristic folate peaks indicating successful attachment of folate. Attachment was further confirmed using dialysis, which resulted in the retention of the characteristic color of folate indicating conjugation to a larger molecule.

Preliminary results showed that the folated eLiposomes attached to HeLa cells to a greater extent than non-targeted eLiposomes. A series of experiments were performed to increase the cell uptake of the eLiposomes and also stimulate cytosolic delivery using ultrasound. Even though definitive evidence of cytoplasmic delivery was not obtained, promising results indicate that it could be possible with longer incubation times, smaller eLiposomes, and altering ultrasound exposure.

Flow cytometer results along with confocal microscopy showed that eLiposomes containing DSPE-PEG-folate in the membrane attached to HeLa cells more than non-targeting eLiposomes. F(ab’)-modified eLiposomes showed increased attachment to HeLa cells over controls but not as high as folated eLiposomes. Although delivery of calcein to the cytosol was not rigorously confirmed, promising results were obtained for cells treated with eLiposomes for 24 hours. The percentage of cells containing calcein fluorescence was as high as 60% for those
treated for 24 hours. Even though increased attachment was observed, the localization of the eLiposomes over time is still unclear. It is possible that the eLiposomes are too large to be internalized through receptor-mediated endocytosis via the folate receptor.

Flow cytometer results show that ultrasound can disrupt eLiposomes enough to release encapsulated calcein; however, limited delivery to the cytosol may indicate that the ultrasound is not reaching a low enough peak negative pressure to increase the probability of nucleating a gas and breaking open the eLiposome. Experiments conducted using calcein and fluorescein dextran both showed the same pattern of decreased cell-associated fluorescence when sonicated. Since the eLiposomes were attached to the outer membrane with no clear signs of internalization the released calcein was washed away in the PBS washes. It could be possible that eLiposomes inside of cells were disrupted and released calcein but the calcein was diluted below a detectable threshold.

6.2 Recommendations

In order to more rigorously prove that the eLiposome is an effective drug carrier, much work still remains to make targeted eLiposomes a functional drug delivery vehicle. As for this work there remain important questions to be answered or clarified.

Attachment of the F(ab’) fragment should be directly verified using radiolabeled F(ab’) fragments or a more sensitive protein assay. A protein assay was attempted but was not sensitive enough to detect the small amounts of protein present. TK1-F(ab’) fragments can be used to target many different cancers. It is an attractive targeting molecule since TK1-targeted-liposomes do not interact with regular healthy cells. Future studies should examine the use of TK1 antibody conjugation to eLiposomes containing doxorubicin or another potent chemotherapeutic.
It is still unknown how extrusion down to 200 nm affects the makeup of the eLiposomes. Other work done in the group showed that extrusion through 800 nm filters still retains emulsions inside a lipid bilayer. However at 200 nm, the size of the outer lipid bilayer approaches the size of the liquid emulsions. Cryo-TEM images should be taken to confirm emulsion retention in the bilayer of 200 nm eLiposomes. Cryo-TEM images of eLiposomes modified with ligands could further elucidate the effects of processing.

Removing external calcein from the eLiposome suspension proved difficult. Spin columns, dialysis and centrifugation were the techniques used in the effort to remove the calcein. Spin columns were inconsistent in that the eLiposomes could get hung up (captured, blocked) in the column. In addition to losing the sample, external calcein removal was not sufficient. Small amounts of external calcein in the sample probably distort flow cytometer results because the external calcein could be pinocytosed into cells. Dialysis takes a long time—in some cases up to 10 days to remove all the calcein. Centrifugation is fast and can remove almost all the external calcein with multiple washes and spins; however, it was observed that after centrifugation large aggregates of eLiposomes formed, and according to dynamic light scattering results, this caused an increase in size of eLiposomes by about 40 nm.

The results of flow cytometry and confocal microscopy indicated minimal internalization of the eLiposomes at the short times examined (one to four hours). It is recommended that longer incubation times be used to determine if more internalization will occur. Preliminary results indicated that more internalization occurs after incubation of 24 hours, but this should be verified. It is also recommended that a rigorous study investigate the time required to achieve optimal uptake. This is necessary so that ultrasound can be applied at the appropriate time to allow escape from the endosome.
Eventually eLiposomes and ultrasound could be used to deliver siRNA. Only small amounts of siRNA are needed in the cell to “knockdown” the target protein, so a cell may not need to internalize more than a few eLiposomes to produce the desired effect. In this study, large amounts of eLiposomes were added ~1,000 eLiposomes per cell (assuming 2,000,000 cells per well), but the fraction internalized was not measurable.

It is recommended that a lower-molecular-weight perfluorocarbon be used as the liquid in the emulsions to increase their sensitivity to ultrasound. Liquid emulsions could be made with perfluoropentane instead of perfluorohexane. Due to the low boiling point of perfluoropentane, care must be taken that the preparation is done with chilled instruments and materials to limit premature nucleation to gas. Once an emulsion is formed, the Laplace pressure should be great enough to prevent spontaneous nucleation of gas.

Negative results for cytosolic delivery of calcein could have been a result of poor concentration choice. It is recommended that higher concentrations of calcein be used. For example, if an eLiposome was internalized and disrupted with ultrasound, the internal calcein could have been diluted to the point of near-zero fluorescence and not show up in flow cytometry data or confocal images. Concentrations within the eLiposome on the order of 100 mM are recommended.
REFERENCES


APPENDIX A. DATA

A.1 eLiposome Preparation Manual

A Lab Manual for the

PREPARATION OF ELIPOSOMES

Dr. Pitt’s Cancer Research Lab 325 CB

Prepared By:
Tara Prasad Pandey and Jon Hartley
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1. MATERIALS REQUIRED

All the materials listed below are not required for any single preparation process but I am listing them just to get an idea about what instruments are required for the whole process of eLiposomes preparation. Materials required for specific method are listed in corresponding section.

- 1 round-bottom flasks
- Spin bar
- temperature bath
- hot plate
- water condenser
- nitrogen purge
- ParaFilm
- Cuvettes
- Micropipettes
- PD-10 Sephadex Spin Columns
- 50 mL round bottom flask (a larger or smaller flask can be used)
- Several sterile vials
- One empty spin column with white filter
- Pipet
- Sterile pipet tips
- Rotavap
- Dry Ice
- Ice bath
- Ultrasound (US) probe sonicator
- Stand and clamp
- 0.05 micron extrusion filter paper
- 0.1 micron extrusion filter paper
- 0.2 micron extrusion filter paper
- 0.4 micron extrusion filter paper
- 0.8 micron extrusion filter paper
- ddH2O (Double Distilled water)
- 2 Extruding Syringes

2. THE PREPARATION OF 30 MG/ML LIPID (DPPC) SOLUTION

Materials and Chemical Required: Rotary Evaporator, 50 mL round bottomed flask, Micropipette, 3 mL cuvette, US probe sonicator, ddH2O, DPPC lipids dissolved in Chloroform (20mg/mL)
➢ Take 1.5 ml of 20mg/ml 1,2-dipalmitoyl-sn-glycero-3 phosphocholine (DPPC) lipid in Chloroform (stored in freezer 325 CB) in a cleaned, sterilized round-bottomed flask
➢ Use the Rotary evaporator (in room 345) to remove Chloroform solvent. (Follow the following procedures to use the Rotary Evaporator)

3. HOW TO USE ROTARY EVAPORATOR

➢ Get a pound of Dry ice pallets from Chemistry Stock Room (Nicholes Building) and pour it in the bucket (see the bucket in figure 1 above)
➢ Insert the round-bottomed flask containing the DPPC sample at the end of the spinning tube by neck and clamp the round-bottomed flask’s neck to the spinning tube using a green clamper found on the desk (see above figure 1)
➢ Lower the round bottomed flask in order to dip it half way through the warm water bath at around 30-40 °C
➢ Turn on the rotary evaporator and turn the spinner to about half way through (spin in moderate speed)
➢ Turn on the vacuum motor (the motor inside a hood by the Rotary Evaporator)
➢ Make sure the vacuum is working by feeling a suction pressure on the rear end outlet of rotary evaporator
➢ Let the flask spin for about 30-60 minutes until all solvent vaporizes generating a thin lipid whitish film attached to the inner wall of the round bottomed flask
➢ Hydrate the thin lipid film with 1mL water (or any other buffer like PBS)(use amount of water to reach a concentration of 30 mg/mL).
➢ Let the solution spin on the same Rotary evaporator for about 20 min (it depends on how long it takes to dissolve the entire lipid solid on the round glass) without vacuum motor turned on under the water bath at 50-60 °C
➢ Sonicate the solution either under the water bath 20kHz sonicator (for about 30 minutes to an hour or use Ultrasound probe at 173 to sonicate the solution at 30% intensity for about 15-20 minutes (the probe works better).
Use DLS (Dynamic Light Scatter) for sizing the lipid formed if sizing is required for the experiment (see Measurement of Nano-Particles section below for sizing procedure)

Transfer the 30mg/ml lipid so formed in a small 5 ml vial and store the solution for the future use in preparing emulsions or lipid sheets.

4. **MEASUREMENT OF NANO-PARTICLES**

- We use Widtsoe Building room # 534 (you need a code to enter the room, see Dr. Pitt for the door code but it’s usually open)
- The machine is on the far west end of the room. The machine used for sizing is called Dynamic Light Scattering)
You might need someone to show how to work on it. However, I have given the basic idea about simple procedures. Once you turn on the machine (find a switch at backside of the machine to turn it on), the computer starts automatically and you have to input password to access the computer. Once you log in the computer, access the characterizing software (icon that looks like a handicap symbol).

Click on parameter and make appropriate changes as per the requirement
- In a new sterilized cuvette, add about 10 μL of the sample that is to be sized, and add double distilled water to make the sample about 2/3 of the cuvette.
- Mix the sample well using a micropipette; make sure there are not any air bubbles in the solution which might invert the results.
- Close the cuvette with a lid and insert the cuvette containing the sample in the machine. (To insert, slide the metal door plate on top of the machine. Then lift the black round lid of you will see once your slide open the door. Then insert the cuvette in the small open extension)
- Close the round lid and then the metal door on top of the machine
- Open the Brookhaven Instruments icon on desktop (or a disabled icon on task bar) and select Particle sizing software icon. This opens the program and the laser on the machine.
- Once the software is open, click the “Parameter” button and check the following areas.
  a. Sample ID: Write a description of the sample and run number.
  b. Operator ID: Write your name
  c. Notes: Add any additional notes
  d. Temperature: Set to 22°C
  e. Angle: Set to 90°
  f. Wavelength: Set to 658nm
  g. Batch#: Set to 0
  h. Minutes: Set to 10 for the full run (1 just for training)
i. **Seconds:** Set to 0

j. **Refractive Index of Particles:** Set Real to 1.59 for biological samples otherwise 1.53. Set Imaginary to 0

k. **Dust Filter:** These values generally given default and we do not have to change unless we know a specific reason to change

l. **Make sure the average count rate is somewhere between 800 kcps to 2 Mcps.** If the counts are less than 800 kcps, stop the run by clicking on stop button on the displayed window and take out the cuvette. Add a drop more sample. Mix the sample well with the micropipette and insert back the cuvette and run the experiment again.

m. **After the end of the run, click on zoom tab to see the results in more detail and analyze them.**

n. **We generally delete the first two sets of data** (the dimmed values in run 1 and run 2 in figure shown below are excluded from the analysis)

*After the complete run the following data are generally useful for the size analysis purposes:*

**Sept 17, DPPC Emulsion (Combined)**

**Effective Diameter:** 339.3 nm  
**Polydispersity:** 0.324  
**Avg. Count Rate:** 988.3 kcps  
**Sample Quality:** 9.6  
**Elapsed Time:** 00:08:00

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**Fig.5.** shows the display windows showing different runs and different parameters for emulsion sizing
Fig. 6 shows the bell curve of diameter distribution of emulsion.

Fig. 7 shows the distribution of diameters and the mean diameter of the sample
5. **PREPARATION OF LIPID SHEETS**

- Use the lipid prepared above to US for about 10-15 minutes until the lipid looks bluish color and size them (the size should be approximately 100 nm or smaller)
- Stir the lipid and add 0.2-0.3 ml of alcohol (about 20%) until the lipid solution becomes really viscous (you can try inverting the vial and see if it is really viscous and does not flow out of the vial)

Fig. 8 Viscous Liposomes formed after the addition of Alcohol

- Dilute the lipid sheet with water and transfer the sheet to spin column
- Centrifuge the sample twice to remove alcohol from the lipid sheets in Dr. Solen’s lab (336CB) at 1000 to 3000 RPM for 2 minutes each time. Caution! Make sure the holders in the centrifuge are balanced on the scale.
- Alcohol is further removed using small centrifuging machine for 2 minutes at 6000 rpm (325 CB, Dr. Pitt’s lab)

6. **PREPARATION OF EMULSION**

**Materials and Chemicals Required**

- 0.1 gm Perfluorohexane(PFC6), double distilled water, 0.333 ml of DPPC lipid solution (30 mg/mL), ice bath, extruding syringe, 3.0 ml glass vial, 2.0 ml plastic vial, US probe

- Mix 0.1 gm of PFC6 (found in a small glass vial in freezer) with 10mg DPPC (1/3 of ml) and add about 1.2 ml water (or PBS to make total volume of 1.5 ml solution in a cuvette

Note: Use a mass balance to measure 0.1 gm PFC6. I usually take the PFC6 and then add DPPC solution (0.333 mL) and mix them to sonicate under ice bath.
- Sonicate the solution under ice bath for 3 minutes (1 minute + break of 1 minute + 2 minutes). If the sizing results do not give very small emulsion, then I would sonicate for 5 minutes instead of 3.

**Fig 9. Use of US probe under ice bath to prepare emulsion**

- Extrude the sample through 50 nm or 100 nm filter (the size depends on the size requirement of emulsion for an experiment). However, it is extremely hard to extrude the solution through 50 nm or even 100 nm. The best way, I think is to extrude first through 200 nm and then 100 nm. It could be further extruded through 50 nm if required.

- Extrusion is done at a transition temperature of DPPC. For example, PFC6/DPPC emulsion, we extrude the sample 15 times (both ways) at 50 °C.

**Fig 10. Extrusion Setup in the lab**

- Once the sample is loaded in a sterilized syringe and the filter paper is sandwiched between the extrusion metal frames, let the syringe along with the sample warm for 10 minutes. This will make the extrusion easier and prevent filter paper from rupture or rearing apart due to bigger size of lipid chunk. Make sure to start extruding only after the temperature of the heating plate is constant at 50 °C.

- Size the emulsion. (Refer the “Measurement of Nano-Particle section for sizing detail)
7. PREPARATION OF ELIPSONES/ENCAPSULATION OF EMULSION OR FLUOROSCENCE MOLECULES

- Mix 1 ml of lipid sheets with 0.2 ml emulsion.
- Add 0.2 ml (calcein is added as fluorescence molecules to test the effect of US to release of calcein) if the size of emulsion is less than 100 nm and add 0.1 ml of calcein if the size is greater than 100 nm (this is done to make sure there is constant number of emulsion particles encapsulated in the eLiposomes).
- Heat the sample in a water bath at 50 °C or incubator. The water bath is prepared in a beaker. The steady state temperature should be 50 °C. It takes about 30 minutes to prepare the water bath. So it is advised to heat the water bath while working on emulsions and lipid sheets.
- The eLiposomes sample is kept in a small capped vial and let it warm under warm water bath for about 30 minutes. The heating plates needs to be at exactly 50 °C steady state. It takes about 30 minutes for the temperature to be at steady state.
- Extrude the sample after the lipid sheets are folded to form eLiposomes for 15 times again following the extrusion technique mentioned earlier.

8. REMOVAL OF EXTERNAL CALCEIN

External calcein can be removed using one of two techniques: Spin column technique or Dialysis technique

a. Centrifugation Technique
b. Dialysis Technique

DIALYSIS TECHNIQUE

- This method takes about at least 10-24 hours for successful separation of outside fluorescence molecules like calcein and emulsion of eLiposomes. (Compare the
time efficiency of few minutes spin column method vs several hours method, this is quite a long method of separation

- Osmotic balance between the sample and the outside solution is very crucial in this technique. The sample osmotic balance depends on the medium it is prepared in. If it is prepared on PBS solution, NaCl solution with the sample osmotic pressure is prepared
- Clamp the solution on a dialysis paper very carefully without spilling it over and dip the sample in the prepared NaCl solution hanging over a clamp. The sample is generally left for about 10-24 hours.

9. VALIDATION OF EMULSION ENCAPSULATION

**Density Cushion**

- This method works on the principle of density difference between emulsion, eLiposomes and the sucrose solution. The emulsion has higher density compared to sucrose or eLiposomes and hence settles on the bottom during centrifugation, eLiposomes forms the middle layer and the sucrose forms the top layer. Separation of eLiposomes (middle layer) is generally obtained by sucking the middle layer using a micropipette.
- Use the eLiposomes so obtained to run Flurometry run and analysis them for encapsulation and drug release. (See Fluorometer section below for detail)

10. FLUOROMETER: TRACKING ENCAPSULATION

- (Have someone show this process)
- Use 30 µl of sample (eLiposomes), 2 ml of water (or PBS is also used instead of water) in a special cuvette for fluorometry.
- Use 300 second run time with US 10% intensity every other 50 second for two times. Titrox 10 is used for the last 50 seconds to release 100% drug (calcein).
- Save the data in Excel file, just copy and paste works fine here.
11. CENTRIFUGATION

CAUTION: It is extremely important to balance a spin column with another column from opposite side, else the machine goes crazy with shaking, vibrating noise which might break the whole centrifugation column machine.

- Use a digital balancer in the Dr. Solen’s lab to balance the columns within ± 0.0001 g
- External calcein can be removed using the ultracentrifuge in room 325
  - Use eppendorf tubes
  - Set centrifuge to 3000 rpm and spin for 8 minutes
    - One spin is enough to remove calcein concentrations up to 0.5 mM

Acknowledgement
Most of the photos were taken from a lab manual prepared by Jwala Adhikari and Enrique Fernández.

A.2 In vitro Experiment Protocol

1. Prepare eLiposomes
   a. Mix fluor and perfluorocarbon emulsions with DPPC sheets.
   b. Extrude eLiposomes down to 200nm
   c. Separate eLiposomes into several eppendorf tubes
   d. Add PEG-micelles and incubate at 50°C for 60 minutes
   e. Centrifuge liposomes at 3000 rpm for 6 minutes
2. Cell culture
   a. Cut cells into a 12 well plate and supplement with folate free media 24 hours before the experiment
   b. A confluent T-25 flask that is resuspended in 5mL of media can be used to seed the 12 well plate. 0.3 mL is placed in each well. After 24 hours confluence is approximately 100%
   c. Cells were used between passage 1 and 20.

3. Adding eLiposomes to cells
   a. 100µL of the eLiposomes is added to the designated wells.
   b. Excess folate is added to designated wells to a final concentration of 1mM
   c. The plate was gently swirled to mix the added solutions
   d. The eLiposomes and cells are incubated together for 4 hours

4. Sonication
   a. 3mL of media was added to the wells to be sonicated.
   b. 20kHz probe is secured on a ringstand
   c. The generator was set to 20% intensity for a 0.2 second pulse

5. Harvest of Cells
   a. After sonication all the media is removed
   b. The cells are washed 4 times with PBS
   c. The cells are then removed from the wells and re-suspended in media (0.5mL)
   d. The cell suspensions are then added to 25 x 75 culture tubes for use at the flow cytometer

6. Flow cytometer
   a. Create an account on the RIC facility website
   b. Schedule a time a few days in advance
   c. Plan ahead so you don’t arrive late to the appointment
   d. Fill out the forms provided
   e. They can only pick up orange or green fluorescent molecules
   f. Bring a negative and positive control to every data acquisition
   g. The tech runs the samples

7. Slide preparation for Confocal
   a. The RIC facility has a machine called the Cytospin which can be used to make nice slides
   b. The cells used for the flow experiment can be used to make the slides
   c. About 200µL of cells at about 1million cells/mL is need for each slide
   d. Have someone show you how to operate the centrifuge
   e. Add a drop of PBS to the cell spot and place a glass coverslip overtop
8. Confocal
   a. Register for an account on the physiology and developmental biology website
   b. Sign up for training (you don’t need to bring a sample the first few times but you can)
   c. Acquire pictures

A.3 F(ab’) Preparation

Preparation of Fab’ fragments

Immobilized Pepsin Equilibration
1. Gently swirl the immobilized Pepsin vial to obtain an even suspension. Seat the spin column frit with an inverted 200 µl pipette tip.
2. Using a wide-bore or cut pipette tip, place 65 µl of the 50% slurry into a 0.8 ml spin column. Cap column and place into a microcentrifuge tube. Centrifuge column at 5,000 x g for 1 minute and discard buffer
3. Wash resin with 130 µl Digestion Buffer. Centrifuge column at 5,000 x g for 1 minute and discard buffer. Cap bottom of spin column with included rubber cap.

IgG Sample Preparation
1. Twist off the bottom closure of a Zeba Desalt Spin Column and loosen red cap. Place column in a collection tube.
2. Centrifuge column at 1,500 x g for 1 minute to remove storage solution. Place a mark on the side of the column where the compacted resin is slanted upward. Place column in centrifuge with the mark facing outward in all subsequent centrifugation steps.
3. Add 300 µl of Digestion Buffer to column. Centrifuge at 1,500 x g for 1 minute to remove buffer. Repeat this step three additional times, discarding buffer from the collection tube.
4. Place column in a new collection tube, remove cap and slowly apply 125 µl sample to the center of the compacted resin bed.
5. Replace cap and centrifuge at 1,500 x g for 2 minutes to collect the sample. Discard the column after use.
6. If IgG sample is 0.2-2 mg/ml, no further preparation is necessary. If sample volume is less than 125 µl, add Digestion Buffer to a final volume of 125 µl.
Fragment Generation

1. Add 125 µl of the prepared IgG sample to the spin column containing the equilibrated Immobilized Pepsin. Place top cap and bottom plug on the spin column. Briefly vortex to mix.
2. Incubate digestion reaction for 2 hours for rabbit or human IgG or 3 hours for mouse IgG with an end-over-end mixier or tabletop rocker at 37 °C. Maintain constant mixing of resin during incubation.
3. Remove bottom cap and place column into a microcentrifuge tube. Centrifuge column at 5,000 x g for 1 minute to separate digest from the Immobilized Pepsin.
4. Wash resin with 130 µl of PBS. Place column into a new tube and centrifuge at 5,000 x g for 1 minute. Repeat this step once.
5. Add both wash fractions to the digested antibody. Total sample volume should be 385 µl. Discard the Immobilized Pepsin.

F(ab’)2 Purification

1. Equilibrate the Nab Protein A column, PBS and IgG Elution Buffer to room temperature. Set centrifuge to 1,000 x g.
2. Snap off bottom closure and loosen top yellow cap on the Protein A Column. Place column in a collection tube and centrifuge for 1 minute to remove storage solution (contains 0.02% sodium azide). Discard the flow-through.
3. To equilibrate column, add 400 µl of PBS and briefly mix. Centrifuge for 1 minute and discard the flow-through. Repeat this step once.
4. Cap bottom of column with the included rubber cap. Apply 25-500 µl sample to column and cap the top tightly. Resuspend the resin and sample by inversion. Incubate at room temperature with end-over-end mixing for 10 minutes.
5. Loosen top cap and remove bottom cap. Place column in a new collection tube and centrifuge for 1 minute. Save the flow-through as this fraction contains F(ab’)2 and Fc fragments.
6. For optimal recovery, wash column with 200 µl of PBS. Centrifuge for 1 minute and collect flow-though. Repeat and combine wash fractions with the F(ab’)2 fraction of Step 5.
7. Measure absorbance at 280 nm. Use an estimated extinction coefficient of 1.4. Assuming complete IgG digestion, F(ab’)2 yields may vary from 50 to 70%, depending on the amount of starting antibody and the protein assays used.
8. If desired, perform dialysis (50K MWCO) to remove the Fc fragments.
Regeneration of the Immobilized Protein A column

1. Apply 400 µl of IgG Elution Buffer to the Protein A column. Centrifuge for 1 minute. Repeat this step two times to obtain three fractions, which will contain undigested IgG. To save the undigested IgG, add 40 µl of a neutralization buffer to each elution fraction.
2. Add 400 µl of IgG Elution Buffer to the column and centrifuge for 1 minute. Discard flow-through and repeat.
3. Add 400 µl of PBS to the column and centrifuge for 1 minute. Discard flow-through and repeat two times.
4. For storage, add 400 µl of 0.02% sodium azide in PBS to column. Replace top and bottom caps. Store column upright at 4 °C. Columns can be regenerated at least 10 times without significant loss of binding capacity.

F(ab’)2 Reduction/ Fab’ Generation

1. Prepare a 100 mL sample of Reduction Buffer with 6.6 g (66mg/mL) of 2-mercaptoethylamine.
2. Add F(ab’)2 solution to 2-mercaptoethylamine reduction buffer in a 10:1 volume ratio (2-mercaptoethylamine will be diluted to 6mg/mL). 5-35mM 2-ME
3. Place the resulting F(ab’)2 solution in shaking water bath at 37°C for 90 minutes.
4. Remove the excess 2-mercapto ethylamine by applying solution to a Sephadex-G25 column.
5. Collect 1 mL fractions and monitor protein content with UV-spectroscopy.
6. Pool fractions with significant protein concentration together.

Antibody Attachment (Do this under nitrogen)

1. Add resulting Fab’ solution to vial containing the liposomes with PEG-maleimide in the membrane or a solution of DSPE-PEG-maleimide micelles.
2. Allow the reaction mixture to mix for 12 hours at room temperature.

A.4 Liposome Calculations

Take the total surface area of the liposome inside and out, then divide by the head group area to obtain the number of lipids per liposome.

\[
N_{\text{lip}} = \frac{4\pi \cdot \left(\frac{d}{2}\right)^2 + 4\pi \cdot \left(\frac{d}{2} - h\right)^2}{a}
\]
The variable, d, is the diameter of the liposomes, h is the lipid bilayer thickness, and a is the head group area of an individual lipid.

The mass of lipid in solution is known from which the moles of lipid in solution can be calculated using the molecular weight of the lipid.

\[
N_{dppe} := \frac{m_{lipid}}{M_{dppe}} \quad N_{dppe} = 1.362 \times 10^{-6} \text{ mol}
\]

\[
N_{lipids.tot} := N_{dppe} \cdot N_a \quad N_{lipids.tot} = 8.204 \times 10^{17}
\]

Once the total number of lipids is known, the number can be divided by the number of lipids per liposome to obtain an estimate of the number of liposomes in solution.

\[
N_{lipo.tot} = \frac{N_{lipids.tot}}{N_{lip}} \quad N_{lipo.tot} = 2.436 \times 10^{12}
\]

A.5 VBA Code for Flow Cytometer Data Analysis

Option Explicit
Dim ssl As Double, ssh As Double, fsl As Double, fsh As Double, Cell As Integer, Cell2 As Integer
Dim GFP(1 To 10000) As Long
Dim sumlog As Double, lmGFP As Double, sstdv As Double, Gval As Double, lmGFP2 As Double, Isdev2 As Double
Dim Isdev As Double, loGFP As Double, zscor As Double
Dim IgFP(1 To 10000) As Long
Dim i As Integer, n As Integer

Public Sub Stats()
Call Gate
Call ttest
Cells(3, 1) = Cell
End Sub
Public Sub Gate()
    ssl = Range("C1")
    ssh = Range("D1")
    fsl = Range("C2")
    fsh = Range("D2")
    'loop over all events and collect points only in the gate
    Cell = 0
    For i = 2 To 10000
        If Cells(i + 3, 3) > ssl And Cells(i + 3, 3) < ssh And Cells(i + 3, 4) > fsl And Cells(i + 3, 4) < fsh Then
            Cell = Cell + 1
            Gval = Cells(i + 3, 5)
            GFP(Cell) = Gval
        End If
    Next i
End Sub

Public Sub ttest()
    'log all gated values
    sumlog = 0
    For n = 1 To Cell
        If GFP(n) > 0 Then
            loGFP = Log(GFP(n))
            lGFP(n) = loGFP
            sumlog = sumlog + lGFP(n)
        End If
    Next n
    lmGFP = sumlog / Cell
    Cells(1, 7) = lmGFP
    'Calculate the summation term for the standard deviation
    sstdv = 0
    For n = 1 To Cell
        sstdv = sstdv + (lGFP(n) - lmGFP) * (lGFP(n) - lmGFP)
    Next n
    lsdev = (sstdv / (Cell - 1)) ^ 0.5 'sample standard deviation calculations
    Cells(2, 7) = lsdev
End Sub

Public Sub Zscore()
    zscor = (lmGFP^2 - lmGFP + 0.5 * (lsdev^2 - lsdev)) / (lsdev / Cell + lsdev^2 / Cell^2 + 0.5 * (lsdev^2 / (Cell - 1) + lsdev^2 / (Cell^2 - 1))) ^ 0.5
    Cells(1, 11) = zscor
End Sub
Private Sub CommandButton1_Click()
Call Stats
Call Samp2
Call Zscore
End Sub

Public Sub Samp2()
ssl = Range("C1")
ssh = Range("D1")
fsl = Range("C2")
fsh = Range("D2")
'loop over all events and collect points only in the gate
Cell2 = 0
For i = 2 To 10000
    If Cells(i + 3, 7) > ssl And Cells(i + 3, 7) < ssh And Cells(i + 3, 8) > fsl And Cells(i + 3, 8) < fsh Then
        Cell2 = Cell2 + 1
        Gval = Cells(i + 3, 9)
        GFP(Cell2) = Gval
    End If
Next i
'log all gated values
sumlog = 0
For n = 1 To Cell2
    If GFP(n) > 0 Then
        loGFP = Log(GFP(n))
        lGFP(n) = loGFP
        sumlog = sumlog + lGFP(n)
    End If
Next n
lmGFP2 = sumlog / Cell2
Cells(1, 9) = lmGFP2
'Calculate the summation term for the standard deviation
sstdv = 0
For n = 1 To Cell2
    sstdv = sstdv + (lGFP(n) - lmGFP2) * (lGFP(n) - lmGFP2)
Next n
lsdev2 = (sstdv / (Cell2 - 1)) ^ 0.5 'sample standard deviation calculations
Cells(2, 9) = lsdev2
Cells(3, 10) = Cell2
End Sub
Figure B-1. Flow results for HeLa cells used as negative control.
Figure B-2. HeLa cells treated with folated eLiposomes. See Figure 2 bar 1.

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Figure B-3. HeLa cells treated with free folate and folated eLiposomes. See Figure 2 bar 9.
Figure B-4. HeLa cells treated with folated eLiposomes and ultrasound. See Figure 2 bar 5.
Figure B-5. HeLa cells treated with folated eLiposomes, free folate, and ultrasound. See Figure 2 bar 12.
Figure B-6. HeLa cells treated with non-targeting eLiposomes (PEGylated). See Figure 2 bar 2.
Figure B-7. HeLa cells treated with non-targeting eLiposomes and free folate. See Figure 2 bar 10.
Figure B-8. HeLa cells treated with non-targeting eLiposomes and ultrasound. See Figure 2 bar 6.
Figure B-9. HeLa cells treated with non-targeting eLiposomes, free folate, and ultrasound. See Figure 2 bar 13.
Figure B-10. HeLa cells treated with TK1-targeting eLiposomes. See Figure 2 bar 4.
Figure B-11. HeLa cells treated with TK1-targeting eLiposomes. See Figure 2 bar 4 (the two values from B-10 and B-11 were averaged to give the value reported in Figure 2.)
Figure B-12. HeLa cells treated with TK1-targeting eLiposomes and ultrasound. See Figure 2 bar 8.
Figure B-13. HeLa cells treated with folated liposomes (no emulsions), free folate, and ultrasound. See Figure 2 bar 14.
Figure B-14. HeLa cells treated with folated liposomes (no emulsions). See Figure 2 bar 3.
Figure B-15. HeLa cells treated with foalted liposomes (no emulsions) and free folate. See Figure 2 bar 11.
Figure B-16. HeLa cells treated with folated liposomes and ultrasound. See Figure 2 bar 7.
Figure B-17. Flow results for HeLa cells used as a negative control. Results of this experiment are in Figure 16.
Figure B-18. Flow results for HeLa cells incubated with folated eLiposomes for 24 hours (See Figure 16).
Figure B-19. Flow results for HeLa cells treated with free folate before adding folated eLiposomes and incubating for 24 hours (See Figure 16).
Figure B-20. Flow results for HeLa cells treated with folated eLiposomes then sonicated 24 hours later (See Figure 16).
Figure B-21. Flow results for HeLa cells treated with folated eLiposomes, free folate and ultrasound. The cells were sonicated after 24 hour incubation time (See Figure 16).
Figure B-22. Flow results for HeLa cells incubated with non-targeting eLiposomes (PEGylated) for 24 hours (See Figure 16).
Figure B-23. Flow results for HeLa cells incubated with non-targeting eLiposomes (PEGylated) for 24 hours then sonicated (See Figure 16).
Figure B-24. Flow results for HeLa cells incubated with folated eLiposomes for 10 hours (See Figure 16).
Figure B-25. Flow results for HeLa cells incubated with folated eLiposomes and free folate for 10 hours (See Figure 16).
Figure B-26. Flow results for HeLa cells incubated with folated eLiposomes for 10 hours then sonicated (See Figure 16).
Figure B-27. Flow results for HeLa cells incubated with folated eLiposomes and free folate for 10 hours then sonicated (See Figure 16).
Figure B-28. Flow results for HeLa cells incubated with non-targeting eLiposomes (PEGylated) for 10 hours (See Figure 16).
Figure B-29. Flow results for HeLa cells incubated with non-targeting eLiposomes (PEGylated) for 10 hours then sonicated (See Figure 16).
Figure B-30. Flow results for HeLa cells incubated with folated eLiposomes for 6 hours (See Figure 16).
Figure B-31. Flow results for HeLa cells incubated with folated eLiposomes and free folate for 6 hours (See Figure 16).
Figure B-32. Flow results for HeLa cells incubated with folated eLiposomes for 6 hours then sonicated (See Figure 16).
Figure B-33. Flow results for HeLa cells incubated with folated eLiposomes and free folate for 6 hours then sonicated (See Figure 16).
Figure B-34. Flow results for HeLa cells incubated with non-targeting eLiposomes (PEGylated) for 6 hours (See Figure 16).
Figure B-35. Flow results for HeLa cells incubated with non-targeting eLiposomes (PEGylated) for 6 hours then sonicated (See Figure 16).