FORMULATION AND IN VITRO CHARACTERIZATION OF PROTEIN-LOADED LIPOSOMES

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Abstract: **Background/Objective:** Protein-based drugs are increasingly used to treat a variety of conditions including cancer and cardio-vascular disease. Due to the immune system's innate ability to degrade the foreign particles quickly, protein-based treatments are generally short-lived. To address this limitation, the objective of the study was to: 1) develop protein-loaded liposomes; 2) characterize size, stability, encapsulation efficiency and rate of protein release; and 3) determine intracellular uptake and distribution; and 4) protein structural changes.

Method: Liposomes were loaded with a fluorescent-albumin using freeze-thaw (F/T) methodology. Albumin encapsulation and release were quantified by fluorescence spectroscopic techniques. Flow cytometry was used to determine liposome uptake by macrophages. Epifluorescence microscopy was used to determine cellular distribution of liposomes. Stability was determined using dynamic light scattering by measuring liposome size over one month period. Protein structure was determined using circular dichroism (CD).

Result: Encapsulation of albumin in liposome was ~90% and was dependent on F/T rates, with fifteen cycles yielding the highest encapsulation efficacy ($p < 0.05$). Albumin-loaded liposomes demonstrated consistent size (<300nm). Release of encapsulated albumin in physiological buffer at 25°C was ~60% in 72 h. Fluorescence imaging suggested an endosomal route of cellular entry for the FITC-albumin liposome with maximum uptake rates in immune cells (30% at 2hour incubation). CD suggested protein structure is minimally impacted by freeze-thaw methodology.

Conclusion: Using F/T as a loading method, we were able to successfully achieve a protein-loaded liposome that was under 300nm, had encapsulation of ~90%. Synthesized liposomes demonstrated a burst release of encapsulate protein (60%) at 72 hours. Cellular trafficking confirmed endosomal uptake, and minimal protein damage was noticed in CD.

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CHAPTER I

INTRODUCTION

Limitations of Conventional Therapy

Medicine has come a long way since the time of Galen in the 1500's. The production of antibiotics, cancer drugs, cardiovascular drugs, and now genomic treatments are a testament to its advancement. These discoveries are allowing treatment of a wide variety of ailments including infectious diseases that once was the number one killer due to lack of aseptic technique (Miller, *et al.*, 2005). Similarly, cancer survival rates have steadily inclined due to the development of new drugs and treatments.

Despite significant advances in human medicine, current treatment approaches rely on systemic delivery with limited organ specificity, and therefore result in adverse side effects in normal tissues and insufficient drug delivery to the target tissue. Drugs can also be filtered from the blood prior to its intended effect. At low doses of drug compounds, the human liver and kidneys can metabolize and clear molecules that are less than 60KDa (Hagenbuch, 2010; Shitara, *et al.,* 2006). However, at higher doses, the clearance of drug is influenced at many levels. For example, absorption of drug molecules from gastrointestinal tract is pH dependent and can be retained differently based on the chemical composition of the drug (Hagenbuch, 2010). The administered drugs can be metabolized in liver, and may create products that are toxic to healthy tissues. Similarly, renal filtration and removal of drugs are helpful if the substance is toxic and needs to be removed rapidly; however, if the drug is removed before it reaches the target organ, or is trapped within a certain organ due to its chemical make-up (hydrophilic drugs vs. hydrophobic drugs), the desired clinical outcomes are not achieved. Thus, new approaches that maintain the efficacy while reducing the toxicity of drugs are needed .

Nanomedicine

Nanotechnology is a multidisciplinary scientific field focused on materials whose physical and chemical properties can be controlled at the nanoscale range (1–1000 nm) by incorporating chemistry, engineering, and manufacturing principles (Kim et al., 2010). The convergence of nanotechnology and medicine, suitably called nanomedicine, can potentially advance the fight against a range of diseases (Sanhai et al., 2008). Nanomedicine can sustain drug release, increase solubility and bioavailability, decrease aggregation and improve efficacy (Ranjan et al 2011). Additionally, the use of nanoparticles may decrease toxicity of drugs (Byrne, *et al.,* 2008) and increase the ability to target the drug to the correct tissue (Jain, 2006; Coti, *et al.,* 2009). Drugs, proteins, or genes have been successfully loaded into differing nanoparticles, and many have made it to clinical trials. For clinical translation, Food and Drug Administration recommends that nanoparticle size be less than 300nm (Food and Drug Administration, *et al.*, 2002). To achieve this, various nanoparticles are created from a variety of materials. For example, some nanoparticles are polymer based and have a cage-like structure while others are created from heavy metals such as gold. In contrast, liposome nanoparticles mimic a cell with a lipid bilayer and aqueous core.

Liposomal Nanomedicine

Liposomes were first described in 1961 by Alec Bangham (Deamer, 2010). Structurally, liposomes are composed of a lipid bilayer with hydrophobic tails in the center and hydrophilic heads on the outside. The aqueous core allows for loading of different materials, and the outside can have different peptides or proteins attached for cellular targeting. Liposomes can be multilamellar (multiple lipid bilayers stacked) or unilamellar (single lipid bilayer), and may have different size and charge chemistry. In general, larger liposomes are relatively quickly taken up by the reticoluendothelial system (Machy & Lesserman, 1983). This can be addressed by decorating liposome surface with polyethylene glycol (PEG)-based polymers. PEGylation helps to increase the half-life of liposomes in a biological system (Maruyama, *et al.,* 1992), prevents uptake by the immune system (Ishida, *et al.,* 2002), and consequently reduces its clearance from the circulation

As a drug delivery system (DDS), liposomes can encapsulate both hydrophilic and hydrophobic molecules, and thus are widely translatable against a variety of clinical conditions (Gregoriadis, 1985). Clinically, liposomes achieve higher volume of distribution and solubilization of drug molecules in blood and thereby can decrease the unwanted side

effects. For example, liposomes can decrease the toxicity of drugs such as gentamicin, which is used for bacterial infections (Frierer, et al., 1990), or doxycycline which is also an antibiotic and doxorubicin for cancer treatments (Green & Rose, 2006).

Liposome mediated site directed drug delivery can be achieved by various methods (Chan & Yeh, 2012). For example, a pH sensitive liposome upon reaching the target tissue will release its content based on the pH difference (e.g. cell endosomes) (Cho, et al., 2008). Alternatively, heat-sensitive liposomes keep the drug in its aqueous core at body temperature, but when a particular spot of the body is heated to 42^0C, the liposomes release the cargo. Other methods of targeting include using cell receptor specific peptides or antibodies (Wang & Thanou, 2010). Finally, the highly pegylated stealth liposome can circulate for long time (-48-72 h) with very slow systemic release, and eventually accumulate in tumors by enhanced permeation to provide targeted drug release (Kim, et al., 1987). Next we discuss the application of liposomes for delivery of protein-based therapeutics.

Protein-Based Treatments

In the last few decades, protein or DNA-based drugs are being increasingly applied in medicine. Examples include the use of thrombokinase for stroke or burtrylcholinesterase against nerve agents. Acetylcholinesterase is an enzyme that breaks down acetylcholine from muscle junctions, but organophosphorus insecticides and nerve agents can inhibit this enzyme. This prevents the breakdown of acetylcholine and leads to a build-up of acetylcholine which leads to excessive salivating, seizures, and respiratory failure (Zimmer, *et al.,* 1998). Drugs such as atropine can reverse these effects. Protein therapeutics have a fast clearance and require administration at the moment of exposure. Additionally, the in vivo usage of protein is limited by its purity and amount (Kimichi-Sarfaty, *et al.,* 2013). Proteins have to be purified from their source before use. As such, it can be challenging to procure a large amount of pure protein from a consistent source. To address this, a major focus in this area of work is on the discovery of recombinant proteins that may last up to a week in the human body (Cohen, *et al.,* 2006) compared to native protein. Alternatively, protein-based therapeutics can be combined with nanocarriers (e.g. liposomes) to reduce frequency and dosage of treatment. For example, in Putney and coworkers (1998) study, they observed the stability of protein-polymers had extended periods in vivo after injection.

Liposomes and Protein-Based treatments

The use of liposomes for protein-based therapy has been extensively investigated and reported previously. One example of liposome use could be in the case of prions, liposomes were combined with a PrP-27-30 protein. By doing this Gabizon contended that this protein was necessary for infectivity of the prion virus (Gabizon, *et al.,* 1988). However, liposomes can also be used to encapsulate other proteins for uses in treatment. These methods of encapsulation can increase the circulation time by preventing the immune system access and increase the targetability of the protein. For example, thrombokinase, which can cause hemorrhage in its native state, could theoretically be encapsulated to prevent unwanted damage to blood vessels and reach its target destination. Similarly, proteins incorporated into the lipid bilayer of liposomes have shown to enhance vaccination response (Bucher, *et al.,* 1980, Theunis, *et al,* 2013,Schwendener, *et al.,* 2010). The blood brain barrier is an effective barrier against most drugs, except those that are lipophilic, so the use of liposomes to bypass it has merit. The addition of proteins to lipid membranes helped to increase targetability to tissues, bacteria, or viruses (Lersman, *et al.,*1980; Bedi, *et al.,* 2011). Proteins can interact with cell-surface markers in both the adaptive and innate immune system. By using proteins, such as antibodies adhered to the liposomes, it is possible to target liposomes away from self-tissues and towards foreign (Schnyder & Huwyler, 2005). For example, Scott's *et al.,* (2007) reported a 92% increase in accumulation of targeted liposomes to myocardial infarction compared to liposome alone.

A variety of methods can be used to encapsulate protein into liposomes (Walde & Ichikawa, 2001; Lasic & Papahadjooulos, 1996; Meyer, *et al.,* 1994; Tan, *et al*. 2006; Gokhale, *et al.,* 1997; Patel, 1976). These are broadly categorized into active and passive loading. Passive loading aims to capture the protein during liposome synthesis. Active loading, on the other hand, uses pH gradient to drive the drug of choice into the aqueous core following liposome synthesis. This method is highly suitable for encapsulation of small molecules (e.g Doxorubicin), but is not feasible for proteins because of their large size and isoelectric points. Near the isolectric point, proteins can precipitate out of solution, and at low or high pH's, denaturing can occur. It may be noted that some structure loss is reversible, but often the activity of protein is impacted at high or low pH.

Despite significant advancement, the current encapsulation approach fails to prevent degradation over time and protein structural damage (Meyer, *et al.,* 1994). New capacity to optimize protein loading into liposome is critically needed. The aim of this thesis project was to address the problems associated with extrusion and pH dependent active loading, through adoption of an older traditional method of freeze-thaw. Fundamentally, freeze-thaw is the use of liquid nitrogen to flash freeze a sample and a warmer thawing temperature, usually done in a water bath, to thaw the sample. The contraction and expansion of the lipid bilayer allows for the capture of certain drugs, molecules, or proteins in the aqueous core or lipid bilayer. Freeze-thaw as a method of protein loading can encapsulate up to 40% of Acetylcholinesterase protein (Colletier, *et al.,* 2002), and create small sized liposomes (< 300nm). This is clinically relevant since large sized nanoparticles are quickly taken up by the immune cells, risk cellular damage and may cause thrombotic diseases by absorption from the red blood cell and disrupting osmotic pressure (Zhao, *et al.,* 2011).

A critical gap in translation of freeze-thaw methodology is lower loading efficiency and temperature induced loss in protein activity. Due to lack of standardized methodology for freeze-thaw, the number of freeze-thaw cycles, timing of each of those cycles, the freezing temperature, and the water bath temperature are highly variable between various laboratories, thereby achieving a range of size, encapsulation efficiency, and cellular interaction profiles. One study extruded first then used a freeze-thaw cycle of 3min in a liquid nitrogen bath, and a thaw at 50°C for three minutes to achieve a mean diameter of 14um (Castile & Taylor, 1999). A different study used liquid nitrogen to freeze for an unreported amount of time before thawing at 23°C for fifteen minutes (Pick, 1981). Studies using different preparations in the lipids, freezing and thawing time and temperatures have yielded different results in encapsulation efficacy and liposome size.

In the last decade, freeze-thaw as a method has been witnessing less use in the more modern labs partly due to availability of extruders that can create uniformly sized liposomes (Benjakul & Bauer, 2000; Xia, *et al.*, 2009). However, modern extruders work at high pressure, and thus may not be suitable for protein with delicate conformation.

The goals of this study were to formulate and characterize protein encapsulated liposomes by freeze thaw and extrusion method, and determine the optimal synthesis methodology for in vivo protein delivery. We chose albumin as a surrogate of protein-based

therapeutics since it is cheap and easily available. Albumin is 66KDa with an isoelectric point of 4.2. Albumin can detoxify toxins and drugs, and increase the circulation time for drugs (Elsadek & Kratz, 2012; Yamasaki, *et al.,* 2013; Jung, *et al.,* 2010; Sleep, *et al.,* 2013). While in these experiments, albumin is a place-holder for other potential proteins or enzymes, there is a potential avenue to use albumin in conjunction with liposomes to further increase circulation time of drugs. Additionally, albumin is not enzymatically active, but can be tagged with fluorescent markers (e.g. FITC or Cy5) to provide a measure of encapsulation efficiency and cellular trafficking.

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CHAPTER II

OPTIMIZATION OF FREEZE-THAW PROCESS FOR LIPOSOMES SYNTHESIS

2.1 Abstract

Background: Freeze-thaw (F/T) can encapsulate proteins into liposomes, however, there is no agreed upon single optimized method. The objective of this study was to: 1. optimize the number of F/T cycles to achieve a liposome under a specific size limit, and 2. determine optimal freeze and thaw time.

Method: Lipid films were hydrated with phosphate-buffered saline (PBS), and subjected to 10-20 F/T cycles as follows: 1. Five min freeze and five min thaw; 2 min freeze, 4 min thaw; and 1 min freeze with a 4 min thaw.

Result: Results indicate that after 15 cycles of freeze-thaw with a 1 min. freeze, 4 min. thaw is the most optimal condition for obtaining liposomes in the size-range of 200-300nm. Shorter cycles with longer F/T yielded larger and non-optimal liposomes (300nm + size).

Conclusion: 15 cycles of freeze-thaw with a 1 min. freeze, 4 min. thaw can achieve desired size range of under 300nm.

2.2 Introduction

Freeze-thaw achieves (MacDonald, *et al.,* 1994) fragmentation of multi-lamellar liposomes into unilamellar vesicles. The advantage of processing multi-lamellar vesicles into unilamillar is size; multi-lamellar liposomes are larger and thus easier for the immune system to functionally remove from the body (Hope, *et al.,* 1986). F/T methodology can effectively encapsulate both drugs as well as proteins into liposomes. F/T methodology is similar to extrusion that also creates unilamellar vesicles by forcing

liposomes through a small diameter filter at high pressure; however, freeze-thaw works on the principles of temperature modules (Costa, *et al.,* 2014; Traikia, *et al.,* 2000; Castile, *et al.,* 1999). Freeze-thaw methodology effectively encapsulates not only drugs but proteins as well in the liposomes (Meyer, *et al.*,1994).

The percentage drug encapsulated can be highly variable among various studies due to differences in the time of annealing, the number of cycles, and lipids used in the procedure (Castile, *et al.*,1999). For optimal encapsulation, 20 cycles of freeze-thaw has been generally adopted as a standard practice (Colletier, *et al.,* 2002). However this process can damage the lipid membranes and increase liposome size (Hincha, *et al,* 1998). Some studies have reported liposomes under 200nm after five or 10 cycles of freeze-thaw (Traikia, *et al.,* 2000; MacDonald, *et al.,* 1994). Attempts to replicate these findings by other groups reported contrasting outcomes with liposome size not decreasing to under 200nm with 5-10 cycles of freeze-thaw (Castile, *et al.,* 1999).

Plausibly, the differences in size could be due to variability in freezing and annealing duration and lipid compositions (Sriwongsitanont & Ueno, 2011). With a goal on achieving liposomes under a certain size (<300nm for systemic use) (Akbarzadeh, *et al.,* 2013), here we discuss a novel freeze-thaw protocol.

2.3 Methods

2.3.1 Liposome thin film preparation

All reagents were obtained from VWR unless noted. Lipids were obtained from Corden pharma (Switzerland). Liposomes were prepared by hydration using phosphate-buffered saline (PBS) of a lipid film followed by freeze-thaw. Three phospholipids (2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearyl-phosphatidyl ethanolamine-methyl-polyethyleneglycol conjugate (DSPE-MPEG), cholesterol) were dissolved in chloroform at a molar ratio of 85.3:9.7:5.0. The chloroform was evaporated off at 60^0C in a rotovap to create a lipid film. This film was dried overnight in a desiccator. All assays were performed using six technical replicates generated in parallel on the same day.

2.3.2 Optimization of freeze-thaw procedure

Freeze-thaw was performed in two stages by optimizing 1) number of cycles of freeze thaw, and 2) the duration of each cycle. A cycle was defined as one round of liquid nitrogen (-196^{0}C) freezing and one round of a 35^{0}C water bath thawing. The 35^{0}C annealing temperature was chosen due to the fragile nature of some thermosensitive proteins.

2.3.3. Optimization of the number of cycles and duration of freeze thaw

The lipid film (as previously stated in 2.3.1) was rehydrated with phosphate buffer saline (PBS) using 5mg of lipids/mL of PBS and transferred into 2mL cryo-protectant vials. Initially, a 5 min. freeze (F), 5 min. thaw (T) was used for size determination. Later, we compared additional groups from a 1 min. freeze, 4 min. thaw and a 2 min. freeze, four min. thaw. Based on this, a 4 min. thaw was found to be the minimum amount of time required to completely thaw a 2mL sample. As controls, half of the hydrated samples were also extruded once through a 200nm filter for size comparison to liposomes that only underwent F/T. Samples were taken at 0 cycles, 5 cycles, 10 cycles, 15 cycles, and 20 cycles. Liposomes were sized using dynamic light scattering (DLS) equipment (Brookhaven instrument Corporation, 90Plus ZetaPALS particle size analyzer).

2.3.4. Statistical analysis

Treatment groups were compared for differences in mean absorbance using ANOVA followed by Tukey's multiple comparisons with GraphPad Prism 6 (GraphPad Software Inc.). A p-value less than 0.05 was used as a threshold for statistical significance.

2.4. Results

2.4.1. Optimization of the number of cycles

To determine if the number of F/T cycles impacted liposome size, we generated liposomes using varied numbers of F/T cycles, and subsequently measured the size of the liposomes generated by each methodology.

Results based on the six technical replicates show that F/T of 15 cycles resulted in a significantly decreased size compared to 10 or 20 cycles (F = 5 min, T = 5 min) ($p < 0.05$). The size of the liposomes dropped from 2000nm to under 500nm after 15 cycles of F/T. Surprisingly, 20 cycles of freeze-thaw caused an increased liposome size compared to 15 cycles of freeze-thaw (Figure 1). Extrusion at any cycle significantly decreased liposome size ~180nm, compared to F/T samples.

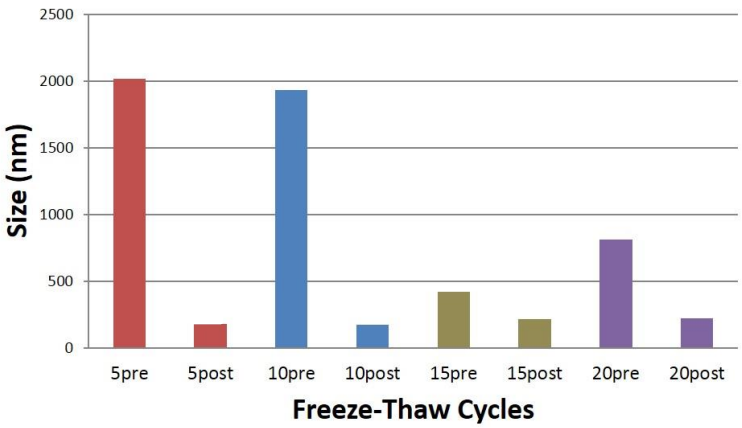

Figure 1. **Effect of F (liquid Nitrogen/T (35^0C) cycles (5-20) on liposome size.** Pre is prior to extrusion; Post is post-extrusion through a 200nm filter. Errors bars reported as SEM.

2.4.2. Optimization of the F/T duration

To determine the effect the F/T duration on liposome size, we generated liposome batch using a varied time on each F/T cycle and measured the size of the liposomes generated by each methodology.

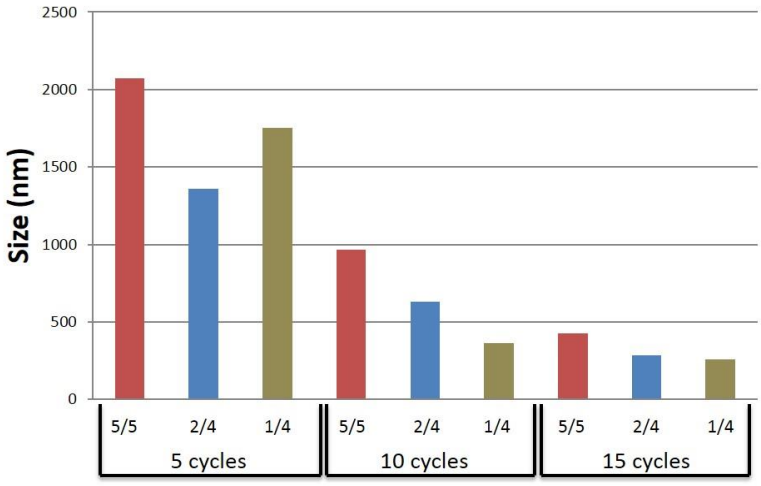

Figure 2. **Effect of F(min)/T(min.) on liposome size**. Errors bars reported as SEM.

Reduced duration of the F/T achieved smaller liposomes (Figure 2). With a 1 or 2 min. freeze and 4 min. thaw, liposome size was consistently decreased from ~450nm to ~200nm than with a 5 min. freeze/5 min. thaw; this trend was particularly prominent from 10 to 15 cycles of F/T. In general, average liposome size decreased significantly ($p < 0.05$) with a 1 min. freeze, 4 min. thaw at 15 cycles. By increasing the cycles of the F/T, liposome size would continue to decrease in diameter. A 1 min. freeze, 4 min. thaw was

under 300nM on average after 15 cycles of F/T and was significantly smaller than both the 2 min. freeze/4 min. thaw and the 5 min. freeze/5 min. thaw.

2.5. Discussion

The advantages and disadvantages of F/T as a viable technique for encapsulating proteins in liposomes has been extensively in the past (Costa, *et al.,* 2014; Sriwongsitanont and Ueno, 2011; Traikia, *et al.,* 2000; Castile and Taylor, 1999; Hope, *et al.,* 1993) (Castile, *et al.,*1999). These published studies report their own methodology, used varying temperatures and yielded different protein encapsulation efficiency and liposomal size. Thus, there is a critical need to standardize freeze-thaw protocol so that the scale-up to clinical use can be achieved.

One approach to standardize synthesis methodology could be to use a set thawing temperature. To do so, we used a set temperature of 35˚C to thaw all of our samples. Our variables, instead, were the duration of each cycle of F/T and the number of cycles of employed for liposome synthesis. An important prerequisite in thawing duration is that it should liquefy the sample completely. As a first step, we chose a 5 min. freeze-5 min. thawing condition that achieved a liposome size of over 300nm after 15 cycles (Figure 1). However after adjusting the freezing time to 1min and thawing to 4min, the size achieved were under 300nm after 15 cycles of F/T (Figure 2). Additionally, extrusion further decreased the size of the liposomes under 200nm in all cases, and there was no significant difference across any of the groups likely due to filters that were 200nm and resulted in a uniform suspension of liposomes. Thus, extrusion was uniform no matter how long or how many cycles of F/T were done. While some studies in the past had achieved a liposome size of under 300nm in less than two F/T cycles (Costa, *et al.,* 2014), other studies reported contrasting outcomes (Castile and Taylor, 1999). Data from our study suggest that increasing the number of cycles to achieve liposomal size below 300nm is important especially when applying shorter freezing and annealing time. Our studies indicated with a 1 min. freeze, 4 min. thaw at 15 cycles, the entire process does not take longer than 1 hour. Thus, our methodology is important in protecting encapsulated protein from thermal damage at higher annealing temperatures.

A consistent finding in our studies was a decrease in liposomal size with up to 15 cycles of freeze-thaw. This is in agreement with Traikia *et al.,* 1999 who have shown that

liposome composition consisting of dioleoylphosphatidylcholine/dioleoylphoshpatidic acid (DOPC/DOPA) can achieve a size under 200nm with greater than 10 cycles of F/T. Interestingly, beyond a certain F/T cycle (e.g. 20 cycles), we noted an increase in NP size suggestive of liposomal damage. Sriwonsitanot's & Castille *et al.,* 2011 have reported that at higher F/T cycles or low levels of DSPE-PEG2000, a loss of spherical shape and membrane damage can occur. This is interesting, but we did not change our lipid composition to explore these effects (see method). Also, the thawing temperatures (60^0C) reported in these studies might not be suitable for protein delivery. We believe that our approach of using a 35^0C thawing might have more widespread use and can enhance our ability to encapsulate a wider range of proteins and warrants further investigation.

2.7 References

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CHAPTER III

CHARACTERIZATION OF FITC-ALBUMIN LOADED LIPOSOMES

3.1 Abstract

Background: F/T methodology currently has a protein encapsulation efficiency that is variable based on methodology. The objective of this study was to evaluate and optimize encapsulation of protein in liposomes to create a desirable product for in vivo use.

Methods: Liposomes were prepared by 15 cycles of a 1 min. freeze, 4 min. thaw. Synthesized liposomes were characterized for encapsulation efficiency, protein release and stability in phosphate buffered saline based physiological fluid.

Results: Results suggest that 15 cycles of a 1 min. freeze and 4 min. thaw can achieve 90% encapsulation efficacy into our liposomes. Synthesized liposome did not differ in size compared to the blank liposomes. Liposome size was stable at both 37^0C and 4^0C to day 28. Release of albumin was minimal till 72 hrs, and at 28 days, liposomes still retained 40% of encapsulated protein.

Conclusion: F/T procedure at 15cycles can achieve high encapsulation efficiency and liposomes are stable for up to 4weeks at 37^0C.

3.2 Introduction

In the last few decades, protein-based therapies are being increasingly applied in treatment of cancer, heart disease, and gene therapy (Weidle, *et al.,* 2013; Khan, *et al,* 2003; Strayer, *et al,* 2005). For therapeutic use, a major challenge is the inability of current methods to acquire proteins with excellent purity and in high amounts. This has been addressed to some extent by discovery of recombinant proteins instead of the native form. However, in vivo, proteins last only for couple of hours before being removed by the body (Schellenberger, *et al,* 2009). There is a critical need to develop alternative approaches to extend the site-specific concentration of chosen protein. To do so, we hypothesize that encapsulating proteins in liposome may protects its function, and enhance its circulation time and targeted delivery. As an initial step, we chose albumin as the protein of interest for encapsulation because: 1) Albumin is easily procured in purity and amount, and 2) Albumin's potential as a scavenger molecule for both endogenous and exogenous molecules has been well established (Evans, 2002).

Albumin is synthesized by the liver and serves as a carrier protein in the blood and performs numerous functions. Upon systemic injection of drugs, albumin binds with them, and this can be leveraged for increasing the circulation time of drugs (Sleep, *et al.,* 2013; Elsadek & Kratz, 2012), and decrease the likelihood of it being removed by the kidneys. It also helps in detoxification by binding to toxins and increasing their ability to be excreted in bile or by renal clearance. Purified albumin is readily available, cheap and can be easily procured commercially especially for large scale studies.

Albumin has also been studied to increase the stability of liposomes themselves (Jung, *et al,* 2010; Vuarchey, *et al,* 2011) and increase their circulation time. However, in these cases, the albumin was tethered to the liposome itself and not loaded (Jung, *et al.,* 2010; Vuarchey, *et al,* 2011). Albumin can be loaded and used as a marker to determine the efficacy of freeze-thaw encapsulation but the reported encapsulation efficacy has been variable between studies. The objective of this study was to develop an optimal method of protein encapsulation for systemic therapy purposes. As surrogate of protein therapeutics, we used bovine serum albumin coupled with a tagged molecule, fluorescein isothiocyanate (FITC), a fluorescent marker (excitation: 495nm, emission: 519nm).

3.3. Methods

3.3.1 Synthesis of liposome-FITC-albumin

Liposomes were prepared as described in 2.2.1. FITC-albumin was loaded by rehydrating the lipid film with 0.17 mg albumin/mL of PBS. Lipid film underwent F/T (1min F/4min Thaw: 1/4 F/T) for 15 cycles. Samples (200uL) were drawn at 0, 5, 10, and 15 cycles. Half of each sample (100uL) at each cycle point was extruded once through a 200nm filter. All samples were purified using a high centrifugation filter (300kDa cut off), and the liposomes were characterized for size via DLS, and encapsulation efficacy using spectrophotometry fluorescence (excitation: 420nm, emission: 520nm). For optimal reading, dequenching was performed by treating the liposomes with 1% Triton-X after purification. Liposome size replicates were technical replicates (n = 6) while encapsulation experiments were conducted independently (n = 2).

3.3.2 Release of FITC-Albumin in physiological media

FITC-albumin from the liposomes was determined as follows. Liposomes were prepared as 3.3.1, and initial encapsulation was determined via fluorescence. The samples (extruded and non-extruded) were placed into separate dialysis bags (300kDa cut off), and kept at 25^0C and 37^0C in 10mL of PBS. One mL samples were collected daily for seven days and samples were measured for fluorescence (excitation: 420nm, cut off: 425nm, emission: 519nm). Each study had 3 technical replicates.

3.3.3 Stability of liposomes in physiological media

Long term stability of the liposomes was determined by measuring its size over time. To do so, the synthesized lipids were stored in 5mL vials and kept at 25^0C or at 37^0C. Liposome size was estimated using DLS on days 1-7, 14, 21, and 28.

3.3.4 Statistical analysis

Treatment groups were compared for differences in mean absorbance using ANOVA and independent t-tests between groups. A p-value less than 0.05 was our threshold for statistical significance.

3.4. Results

3.4.1 Liposomal Loading with FITC-Albumin

To determine the encapsulation efficacy (EE) of albumin into liposomes, we generated liposomes as previously described and loaded them with 0.17mg/mL of albumin. After removing free albumin from the sample via centrifugation, we measured the excitation and emission of the FITC-albumin to determine the EE.

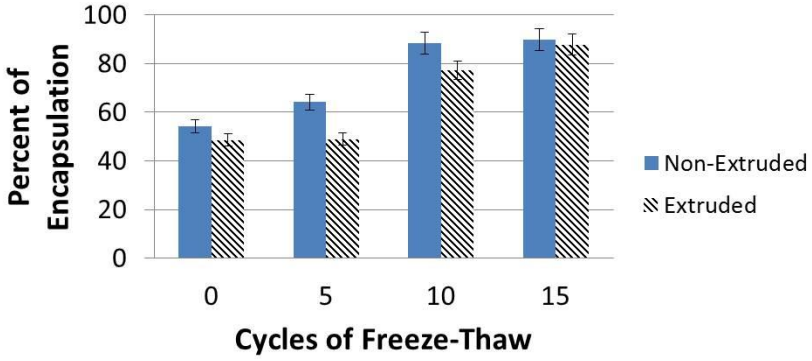
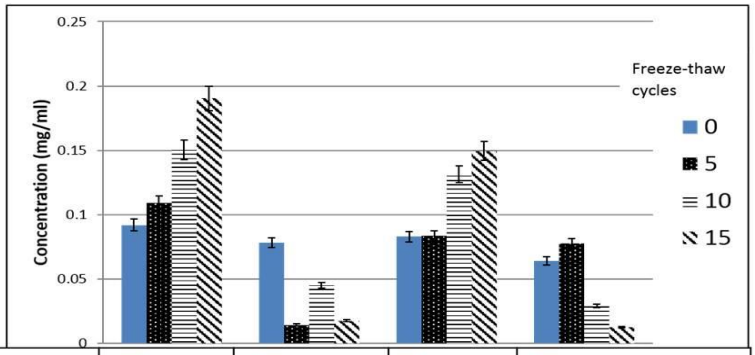


Figure 3. **Encapsulation efficiency of albumin into liposomes (mg/mL) over F/T cycles**.

At 10 cycles of F/T or greater, non-extruded liposome had an EE of~ 90% (Figure 3). With extrusion, a significant loss in EE was noted at 0, 5, and 10 cycles ($p < 0.05$) with 5 F/T cycles showing the greatest loss. For the extruded group of liposomes, there was no significant difference between 0 and 5 F/T cycles, and none between 10 and 15 F/T cycles. However, there was a significant increase in encapsulation efficacy with regards to extrusion from 5 to 10 F/T cycles (Figure 4).

3.4.2 Release of FITC-Albumin in physiological media

To determine release of the FITC-albumin from the liposomes over the course of a week, we took a batch of liposomes with a pre-determined EE, and separated them into dialysis bags at 25°C or 37°C. Over the course of a week, samples of the buffer were analyzed for protein release.



Extruded		Free Protein	x	Free Protein
Purified	x		x	

Figure 4. **Encapsulation efficiency of albumin into liposomes (mg/mL).** Error bars reported as ±SEM

FITC-albumin was noted on day 3 from both the extruded liposomes and non-extruded liposomes (60% and 53% respectively). For the non-extruded liposomes, at day 2 about 10% loss was observed that increased to almost ~ 50% on day 3. In contrast, extruded liposomes demonstrated no release on day 1 or 2, but had about 60% loss on encapsulated protein on day 3 (Figure 5). Following the burst release noted at day 3, non-extruded liposomes demonstrated significantly less release compared to the extruded liposomes over the subsequent days (47% retained). The extruded liposomes following burst release only maintained 40% of their original FITC-albumin.

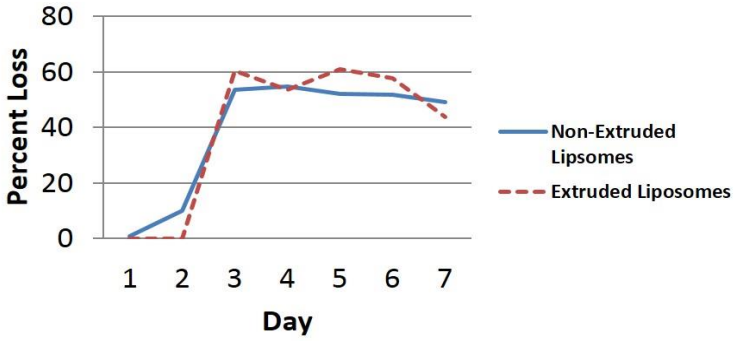


Figure 5. **Percent release comparison of non-extruded and extruded FITC-Albumin liposomes over the course of seven days.**

3.4.3 Stability

To determine the stability of our liposomes when loaded with albumin, we generated liposomes and stored at 25°C and 37°C. Over the first week, we took a sample per day before changing to once per week subsequently.

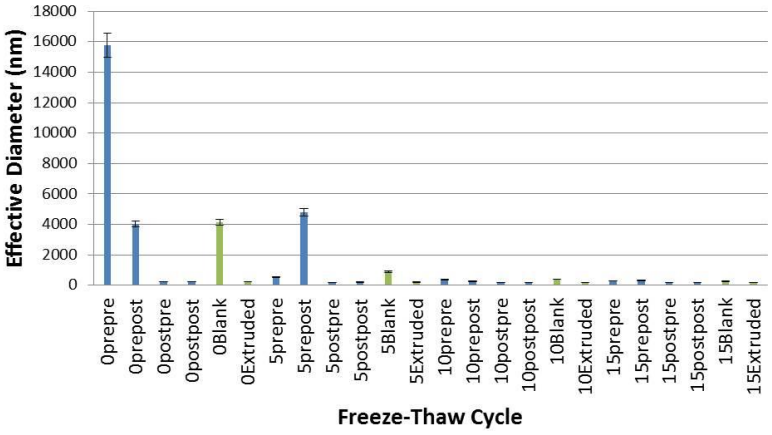


Figure 6. **Liposome size (loaded (blue) and blank liposomes- green).**

FITC-albumin loading did not change liposome size (Figure 6). Liposome size over 28 days remained stable at both 25^{0}C and 37^{0}C (<200nm for extruded samples, <300nm for non-extruded FITC-albumin liposomes). Day 1 size when compared to day 28 size for all groups (blank liposomes or FITC-loaded) was not significantly different (Figure 6). Day 1 size for non-extruded FITC-albumin liposomes at 25˚C, for example, was 300nm, and after 28 days, the size was still 289nm (Figure 6).

Extrusion of liposomes resulted in smaller liposomes over 28 days in that their size was consistently under 200nm. At day 1, extruded liposomes started under 200nm, and after 28 days of incubation at either 37˚C or 25˚C, their size remained consistently <200nm.

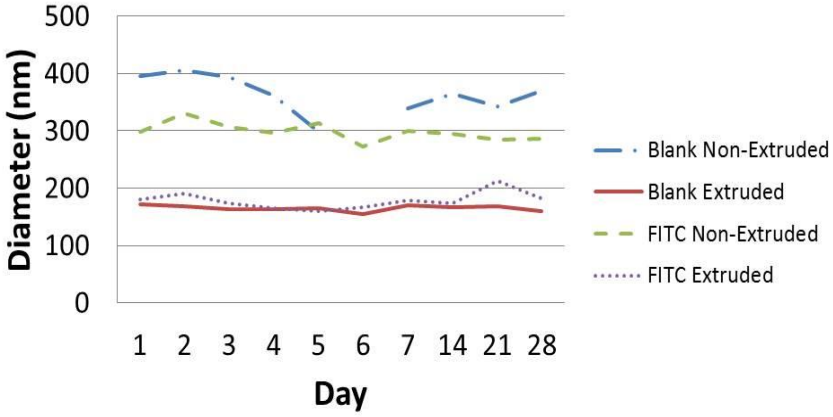


Figure 7. **Liposome size over the course of 30 day period at 25˚C**

In comparison between the two temperatures, there was no significant difference between the FITC non-extruded groups, FITC extruded, and the blank extruded groups. Temperature did not factor in to size changes between the groups of liposomes. Liposomes kept at 37˚C that were extruded did not differ in size after 28 days from liposomes that were extruded and kept at 25˚C (Figure 7 & 8).

To determine whether our liposomes were of a single population or multiple populations in size, we analyzed the polydispersity index. Polydispersity is the measure of a liposome's population size- whether or not a group of liposomes are uni-sized or have multiple populations of multiple sizes. For polydispersity index, there was no significant difference in that population size and was consistent across the entire month period within a group of

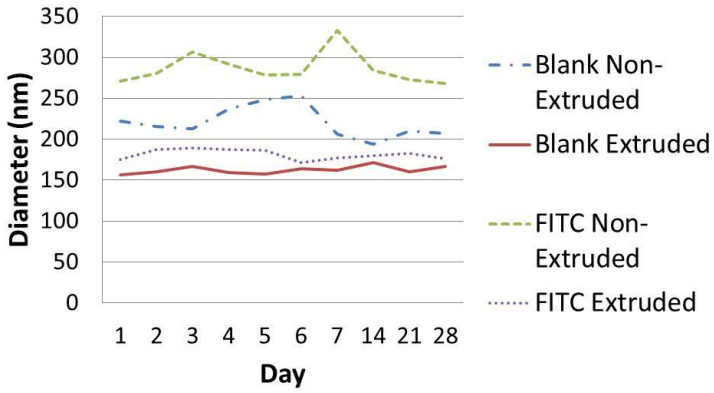


Figure 8. **Liposome size over the course of 30 day period at 37˚C**

liposomes at one temperature or when compared between liposomes kept at 25°C and 37°C (i.e. extruded at 25^0C and extruded at 37^0C) (p = .15)(Table 1 & 2).

Day	Blank Non-Extruded	Blank Extruded	FITC Non-Extruded	FITC Extruded
1	0.203 ± 0.022	0.122 ± 0.022	0.194 ± 0.022	0.053 ± 0.022
2	0.237 ± 0.031	0.047 ± 0.017	0.211 ± 0.025	0.059 ± 0.019
3	0.23 ± 0.030	0.114 ± 0.015	0.241 ± 0.050	0.118 ± 0.022
4	0.193 ± 0.021	0.128 ± 0.016	0.216 ± 0.016	0.133 ± 0.021
5	0.233 ± 0.021	0.126 ± 0.017	0.209 ± 0.030	0.167 ± 0.010
6	n/a	0.163 ± 0.013	0.249 ± 0.011	0.138 ± 0.004
7	0.2 ± 0.027	0.134 ± 0.030	0.185 ± 0.028	0.121 ± 0.024
14	0.225 ± 0.018	0.147 ± 0.015	0.225 ± 0.050	0.124 ± 0.022
21	0.245 ± 0.025	0.15 ± 0.029	0.196 ± 0.031	0.139 ± 0.023
28	0.165 ± 0.020	0.193 ± 0.010	0.273 ± 0.018	0.066 ± 0.021

Table 1. **Polydispersity of liposomes at 25^0C over a month period.**

Day	Blank Non-Extruded	Blank Extruded	FITC Non-Extruded	FITC Extruded
1	0.23 ± 0.027	0.12 ± 0.023	0.2 ± 0.018	0.0065 ± 0.018
2	0.25 ± 0.013	0.168 ± 0.023	0.229 ± 0.009	0.092 ± 0.029
3	0.271 ± 0.010	0.118 ± 0.009	0.221 ± 0.030	0.058 ± 0.015
4	0.195 ± 0.009	0.159 ± 0.016	0.235 ± 0.018	0.096 ± 0.023
5	0.229 ± 0.031	0.186 ± 0.008	0.224 ± 0.024	0.131 ± 0.021
6	0.239 ± 0.023	0.176 ± 0.009	0.227 ± 0.012	0.14 ± 0.016
7	0.218 ± 0.021	0.155 ± 0.018	0.146 ± 0.022	0.138 ± 0.017
14	0.284 ± 0.030	0.068 ± 0.017	0.218 ± 0.023	0.162 ± 0.022
21	0.274 ± 0.010	0.114 ± 0.010	0.207 ± 0.018	0.102 ± 0.015
28	0.295 ± 0.018	0.154 ± 0.016	0.267 ± 0.019	0.161 ± 0.023

Table 2. **Polydispersity of liposomes at 37^0C over a month period.**

3.5 Discussion

Effects of serum/plasma components on the integrity of liposomes are not readily generalized, as they vary among individual liposomal formulations and preparation methodology, and thus must be determined empirically. Therefore, to yield a consistent product of desired size and stability we determined the stability of albumin-liposome in PBS over one month. Our data suggest that the liposome size (F/T average = 300nm; blank average = 400nm) and the polydispersity index (markers of structural stability) did not change at either 25^0C or 37^0C for a period of one month (extruded or non-extruded). This was in contrast to encapsulation efficiency that showed only 40-50% of the protein retention in the liposomes after 72hrs; although the non-extruded liposomes (15 cycles F/T) had less protein release (~7%) over the week period. The discrepancy in size and albumin release needs further investigation, but we speculate that if the liposome is stored frozen within the first 72hrs post-freeze-thaw, then the release can be minimized/controlled.

Our encapsulation efficiencies were comparable to those observed in previous studies (Zhao & Lu, 2009; Colletier, *et al.,* 2002; Xu, *et al.,* 2012), In our study, 15 cycles of F/T yielded the best encapsulation efficacy (~90%) of the protein, and least loss upon extrusion. This method of F/T is relatively superior to a previous study (Dhoot, 2003), wherein FITC-albumin encapsulated via sonication achieved an encapsulation efficiency of 50-55%. During this process, similar to sonication, albumin is captured and retained; however unlike sonication, F/T includes the energy change of multi-lamellar to unilamellar liposomes and size changes associated with heating and freezing that likely was more effective in encapsulation under our conditions. Protein release pattern was consistent with Dhoot (2003) *et al.* that suggested a significant loss of encapsulated protein upon extrusion at 0, 5, or 10 F/T cycles. Further, like previous studies, cholesterol incorporation allowed more release of their cargo (Dhoot, *et al.,* 2003). However, unlike previous studies that notice burst release of FITC-albumin after 24hrs (Hua, 2014) from the liposomes prepared with sonication, our data suggest a more robust encapsulation of proteins by F/T method (Figure 9), and suggest retention up to 50% of encapsulated protein for 30 days. The differences in results could be due to higher amounts of cholesterol (7:3 ratio of phosphatidylcholine and cholesterol) relative to our formulation that had a molar ratio of 85.3:9.7:5.0 ratio (cholesterol being the minimum amount added). We also speculate that the albumin may not have been

entirely encapsulated but was retained on the surface of the liposome in liposomes with higher cholesterol content. Albumin, due to its size and anionic nature, is cell membrane impermeable, and can associate with positive groups on the liposomes surface. Presence of the PEG theoretically can prevent such interactions and may improve liposome retention for a relatively longer period.

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CHAPTER IV

LIPOSOMAL INTERACTIONS WITH CELLS IN VITRO, AND FREEZE-THAW MEDIATED PROTEIN STRUCTURAL CHANGES

4.1. Abstract

Background: Targeted cell-specific therapy of albumin-liposome requires an understanding of the structure altering properties of the freeze-thaw on proteins, and uptake and trafficking rates by various cell types. The objectives of this study were the following: 1) Determine the interaction of liposomes with red blood cells (RBCs), macrophages and lung epithelial cells; 2) Quantify the rate and mechanism of uptake, and 3) Determine protein structure and activity upon freeze thaw.

Methods: Liposomes were incubated with red blood cells (RBCs), macrophage and lung epithelial cells at 37^0C. Epifluorescence microscopy was performed at 1hr following co-incubation of liposomes with cell. Flow cytometry was conducted to quantify cellular uptake over time (15min, 30min, 1hr, and 2hr). Protein structure in liposomes upon F/T was analyzed using circular dichroism (CD).

Results: Liposomes were taken up by an endosomal route. The rate of uptake varies significantly between the extruded, non-extruded liposomes, and free albumin. At 2 hours, free albumin demonstrated relatively greater phagocytosis than liposomal albumin. Additionally, the rate of uptake of albumin liposomes was highest in macrophages with intermediate to minimal phagocytosis in lung cancer and RBC cells. Protein structure was negatively impacted by freeze-thaw and the damages were more profound compared to encapsulated form.

Conclusions:. Liposomal encapsulation of protein reduces its cellular uptake. F/T can impact protein structure if in free from. Protein encapsulation in the liposome can preserve some of the secondary structure and prevent protein damage.

4.2. Introduction

Conventional drug therapy has limited specificity and can cause toxic side effects. To address this problem, currently drugs are encapsulated in nanoparticles as a means to achieve site specific delivery. A variety of drug delivery system are currently under investigation. This includes gold nanoparticles (Ghost, *et al.,* 2008, Zensi, *et al,* 2009), polymers (Davis, 2009), and lipid based nanoparticles (Xu, *et al,* 2009). Of these, liposomes have a bilayer structure with an aqueous core, and can encapsulate both hydrophobic and hydrophilic drugs.

Liposomes can enter a cell by a variety of mechanisms including: endocytosis; absorption; and lipid transfer (Pagano, & Weinstein, 1978). However, the rate of its uptake is highly variable between cells (macrophages, cancer cells etc.). In general, no uptake is seen in the red blood cells due to absence of endosomes in RBCs, however, liposomes may cause RBC hemolysis, and thus can serve as a marker for cytotoxicity (Iren, *et al.,* 2003, Stork, *et al.,* 2013). Hemolysis below 10% is considered safe in the human body (Mocan, 2013), and thus, optimal dosing is critical to avoid cytotoxic events upon nanoparticle therapy.

One approach to prevent hemolytic interaction and dissuade preferential uptake by the phagocytic cells is by modulation of the physicochemical properties of the liposomes (e.g. size). For example, larger (>80nm) liposomes tend to be taken up by the immune cells (e.g. macrophages)(Badiee, *et al.*, 2012); however, macrophages cannot identify liposomes or nanoparticles under 80nm. Thus, an elegant approach to avoid phagosomal uptake can be by creation of small-sized liposomes using extrusion method. In some cases especially for protein delivery, extrusion is not the preferred methodology since it can cause conformational changes in protein structure. One approach to address this issue has been loading of proteins using F/T methodology. However, F/T creates larger and less uniformly shaped liposomes causing it to interact with cells in a different

manner (Traikia, *et al.,* 2000). To verify these anomalies, in this study we quantified the uptake mechanisms of liposomes by fluorescent microscopy, flow cytometry, and hemolysis assay.

As noted earlier, liposome synthesis methods (F/T vs extrusion) can also impact protein structure that may influence the activity and encapsulation. There is some evidence of proteins denaturing even with F/T (Chang, *et al.,* 1995), and the loss in activity was proportional to protein concentration in the sample (Jian & Nail, 1998). In contrast, Chaize et al. observed least amount of damage compared to other methodologies of encapsulation (Chaize, *et al.*,2003). Thus, it is important that F/T needs optimization prior to clinical use.

To evaluate liposomal protection of protein, in this study we used circular dichroism (CD). CD was discovered in early 19th century by Jean-Baptiste Biot. The concept is based on the light that is absorbed differently by the alpha helixes of a protein compared to the β-sheets of a protein. This allows to help determine changes in a protein's secondary structure in liposomes and other proteins (Jullien, *et al.,* 1988; Hirak & Lentz, 2012). In our CD studies, we determined secondary structure of both free and liposome encapsulated liposomes to determine nanoprotection. Although damage to secondary structure cannot predict potential damage in tertiary form, yet it may still serve as a good basis to understand the impact of synthesis methodologies.

4.3. Methods

4.3.1 Cell Culture

A549 alveoli cancer cell line and the RAW 264.7, macrophage cell lines were kindly provided by Dr. Lin Liu (Oklahoma State University). A549 cells were grown in RPMI media. RAW 264.7 cells were grown in DMEM media (Life Technologies). Cells were incubated at 37°C at 5% CO_2. Both media contained 10% fetal bovine serum (Sigma-Aldrich). Both media were supplemented with 1% Penstrep. Red blood cells were obtained from a bovine donor (OSU IAUCC approval), and were stored at 25°C until use (within 24hrs).

4.3.2: Uptake mechanism determination by fluorescent imaging:

To determine liposomal interaction with cells, we used FITC-albumin encapsulated liposomes.

Liposomes were prepared as described in section 3.3.1. A549 cells were grown overnight (90% confluence) in 6-well plates in a 5% CO_2 at 37^0C. Cells were incubated for one hour at 37^0C with: free FITC-albumin (0.17mg/mL), FITC-albumin loaded liposomes that underwent just 15 cycles of freeze thaw (non-extruded group), FITC-albumin loaded liposomes that were formed by both 15 cycles of freeze thaw and one round of extrusion (extrusion group), and a non-liposomal control with just PBS. Prior to imaging, cells were rinsed with PBS three times and the nucleus was stained with 4,6-diamidino-2-phenylindole (DAPI). Additionally, the cell membrane and the endosomes were dual-labeled with a green membrane stain: pkh 67 (Sigma-Aldrich, catalog PKH67GL-1KT) according to the manufacturer's protocol to determine endosomal uptake.

Red blood cells were not stained due to their natural fluorescence. Cells were imaged using an IX-81 confocal fluorescent microscope using a DAPI (exposure = 10ms, excitation: 358nm, emission: 461nm), TRITC (exposure: 100ms, excitation: 545nm, emission: 600nm), and FITC (exposure: 100ms, excitation: 493nm, emission: 513nm) filter.

4.3.3: Quantification of liposome uptake by flow cytometry

Flow cytometry was used to quantify the rate of uptake of the liposomes into the RAW 264.7 macrophage cells. Cells were incubated with extruded and non- extrusion FITC-albumin liposomes (15 F/T cycles at 1 min F, 4 min T) for 15 min., 30 min., 1 hour, and 2 hours. Prior to imaging, the cells were washed twice with phosphate-buffered saline to remove any nonphagocytosed nanostructures. The fluorescence intensity of each sample was analyzed by fluorescence-activated cell sorter flow cytometry (BDFACS Aria). Assays were performed at room temperature, with time run averaging under a min. per sample.

4.3.4 Hemolysis assay to determine cellular toxicity

To determine liposome toxicity, RBCs were centrifuged at 1500rpm for 15 min., and then washed three times with PBS. Then, FITC-albumin liposomes (extruded and

non-extruded) at 5mg FITC-albumin/mL in PBS were incubated with the red blood cells along with appropriate controls (free albumin and PBS) for four hours at 37^0C with gentle shaking. After incubation, the cells were centrifuged at 1300rpm for 15 min. The supernatant was collected and the absorbance was measured at 394nm. PBS and water was used as positive and negative control markers of hemolysis (Chen, *et al.*, 2008).

4.3.5 Assessment of protein damage by circular dichroism

Liposomes for CD were prepared and loaded with FITC-albumin as described in Section 2.3.1. and Section 3.2.3. As additional control, free bovine serum albumin was used and underwent the same procedure. Samples were analyzed at 0, 5, 10, and 15 cycles by CD via spectra-analysis which analyzed secondary structure of albumin using light rotation. After initial results were obtained, liposomes were lysed with 10% acetonitrile to observe the encapsulated protein in a free state.

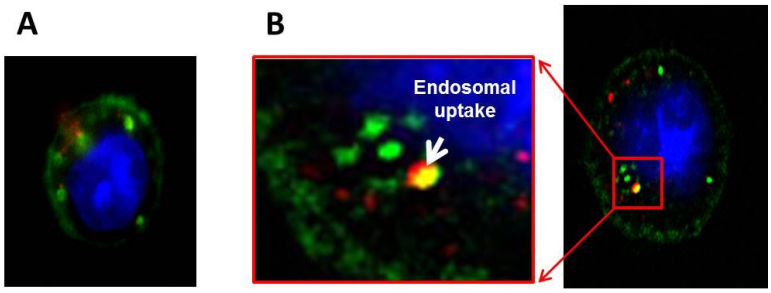

Figure 9. **Fluorescent images of A549 cells.** The nucleus of the cells are stained in blue (DAPI), the membrane is stained in green (FITC), and the liposomes are red (TRITC).

4.3.6 Statistical methods

For pairwise comparison, a 2- tailed independent t-test was conducted ($p < 0.05$). To compare across multiple treatment groups, an ANOVA followed by pair-wise comparison was used ($p < 0.05$).

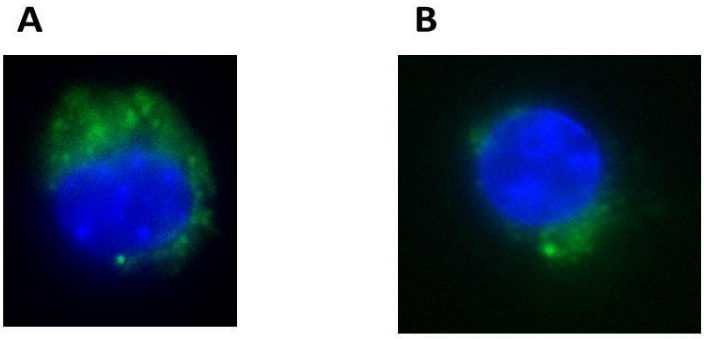

Figure 10. **RAW 264.7 cells containing albumin-lip-FITC.** The nucleus of the cells are stained in blue (DAPI), and the liposomes are green (FITC).

4.4. Results

4.4.1 Uptake mechanism determination by fluorescent imaging

FITC-albumin liposomes were phagocytosed successfully upon incubation with the macrophage and A549cells (Figures 9A & B) by endosomal mechanism. Liposomes

were localized in the endosomes/lysosomes as indicated by distinct yellow-to-orange spots formed by co-localization of green nanoparticles and red membrane (Figure 10A & B). No nuclear infiltration in either cell line was observed, and there was no uptake in red blood cells (data not shown due to absence of fluorescence).

4.4.2: Rate of liposome uptake by flow cytometry:

The median fluorescence intensity of FITC-liposome in RAW 264.7 macrophages showed a significant increase uptake over time ($p < 0.003$) (Figure 11). Figures 12 & 13 are representative images of flow cytometry data acquired showing the threshold for positive cells (i.e. cells that have actively taken up the FITC-albumin

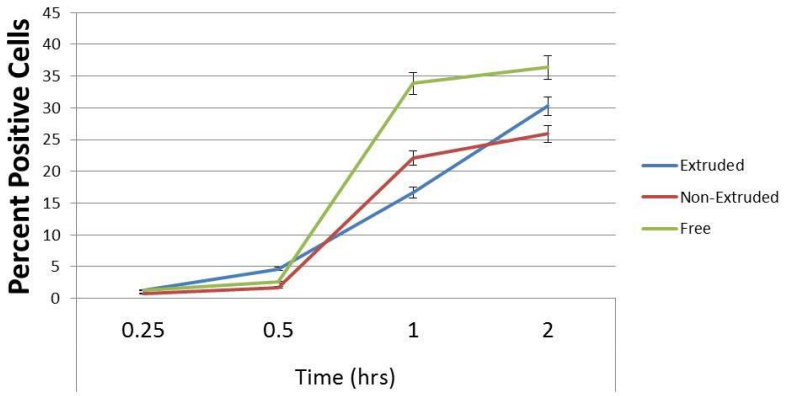

Figure 11. **Cellular uptake of liposomes (15 F/T) over a period of 2 hours in RAW 264.7.**

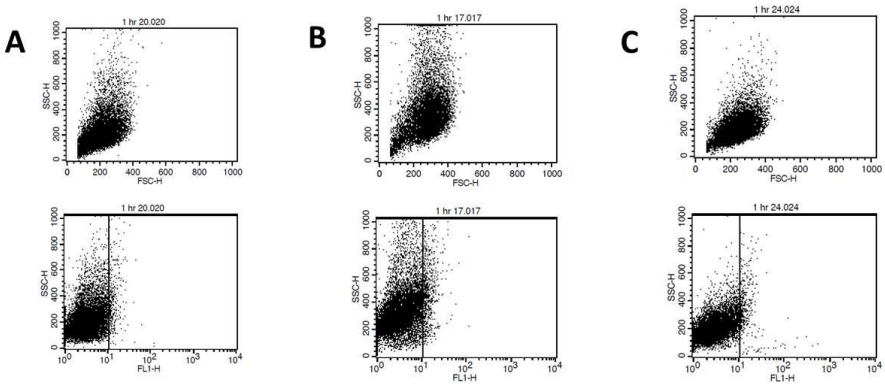

Figure 12. **A**: A549 cellular uptake of extruded liposomes (representative image). **B**: A549 cellular uptake of free albumin. **C**: A549 cellular uptake of non-extruded liposomes.

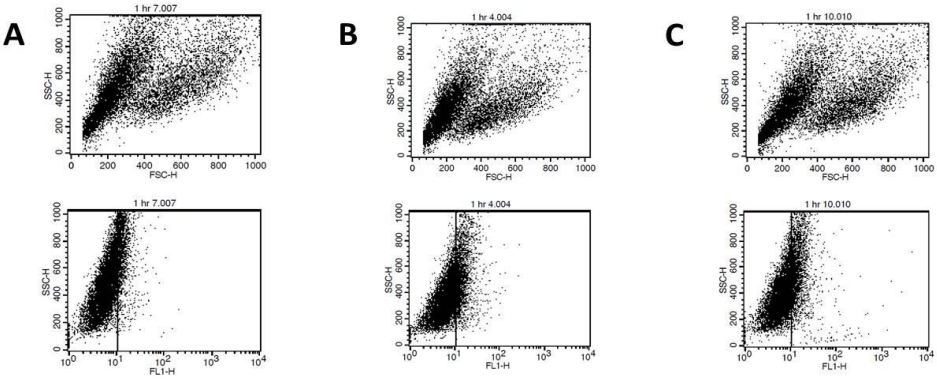

Figure 13. **A**: RAW 264.7 cellular uptake of extruded liposomes (representative image). **B**: RAW 264.7 cellular uptake of free albumin. **C**: RAW 264.7 cellular uptake of non-extruded liposomes.

liposome). The rate of uptake varied slightly between extruded (~30%) and non-extruded liposomes (~26%) at 2 hours.

4.4.3 Hemolysis assay to determine cellular toxicity

Both FITC-albumin liposomes and the free FITC-albumin demonstrated no hemolysis (Figure 14), and the percent hemolysis was less than 1%, between groups (data not shown).

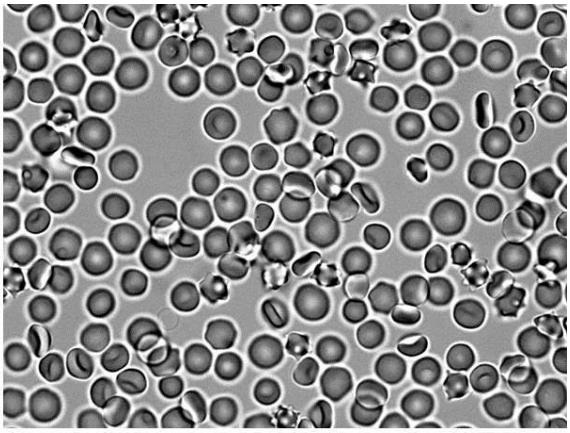

Figure 14. **Confocal image of bovine red blood cells showing no hemolysis**

4.4.4 Protein Structure

To determine the potential effects F/T had on albumin, we generated a batch of liposomes under conditions described in 3.2.3. and measured the protein change via CD.

Significant change in secondary structure of free albumin at 5 (~18%), 10 and 15 (26.5%) cycles of freeze-thaw ($p < 0.05$) (Figure 15), compared to the free control albumin was noted. An increased damage of protein was noted upon extrusion (~63% at 15 cycles) when compared to the protein that only underwent freeze-thaw ($p < 0.05$) (Figure 16). In contrast to free albumin, albumin-encapsulated liposomes demonstrated minimal damage at 5 cycles, however at 15 cycles, the secondary structure (25.8%) change was similar to free albumin (Figure 17). When extruded after 15 cycles of F/T, there was 38.1% change in structure, thereby suggesting that encapsulation afforded about 50% protection in protein structure (Figure 17). When acetonitrile was incubated with the liposomes and free albumin, we observed denaturing of the free albumin to a complete loss of secondary structure; however, when encapsulated with no extrusion, the albumin was able to retain ~45% of its original structure (Figure 18). Figures 19-22 are breakdowns of Figure 18.

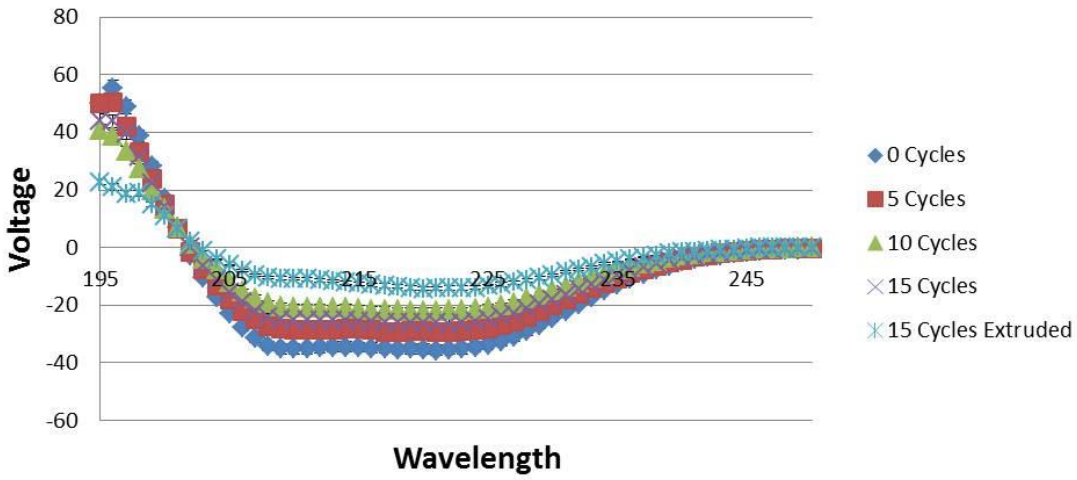

Figure 15. **Circular Dichroism of free albumin following freeze-thaw treatment.** Significant protein structural changes was observed following freeze-thaw.

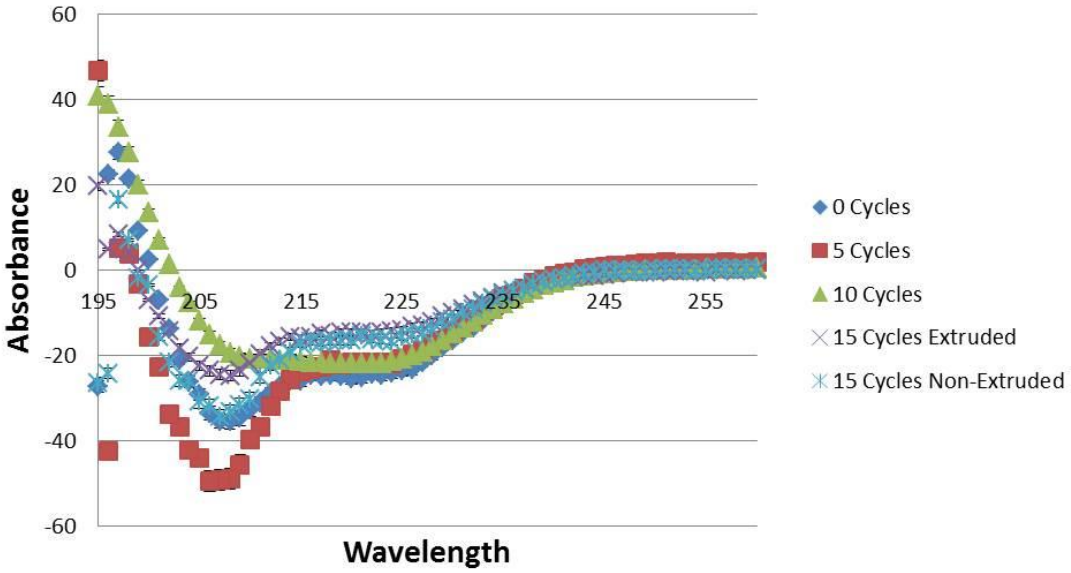

Figure 16. **Circular Dichroism of encapsulated albumin following freeze-thaw treatment.**

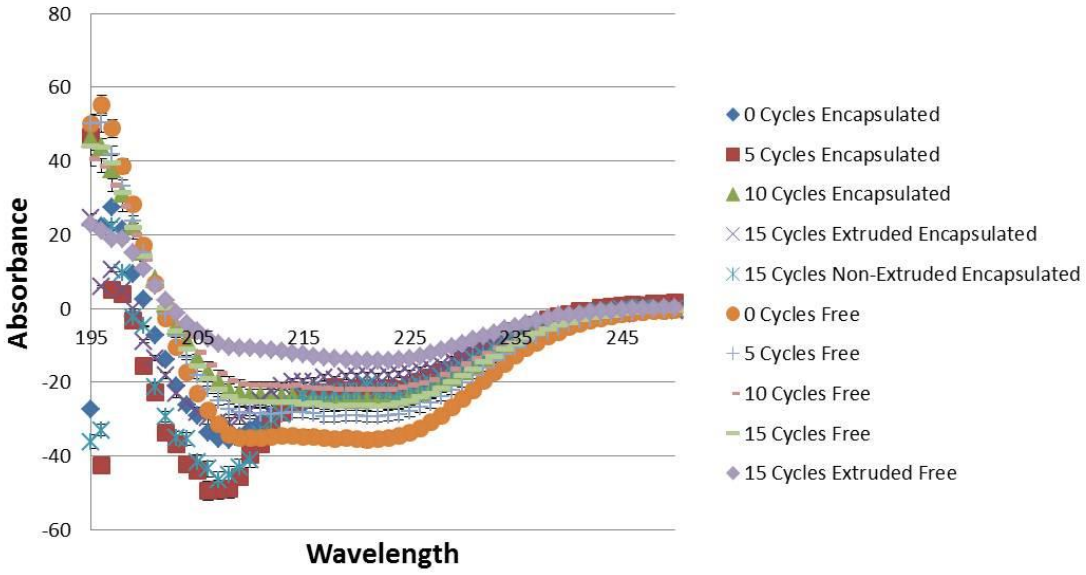

Figure 17. **Circular Dichroism of encapsulated and free albumin following freeze-thaw treatment.**

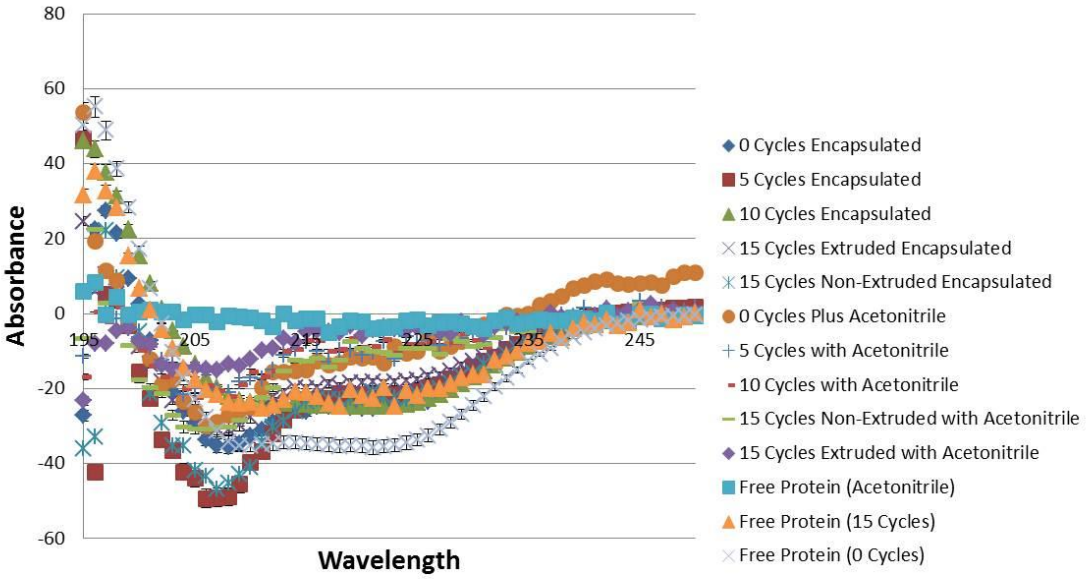

Figure 18. **Circular Dichroism of encapsulated and free albumin following freeze-thaw and acetonitrile treatment.**

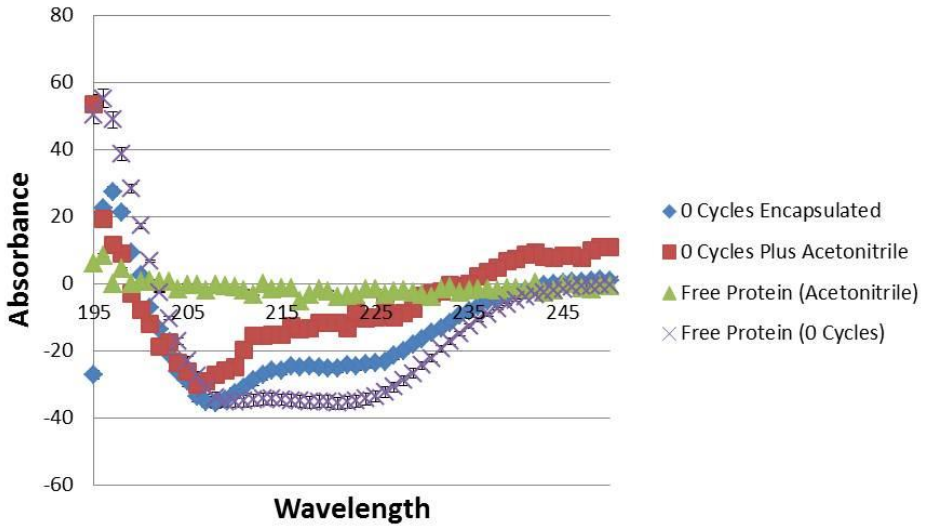

Figure 19. **Circular Dichroism of encapsulated and free albumin following freeze-thaw and acetonitrile treatment at 0 cycles.**

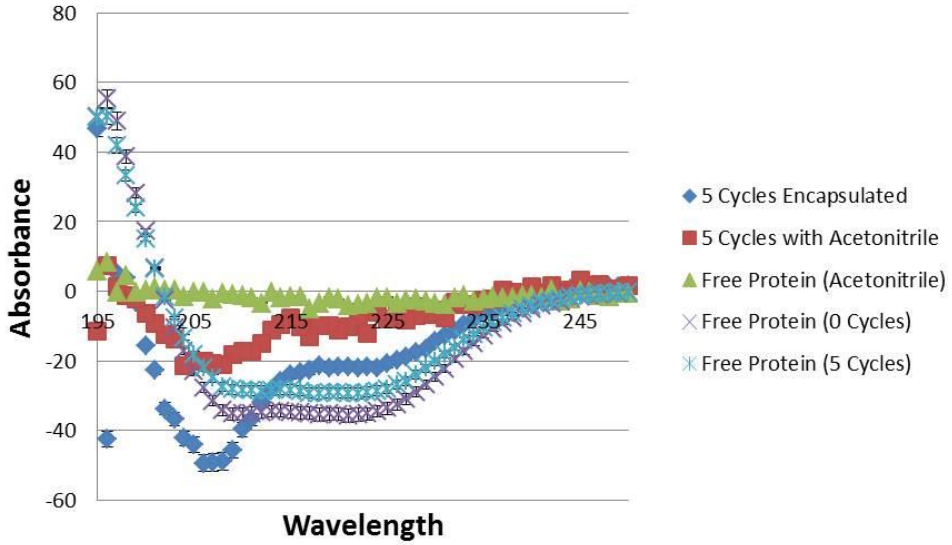

Figure 20. **Circular Dichroism of encapsulated and free albumin following freeze-thaw and acetonitrile treatment at 5 cycles.**

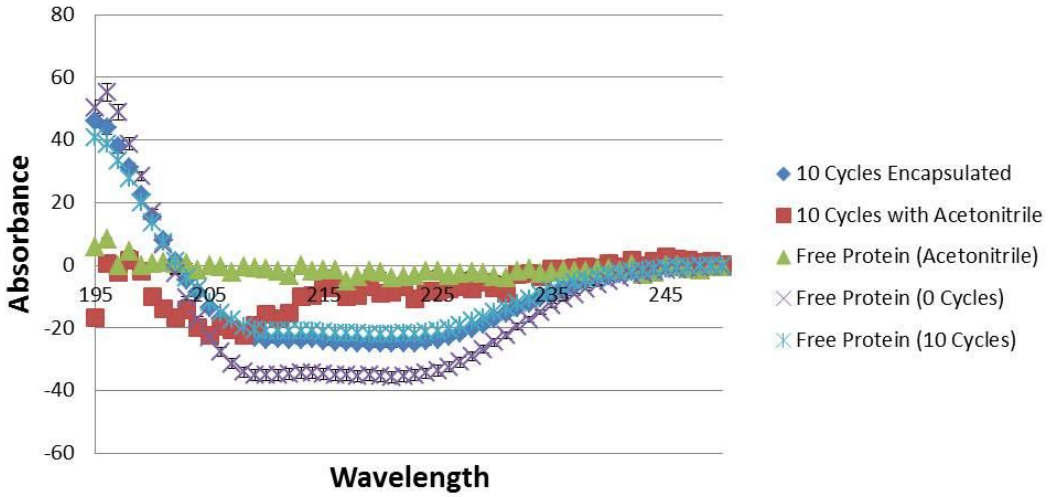

Figure 21. **Circular Dichroism of encapsulated and free albumin following freeze-thaw and acetonitrile treatment at 10 cycles.**

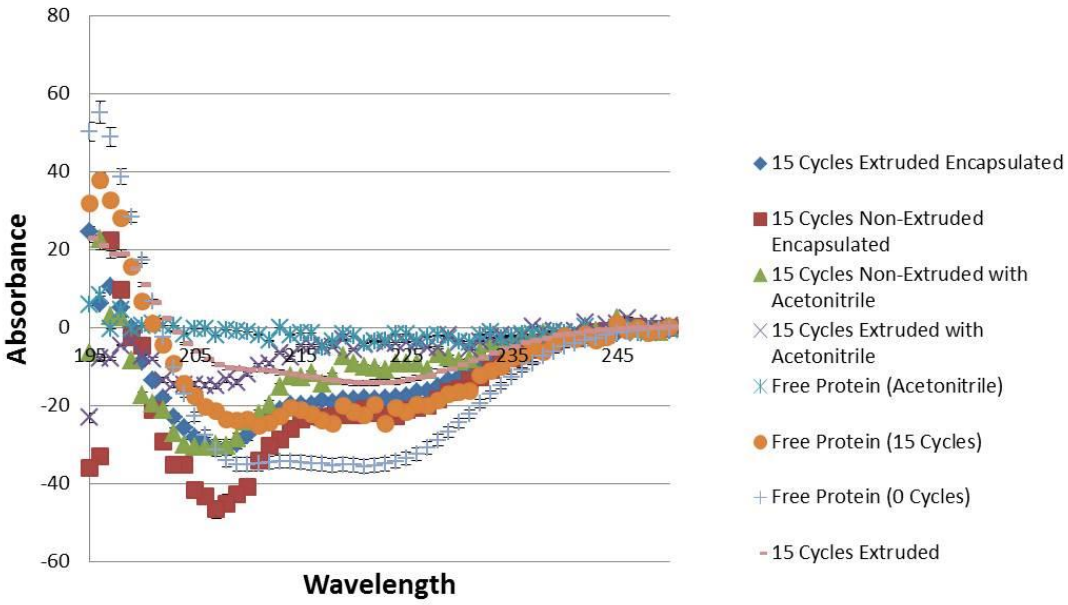

Figure 22. **Circular Dichroism of encapsulated and free albumin following freeze-thaw and acetonitrile treatment at 15 cycles.**

4.5 Discussion

The overall goal of our study was to understand the liposomal cellular interaction and structural changes in the encapsulated protein upon F/T. For liposomal cellular trafficking studies, A549 lung epithelial cells and RAW 264.7 macrophages were used.

Fluorescence microscopy was employed to understand the route of uptake in cells and the rate of uptake was quantified by flow cytometry. Data suggest that FITC-albumin liposomal uptake is mainly by endosomal route which is consistent with previous reports (Lee & Low, 1994; Pagano, & Weinstein, 1978; Chan, *et al.,* 2012. Further, in immune cells such as RAW 264.7, FITC-albumin accumulation was also noted in the cytoplasm, likely by micropinocytosis (Makino, *et al.,* 2003). Quantitatively, the rate of uptake of FITC-ALB -liposomal in macrophage was greater than the A-549 (~25%, Figure 20). This is expected since the immune cells are natural phagocytic cells and are adept at removing foreign material, and have a propensity to take-up foreign materials. Previous studies have shown that liposomal coating of protein by PEGylation minimizes its interaction with immune cells, and enhances the circulation time of drug molecules. We also noted similar findings since the rate of uptake of free albumin was greater than the liposome encapsulated protein at 2h of incubation. Metabolically active cells such as macrophages can ingest foreign molecules by phagocytosis or micropinocytosis. In contrast to macrophages, RBCs cannot perform endosomal uptake since their primary function is in oxygen transport. To verify this hypothesis, we incubated the RBCs with the liposomes. Results indicated no uptake as expected.

To determine the effect of F/T on encapsulated albumin (mostly an alpha-helical protein (67%)), we measured the structural change via CD. Data suggest that structural changes initiate at 5 F/T cycles. These changes were more pronounced for the free albumin that frequently transitioned from an alpha helical nature to beta sheet. Similarly, the encapsulated albumin also showed random coil generation, however, the difference in elipticity from the different cycles was more limited when the FITC-albumin was encapsulated compared to the free FITC-albumin in that the fold difference when encapsulated was limited (no change up to 5 cycles, <less than 1-fold change from 5- 15 cycles of freeze thaw). In contrast, the fold change in free albumin structure was greater than 1x. An unexpected finding in our study was the preservation of protein structure in liposomes in the presence of organic solvents (e.g. acetonitrile, Figure 27). This protective effect was also seen in the case with the free protein that underwent extrusion, and requires more evidence to confirm these findings. Regardless, an increase in F/T cycles is associated with a proportional increase in damage, and this agrees with previous studies (Cao, *et al.,* 2003; Benjakul, *et al.,* 2000). We also observed protein damage via extruding of the free BSA through a filter at high pressure one time may have

caused a significant change in protein structure. Interestingly, encapsulating the free albumin into liposomes can prevent this effect by decreasing the damage by 1.6x fold. In summary, it is clear that both F/T and extrusion cannot prevent protein damage (Pikal-Cleland, *et al.,* 2000). Methods to limit damage should include an optimal buffer type with low salt crystal, an optimize protocols that can achieve fast freezing to partially limit the damage (Cao, *et al.,* 2003, Pikal-Cleland, *et al.,* 2000). For dosing, such damage should be accounted for prior to administering in a patient.

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CHAPTER V

SUMMARY

F/T has been shown to be an efficient method of protein encapsulation. (Costa, *et al.,* 2014). Despite extensive use, widespread variation exists in the encapsulation efficiency (EE) and size of liposomes. Our studies indicate that the number of F/T cycles is a crucial factor since F/T beyond the optimal numbers (~ 15 cycles) may damage lipid membrane, thereby preventing the liposomes from holding a spherical shape. The ability to maintain spherical bilayer shape is also dependent on adequate freezing and thawing of the sample. In general, a 1 - 2 min. thaw is insufficient to completely liquefy a frozen liposome sample (Castile & Taylor, 1999). Our data suggest that a 4 min. thaw at 35°C can optimally thaw a sample in a time efficient manner, and achieve size controlled liposome similar to that of extrusion process. Loading of small molecules (e.g. anticancer drugs) in liposomes is relatively different than large molecules (e.g proteins such as albumin), and it is conceivable that this may impact liposome size. Our data suggest that F/Ting the albumin-liposome samples for 5 cycles or greater achieves a conversion from multilamellar to unilamellar liposome form and achieves a dramatic decrease in size from 16000μm to less than 1μm. Further, at 10- 15 cycles of F/T, an average size of less than 300nm is achieved. Thus, the loading of albumin seems to have no detrimental effect on the liposome size.

Compared to previous study that reported 40-50% EE after 20 cycles F/T (Costa, *et al.,* 2004), we observed an EE of 90% after 15 cycles. It appears that a 4 min. thawing at 35^0C is optimal for wrapping lipids around the protein and for achieving a higher encapsulation efficacy. We also believe that this method of thawing is a significant improvement over extrusion methodology that also achieve smaller size but is often has lower EE (Costa, *et al.,* 200).

An ideal goal of liposome loading of proteins is the minimal release of the encapsulated content in physiological fluids. Our data suggests that at 72 hours, ~40-50% of the encapsulated protein is released in physiological media. Interestingly, this loss of encapsulated protein did not coincide with any changes in liposome size or polydispersity index. Although the reasons are not known, we speculate that there could be partial damage to lipids during F/T and that exacerbates in physiological fluid, resulting in a greater protein loss over time. Alternatively, the protein may not be encapsulated but remain attached to the outside membrane, and this could break off into free form in PBS; a future study using a protease should be conducted to eliminate this potential hypothesis.

Cell specific nanoparticle therapy requires precise targeting. Since PEGylation minimizes liposome interaction with immune cells, this may consequently increase uptake by other cell types. Our studies show that the uptake of liposome-albumin by the macrophages is relatively lower compared to free albumin, thereby validating the prevailing hypothesis. We also noted that both F/T and extruded liposomes are taken up by the macrophages by endosomal route, thereby indicating that uptake rates are independent of synthesis methodology. In some cases , cytoplasmic localization of NPs was noted in the macrophages at 2h of incubation. This is likely due to high rate of pinocytosis mediated uptake by the macrophage cells. Interestingly, the RBCs showed no interactions or uptake as reported elsewhere (Vranic, *et al.,* 2013). It is conceivable that the lipid bilayer of the RBC can physically interact with liposome, but such contacts may be very weak in the absence of endosomal mechanism, presence of PEG on liposome surface, and at shorter incubation time (<2-3 hr). There are some report in literation that points suggest induction of hemolysis by PEG molecules (Mocan, 2013; Chen, *et al.,* 2008), but this was not observed in our studies. Future experiment should include longer incubation protocols to throw more light on this observation. Similarly, nuclear infiltration in both macrophage and lung epithelial cancer cell line were not observed since the nuclear pores are ~40nm, and the liposomes with a > 150nm and above (Stewart, 1992) may not escape the endosomes efficiently. In summary, F/T achieves optimal size and EE, without significant hemolysis.

Finally, circular dichroism data suggest protein structural changes with freeze-thaw. In the native protein at 5 cycles of freeze-thaw, the structure was 82% similar, but

at 10 cycles, it dropped to 61%. Encapsulating the protein in liposome can help stabilize the protein, and the resulting damage is not as pronounced as the free form of albumin protein. Thus, by creating a method that can optimally load protein in liposomes, this study can ease translation of many protein therapeutics, and thus has high translational potential.

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