Ultrasound-Mediated Leakage from DMPC-Cholesterol Model Membrane

A Thesis

Submitted to the Faculty

of

Drexel University

by

Stephanie Alyssa Carroll

in partial fulfillment of the

requirements for the degree

of

Master of Science in Chemical Engineering

June 2008

Dedications

I would like to dedicate this work to my parents, Andrew and Kim Toth, in memory of my grandfather, Jack Weaknecht and to my husband, Jason Carroll, currently serving overseas in Iraq.

Acknowledgments

I would like to thank my colleagues in Dr. Steven Wrenn's lab for their support and guidance. Special thanks to Dr. Steven Wrenn and Eleanor Small for all their help throughout my time in the lab. I also would like to thank the faculty of the Chemical and Biological Engineering Department for giving me the opportunity to pursue my education at Drexel University.

Table of Contents

List of Tables

List of Figures

Abstract

Ultrasound-Mediated Leakage from DMPC-Cholesterol Model Membranes
Stephanie Alyssa Carroll
Advisor: Steven P. Wrenn

The application of ultrasound for targeted drug delivery offers a convenient, universal, and affordable method for drug delivery. Targeted drug delivery through the use of lipid vesicles coupled with ultrasound is beneficial for the treatment of diseases because it reduces the amount of effective dosage necessary and toxic side effects compared to traditional systemic treatments. Ultrasound-mediated leakage from model membranes occurs when the lipid vesicles are exposed to low ultrasound frequencies of approximately 20 kHz which causes transient cavitation of gaseous voids in the sample. This work investigated a binary, DMPC-cholesterol, model membrane to study the effects of lipid composition and lipid phase behavior on vesicle leakage by ultrasound. A self-quenching dye, calcein, was encapsulated within the aqueous core of lipid vesicles. These vesicles were exposed to ultrasound at a frequency of 20 kHz via a probe tip transducer in small intervals with resting time. Steady state fluorescence spectroscopy was used to quantify the dye leakage resultant from the exposure to ultrasound. Leakage profiles obtained indicate that different mole fractions of cholesterol have different effects on the membrane's ability to resist leakage by ultrasound.

Chapter 1: Introduction

Cholesterol is the most abundant and widely distributed sterol in eukaryotic membranes [1]. This amphiphilic sterol can comprise of up to 0.50 of the total lipid composition of a membrane [2-5]. A physiological effect caused by cholesterol molecules in a membrane is increased bilayer cohesion where saturated lipids are present [2]. The study presented here investigates the contribution of this cholesterol effect on membrane strength by steady state fluorescence spectroscopy.

In past work, the orientation of cholesterol in a phospholipid bilayer has been shown to be similar to lipids. Cholesterol will orientate with its polar headgroup towards the aqueous phase of a sample and its hydrophobic end becomes parallel with the adjacent phospholipid acyl chains [2]. Studies by Almeida *et. al*. investigated the behavior of sterol-phospholipid systems such as 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC) or 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC) resulting in the creation of phase diagrams [3,5].

. Liposomes, are membranes composed of a lipid bilayer. Similar to cell membranes, a liposome membrane determines which molecules will diffuse through [6]. Liposomes present many advantages for targeted drug delivery in treatment of diseases with toxic medicines [7]. Membrane integrity of liposomes is important for control of the diffusion of hydrophilic drugs, encapsulated in the aqueous core of the vesicles. Recent studies have used calcein, a self-quenching fluorescent dye, to mimic drug release from lipid/cholesterol systems [8]. Many mechanisms for drug release from liposomes have been studied but ultrasound has recently come to the forefront of non-invasive release for

encapsulated drugs [7]. The exposure to ultrasound changes the membrane permeation, triggering leakage of the drug. Targeted drug delivery using ultrasound as the release trigger can greatly reduce the amount of effective and toxic side effects [9-10]. It was this potential benefit to those taking dangerous medicines that motivated this study.

To optimize the application of drug delivery, further understanding of ultrasound and cell membrane interaction must be gained. The number of variables that alter membrane properties such as protein and sterol content, have prevented many research groups from studying the interaction between ultrasound and cells [9]. To study this interaction, phospholipid vesicles, have been chosen as the membrane models because the structures are similar to cell membranes, can be prepared in laboratory environment and can be altered in a controlled manner. These model membranes simplify the system allowing for investigation of key parameters influencing the effect of ultrasound field on membrane permeability.

This work investigated a binary (DMPC and cholesterol) model membrane system to study the effects of lipid composition and lipid phase behavior on vesicle leakage by ultrasound.

Chapter 2: Background

2.1 Lipid Phase Behavior and Cholesterol Induced Phase Separation

Glycolipids contain fatty chains that range from 14 to 24 carbons. Glycerophospholipids, which are commonly found in biological membranes, are structured with at two acyl chains that can be saturated, unsaturated, or one of each and a head group of either a phosphate group or an alcohol attached to a phosphate group [11]. 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC), is a glycerolphospholipid a choline head group and two fully saturated 14 carbon chains, shown in Figure 2.1a.

Lipids can be categorized by their melting temperature (T_m). Below the T_m, lipids are in the solid phase (s_o), containing minimal cross sectional area and maximal bilayer thickness, reducing the movement of hydrocarbon chains. Above the T_m, lipids are present in the liquid phase (l) where hydrocarbon chains are not as tightly packed or extended. In this state, the cross sectional area of the lipid is increased and the bilayer thickness is decreased [11]. Lipids with a low T_m contain double bonds or short acyl chains that take less energy to order than longer acyl chains. Lipids with high T_m have longer, saturated acyl chains. The lipid used in this study, DMPC, is an example of a lipid with a high T_m of 23.9˚C that exists in the liquid phase at natural body temperature of 37˚C.

Lipid membranes undergo three different phases: a solid phase (s_o), also known as the gel phase, a liquid disordered phase (l_{do}) and a liquid ordered phase (l_o). Many studies have examined the properties of two component lipid bilayers, with special

interest in the region where two lipid phases can coexist at the same temperature and pressure [3,5,12]. The published phase diagrams are results of thermodynamic data which describe the molecular interactions that cause a particular phase behavior.

Binary mixtures with cholesterol as one component, have received much attention in the past [3,5]. Cholesterol, the most common sterol, is an important constituent of cell membranes [1]. Cholesterol has a planar structure with an OH as the polar head group, shown in Figure 2.1b. The addition of cholesterol to the bilayer forces the neighboring lipid hydrocarbon tails to become fully extended which is known as the cholesterol condensing effect [7]. Vesicle aggregation, fusion, curvature, and interactions between lipids, change when cholesterol is incorporated [13]. The extent to which these behaviors change depends on the types of lipids in membrane.

l_{do} and l_o coexist in the same region in binary mixtures of saturated lipids and cholesterol. The DMPC-cholesterol system phase diagram, from [5], is displayed in Figure 2.2. DMPC packs well with cholesterol because of its two fully saturated acyl hydrocarbon chains. As shown in the diagram, above the T_m, l_{do} and l_o can coexist or outside the two phase region the bilayer exist either as l_{do} or l_o depending on the cholesterol mole fraction [3,5,14]. At low cholesterol concentrations the membrane experiences high lateral mobility and lipids can "flip-flop" from one leaflet to another. This l_{do} phase is also characterized by disordered hydrocarbon chains. As the cholesterol concentration is increased, more DMPC molecules are driven into the extended chain formation, which decreases the total surface area [7]. Therefore, as the cholesterol concentration is increased, pockets of liquid ordered are formed in a liquid disordered environment. These pockets are known as rafts. As the membrane transitions into the l_o

phase, which is characterized by ordered hydrocarbon chains, lipids that are not fully extended will group together to form domains of l_{do} in a majority of l_o [7,15]. The height difference between the two phases exposes parts of the acyl chains to water creating areas of line tension.

2.2 Line Tension

Domains of the model membrane can be explained when compared to natural membranes through the explanation of line tension. In these domains, lipids in l_o are misaligned with lipids in l_{do} because of height difference, which is created by ordered and disordered hydrocarbon chains [16]. Line tension, which occurs at the edge of these domains, as shown in Figure 2.3a, is a result of the exposure of the hydrocarbon chains in l_o exposed to water which creates an unfavorable interaction between the hydrophobic and hydrophilic molecules [7,16]. To reduce line tension, the domains coalesce together to form a minimum number of domains. Natural membranes, as shown in Figure 2.3b, contain many phospholipids of different chain lengths and saturations which alleviate the line tension. In a multi-component system, the components with varying chain length act as lineactants to relieve line tension.

2.3 Stable and Transient Cavitation

In recent years, the use of ultrasound as a diagnostic imaging modality has continued to grow [17,18]. Since targeted drug delivery methods are limited by passive diffusion across membranes, ultrasound is becoming the preferred method to increase membrane permeability [19]. The application of ultrasound for targeted drug delivery offers a convenient, universal, and affordable method for drug delivery [14,17]. Briefly,

a transducer emits a short pulse sound wave through the human body that is absorbed by some tissues, scattered by others, and reflected by boundaries between tissues of different sound speeds [7]. The mechanism by which ultrasound temporarily increases membrane permeability is suspected to be acoustic cavitation [20,21]. There are two types of acoustic cavitation; transient and stable [22,23]. In both stable and transient cavitation, gas filled bubbles injected into the blood stream of a patient, known as contrast agents, oscillate non-linearly because of ultrasound radiation [7,10,17,24].

Stable and transient cavitation depends on frequency, exposure time, temperature and pressure [20,22]. Bubble oscillation can be described by the following Rayliegh-Plesset-Noltingk-Neppiras-Poritsky (RPNNP) equation:

$$R\ddot{R} + \frac{3}{2}\rho\dot{R}^2 = \frac{1}{\rho}\{\rho_{go}\left(\frac{R_o}{R}\right)^{3\Gamma} + \rho_v - P_o - \frac{2\sigma}{R} - 2S_P\left(\frac{1}{R_o} - \frac{1}{R}\right) - \delta\omega\rho R\dot{R} - \rho_a(t) \quad (1)$$

where ρ is the density of the surrounding medium, Γ is the polytropic exponent of the gas, ω is the driving frequency, and δ is the total damping parameter which is equal to the summation of viscous damping, reradiation damping, thermal conduction damping, and friction damping. R is the instantaneous radius of the bubble, R_o is the equilibrium bubble radius, P_o is the static pressure of the surrounding medium, σ is the surface tension, p_v is the vapor pressure, p_{go} is initial internal pressure of the bubble and S_p is the shell stiffness parameter. S_p and p_{go} are defined by the following equations respectively:

$$S_P = \frac{8\pi E h_s}{1-\nu} \quad (2)$$

where E is young's modulus, h_s is the shell thickness and ν is the Poisson ration which is roughly 0.500 for lipid shells [7,25].

In stable cavitation, contrast agents sustain stable oscillations. Studies from Wamel *et. al*. explain that contrast agents experiencing stable cavitation generate fluid movement around them, known as microstreaming. Microstreaming applies shear stress on any surface within the boundary layer of moving fluid [7,22,26]. Although stable cavitation does not collapse the bubble, studies have proved that leakage from liposomes can be induced.

Another form of stable cavitation is transient cavitation in which the bubble collapses. Transient cavitation is the result of the bubble's kinetic energy exceeding the instantaneous surface energy of the bubble [7,27]. In transient cavitation, the bubbles are exposed to two types of pressure cycles known as rarefraction (negative pressure) and condensation (positive pressure) [7,22]. The rarefraction pressure forces the bubble to expand through gas diffusion, which then enters the condensation pressure cycle. The condensation pressure cycle occurs when the volume of the gas bubble contracts due to positive pressure applied to the system. Eventually the bubble will collapse once it reaches its critical radius [7]. Transient cavitation can be prevented by increasing the intensity frequency which reduces the time between pressure cycles thus decreasing the amount of diffusion into the bubble in each cycle. Therefore, low frequencies were used, approximately 20 kHz, to produce transient cavitation which allows leakage for vesicles [7]. This suggests that ultrasound-induced permeation of membranes is a promising method to enhance drug delivery from liposomes [14,20,28-30].

2.4 Fluorescent Assays

Leakage of the encapsulated fluorophores from the aqueous core of the vesicle can be measured by fluorescence spectroscopy [7,22]. Encapsulation of a self-quenching

dye, such as calcein, enables the fluorescence spectrometer to better detect the increase of fluorescence intensity as leakage increases. Calcein has been used in previous experiments, at a concentration of 70mM or higher because of its ability to self-quench through collision transfer at these concentrations [7]. When calcein is encapsulated within the aqueous core of the vesicle, it is unable to fluoresce. As the calcein is released from the liposome, due to the application of ultrasound, the molecules are able to spread out and fluoresce. It is this fluorescence that is measured by the fluorescent spectrometer. Steady state fluorescence spectroscopy measures the intensity of light emitted by a fluorophore as it returns to the ground state energy level after being excited by a laser [7,31]. The fraction of dye released is determined by:

$$\text{Fraction Release (\%)} = \frac{F_{obs} - F_{\text{int}}}{F_{TX} - F_{\text{int}}} \times 100\% \qquad (3)$$

where F_{obs} is the observed fluorescence after ultrasound has been applied, F_{int} is the initial fluorescence before the application of ultrasound (0% leakage), and F_{TX} in the intensity after a detergent, Triton X, has been added (100% leakage). Triton X-100 is a surfactant which, owing to its small hydrophobic tail, can partition readily into lipid membranes which causes a destabilization of the membrane [9]. At concentrations greater than 0.3 mole%, Triton X-100 has been found to cause catastrophic solubilization of the membrane [9]. Based on this knowledge, the fluorescence measured after Triton-X is assumed to be 100%.

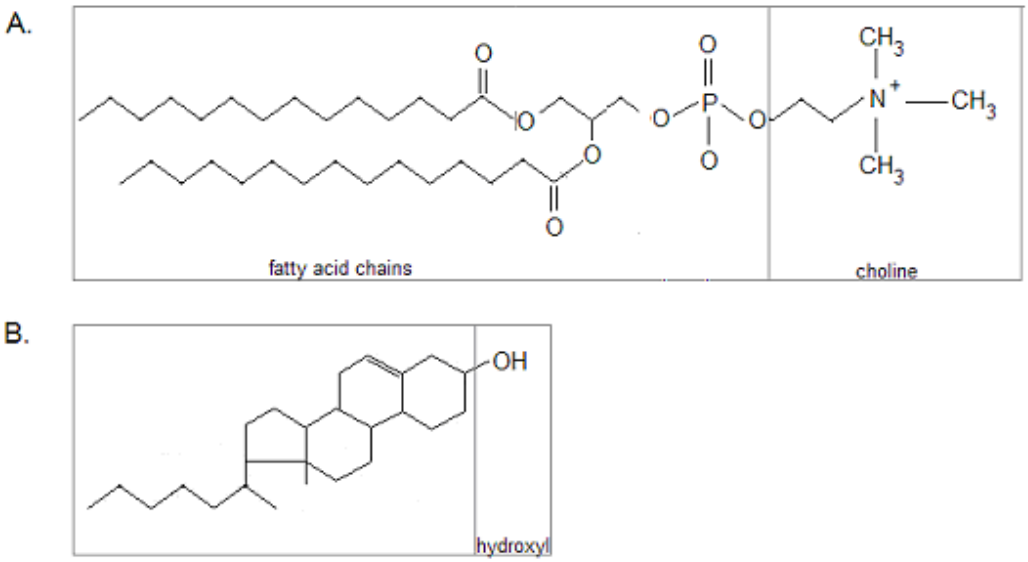


Figure 2.1 Chemical structures of DMPC and cholesterol (a) is a phospholipids with a choline headgroup and two fully saturated 14-carbon chains [11]. Cholesterol (b) is a planar structure with a hydroxyl headgroup.

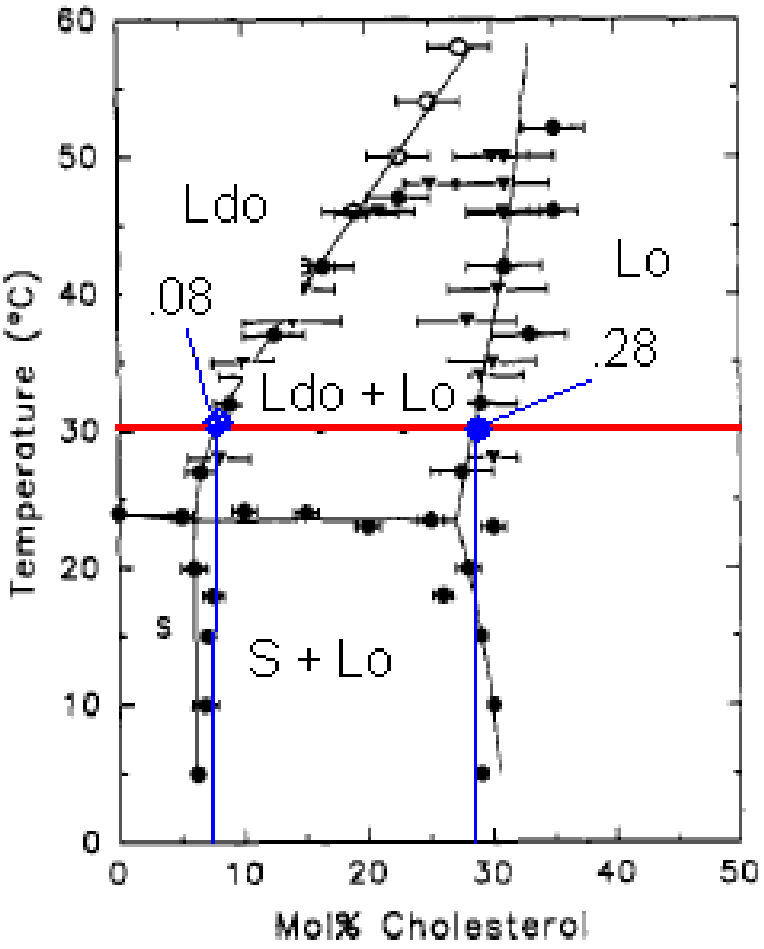
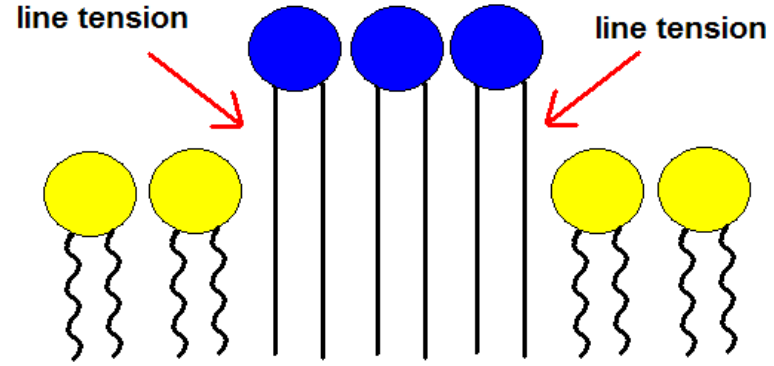


Figure 2.2 DMPC-cholesterol phase diagram [5]. At 30˚C, the two phase region, the l_{do} and l_o phase, exist approximately between 8% and 28%. The outside regions exist either in the l_{do} or the l_o phase.

A.



B.

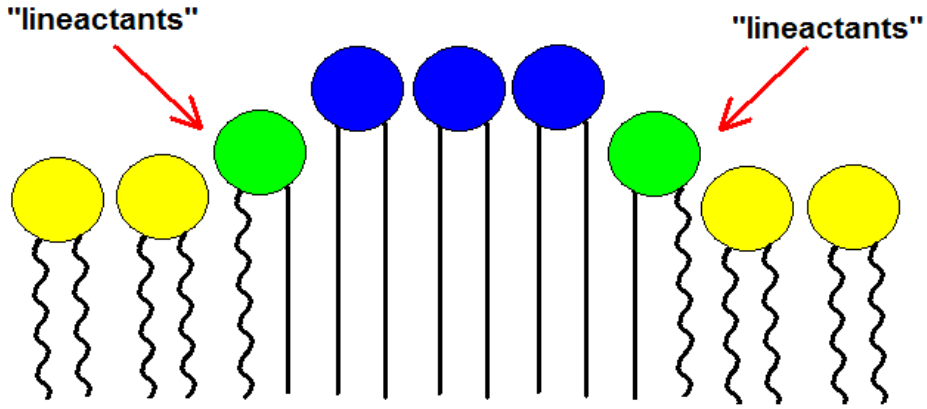


Figure 2.3 Cartoons of line tension of phase boundaries in a binary system (a.) and "Lineactants" which alleviate line tension. Lineactants are found in multicomponent systems [32].

Chapter 3: Research Goals

The purpose of this work was to investigate whether lipid composition and therefore lipid phase behavior, influence the membranes susceptibility to leakage with application of ultrasound. The two phase region, where l_{do} and l_o phases coexist, was the primary focus and a range of cholesterol compositions were used to obtain the different phase. The extend of the cholesterol condensation effect in a binary membrane was also studied.

Model membrane systems consisting of various concentrations of DMPC and cholesterol were studied. Calcein fluorescence was used to detect leakage from the liposome. Steady state fluorescence spectroscopy was used to quantify the leakage.

Chapter 4: Materials and Methods

The model membranes, phospholipid vesicles consisting of DMPC and cholesterol were extruded to create large unilamellar vesicles. The model membrane preparation and analysis is described below.

4.1 Chemicals

NaCl, EDTA (anhydrous), cholesterol, calcein, Triton X-100, and Sephadex G-50 were purchased from Sigma Aldrich (St. Louis, MO). Tris base salt was purchased from Fisher Scientific Inc. (Fair Lawn, NJ). 1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine (DMPC) was purchased from Lipoid LLC. (Newark, NJ). Drain disks 25mm PE and poly carbon filters 0.4μm and 0.2μm pores were purchased from Nuclepore, Whatman Inc. (Clinton, NJ).

4.2 Model Membrane Preparation Method

Model membranes were created in the lab through a technique in which various lipid mixtures are exchange from chloroform into an aqueous buffer solution [11]. Lipid and cholesterol components are stored in chloroform. The buffer solution will contain multilamellar vesicles (MLV) and is pressure extruded though polycarbon filters to make large unilamellar vesicles (LUV).

4.3 Rapid Solvent Exchange

To create MLVs, the rapid solvent exchange method (RES) is used [33]. This method is used to replace the cholorform from the stoke solutions with aqueous calcein

buffer (150mM NaCl, 10mM Tris, 1mM EDTA and 70mM calcein; pH = 7.4). The calcein fluorophore used is shown in Figure 4.1. By using this method the lipid mixture can be directly transferred from the organic to aqueous phase [11]. In RES, 3mL of aqueous calcein buffer, heated to 60˚C, is added to the lipid mixture. The buffer is heated to 60˚C so the MLVs that are created are in the liquid phase. This mixture is then vortex-mixed while, simultaneously, a vacuum is pulled. As the chloroform solvent evaporates, lipids are transferred into the calcein buffer where MLVs are formed. The solution is then diluted with an additional buffer [11].

4.4 Pressure Extrusion and Size Exclusion Chromatography

In pressure extrusion, the MLV solution, held at 30˚C, is forced through two stacked polycarbonate filters in an extruder. The solution is first passed through 0.4μm pores five times followed by five times through 0.2μm pores to create LUVs. The LUVs are then passed through a Sephadex G50-packed column to remove unencapsulated calcein dye. The LUVs were washed through with an ultrasound buffer solution (150mM NaCl, 10mM Tris and 1mM EDTA) and samples were collected.

4.5 Preliminary Analysis

4.5.1 Dynamic Light Scattering

Vesicle diameter sizes were analyzed using a Brookhaven 90Plus dynamic light scattering (DLS) apparatus. This system consists of a 15 mW, solid-state laser operating at a 678 nm wavelength and a BI-9000AT digital autocorrelator. The measured autocorrelation functions were analyzed for the first and second cumulants of a cumulant fit, which provide measures of the apparent diffusivity and the polydispersity,

respectively. Table 4.1 displays the average vesicle diameters of each sample. The data are given in terms of an effective diameter using the Stokes-Einstein equation:

$$\text{Effective diameter} = \frac{k_B T}{3\pi\eta D} \qquad (4)$$

where k_B is the Boltzmann constant, T is the temperature (25 °C), η is the solvent viscosity, and D is the diffusivity from the first cumulant.

4.5.2 UV VIS Spectrometer

Turbidities were measured as absorbance (or optical density) at 600 nm using a Perkin-Elmer BUV40XWO UV-visible absorbance spectrometer. The UV Vis Spectrometer was used to indicate which samples would be run on the DLS.

4.6 Sonication

The ultrasound source used was a commercial (Misonix Inc., XL 2020 model from Farmingdale, NY) 20 kHz unit with a 3.18mm (diameter) transducer (Misonix Inc., model 419 from Farmingdale, NY). The microtip was inserted into a cuvette containing 3 mL of vesicle solution, and was sonicated in a cold water bath to reduce the thermal effects from the ultrasound. The vesicle solution was sonicated in intervals of thirty seconds with three minutes between intervals until a total sonication time reached twenty minutes. The setting for the ultrasound experiments using 20 kHz was 40% of the duty cycle and 25 Watts. Additional parameters are listed in Table 4.2.

4.7 Fluorescence Spectroscopy

Fluorescence Spectroscopy involves emission of light from single excited states. A fluorescence molecule will first be excited to a higher energy level during a process

called absorption. Next, the molecule will return to its ground state energy level during processes called internal conversion and vibrational relaxation. Energy is released as the molecule returns to the ground energy level in the form of light. The emission light from the fluorophore, in this case calcein, is detected by the fluorescence spectrometer. Samples were analyzed using a fluorescence spectrometer after the application of ultrasound to measure the leakage from liposomes. Fluorescence intensities were measured using a Photon Technology International, Inc., A-710 steady-state fluorescence spectrometer. This technique produced a release profile of the fluorophore, calcein [11]. The release profile was created as more calcein leaks from the vesicle and fluoresces, causing the emission intensity to increase over time. The excitation wavelength of calcein was 470 nm with an emissions range from 485nm to 530nm. The emissions intensity used to determine fraction of leakage from liposomes was 514 ± 1nm.

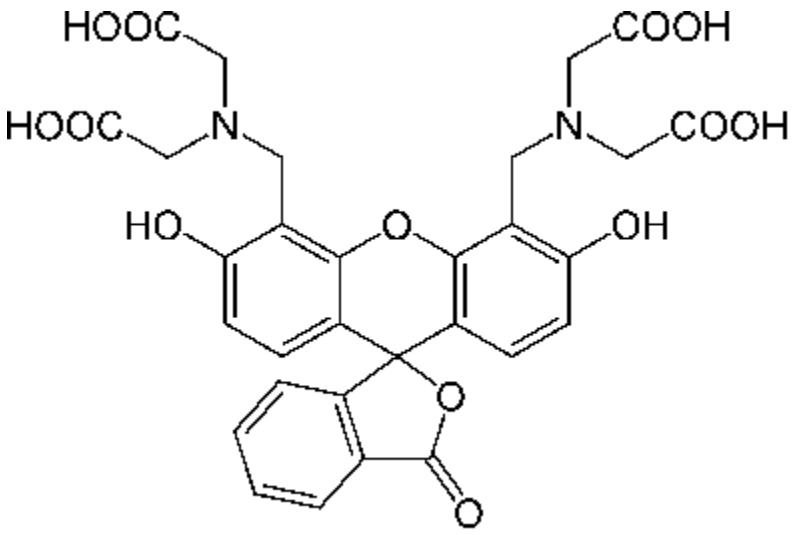


Figure 4.1 Chemical structure of a calcein molecule, which is the fluorescent assay for these experiments [34].

Table 4.1 Average vesicle diameters with standard deviation for each mole fraction of cholesterol.

mole fraction of cholesterol	Average Diam (nm)	stdev (nm)
2.5	163.2	2.11
5	153.9	25.8
10	130.2	21.7
15	130.6	13.2
20	143.1	4.3
25	154.7	32.8
30	167.4	1.8
35	172.1	0.12

Table 4.2 Setting and parameters of ultrasound transducer: setting of 2 out of 5 was used during the experiments and setting 5 out of 5 is the max setting for the sonicator.

Setting (out of 5)	Microtip Probe	Tip Diam (mm)	Max Amp (μm)	Amplitude (μm)	Power (W)	Acoustic Power Density (W/cm^2)
2	419	3.2	240	96	25	310.8

Setting (out of 5)	Microtip Probe	Tip Diam (mm)	Max Amp (μm)	Amplitude (μm)	Power (W)	Acoustic Power Density (W/cm^2)
5	419	3.2	240	240	60	746

Chapter 5: Ultrasound-Mediated Leakage Studies

5.1 Introduction

Understanding how lipid composition and lipid phase behavior influence the susceptibility to leakage with the application of ultrasound is key in the design of a triggered drug delivery method. The set of experiments that follow investigated how various cholesterol compositions in model membrane systems influenced membrane phase behavior, as well as the interaction of ultrasound and the membrane.

5.2 Theory

As mentioned previously, cholesterol has a unique effect on lipid membranes known as the cholesterol condensing effect [7]. Previous studies in this lab have investigated model membranes for ultrasound-mediated leakage. These studies used fluorescent 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt ANTS and its quencher, p-xylene-bis-pyridium bromide DPX in a model membrane with 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol) (DPPE-PEG) and cholesterol [9,22]. Pong *et. al*. determined that as PEG concentrations increase, the membrane becomes less stable and more receptive to leakage. These studies indicate that 5 mol% PEG and 8 mol% PEG using a 20 kHz transducer showed no difference in leakage profile [22]. Although this pair has been successful, calcein has been selected to replace ANTS-DPX because it is more cost effective and is self-quenching at concentrations of 70mM or higher [7]. A model membrane system containing DMPC-cholesterol has been chosen because the phase diagram is well

established [3,5] and the lipids are in the liquid phase at room temperature. This model membrane was studied to determine how cholesterol impacts the membrane phase behavior.

5.3 Experimental Setup

Calcein was used as the encapsulated fluorescent dye in a DMPC-cholesterol model membrane. Eight vesicle solutions were created with varying concentrations, (2.5, 5, 10, 15, 20, 25, 30, 35 mole%) of cholesterol and DMPC. The LUVs are passed through size exclusion chromatography column. Each vesicle sample was sonicated with a 20 kHz transducer in intervals of thirty seconds with three minutes of off time until total sonication time reached twenty minutes. In between measurements the vesicle solution was placed in a circulating water bath to maintain sample temperature at 30 ± 0.5˚C. The samples were excited at 470 nm and the emissions scans were measured from 485 to 530nm. The experiments were repeated and the data was smoothed and averaged for analysis. The sample sets used in this study are summarized in Table 5.1.

5.4 Analysis of Experimental Data

Figure 5.1 is an example of an intensity profile of a LUV sample subjected to ultrasound by a 20 kHz transducer. The graph reveals an increase in intensity as sonication time increases. Maximum intensity was reached after the addition of Triton X-100. The maximum intensity for the data series seen in this figure was at a wavelength of 514 nm. Figure 5.2 compares the emissions of a sample sonicated with a 20 kHz transducer to the control sample which was not subjected to a 20 kHz transducer. Once again, the maximum was at a wavelength of 514 nm. This figure clearly shows how the

application of ultrasound permeates the membrane which results in the release of calcein dye. In this figure, the control sample was also stored in the circulating water bath at 30˚C, and shows no photobleaching has occurred. The set of experiments were analyzed using the emission intensity at a wavelength of 514 ± 1nm to determine the fraction of release from the liposomes.

The results of the ultrasound-mediated study in this DMPC-cholesterol model membrane are shown in Figure 5.3, Figure 5.4 and Figure 5.5. As mentioned above, the fraction of release was calculated with emissions intensity located at 514 ± 1nm. Figure 5.3 and shown again in Figure 5.4 with error bars, shows the release profile of ultrasound-induced leakage from DMPC-cholesterol LUVs with varied mole fraction of cholesterol. Figure 5.5 shows the fraction of release from DMPC-cholesterol LUVs as a function of cholesterol mole fraction. The general trend shows that ultrasonically induced leakage reached maximum values near the phase boundaries, at 10 mol% cholesterol, 15 mol% cholesterol and 30 mol% cholesterol. The minimum leakage occurred outside the two phase region, at values of 2.5 mol% cholesterol and 35 mol% cholesterol. Within the two phase region, there was a local minimum at 20 mol% cholesterol.

5.5 Results and Discussion

The goal of targeted drug delivery through the use of ultrasound and liposomes is beneficial for the treatment of diseases because it reduces the amount of effective dosage necessary and toxic side effects [9,10]. The binary system of DMPC-cholesterol was selected for this ultrasound-mediated leakage study. The results indicate that different mole fractions of cholesterol have different effects on the membrane strength. The effect

that cholesterol has on a DMPC-cholesterol membrane can be explained by referring to Figure 2.2 and Figure 5.5.

As seen in Almeida's phase diagram, l_{do} and l_o coexist in the same region roughly between 8 mol% and 28 mol% cholesterol at 30˚C. These phase boundaries are important in the investigation of the unique trend of the fraction of release as a function of cholesterol mole fraction. Experiments performed with membranes in the l_{do} region, 2.5 mol% and 5 mol% cholesterol, do not result in a high fraction of release. The addition of cholesterol to the system, in this case, does not perturb the vesicles from the l_{do} phase because the membrane is so fluid in this stage.

Additional cholesterol is added to the system and the membrane transitions from the l_{do} region to the l_{do} and l_o region. It is at this first phase boundary that the fraction of release increases as shown in the experiment containing 10 mol% cholesterol. At the phase boundary, cholesterol has formed l_o domains, known as rafts. The membrane was easily disturbed by the 20 kHz applied ultrasound. This may be caused by the presence of line tension. In these domains, lipids in l_o are misaligned with lipids in l_{do} because of height difference, which is created by the straightening of some of the hydrocarbon chains [16]. As the cholesterol concentrations increase, l_o rafts are formed and the fraction of release decreases. Although additional rafts were formed, the driving force to coalesce was able to overcome the polar head group's expansion force. At lower cholesterol percentage, the driving force was not strong enough allowing for an increase in line tension.

As seen in Figure 5.5, the fraction of release decreases at 20 mol% cholesterol. On Almeida's phase diagram, 20 mol% cholesterol is roughly the center of the tie line in

the l_{do} and l_o region in which the membrane is 50/50 l_{do} and l_o. The minimum fraction of release may be a result of the rafts tightly coalescing because of the natural driving force to reduce tension. When this occurs, the total vesicle parameter and line tension are reduced resulting in a membrane that is more stable. Previous studies, by this group have investigated a dehydroergosterol (DHE)/1-myristoyl-2-[12-[(5-dimethylamino-1-naphthalenesulfonyl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (DAN-PC) Förster resonance energy transfer (FRET) pair in a DMPC-cholesterol system at 30˚C [16]. Brown has concluded, as seen in Figure 5.6, that the minimum efficiency of energy transfer value was observed to be in the two phase region at 20 mol% cholesterol, meaning the largest rafts were present at this cholesterol concentration. This analysis agrees with the minimum found via fraction of release and further strengthens the conclusions made.

Detailed investigation of this study, within the two phase region, indicates an increase in the fraction of release at 25 mol% and 30 mol% cholesterol. These compositions surround the phase transition from two phases to l_o phase. Around the phase boundary, the total vesicle parameter increases because more l_{do} pockets are formed that are unable to coalesce. As the mole fraction of cholesterol is further increased, the membranes enter the single l_o phase. In this phase, as previously mentioned, the hydrocarbon chains are fully extended because of the cholesterol condensing effect. It is within this l_o phase that a decrease in the fraction of release occurs, as shown by the release results at 35 mol% cholesterol. Therefore, as the membrane becomes cholesterol-enriched, it is more cohesive and less easily disturbed than the membrane with concentrations at the phase boundaries.

Table 5.1 Binary mixtures of DMPC-cholesterol samples

2.5% Chol 97.5% DMPC

Lipid	Molarity (mM)	Mol %	Vol Needed (µL)
DMPC	0.5	97.5%	165.2
Cholesterol	0.5	2.5%	1.9

5% Chol 95% DMPC

Lipid	Molarity (mM)	Mol %	Vol Needed (µL)
DMPC	0.5	95%	161.0
Cholesterol	0.5	5%	3.9

10% Chol 90% DMPC

Lipid	Molarity (mM)	Mol %	Vol Needed (µL)
DMPC	0.5	90%	152.5
Cholesterol	0.5	10%	7.7

15% Chol 85% DMPC

Lipid	Molarity (mM)	Mol %	Vol Needed (µL)
DMPC	0.5	85%	144.1
Cholesterol	0.5	15%	11.6

20% Chol 80% DMPC

Lipid	Molarity (mM)	Mol %	Vol Needed (µL)
DMPC	0.5	80%	135.6
Cholesterol	0.5	20%	15.5

25% Chol 75% DMPC

Lipid	Molarity (mM)	Mol %	Vol Needed (µL)
DMPC	0.5	75%	127.1
Cholesterol	0.5	25%	19.3

30% Chol 70% DMPC

Lipid	Molarity (mM)	Mol %	Vol Needed (µL)
DMPC	0.5	70%	118.6
Cholesterol	0.5	30%	23.2

Table 5.1 (Continued)

35% Chol 65% DMPC

Lipid	Molarity (mM)	Mol %	Vol Needed (µL)
DMPC	0.5	65%	110.2
Cholesterol	0.5	35%	27.1

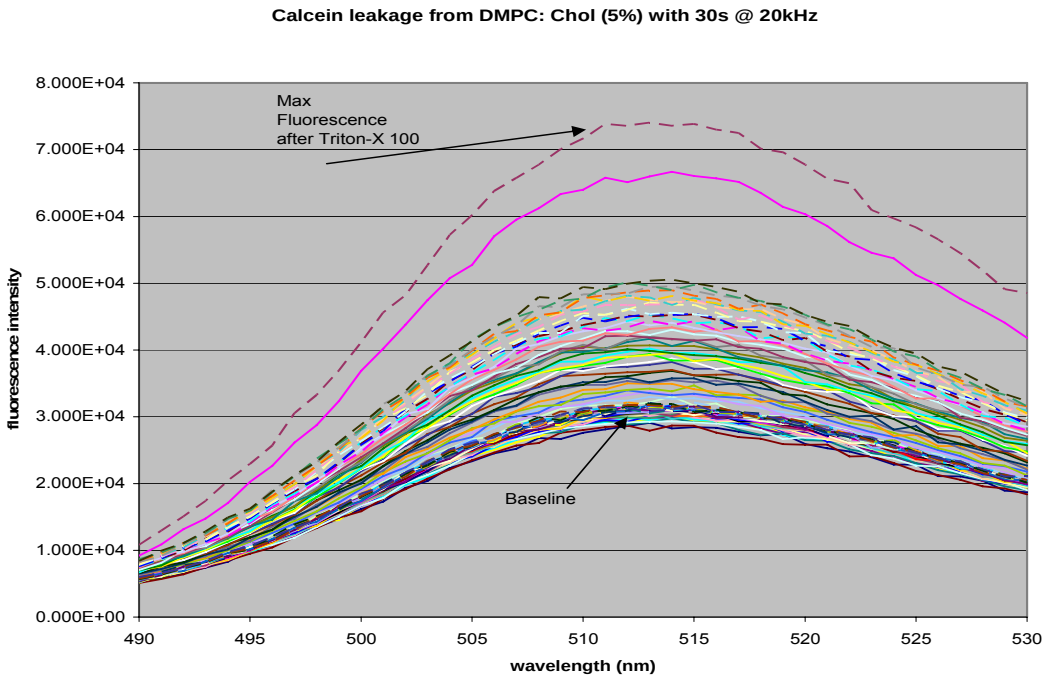


Figure 5.1 Intensity profile of calcein leakage from DMPC-cholesterol: A LUV sample subjected to ultrasound by a 20 kHz transducer. The graph reveals an increase in intensity as sonication time increases. The maximum intensity was reached, after the addition of Triton X-100.

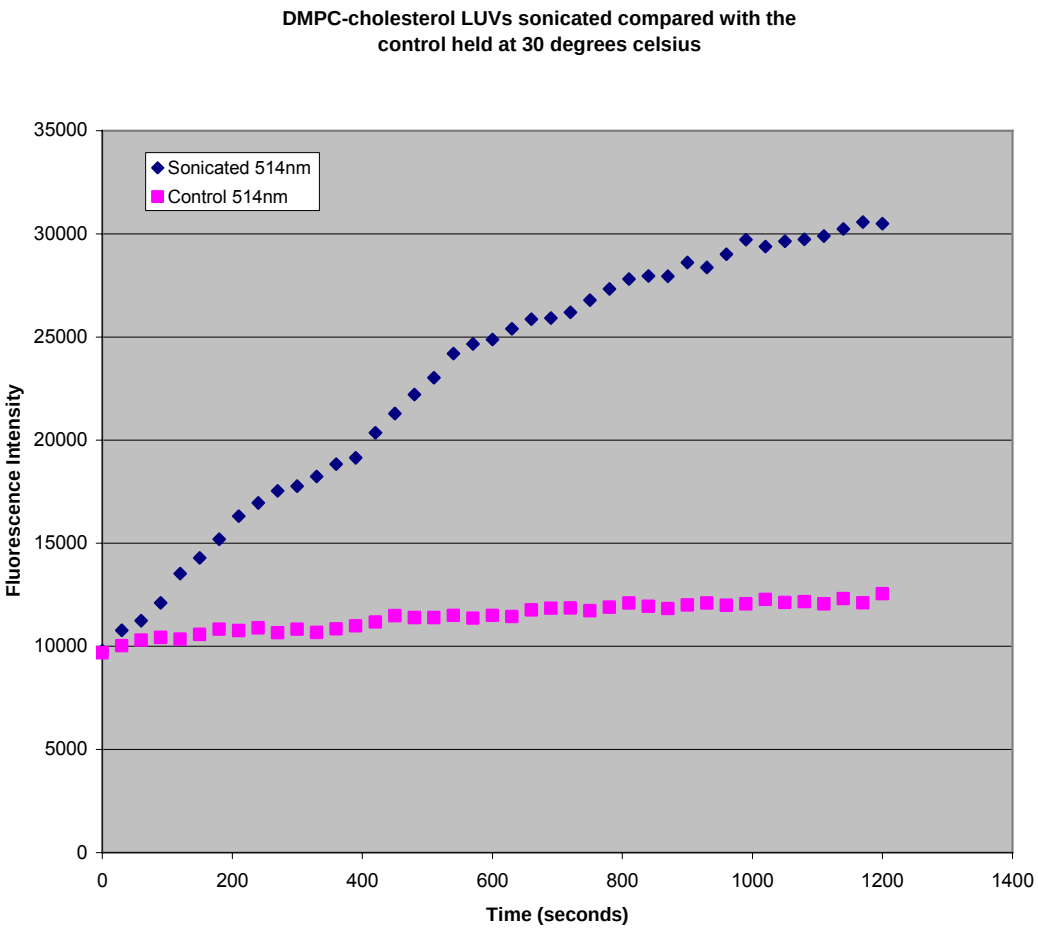


Figure 5.2 DMPC-cholesterol LUVs sonicated compared to control**:** Emissions of a sample sonicated sample with a 20 kHz transducer compared to the control sample which was not subjected to a 20 kHz transducer. Once again, the maximum intensity for the data series was located at a wavelength of 514 nm.

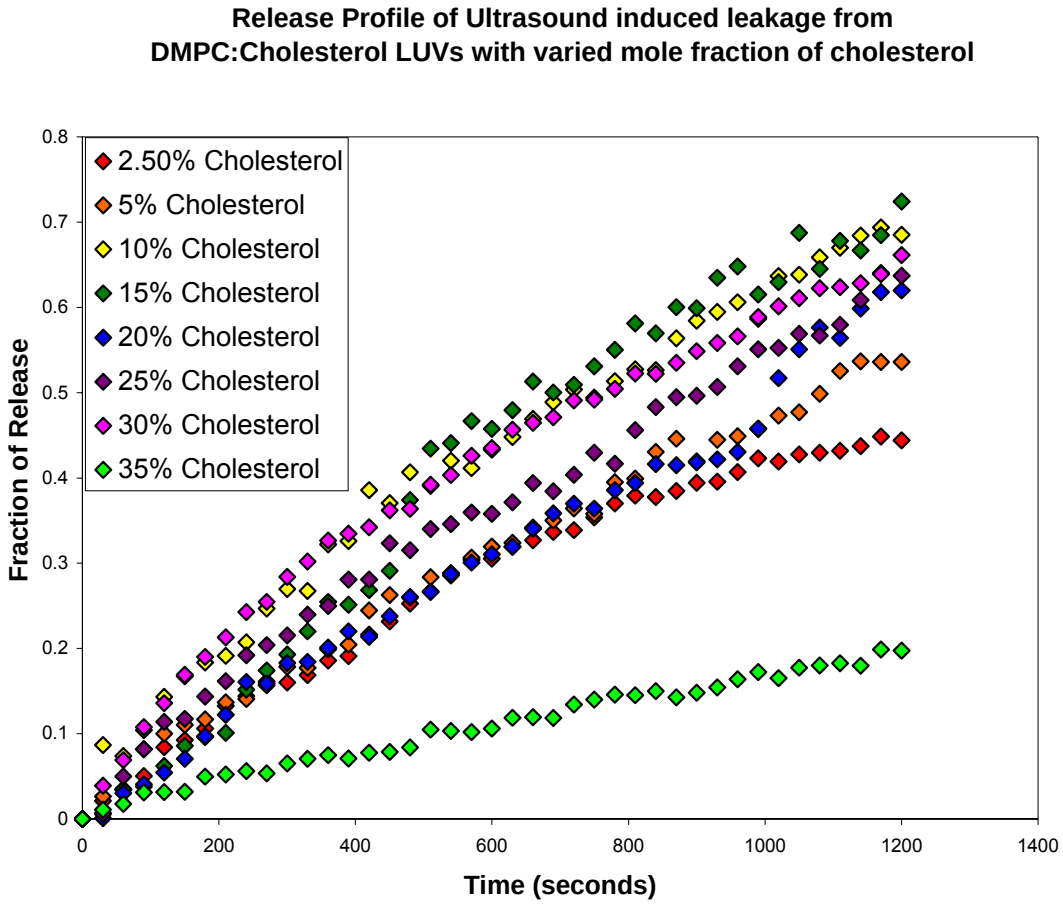


Figure 5.3 Release profile of DMPC-cholesterol LUVs: Maximum values at 10, 15, and 30 mol% cholesterol. The minimum leakage occurred at values of 2.5 mol% cholesterol, 20 and 35 mol% cholesterol.

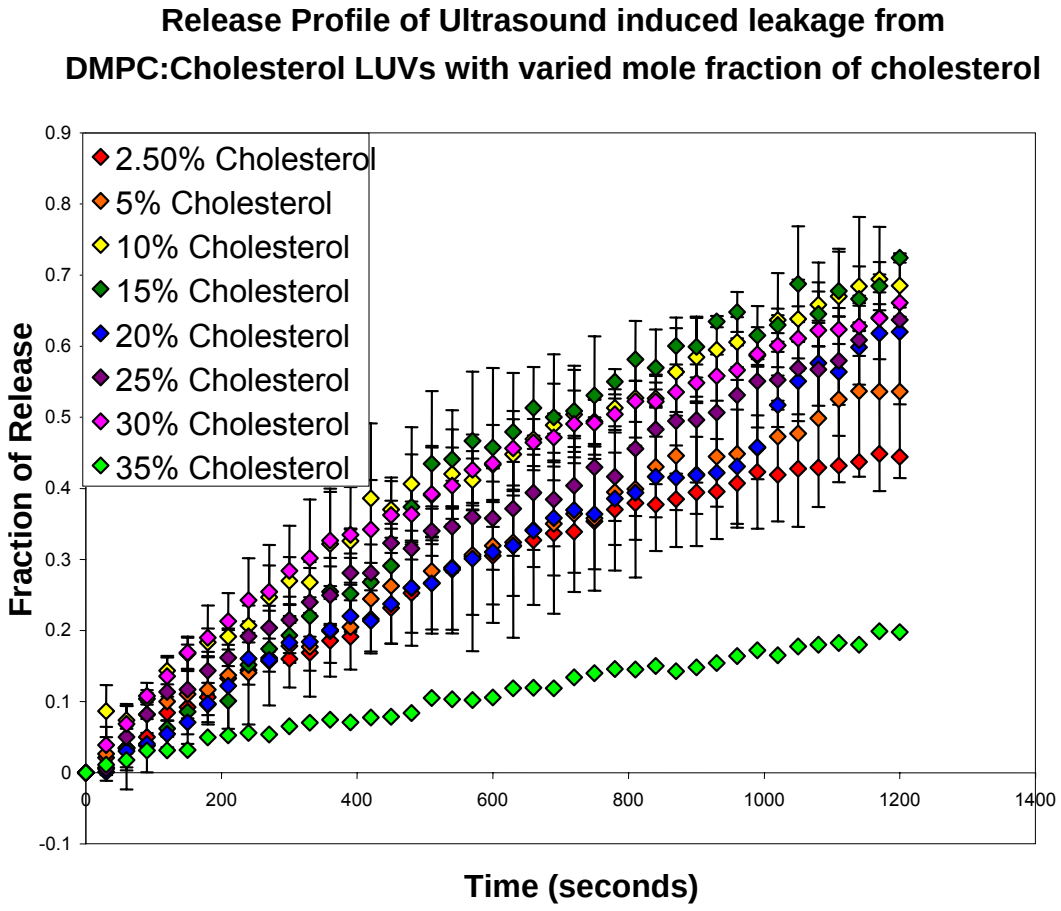


Figure 5.4 Release profile of DMPC-cholesterol LUVs with error bars: Maximum values at 10, 15, and 30 mol% cholesterol. The minimum leakage occurred at values of 2.5 mol% cholesterol, 20 and 35 mol% cholesterol.

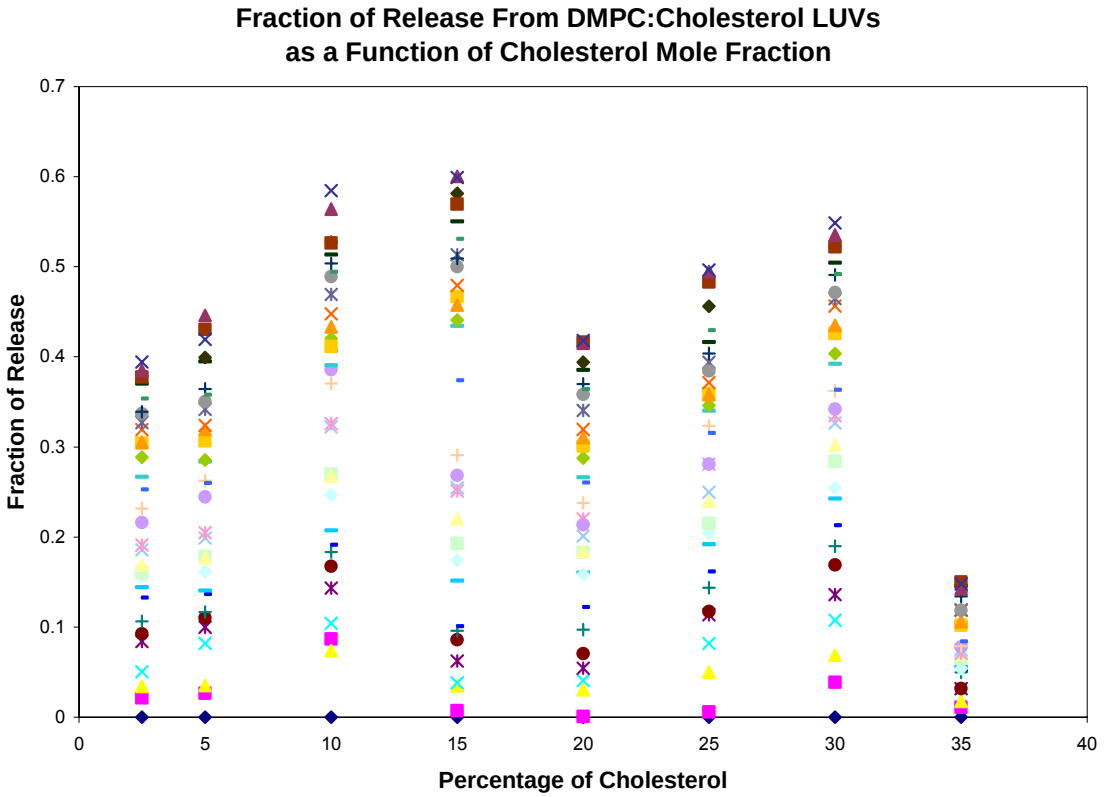


Figure 5.5 Fraction of release as a function of cholesterol mole fraction: This figure clearly shows increases of leakage from 2.5 mol% to 15 mol% and 25 mol% to 30 mol%, with decreases of leakage at 20 mol% and 35 mol% cholesterol.

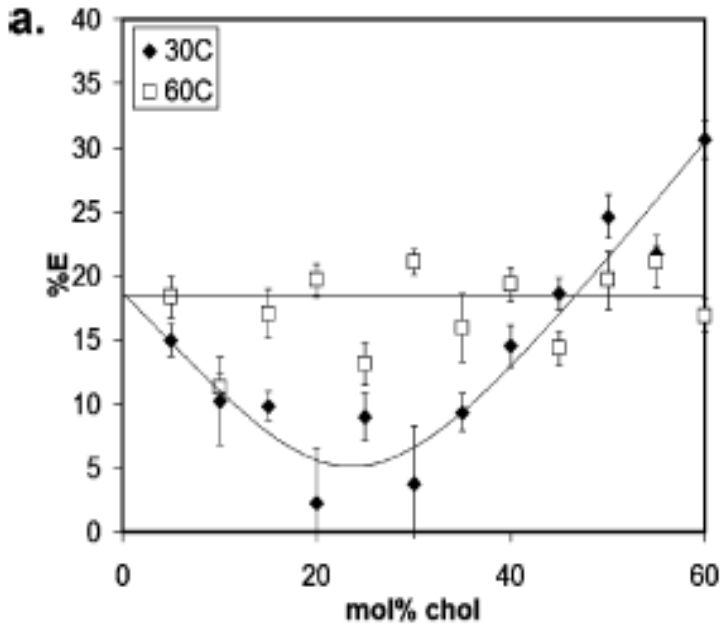


Figure 5.6 DAN-PC/DHE FRET in DMPC- cholesterol system: A 1-myristoyl-2-[12-[(5-dimethylamino-1-naphthalenesulfonyl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (DAN-PC)/ dehydroergosterol (DHE) Förster resonance energy transfer (FRET) pair in a DMPC-cholesterol system at 30˚C. The minimum efficiency of energy transfer value was observed to be in the two phase region at 20 mol% cholesterol [16].

Chapter 6: Conclusions and Recommendations

The goal of this work was to determine whether lipid composition and lipid phase behavior, in a binary system, influence the membrane susceptibility to leakage with the application of ultrasound. The results, as discussed in Chapter 5 indicate that membrane systems with mole fractions of cholesterol near the phase boundaries result in maximum ultrasound-induced leakage due to maximal line tension. The results also indicate that a local minimum in leakage within the two phase region occurs near the 50/50 composition. Since the results have such a unique trend, influence of the phospholipid composition should be investigated. In ternary systems, with the addition of cholesterol, the membrane may become more resistant to ultrasound induce leakage [7]. This phenomenon if it is found true might occur due to the reduction of line tension because the third lipid can act as a lineactant. For this reason, ternary model membranes in conjunction with ultrasound need to be studied, both in the effect of cholesterol composition and the effect of different lipids

Lists of References

[1] H.Y. Lin and J.L. Thomas, "Factors affecting responsivity of unilamellar liposomes to 20 kHz ultrasound," *Langmuir*. 20 (2004) 6100-6106.

[2] K. Gong, S. Feng, M.L. Go, P.H. Soew, "Effects of pH on the stability and compressibility of DPPC/Cholesterol monolayers at the air-water interface," *Colloids and Surface*. 207 (2002) 113-125.

[3] R. Tampé, A. von Lukas, H.J. Galla, "Gycophorin-Induced Cholesterol-Phospholipid Domains in Dimyristoylphosphatidylcholine Bilayer Vesicles," *Biochemistry*. 30 (1991) 4909-4916.

[4] D. Needham and R.S. Nunn, "Elastic deformation and failure of lipid bilayer membranes containing cholesterol,"*Biophysical Journal* 58 (1990) 997-1009.

[5] P.F.F. Almeida, W.L.C Vaz and T.E. Thompson, "Lateral diffusion in the liquid phases of Dimyristoylphosphatidylcholine/Cholesterol Lipid Bilayers: A Free Volume Analysis," *Biochemistry*. 31 (1992) 6739-6747.

[6] A. Kheirolomoom, P.A. Dayton, A.F.H. Lum, E. Little, E.E. Paoli, A. Zheng, K.W. Ferrara, "Acoustically-active microbubbles conjugated to liposomes: Characterization of a proposed drug delivery vehicle," *Journal of Controlled Release*. 118 (2007) 275-284.

[7] E. Glifort, "Ultrasound controlled leakage of liposomes bound to contrast agents," *Research Proposal I*. Sept 2007, 1-15.

[8] S. Mouras, S. Fotopoulou, S. Duraj, V. Sfika, C. Tsakiroglou and S.G. Antimisiaris, "Liposomal drugs dispersed in hydrogels effect of liposome, drug and gel properties on drug release kinetics," *Colloids and Surfaces B: Biointerfaces*. 55 (2007) 212-221.

[9] M. Pong, S. Umchid, A.J. Gaurino, P.A. Lewin, J. Litniewski, A. Nowicki and S.P. Wrenn, "In vitro ultrasound-mediated leakage from phospholipids vesicles," *Ultrasoinc*. 45 (2006) 133-145.

[10] A.F.H. Lum, M.A. Borden, P.A. Dayton, D.E. Kruse, S.I. Scott and K.W. Ferrara, "Ultrasound radiation force enables targeted deposition of model drug carriers loaded on microbubbles," *Journal of Controlled Release*. 111 (2006) 128-134.

[11] A.C. Brown, "A systematic study of cholesterol domains in model membranes," *Master's Thesis*. Nov 2005, 1-75.

[12] M.B. Sankaram and T.E Thompson, "Cholesterol-induced fluid-phase immiscibility in membranes," *Biophysics*. 88 (1991) 8686-8690.

[13] D-Z. Liu, W-Y. Chen, L-M. Tasi and S-P. Yang, "Microcalorimetric and shear studies on the effects of cholesterol on the physical stability of lipid vesicles," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 172 (2000) 57-67.

[14] S.P. Wrenn, "Interactions of ultrasound with cholesterol nanodomains in lipid membranes," *Humboldt Research Fellowship*. (2007) 1-17.

[15] S.L. Veatch and S.L. Keller, "Seeing spots: Complex phase behavior in simple membranes," *Biochimica et. Biophysica Acta.* 1746 (2005) 172-185.

[16] A.C. Brown, K.B. Towles and S.P.Wrenn, "Measuring raft size as a function of membrane composition in PC-Based systems: Part I- Binary Systems," Research Proposal II. March 2007, 1-8.

[17] A. Klibanov, "Targeted delivery of gas-filled microspheres, contrast agents for ultrasound imaging," *Advanced Drug Delivery Reviews*. 37 (1999) 139-157.

[18] M.A. Borden, P. Dayton, S. Zhao, and K. Ferrara, "Physiochemical properties of the microbubble lipid shell," *Proceedings of the 2004 IEEE Ultrasonics Symposium*. 1 (2004) 20-23.

[19] R.K. Schlicher, H. Radhakrishna, T.P. Tolention, R.P. Apkarian, V. Zarnitsyn, M.R. Prausnitz, "Mechanism of intracellular delivery by acoustic cavitation," *Ultrasound in Medicine and Biology*. 32 (2006) 915-924.

[20] D.M. Hallow, A.D. Mahajan, T.E. McCutchen and M.R. Prausnitz, "Measurement and correlation of acoustic cavitation with cellular bioeffects," *Ultrasound in Medicine and Biology.* 32 (2006) 1111-1122.

[21] C.T. Chin, A.V. Wamel, M. Emmer, N.D. Jong, C.S. Hall and A.L. Klibanov, "Mechanisms of ultrasonically-mediated drug delivery: high-speed camera observations of microbubbles with attached microbeads," *IEEE Ultrasonics Symposium*. (2005) 1-4.

[22] M. Pong, "Ultrasound mediated leakage from PEG incorporated vesicles," *Master's Thesis.* Aug 2005, 1-39.

[23] A.A. Doinikov and P.A. Dayton, "Maxwell rheological model for lipid-shelled ultrasound microbubble contrast agents," *Acoustical Society of America*. 121 (2007) 3331-3340.

[24] A.V. Wamel, K. Kooiman, M. Harteveld, M. Emmer, F.J. ten Cate, M. Versluis and N.D. Jong, "Vibrating microbubbles poking individual cells: Drug transfer into cells via sonoporation," *Journal of Controlled Release*. (2006) 1-7.

[25] M. Postema, N.D. Jong and G. Schmitz, "Shell rupture threshold, fragmentation threshold, Blake threshold," *Ultrasonics Symposium IEEE*, 3 (2006) 1708-1711.

[26] B. Krasovitski and E. Kimmel, "Shear stress induced by a gas bubble pulsating in an ultrasonic field near a wall," *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*. 51 (2004) 973-979.

[27] A. Klibanov, "Ligand-carrying gas filled microbubbles: Ultrasound contrast agents for targeted molecular imaging," *Bi conjugate Chemistry*. 16 (2005) 9-17.

[28] M. Postema, P. Marmottant, C.T. Lancée, S. Hilgenfeldt and N.D. Jong, "Ultrasound-induced microbuble coalescence," *Ultrasound in Medicine and Biology*. 30 (2004) 1337-1344.

[29] P.A. Dayton, K.E. Morgan, A.L. Klibanov and G.H. Brandenburger, "Optical and acoustical observations of the effects of ultrasound on contrast agents," *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*. 46 (1999) 220-232.

[30] J. Wu, J.P. Ross and J-F. Chiu, "Reparable sonoporation generated by microstreaming," *Acoustical Society of America* 111 (2002) 1460-1464.

[31] M. Hara, Y. Yamano, Y. Sakai, E. Kodama, T. Hoshino, M. Ito and J. Miyake, "Stabilization of liposomal membranes by carotenoids: Zeaxanthin, zeaxanthin glucoside and thermozeaxanthin," *Materials Science and Engineering*. (2007) 1-6.

[32] A. Brown, "Development of a Fluorescence Assay to measure the size of 'Model Rafts' *in Vitro*," *Research Proposal I*. Jan 2006, 1-17.

[33] H. Huang, J.T. Buboltz and G.W. Feigenson, "Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers," *Biochimica et Biophysica Acta.* 1417 (1999) 89-100.

[34] "Calcein." Wikipedia. Apr. 2008. Wikimedia Foundation Inc. 15 May 2008 <http://upload.wikimedia.org/wikipedia/commons/archive/7/70/20070918170722!Calcein.png>.