Structural Investigations of Liposomes: Effect of Phospholipid Hydrocarbon Length and the Incorporation of Sphingomyelin

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By

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ABSTRACT

The liquid crystal morphologies of symmetrical diacyl phosphatidylcholine liposomes examined in this research were found to be dependent on saturated hydrocarbon chain length. Both powder x-ray diffraction and synchrotron mid-IR microscopy indicate that phosphatidylcholines with short hydrocarbon tails (i.e. ten and twelve carbons) were more likely to form unilamellar liposomes while those with long hydrocarbon tails (i.e. eighteen and twenty carbons) are more likely to form multilamellar liposomes. Hydrocarbon chain lengths of fourteen and sixteen represented a transitional zone between these two liquid crystal morphologies. The Fourier transform infrared (FTIR) spectra where a shoulder developed on the peak at wavenumber 1750 cm^{-1} particularly highlights the change in the packing of adjacent molecules in the transitional zone. Due to structural similarities between phosphatidylcholine and sphingomyelin, further investigations were conducted on liposomes formed of a combination of these compounds. Sphingolipid containing liposomes have the potential to enhance anti-cancer drug delivery systems due to possible syngergistic effects between bioactive sphingolipids and the drug itself. Using the gentle hydration method, liposomes composed of different ratios of 1,2-distearoyl-sn-glycero-3-phosphocholine (PC) and N-(tricosanoyl)-sphing-4-enine-1-phosphocholine (SM) were formed. Based on synchrotron mid-IR microscopy, the addition of SM to a PC liposome at ratios of 1PC:1SM, 2PC:1SM and 4PC:1SM variations in the packing of adjacent molecules were detected but were not found to impact the ultimate supramolecular structure of the mixture. Slight variations in packing are the result of the enhanced hydrogen bonding capability of the SM headgroups and differences in hydrocarbon chain length.

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To the graduate students of the FABS department, thanks for all your help, in school and out, and best of luck.

To the Canadian Dairy Commission, I am so grateful for your financial support and the opportunities with which it has provided me.

DEDICATION

This thesis is dedicated to all the wonderful humans who have helped me keep my head up over the last two years. Much love and thanks to my beautiful family, Bacon Nation, groovy Guelph cats, Ridgeway ladies and my stellar Saskatoon support network. I am lucky to have each and every one of you in my life.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

EPR	Enhanced permeability and retention
FTIR	Fourier transform infrared
G′	Storage modulus
G″	Loss modulus
PC	Phosphatidylcholine
PC 10	1,2-didecanoyl-sn-glycero-3-phosphocholine
PC 12	1,2-dilauroyl-sn-glycero-3-phosphocholine
PC 14	1,2-dimyristoyl-sn-glycero-3-phosphocholine
PC 16	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
PC 18	1,2-distearoyl-sn-glycero-3-phosphocholine
PC 20	1,2-diarachidoyl-sn-glycero-3-phosphocholine
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PI	Phosphatidylinositol
PS	Phosphatidylserine
SM	Sphingomyelin

1. INTRODUCTION

In aqueous solution the contrasting effects of the hydrophobic and hydrophilic moieties of amphiphilic molecules cause the molecules to assemble into supramolecular liquid crystal structures (Tanford 1980). In the case of phospholipids, the hydrophilic head groups associate with the aqueous phase whereas the hydrophobic hydrocarbon chains interact with each other. Characteristics of the particular phospholipid, or combination of phospholipids, also contribute to the type of supramolecular structure. For example, the charge of head group can contribute to ionic repulsion of adjacent molecules (Israelachvili 1991).

Lipsomes (i.e. phospholipid bilayer vesicles) have been the subject of investigation since the early 1960's. The potential for liposomes to encapsulate both water and lipid soluble compounds has also been explored since the advent of liposome research (Bangham 1980; Gregoriadis 1980; Drummond et al. 1999). In the initial investigations as well as current research, multiple bilayer structures have been observed (Bangham 1980; Gregoriadis 1980). As research in the area of liposomes began to focus on using them as mimics of a cell membrane, unilamellar liposomes became the standard system for observation (Bangham 1980; Gregoriadis 1980; Drummond et al. 1999). Unilamellar liposomes also represent the standard for liposomes prepared for research into drug delivery capabilities of liposomes, thus many researchers employ methods to reduce lamellarity. Membrane extrusion and microfluidization are both employed to ensure the homogeneity of liposomal suspensions.

Characteristics of both the hydrophobic hydrocarbon tails and hydrophilic head groups of the phosopholipid can be used to predict which structures will form (Israelachvili 1991). These parameters most efficiently predict the structures of unilamellar, single component supramolecular structures (Israelachvili 1991). In a multilamellar structure the interaction of head groups between separate lamellae, the behaviour of the aqueous solution within the multilamellar system and the interaction of adjacent molecules within in each lamellae must all be considered. The inclusion of multiple phospholipid components in a single supramolecular

structure provides an opportunity to investigate the effects of head group charge, hydrocarbon chain length and hydrocarbon saturation.

Liposomes have been widely investigated as drug delivery systems, particularly for the delivery of anti-cancer drugs (Bangham 1980; Gregoriadis 1980; Drummond et al. 1999). Liposomes that have been sterically stabilized with compounds such as polyethylene glycol (PEG) have contributed to increased therapeutic indices of anti-cancer and anti-fungal drugs (Drummond et al. 1999). This is the result of increased circulation times and reduced toxicity compared to the free drug (Allen 1996; Johnston, Semple et al. 2006). The versatility of liposomes extends to the encapsulation of both lipid and water soluble drugs as well as the inclusion of bioactive amphiphilic lipids into the structure itself.

Sphingolipids are a class of bioactive amphiphilic lipids that play roles in the cell signaling pathways associated with senescence and apoptosis (i.e. programmed cell death) (Ogretmen and Hannun 2004; Hannun and Obeid 2008). Sphingomyelin, sphingosine and ceramide belong to this class. It is possible that this function can be exploited to increase the rate of cell death in cancer treatment. Supramolecular structures of phosphatidylcholine (PC) and sphingomyelin (SM) were investigated. Both PC and SM possess a phosphocholine head group and two hydrocarbon chains, there are differences in their structure that in turn effect their behaviour in phospholipid bilayer systems (Terova et al. 2004). A drug delivery liposome that contains sphingolipids, namely SM, has the potential to create a drug delivery system that can combine the effect of liposomally encapsulated anti-cancer drugs with the anti-cancer effects of the released sphingolipids. Investigations of their compatibility in liposomal structures must precede further research.

2. LITERATURE REVIEW

2.1 Phospholipids

Phospholipids are a class of amphiphilic lipids characterized by their negatively charged phosphate head group (at physiological pH) and non-polar fatty acid components (Kent 1995; Drummond et al. 1999; Li and Vance 2008). Reference to phospholipids in this research particularly means diacyl-glycerophospholipids which are composed of a phosphate head group, a diacylglycerol, and an additional simple organic molecule as part of the head group structure. The addition of organic molecules to the phosphate head group creates a variety of phospholipid species such as phosphotidylethanolamine (PE), phosphotidylserine (PS), phosphatidylinositol (PI) and phosphatidylcholine (PC) (Kent 1995). Phospholipids play structural roles in cell membranes and are capable of orienting in a variety of supramolecular structures in aqueous solution (Israelachvili 1991; Kent 1995; Li and Vance 2008). The formation of supramolecular structures in aqueous solution is driven by the hydrophobic interactions of the hydrocarbon chains (Tanford 1980; Israelachvili 1991; Kent 1995).

The biosynthetic precursor for the diacyl-glycerophospholipids is phosphatidate (i.e. 1,2-dicacyl-sn-glycero-3-phosphate). The pathway described in Figure 2.1 occurs in mammalian cells (Kent 1995; Li and Vance 2008). Phosphatidate is synthesized *de novo* from glycerol-3-phosphate, a product of glycolysis, and two acyl-CoA molecules via the action of glycerol-3-phosphate acyltransferase. There are two forms of this enzyme, one catalyzing the reaction at carbon-1 and the other at carbon-2 (Kent 1995; Li and Vance 2008).

A divergence in the synthesis pathway occurs here: 3-sn-phosphatidate phosphohydrolase may cleave the phosphate group from carbon-3 leaving a diacylglycerol (DAG) or cytidine diphosphate-diacylglycerol (CDP-DAG) may be formed by the action of CDP-DAG synthase which catalyzes the addition of CDP to the existing phosphate group from cytidine triphosphate (CTP) (Kent 1995; Li and Vance 2008). The DAG branch leads to the production of PC, PE and PS while the CDP-DAG branch leads to the production of PI, PS, and other lipid molecules. This

research utilized PC, as many previous studies have to investigate phospholipid structures (Kent 1995; Li and Vance 2008).

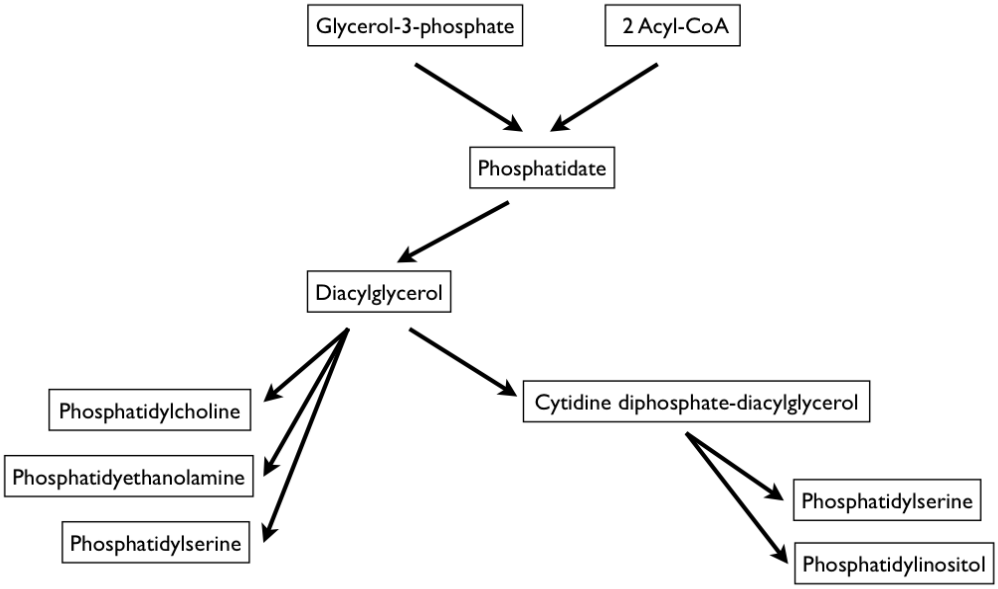


Figure 2.1 Selected pathways in phospholipid metabolism.

2.1.1 Phosphatidylcholine

Phosphatidylcholine (Figure 2.2) is abundant in both plant and animal cell membranes; it may account for 50-90% of cell membrane phospholipids and is largely located in the outer leaflet of the cell membrane (Kent 1995; Li and Vance 2008). It has been used as a food additive, emulsifier and in the liposomal encapsulation of flavours, textile dyes and pharmaceuticals (Drummond et al. 1999). Phosphatidylcholine in mammals is primarily synthesized from the DAG branch of the phospholipid synthetic pathway. Phosphocholine is transferred to carbon-3 via the action of choline:1,2-diacylglycerol cholinephosphotransferase (Kent 1995; Li and Vance 2008).

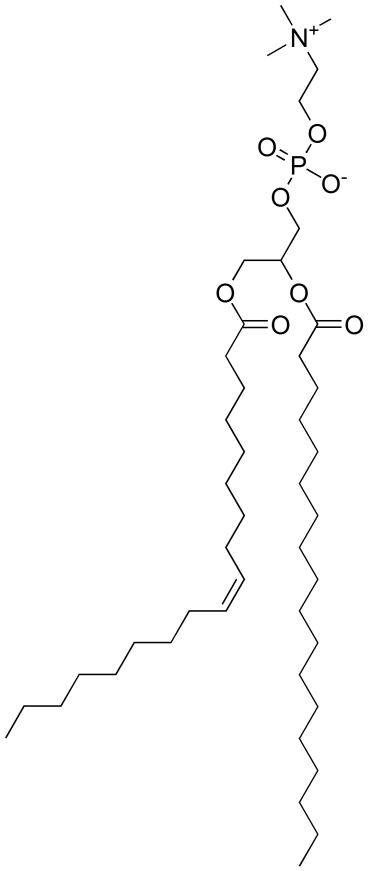


Figure 2.2 Representative chemical structure of phosphatidylcholine.

2.2 Sphingolipids

Sphingolipids are a class of lipids that contain a sphingoid base; an amino-alcohol molecule. These compounds share their amphiphillic characteristics with and have similar structural roles in the cell membrane as phospholipids (Ogretmen and Hannun 2004; Hirabayashi et al. 2006; Hannun and Obeid 2008). Typically, they are concentrated in microdomains of the cell membranes called lipid rafts. These domains have a higher concentration of sphingolipids, cholesterol and membrane bound lipoproteins than the surrounding cell membrane. These raft structures play an important role in cell-cell recognition, ligand interaction and cell attachment (Ogretmen and Hannun 2004; Hirabayashi et al. 2006; Hannun and Obeid 2008). This understanding of membrane structure led to further investigations of the nature of sphingolipids and revealed their importance as signaling molecules when previously they had been thought to

serve only a structural role (Ogretmen and Hannun 2004; Hirabayashi et al. 2006; Hannun and Obeid 2008).

Sphingolipids are associated with the signaling pathways that influence cell proliferation, senescence, inflammatory responses and apoptosis. Effector molecules from the sphingolipid family include ceramide, sphingosine and sphingosine-1-phosphate. (Ogretmen and Hannun 2004; Hirabayashi et al. 2006; Hannun and Obeid 2008)

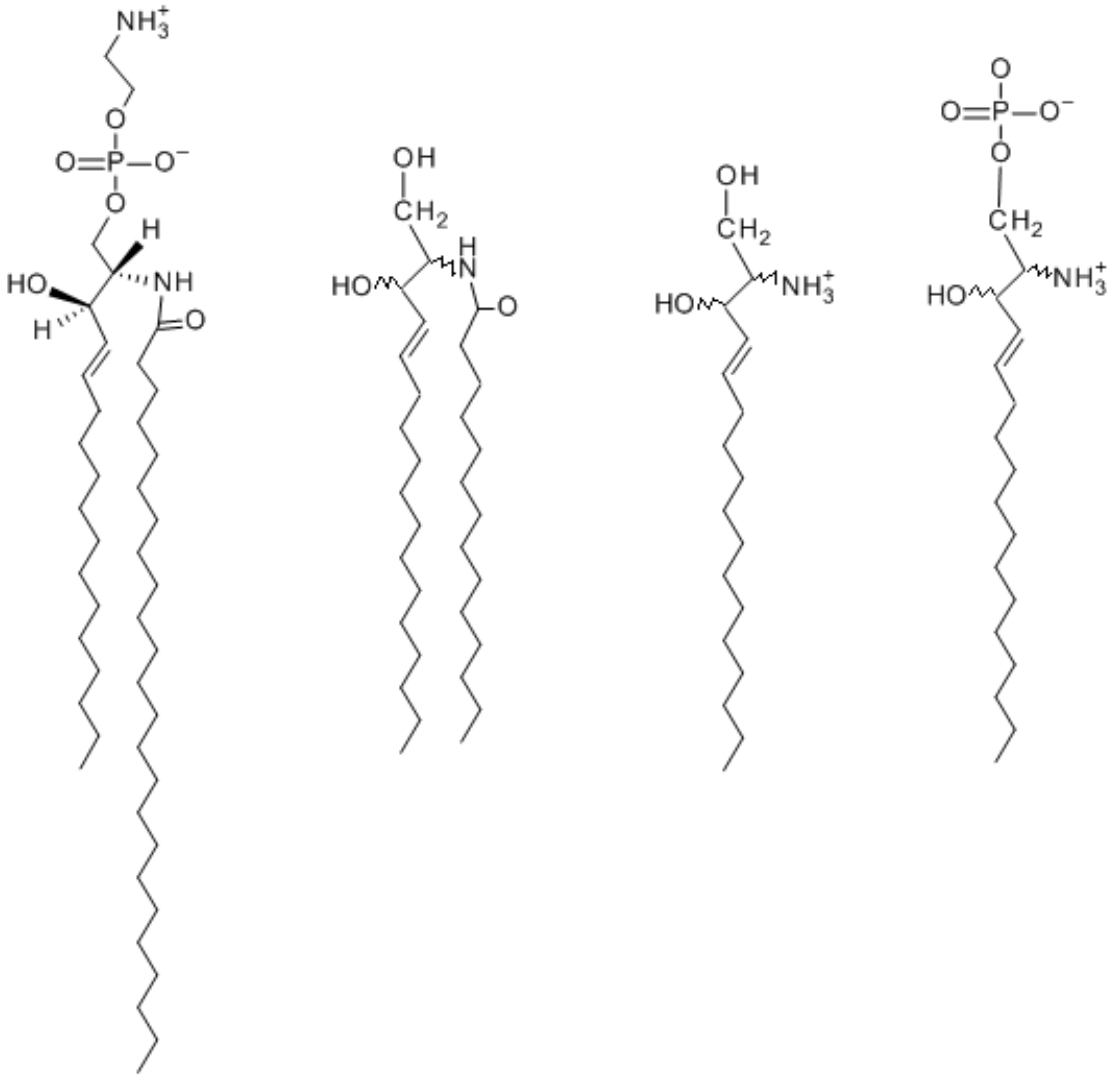


Figure 2.3 Representative chemical structures of sphingomyelin (A), ceramide (B), sphingosine (C) and sphingosine-1-phosphate (D).

Sphingolipid metabolism is highly diverse and its full complexity has yet to be entirely elucidated. Ceramide is considered to be a central molecule in sphingolipid metabolism because of its role in an array of anabolic and catabolic reactions (Ogretmen and Hannun 2004). Figure 2.4 illustrates some selected pathways in sphingolipid metabolism.

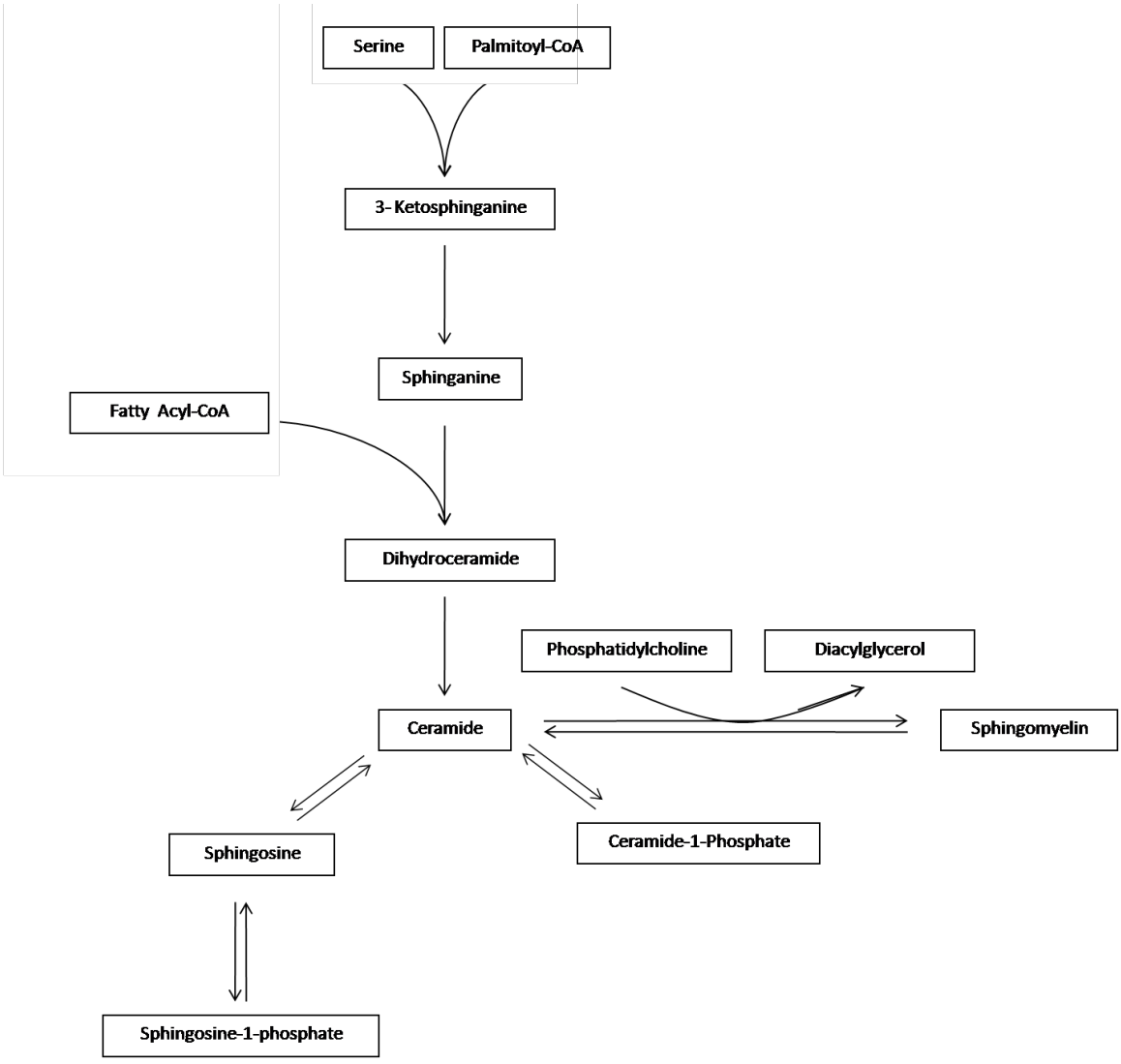


Figure 2.4 Selected pathways in sphingolipid metabolism. Adapted from Ogretman and Hannun (2004).

2.2.1 Sphingomyelin

Sphingomyelin (SM) is the most common sphingolipid in mammalian cell membranes. Like PC, it is typically located on the outer leaflet of the cell membrane(Niemela et al. 2004; Hirabayashi et al. 2006). Also as with PC, the fatty acid composition of SM in mammalian cells differs based on location in the body. Sphingomyelin is synthesized de novo from phosphatidylcholine and ceramide according to the following reaction catalyzed by sphingomyelin synthase:

ceramide + phosphatidylcholine ⇔ sphingomyelin + 1,2-diacyl-sn-glycerol

A pathway for ceramide synthesis is the cleavage of the phosphocholine group of SM by sphingomyelinase (Ogretmen and Hannun 2004; Hirabayashi et al. 2006; Hannun and Obeid 2008). Since SM represents a large portion of the sphingolipids found in mammalian cells, it is a significant source for the production of ceramide from the cell membrane for signaling purposes (Ogretmen and Hannun 2004; Hirabayashi et al. 2006; Hannun and Obeid 2008).

2.2.2 Sphingolipid Metabolism in Cancer Pathogenesis and Treatment

A number of sphingolipids have been shown to be of significant importance in cell signaling pathways associated with senescence and apoptosis (Ogretmen and Hannun 2004; Hannun and Obeid 2008). Apoptosis is the collection of biochemical signaling pathways that lead to programmed cell death (Ogretmen and Hannun 2004; Hannun and Obeid 2008). Cancer pathogenesis involves disruptions in the apoptitic pathway (Ogretmen and Hannun 2004; Hannun and Obeid 2008). Without the regulation of cell death, cancer cells have an increased capacity to proliferate and induce angiogenesis (i.e. the formation of new blood vessels) (McDonald and Baluk 2002). In a normal cell, ceramide is involved in the regulation of apotosis as well as senescence and growth arrest. Ceramides have specific enzymatic targets, such as ceramide-activated protein phosphatases and D-cathespin, that in turn participate in cascades that have the downstream effects of cell growth arrest, senescence and apoptosis (Ogretmen and Hannun 2004; Hannun and Obeid 2008).

Atypical levels of sphingolipids and enzymes involved with their metabolism, compared with normal cell metabolism, have been noted in different stages of cancer development (Hannun and Obeid 2008). The application of this knowledge has led to research in the area of cancer pathogenesis and the use of bioactive sphingolipids in cancer treatment (Stover and Kester 2003;

Hannun and Obeid 2008; Simon et al. 2010). In the past three decades, sphingolipid research has been an extremely active area however, due to the complex nature of sphingolipid metabolism only a small number of highly significant mediators and signalers have been the research focus (Hannun and Obeid 2008).

Changes in cellular levels of sphingolipids have also been observed at different stages in cancer pathogenesis (Ogretmen and Hannun 2004). Ceramide and sphingosine-1-phosphate, particularly, have been identified for their roles in mediating apoptosis and cell proliferation, respectively (Ogretmen and Hannun 2004). Decreased levels of total ceramides and/or specific long chain ceramides have been noted in several types of cancer. Total ceramide content, when compared to normal ovarian cells, is less in ovarian tumors (Ogretmen and Hannun 2004). A decrease in ceramides with acyl chains of 16, 18 and 24 carbons was noted in human lung, head and neck cancers (Ogretmen and Hannun 2004). When coupled with observations that sphingosine-1-phosphate levels increase, this may indicate that ceramide metabolism is accelerated in cancerous cells producing higher amounts of sphingosine-1-phosphate (Ogretmen and Hannun 2004; Hannun and Obeid 2008).

Exogenous ceramides have been introduced into a variety of cancer treatment scenarios with positive results. The application of liposomally delivered ceramides can increase the rate of apoptosis in cancer cells *in vivo* (Shabbits and Mayer 2003; Stover and Kester 2003). Stover and Kester (2003) observed the effect of liposomally delivered ceramides on MDA-MB-231 breast adenocarcinoma cells. When compared to free ceramide, liposomally encapsulated ceramides that were delivered as part of a liposome contributed to a larger decrease in cancer cell proliferation (Stover and Kester 2003). Shabbits and Mayer (2003) observed a similar trend with liposomally introduced ceramides in their investigations of MDA435/LCC6 human breast cancer in J774 mouse macrophage cells.

2.3 Supramolecular Strucures of Amphiphillic Lipids

Driven by the hydrophobic effect, amphiphilic lipids can orient in a variety of supramolecular structures in aqueous solution (Tanford 1980; Israelachvili 1991). In order to minimize or eliminate hydrocarbon-water interactions, phospholipids orient themselves so that the hydrophilic headgroups ‘shield’ the hydrophobic hydrocarbon tails from the aqueous solution (Israelachvili 1991). Characteristics of the molecule such as the charge and size of the headgroup

and the length and degree of saturation of the hydrocarbon tails effect how the molecules pack together (Israelachvili 1991).

The favoured structure of amphiphilic molecules is determined by: the optimal effective area of the head group (a); the volume of the hydrocarbon chain(s) (v), which is/are fluid and incompressible; and the maximum effective length of the chain (l_c) (Israelachvili 1991). Depending on these packing considerations, five possible liquid-crystal configurations are possible: spherical micelles, cylindrical micelles, flexible bilayer vesicles (i.e. liposomes), planar bilayers and inverted micelles (Israelachvili 1991). Novel structures are also capable of forming; these include both cubic and hexagonal liquid crystal structures as well as biphasic lipid structures (Gan, Han et al., 2009).

Israelachvili has shown that in order for amphiphilic molecules to assemble into spherical vesicles, the surface area (a_o) must be sufficiently large, while the hydrocarbon volume (v) must be sufficiently small and the radius of the supramolecular structure (R) must not exceed the critical length of the amphiphilic molecule (Israelachvili 1991). Based on geometric considerations, for a sphere of radius, R, the mean aggregation number is equal to:

$$M = \frac{4\pi R^2}{a_o} = \frac{4\pi R^3}{3v} \qquad \text{(Equation 2.1)}$$

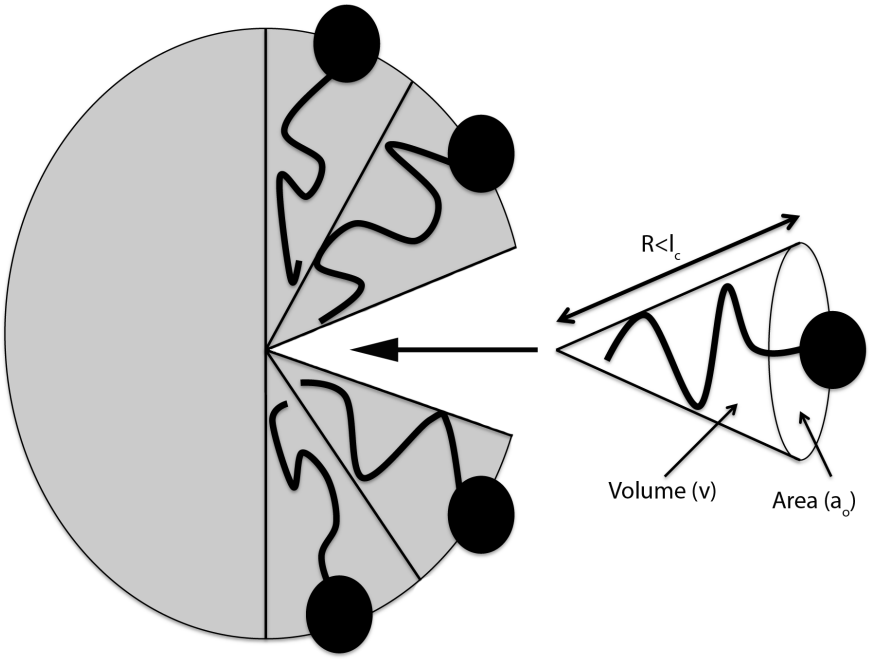
this may be rearranged to:

$$R = \frac{3v}{a_o} \qquad \text{(Equation 2.2)}$$

hence, the geometric constraint for a spherical vesicle may be calculated by:

$$\frac{v}{a_o l_c} \qquad \text{(Equation 2.3)}$$

where $R=l_c$. Israelachvili has determined the mean dynamic packing parameters for different liquid crystal morphologies (Israelachvili, 1991; Figure 2.2). Amphiphilic lipids with two hydrocarbon chains are expected to form bilayers and liposomes due to their large hydrocarbon volume resulting in a geometric constraint in the range of 1/2 to 1 (Israelachvili 1991).



Critical Packing Parameter $v/a_o l_c$	Critical Packing Shape	Liquid Crystal Structure
1/3	Cone	Spherical Micelles
1/3 - 1/2	Truncated Cone	Cylindrical Micelles
1/2 - 1	Truncated Cone	Flexible Bilayers, Vesicles
1	Cylinder	Planar Bilayers
>1	Wedge	Inverted Micelles

Figure 2.5 Critical packing parameters for amphiphillic molecules and the resulting critical packing shapes and resulting liquid crystal structures. Adapted from Israelachvili (1991).

2.3.1 Liposomes

Liposomes were first characterized in the early 1960's by Bangham as bilayer vesicles in aqueous solutions as formed by phospholipids (Bangham and Horne 1964; Bangham et al. 1965; Bangham 1980). The characterization of liposomes occurred at a time when Bangham and colleagues were investigating universal membrane structures (Bangham 1980).

The structure of phospholipids creates compartments within the liposome bilayer that may contain exclusively lipid or aqueous solutions. This characteristic makes liposomes a versatile tool for the encapsulation of compounds. As early as the 1970's, the use of liposomes as drug delivery systems was investigated (Gregoriadis 1980; Chonn and Cullis 1995). Initially, the limitations of liposomal drug delivery pertained to the circulation time of the drug. In early 1990's the *in vivo* circulation time of liposomal drugs were improved via the addition of polyethylene glycol (PEG) (Klibanov et al. 1990). Current commercially available and clinical trial versions of liposomal drugs are PEG-liposomes (Gregoriadis 1980; Chonn and Cullis 1995).

2.3.1.1 Advantages of Liposomal Drug Delivery Systems

The major downfall of chemotherapeutic drugs used in the treatment of certain types of cancer is their lack of specificity. The same mechanisms by which wide spectrum chemotherapy drugs such as doxorubicin or daunorubicin induce cancer cell apoptosis for cancer cells also initiate the same events in healthy cells, which leads to vast complications including decreased immune activity and subsequent secondary infections, chronic pain and fatigue (Strohl 1991). Doxorubicin, for example, is believed to work via intercalation; the insertion of the drug into the DNA of the cell. This interruption of the DNA sequence adversely affects the cancer cell's ability to correctly translate DNA into proteins, which ultimately reduces the rate at which the cell successfully divides (Strohl 1991). Although doxorubicin is effective at destroying cancer cells, it cannot distinguish between cancer cells and healthy cells thus doxorubicin is also a potent mutagen. As a result, a potential side effect of doxorubicin used for chemotherapy is the induction of other forms of cancer (Strohl 1991). As a drug that exclusively destroys cancer cells has yet to be developed, using liposomal drug delivery systems to increase the specificity of existing drugs provides the potential for a more effective cancer treatment strategy.

Liposomes are used clinically for the delivery of both water and lipid soluble drugs (Allen 1996; Drummond et al. 1999). Liposomes have been shown to increase the circulation

time of drugs when compared to the free drug, however, the standard for drug delivery liposomes are those that are sterically stabilized by the addition of PEG (Drummond et al. 1999). This increase in circulation time of PEG-liposomal drugs over the free drugs utilized in cancer chemotherapy regimes allows the exploitation of leaky tumor vasculature and increased blood flow to the tumor, collectively know as enhanced permeability and retention (EPR) effects. Cancer cells grow rapidly and without regulation of cell size or shape. Irregularities in cellular properties result also create irregularities in the vasculature resulting in large blood vessels that allow larger molecules such as liposomes to permeate the interior of the tumor.

Angiogenesis is the process by which new blood vessels are created. Tumors induce blood vessel growth by releasing biochemical triggers that result in the creation of new vessels that supply blood exclusively to the tumor. (Huang et al. 1992; Yuan et al. 1995; McDonald and Baluk 2002). This increased blood flow to cancer cells allows for rapid growth that results from increased nutrition and gas exchange (Huang et al. 1992; Yuan et al. 1995; McDonald and Baluk 2002). Liposomal drug delivery systems may exploit the utility of increased blood flow which, based on their prolonged circulation, would result in a higher probability of drug-tumor interactions (Huang et al. 1992; Yuan et al. 1995; McDonald and Baluk 2002).

The nature of tumor structure also results in decreased efficacy of lymph drainage resulting in liposomes being retained within the tumor (Huang et al. 1992; Yuan et al. 1995; McDonald and Baluk 2002).

2.3.1.2 Preparation Methods

Depending on the research focus and ultimate application of the liposome a variety of production methods may be used to achieve the desired result. Size, lamellarity and the homogeneity of the liposomal suspension are all end goals that influence preparation choice. The first method used to prepare liposomes is referred to as the Bangham method, gentle hydration method or lipid film hydration (Bangham 1980; Tsumoto et al. 2009; Lesoin et al. 2011). Phospholipids are distributed in a thin film on the surface of glassware by dissolving the phospholipids in an organic solvent, like choloroform, and later evaporating the solvent (Bangham 1980; Tsumoto et al. 2009; Lesoin et al. 2011). The addition of an aqueous solution under conditions of vortexing or agitation results in the production of liposomes (Bangham 1980; Tsumoto et al. 2009; Lesoin et al. 2011).

The gentle hydration method often serves as the first step in other liposome production methods. The phospholipids are suspended in the manner previously described and can be physically processed with membranes or homogenization equipment to create liposomal suspensions with specific physical properties (Gregoriadis 1995; Tsumoto et al. 2009; Lesoin et al. 2011). The majority of experimentally produced and studied liposomes are extruded through membranes under pressure in order to create a homogenous size distribution. Liposomes with diameters between 100-200 nm are the most desirable as there is evidence that this size is the most physiologically active (Gregoriadis 1995; Drummond et al. 1999). This size range does not induce an immune response yet the liposomes are of sufficient size to carry an appropriate concentration of the desired drug (Drummond et al. 1999).

In an effort to eliminate organic solvents, and any potential harmful residues that may result, from the preparation of liposomes for drug delivery, novel preparation methods have been developed (Lesoin et al. 2011). These include the preparation of liposomes using supercritical fluids (Lesoin et al. 2011). In this process a phospholipid-solvent solution is sprayed through supercritical carbon dioxide which causes the rapid evaporation of the solvent (Lesoin et al. 2011). This causes the phospholipids to precipitate out as empty vesicles that can then be rehydrated using a variety of aqueous solutions (Lesoin et al. 2011). In cases where liposomes are not being prepared for use in food or drug systems, the gentle hydration method represents an opportunity to investigate phospholipid self assembly (Tsumoto et al. 2009).

3. DEPENDENCE OF LIQUID CRYSTAL MORPHOLOGY ON PHOSPHOLIPID HYDROCARBON LENGTH[1]

3.1 Abstract

The liquid crystal morphologies of symmetrical diacyl phosphatidylcholine liposomes examined in this research study were found to be dependent on saturated hydrocarbon chain length. Both powder x-ray diffraction and synchrotron mid-IR microscopy indicate that phosphatidylcholines with short hydrocarbon tails (i.e. ten and twelve carbons) are more likely to form unilamellar liposomes while those with long hydrocarbon tails (i.e. eighteen and twenty carbons) are more likely to form multilamellar liposomes. Hydrocarbon chain lengths of fourteen and sixteen represent a transitional zone between these two liquid crystal morphologies. The FTIR spectra where a shoulder develops on the peak at wavenumber 1750cm^{-1} particularly highlights the change in the packing of adjacent molecules in the transitional zone.

3.2 Objective and Hypothesis

The objective of this research study is to assess the structural effects on liquid crystal supramolecular structures of phospholipids caused by changing the length of the hydrocarbon chains.

It is hypothesized that liposome diameter will increase in a positive linear fashion with an increase in hydrocarbon length.

3.3 Introduction

Amphiphilic molecules have the ability to associate into numerous structures in aqueous solutions depending on molecular packing considerations. The ability to pack into

[1] Reprinted from Colloids and Surfaces B: Biointerfaces, 87 (1), H. Rutherford, N.H. Low, F. Borondics, T. Pederson, M.A. Rogers. Dependence of liquid morphology on phospholipid hydrocarbon length, 116-121, Copyright (2011), with permission from Elsevier.

supramolecular structures is governed by the hydrophobic attraction at the hydrocarbon-water interface. As the molecules associate, the hydrocarbon chains pack together while the polar entity of the molecule remains in contact with the aqueous phase. Hydrophilic, ionic or steric repulsion of the polar head groups can further alter the packing of the molecules (Israelachvili 1991).

These contrasting parameters are a result of the amphiphilic nature of the molecules and dictate the supramolecular structure (Tanford 1980). The favoured structure of amphiphilic molecules is determined by: the optimal effective area of the head group (a); the volume of the hydrocarbon chain(s) (v), which is/are fluid and incompressible; and the maximum effective length of the chain (l_c) (Israelachvili 1991). Depending on these packing considerations, a variety of supramolecular structures are possible (Israelachvili 1991). Liposomes are spherical supramolecular structures of phospholipids composed of at least one phospholipid bilayer and are the structures examined in this study (Israelachvili 1991).

Israelachvili has shown that in order for amphiphilic molecules to assemble into spherical vesicles, the surface area (a_o) must be sufficiently large, while the hydrocarbon volume (v) must be sufficiently small and the radius of the supramolecular structure (R) must not exceed the critical length of the amphiphilic molecule (Israelachvili 1991). Based on geometric considerations, for a sphere of radius, R, the mean aggregation number is equal to Equation 2.1 this may be rearranged to Equation 2.2 hence, the geometric constraint for a spherical vesicle may be calculated by Equation 2.3. Israelachvili has determined mean dynamic packing parameters for different liquid crystal morphologies (Israelachvili, 1991). Amphiphilic lipids with two hydrocarbon chains are expected to form bilayers and liposomes due to their large hydrocarbon volume resulting in a $v/a_o l_c$ in the range of 1/2 to 1 (Israelachvili 1991).

From the earliest investigations of phospholipid liquid crystal structures, particularly liposomes, multiple bilayers structures have been observed (Bangham 1980; Gregoriadis 1980). Research in the area of liposomes quickly began to focus in the ability of these structures to effectively encapsulate compounds for a variety of applications including drug delivery (Bangham 1980; Gregoriadis 1980; Drummond et al. 1999). Undoubtedly, the most resource efficient way to study liposomes for encapsulation purposes is to prepare homogeneous suspensions of unilamellar liposomes, often this is accomplished using sonication, membrane extrusion or microfluidization (Gregoriadis 1995; Drummond et al. 1999; Lapinski, Castro-

Forero et al. 2007). Particularly when conducting research on liposomes for drug delivery applications, control of both size and structure is of great concern since these parameters will effect the performance of the drug delivery system *in vivo* (Allen 1996; Drummond et al. 1999).

Hydrocarbon chain length has been shown to have a positive linear relationship with bilayer thickness (Lewis and Engelman 1983). The influence of hydrocarbon length on liposome size can be overcome with processing; liposomes of a minimal size can be formed of phospholipids with differing hydrocarbon chain lengths (Israelachvili et al. 1977). The effect of the hydrocarbon chain length has not only been shown to affect the morphology of the liquid crystalline structure but also the fluidity of the bilayer (Israelachvili 1991). While the formula for geometric packing parameter (Equation 2.3) can be used to predict supramolecular structures, it does not allow for the prediction of the structure of multilamellar liposomes. While unilamellar liposomes have been considered to have more utility than their multilamellar counterparts, there is great value in investigating the preferential aggregation of phospholipids with differing hydrocarbon chain lengths into supramolecular structures without the aid of external factors such as homogenization, filtration or ultrasonics.

3.4 Materials and Methods

The following synthetic symmetrical diacyl phosphatidylcholines were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and used as received: 1,2-didecanoyl-sn-glycero-3-phosphocholine (PC10), 1,2-dilauroyl-sn-glycero-3-phosphocholine (PC12), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (PC14), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC16), 1,2-distearoyl-sn-glycero-3-phosphocholine (PC18), and 1,2-diarachidoyl-sn-glycero-3-phosphocholine (PC20). Liposomes were formed using a dry film gentle hydration method (Bangham et al. 1965; Tsumoto et al. 2009). Samples were prepared in triplicate, all measurements were performed on each sample. The film was prepared by dissolving 0.1 mM of phosphatidylcholine in 2 mL of chloroform (Sigma-Aldrich, St. Louis, MO, USA) in a 50 mL round bottom flask. A Buchi rotary evaporator (Flawil, Switzerland) with the water bath at 55^{o}C was used to evaporate the chloroform under reduced pressure leaving a thin dry film of phosphtidylcholine. Two milliliters of HEPES buffered saline (20 mM HEPES, 150 mM NaCl) (Sigma-Aldrich, St. Louis, MO, USA) was added to the film and was mixed using the rotary action of the Buchi rotary evaporator with the water bath temperature set above the transition

temperature of the particular phosphatidylcholine being used. The resulting liposome suspensions were sonicated using a Branson 2510 bath sonicator (Danbury, CT, USA) at room temperature for 10 minutes at 40 kHz (i.e. until no large aggregates were visible in the suspension).

A Nikon Eclipse E400 light microscope (Nikon Instruments Inc., Melville, NY, USA) equipped with a Nikon DS-Fi1 color camera (Nikon Instruments Inc., Melville, NY, USA) and a 40x lens and condenser (Nikon Instruments Inc., Melville, NY, USA) were used for light microscopy. Images were captured at a resolution of 2560 by 1920 pixels using a Nikon DS-FiL color camera. Images were analyzed using Adobe Photoshop Extended CS4.0 (Adobe Systems Inc., San Jose, CA, USA).

Light scattering measurements (hydrodynamic diameter and surface area) were performed using a Malvern Hydro 2000S Mastersizer (Malvern, Worcestershire, UK). Rheological experiments were performed at 25^{o}C with a Thermal Analysis AR-G2 rheometer (New Castle, DE, USA) using a 2^{o} 40 mm acrylic cone and plate geometry. Oscillatory frequency sweep measurements were conducted at room temperature and a constant stress of 1 Pa through a range of frequencies from 1 to 100 rad/s to assess storage modulus (G') and loss modulus (G'') in the viscoelastic region.

Ten randomly selected vesicles were analyzed using Photoshop; the initial measurement of diameter was taken in pixels and converted to microns using a calibrated scale bar. This number-mean diameter (D[1,0]) was converted to a volume-moment mean diameter (D[4,3]) for comparison with the collected light scattering data.

Fourier transform infrared (FTIR) spectra were collected at the Canadian Light Source (Saskatoon, SK, Canada) at the mid-IR spectromicroscopy beamline (01B1-1). The end station was comprised of a Bruker Optics IFS66v/S interferometer coupled to a Hyperion 2000 IR microscope (Bruker Optics, Billerica, MA, USA). A drop of sample was placed between two CaF2 optical windows (25 mm diameter, 2 mm thick). Light is focused on the sample using a 15X magnification Schwarzschild condenser, collected by a 15X magnification Schwarzschild objective with the aperture set to a spot size of 40 mm by 40 mm and detected by a liquid nitrogen cooled narrowband mercury cadmium telluride (MCT) detector utilizing a 100 mm sensing element.

A KBr-supported Ge multilayer beamsplitter was used to measure spectra in the mid-infrared spectral region. Measurements were performed using OPUS 6.5 software (Bruker Optics, Billerica, MA). The measured interferograms were an average of 512 scans and were recorded by scanning the moving mirror at 40 kHz (in relation to the reference HeNe laser wavelength of 632.8 nm). The wavelength range collected was 690 to 7899 cm-1 with a spectral resolution of 4 cm-1. Single channel traces were obtained using the fast Fourier transform algorithm, with no zero-filling, after applying a Blackman–Harris 3-Term apodization function.

A Rigaku Multiplex Powder X-ray Diffractometer (Rigaku, Japan) with a ½ degree divergence slit, ½ degree scatter slit, and a 0.3 mm receiving slit, was set at 40kV and 44 mA to determine the polymorphic form and long spacings of the phosphatidylcholine vesicles. Scans were performed from 1 to 30 degrees 2 theta at 0.2 degree/min.

Linear regression for Table 3.1 and ANOVA performed with Graphpad Prism 5 (San Diego, California, USA).

3.5 Results and Discussion

The effect of sonication time on liposome size was assessed prior to deciding the final preparation method. Light scattering measurements of preliminary samples were taken every minute from zero to ten minutes, followed by measurements at five minute intervals up to 30 minutes. Ten minutes was sufficient to break apart large visible aggregates in the suspension, and after ten minutes no significant changes in the size of the vesicles was noted. The application of heat to levels above the transition temperature of the phosphatidylcholines during the mechanical agitation of the suspension ensured that the entire lipid film was removed from the glass surface and suspended in aqueous solution.

Regardless of hydrocarbon chain length, phosphatidylcholines from PC 10 to PC 20, were observed to form spherical aggregates (Figure 3.1). Visualization, using light microscopy, of these spherical aggregates could not conclusively discern whether the vesicles were composed of single or multiple bilayers. The lower hydrocarbon chain lengths, PC 10-14, appear to have small spherical particles which have aggregated into clumps during sample preparation (Figures 3.1A-C). Figures 3.1D-F, representing phosphatidylcholines with hydrocarbon chain length greater than 16 carbons, appeared to have a slightly different morphology namely larger particles with greater spaces between individual particles. As a note, it is not surprising to see a cylindrical

and spherical structures, such as in Figure 3.1D, as it is possible that both structures can exist in thermodynamic equilibrium (Israelachvili 1991).

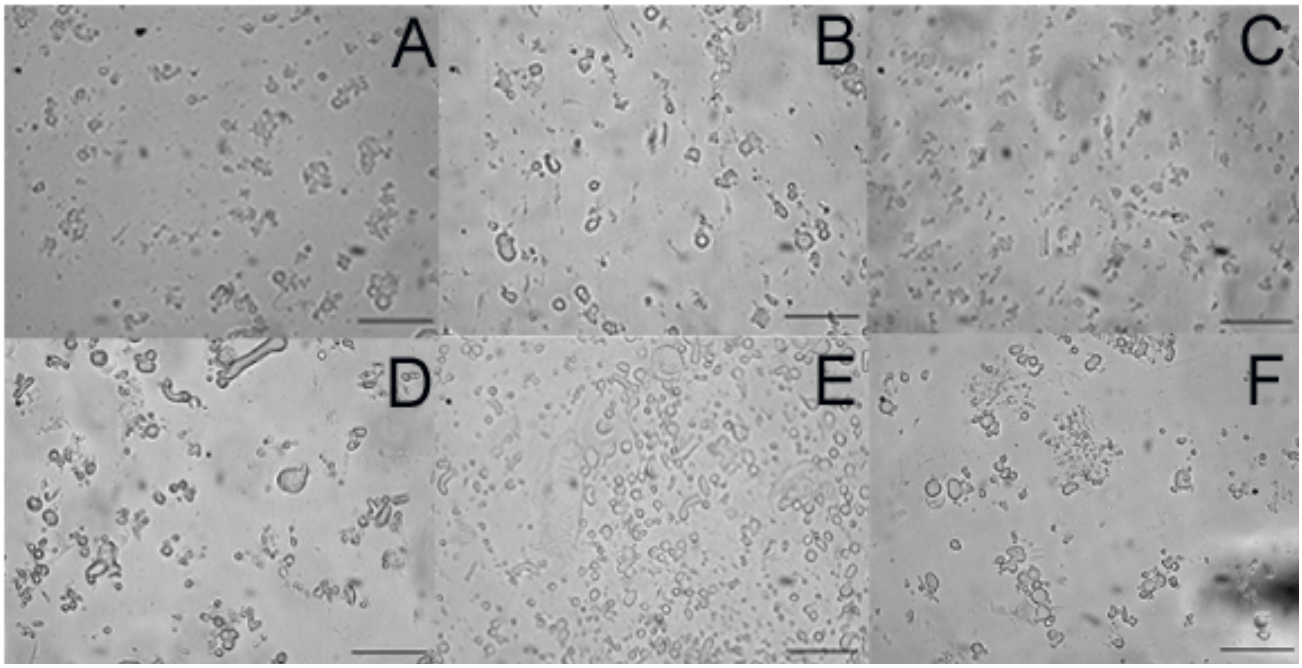


Figure 3.1 Light micrographs of vesicle suspensions composed of PC10 (A), PC12 (B), PC14 (C), PC16 (D), PC18 (E), and PC20 (F). Scale bar indicates 5 μm.

Previous research has revealed a positive linear relationship between hydrocarbon chain length in phospholipids and bilayer thickness (Lewis and Engelman 1983; Allen 1996). Assuming that a thicker bilayer would require a larger radius of curvature, it was expected that liposome size would increase with increasing hydrocarbon chain length. Two separate trends within the vesicle diameter collected using light microscopy were observed: the first being a plateau from PC 10 to PC 14 and a second higher plateau from PC 16 to PC 20 (Figure 3.2).

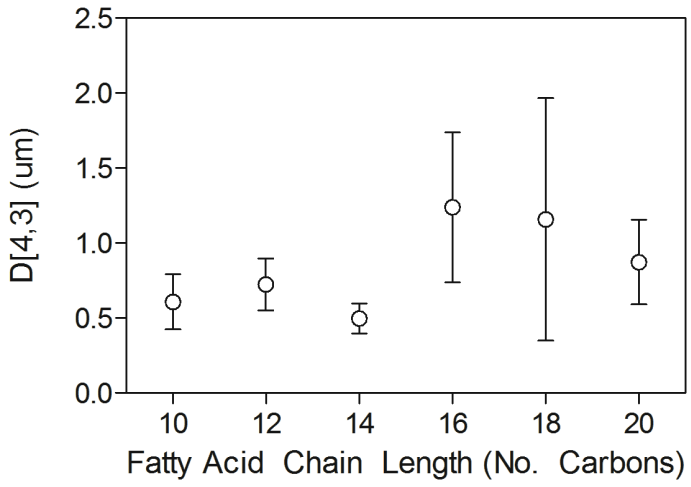


Figure 3.2 Volume-moment mean diameters of phosphatidylcholine vesicles calculated using pixel measurements obtained from light micrographs. Data points represent mean values ± one standard deviation.

To further assess the vesicle size trend, light scattering was used to determine the size of the vesicles in solution. The raw percent volume versus particle size (Figure 3.3) indicated that for short hydrocarbon chains the distribution of vesicle size was similar for chains shorter than C14 (Figures 3.3A-C). A bimodal distribution was observed which suggests that, in concordance with the light micrographs, that there were small vesicles and aggregates of small vesicles. Above PC 14 (Figures 3.3D-F) the distribution shifts to larger particle sizes.

The calculated D[4,3] determined using light scattering (Figure 3.4A) showed similar trends to those values determined using light microscopy (Figure 3.2). A linear tread in vesicle size was observed above PC 14. Below a hydrocarbon chain length of PC 14, no significant effect ($p<0.05$) of chain length on vesicle size was observed.

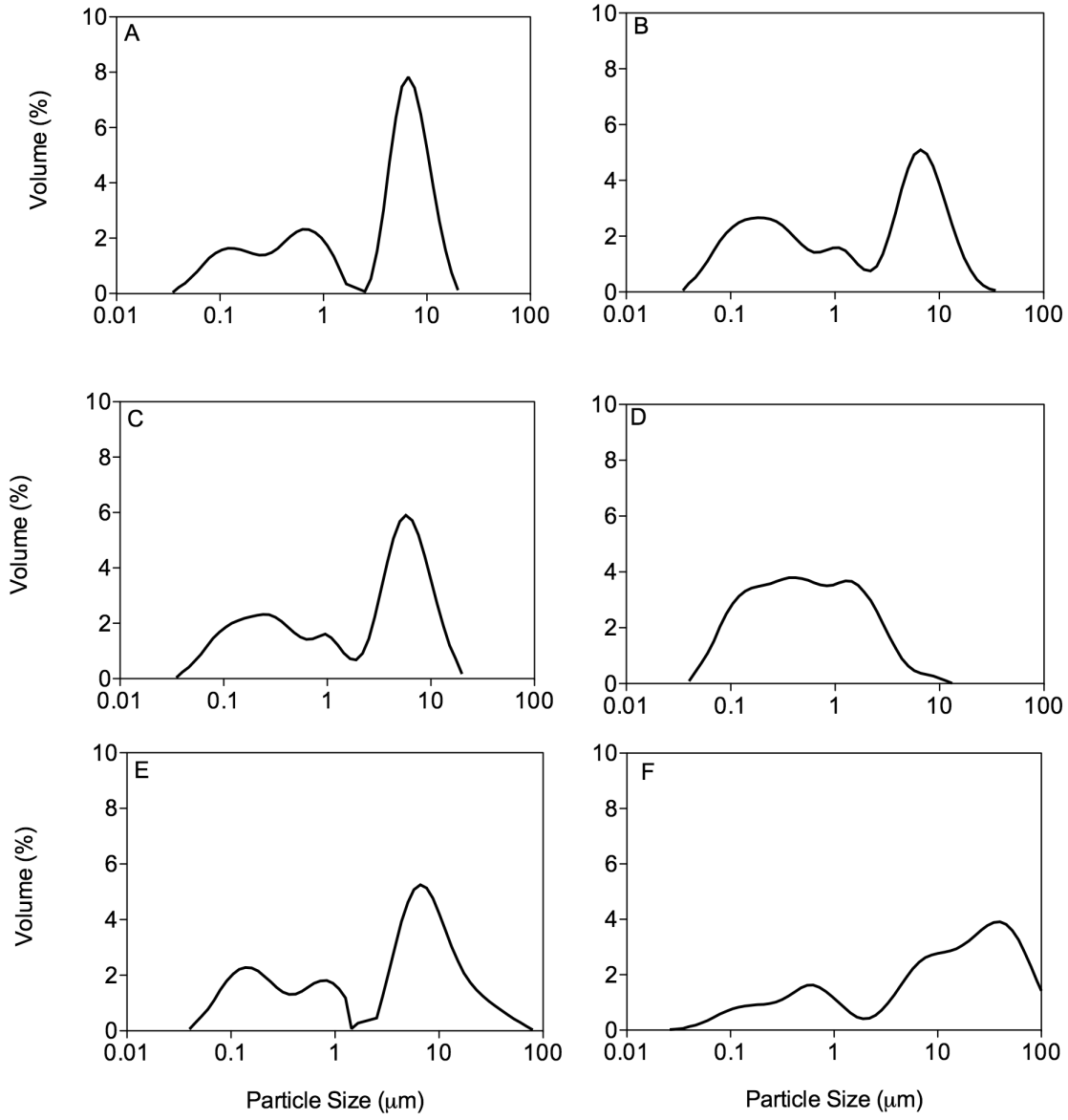


Figure 3.3 Light scattering intensities of phosphatidylcholine vesicle suspensions with varying hydrocarbon chain lengths (A) PC10, (B) PC12, (C) PC14, (D) PC16, (E) PC18 and (F) PC20.

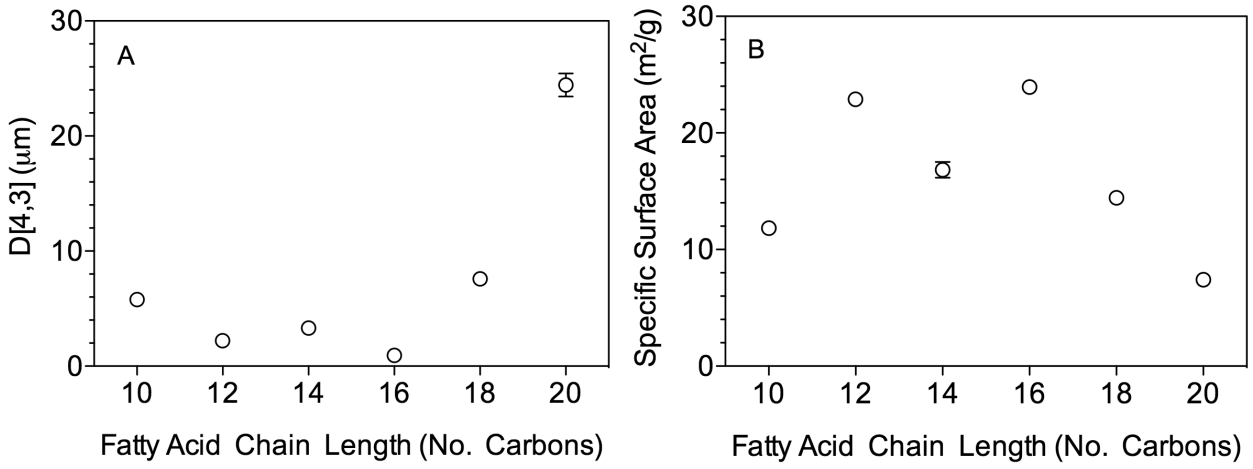


Figure 3.4 Volume-moment mean diameters and surface area of phosphatidylcholine vesicles calculated from dynamic light scattering measurements. Data points represent mean values ± one standard deviation.

The parabola-like relationship of surface area and hydrocarbon chain length indicates that the same type of structure is not being formed by the different chain length phosphatidylcholines. Particularly, the increase in surface area from PC 10 to PC 12 and the abrupt decrease at PC 14 indicates a change in the structural dynamics of the vesicle structure. This suggests that the packing arrangement may be affected by the hydrocarbon chain length resulting in a transition from a unilamellar to multilamellar liposomes.

X-ray powder diffraction data indicated that the phosopholipid head groups were arranged with a short spacing observed at ~ 4.2Å (Figure 3.5). However, phospholipids with longer hydrocarbon showed a slightly smaller head group spacing (d~4.1 Å) (Figure 3.5A) compared to the shorter hydrocarbon side chains (d~4.3 Å) (Figure 3.5B). This is attributed to the smaller vesicle, which in turn results in a greater surface curvature, resulting in an increase in the short spacing.

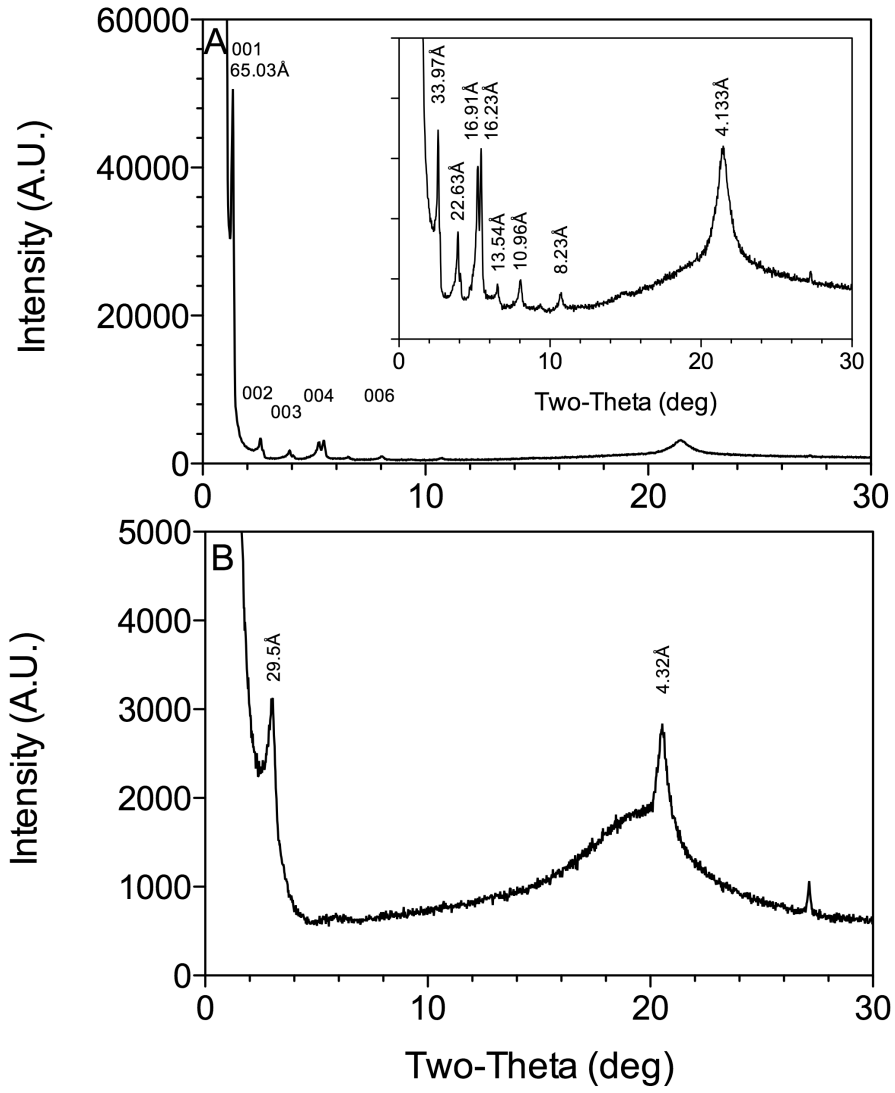


Figure 3.5 Powder X-ray diffraction data for PC18 (A) and PC12 (B).

X-ray diffraction patterns confirm the transition from unilamellar to multilamellar structure with an increase in hydrocarbon chain length. This is evident by the 001 peak at 65.03 Å in Figure 3.5A. This indicates that the liposome has a bilayer which is equivalent to two times the length on a single phospholipid. Further, the presence of the higher order reflections at 33.9 Å (002), 22.6 Å (003), and 16.9 Å (004) and 10.9 Å (006) indicate a liposome with multiple bilayers (Figure 3.5A). As well, there are doublets peaks observed at approximately 22 Å and 16 Å which suggest two species are present. Conversely, a single long spacing at 29.5 Å was observed for the C12 phospholipid suggesting that the liposome consists of a single bilayer of

phospholipid. This is confirmed by the long spacing being equal to the length of the phospholipid as well as the lack of higher order reflections (Figure 3.5B). Synchrotron mid-IR spectroscopy reveals a change in the short spacing of the head groups at the low and high end of the hydrocarbon lengths examined. The FTIR data indicates differences in both the head group and hydrocarbon chain packing for saturated phosphatidylcholines (Figure 3.6).

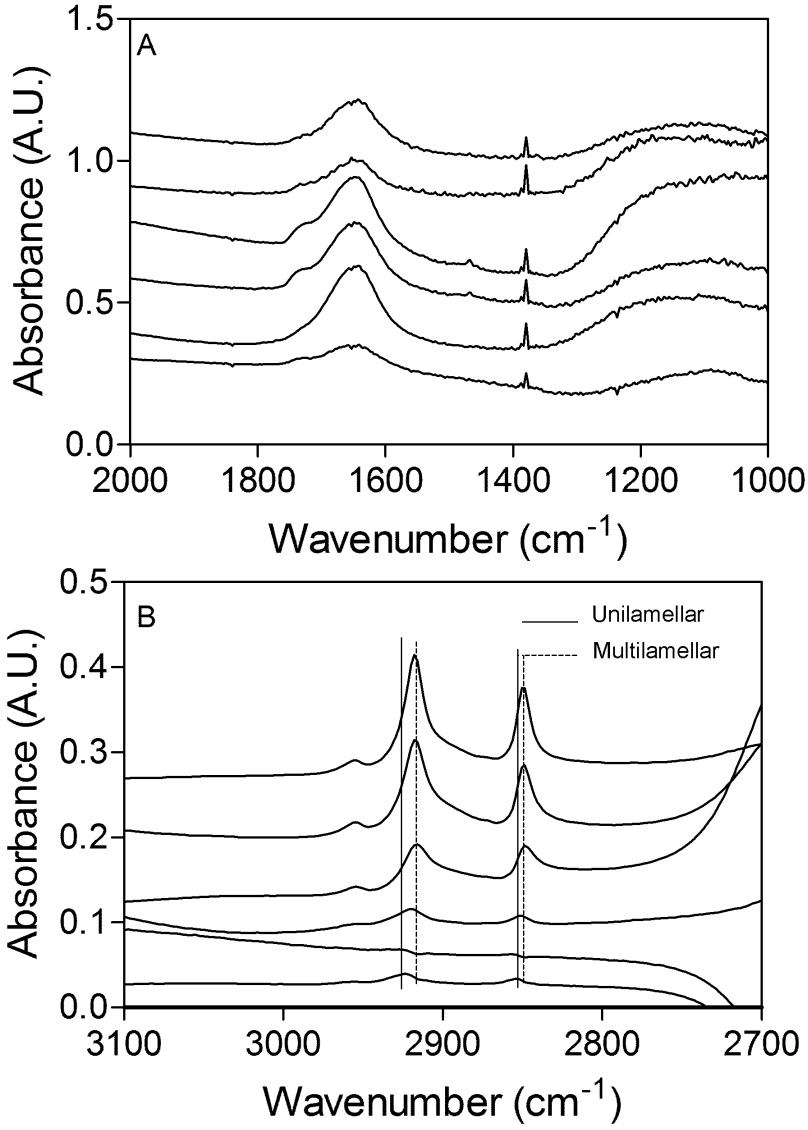


Figure 3.6 FTIR spectra for liposomal suspensions of PC 10 (bottom spectrum) to PC 20 (top spectrum) vesicle suspensions in water (A) and deuterated water (B).

The peak at wavenumber ~1750cm^{-1} represents the C=O stretching of the ester bond where the hydrocarbon chain meets the head group (Jiang et al. 2005). The appearance of a shoulder on the 1750 cm-^{1} peaks for PC 14 and PC 16, indicates that the head group is more highly confined (i.e., have a greater amplitude of hydrogen bonding). This indicates a transition in the structure of liposomes formed below a chain length of 14 and above a chain length of 16. PC 14 and PC 16 can be considered to have chain lengths within a transitional zone. This result supports the observation in X-ray diffraction patterns (Figure 3.5A) where the phospholipids with long hydrocarbon chains have a smaller headgroup spacing (i.e. pack more closely). Logically, the multilamellar liposomes have a larger radius than the unilamellar liposomes, resulting in less torsion applied to the adjacent molecules. As a result, in a multilamellar liposome configuration adjacent molecules are closer and interact more highly with each other.

The hydrogen bonding associated with water make it difficult to discern the CH_2 (symmetric stretch ~2850 cm^{-1}; antisymmetric stretch ~2920 cm^{-1}) and CH_3 (symmetric stretch was not evident; antisymmetric stretch ~2957 cm^{-1}) bands of the hydrocarbon chain therefore deuterated water was used to eliminate the interference of this spectral region (Figure 3.6B) (Jiang et al. 2005).

Jiang et al. observed the formation of phospholipid bilayers on a titanium dioxide surface (2005). The system presented here differs, though the differences caused by the curvature of the bilayers in a liposomal system is considered to be negligible. Jiang et al. observed that when PC16 formed a monolayer the CH_2 symmetric stretch was observed at 2851 cm^{-1}, when the phosopholid layer increased to 3 layers the band shifted to 2850 cm^{-1} and at 5 layers it band was observed at 2849 cm^{-1} (Jiang et al. 2005). Similar trends were observed for the CH_2 antisymmetric stretch where 1 layer had a band at 2919 cm^{-1}, at 3 layers 2919 cm-^{1} and at 5 layers it shifted to 2918 cm^{-1} (Jiang et al. 2005). Figure 3.6B indicates that above C14 there is a shift of both the CH_2 antisymmetric and symmetric stretches to lower wavenumbers corresponding to those observed as the layer thickness went from 1 phospholipid to multiple phospholipid layers. The shift in the CH_2 bands, along with the powder x-ray diffraction, concur and suggest, in this system, that there is a transition form unilamellar to multilamellar structures above a hydrocarbon chain length of 14 carbons.

While the majority of liposomes in the PC 10 sample are unilamellar, it is likely that within each suspension both unilamellar and multilamellar liposomes exist. Previous research

has shown that multilamellar liposomes are present when samples are prepared using the gentle hydration method. Further investigation would be required to assess the composition of samples based on lamellarity. For the purposes of this study, the trends discussed will be determined using the mean lamellarity indicated by the both the X-ray diffraction data and FTIR.

Oscillatory measurements were used to assess the storage and loss modulus of the phospholipid suspensions, G′ and G″ respectively (Figure 3.7).

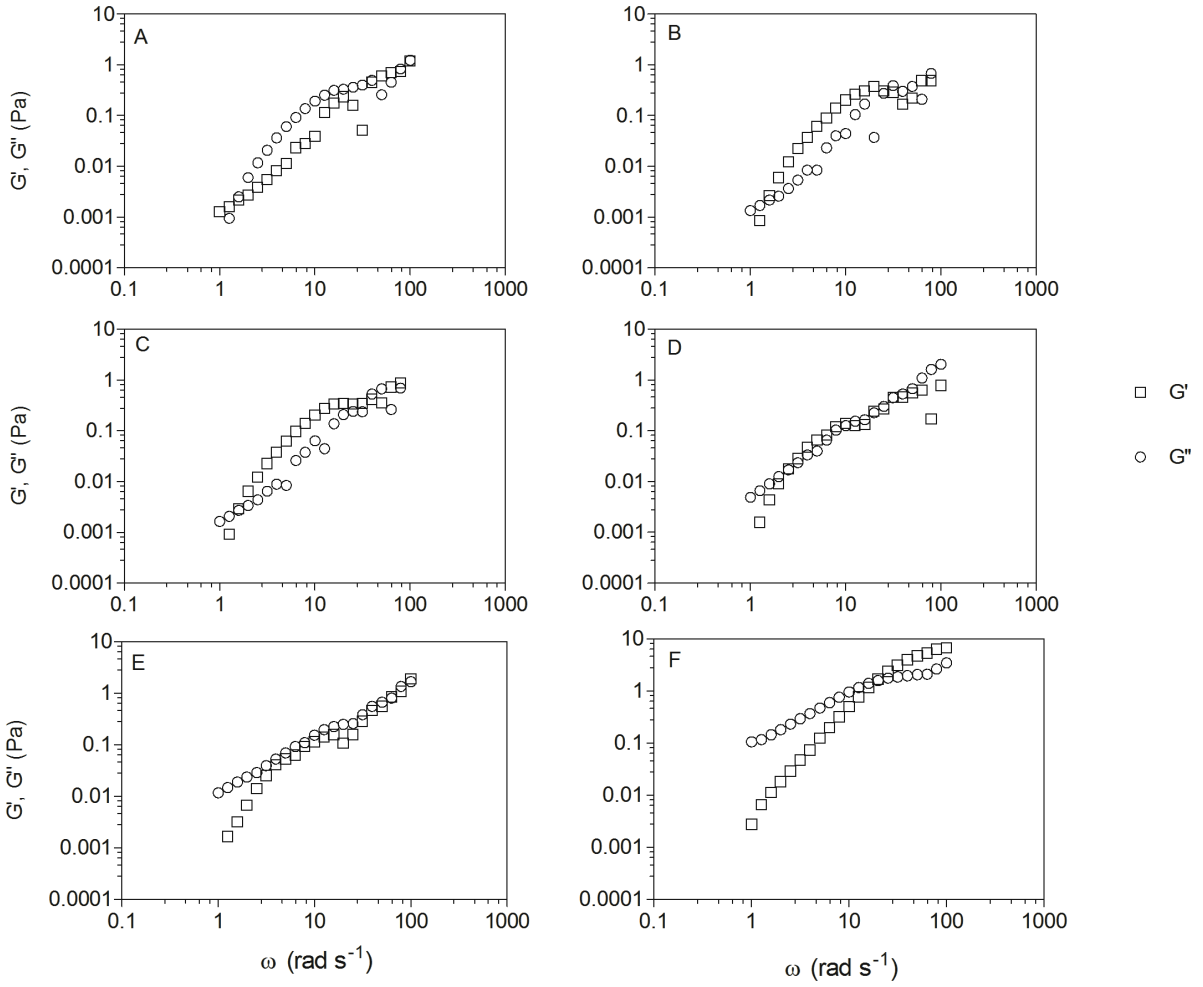


Figure 3.7 Oscillatory frequency measurements within the linear visco-elastic region for PC10 (A), PC12 (B), PC14 (C), PC16 (D), PC18 (E) and PC20 (F).

From Steffe's work with rheology in soft matter systems, the behaviours of the liposomal suspensions behave as dilute solutions (Steffe 1996). The crossover of the G' and G" values at the middle of the frequency range indicates that the suspension is behaving more like a

solid as it is subjected to higher frequencies. PC16, PC18 and PC20 suspensions exhibit this transition. This indicates that multilamellar structures exhibit more solid-like properties under oscillatory stress than the lipoomes formed using PC10, PC12 and PC14. By using simple scaling relationship established by Steffe a transition occurs and the hydrocarbon length increases where an order of magnitude decrease in the G' scaling constant (a) and an increase in the order of magnitude in G" (b) is observed (Table 1). This observation confirms that regardless of the phospholipid utilized it has a solution behaviour as a dilute solution. Further, phospholipids with side chains less than C16, form numerous, small diameter liposomes which are more capable in setting up an elastic network compared to the few large multilayer liposomes formed with long phospholipid side chains.

Table 3.1 Power law coefficients of the storage ($G' = a\omega^b$) and loss modulus ($G'' = c\omega^d$) of liposomal suspensions compared with those for typical materials as determined by Steffe (1996).

Hydrocarbon Length	a ($Pa\ s^b$)	b (n.u.)	c ($Pa\ s^d$)	d (n.u.)
10	1.12×10^{-2}	1.17	5.96×10^{-4}	2.02
12	1.33×10^{-2}	1.10	8.10×10^{-4}	1.89
14	1.19×10^{-2}	1.17	3.69×10^{-3}	2.12
16	1.18×10^{-2}	0.98	9.09×10^{-2}	1.08
18	3.60×10^{-3}	1.88	1.07×10^{-1}	0.94
20	4.00×10^{-3}	2.15	1.43×10^{-1}	1.02
Dilute Solution*	2.80×10^{-3}	1.66	1.18×10^{-2}	9.34×10^{-1}
Concentrated Solution+	16.26	0.84	27.78	5.20×10^{-1}
Gel#	5626	3.70×10^{-1}	344.70	1.45×10^{-2}

* 5% dextrin; + 5% lambda carrageenan; # 1% agar (Steffe 1996)

3.6 Conclusions

Phospholipid supramolecular aggregates may be modified from unilamellar to multilamellar liposomes by altering the carbon number of the hydrocarbon chain. There is a transition where below a chain length of 14 unilamellar liposomes form and above a chain length of 16 multilamellar liposomes form. Evidence from FTIR spectra particularly indicates that

chain lengths of 14 and 16 represent a transitional zone in the liquid crystal morphology of phospholipid liposomes. Understanding and characterizing this structural transition may have utility in investigation the aggregation of amphiphilic molecules.

3.7. Rationale Linking Research Studies

Due to the similarities in the structures of PC and SM, research on the pairing of these two phospholipids in liposomal structures precedes this study (Webb et al. 1995; Semple et al. 2005). Investigations of SM containing liposomes have been conducted in efforts to stabilize the resulting liposomes and to mimic lipid raft structures found in cell membranes (Webb et al. 1995; Semple et al. 2005). However, what remains unexamined in literature is the effect of the addition of SM to a liposomal drug delivery system on the overall efficacy of the treatment. In an effort to facilitate further research in this area, a structural investigation was undertaken to determine to what degree a two component system comprised of PC and SM would have on the packing of adjacent molecules and effect on the overall supramolecular structure of the phospholipids were altered in this two component system when compared to pure systems of PC and SM.

Sphingomyelin is obtainable from both plant and animal sources, e.g. canola, eggs and milk. Milk and its products (e.g. cheese) are a good source of sphingolipids (Vesper et al., 1999). A possible waste stream for sphingomyelin isolation and utilization as a structural component of liposomes is buttermilk (Morin et al., 2007). Phospholipids can be extracted from buttermilk by filtration methods or by supercritical fluid extraction and sphingomyelin can be isolated from this phospholipid fraction by the use of high performance liquid chromatography (Astaire et al., 2003).

4. STRUCTURE OF SPHINGOMYELIN-PHOSPHATIDLYCHOLINE LIPOSOMES

4.1 Abstract

Using the gentle hydration method, liposomes composed of different ratios of 1,2-distearoyl-sn-glycero-3-phosphocholine (PC) and N-(tricosanoyl)-sphing-4-enine-1-phosphocholine (SM) were formed. The addition of SM to a phosphatidylcholine (PC) liposome at ratios of 1PC:1SM, 2PC:1SM and 4PC:1SM showed slight variations in packing. These are the result of the enhanced hydrogen bonding capability of the SM headgroups and differences in hydrocarbon chain length. Optimal packing in this study was found to occur at a ratio of 4PC:1SM.

4.2 Objective and Hypothesis

The objective of this research study is to assess the structural effects on liquid crystal supramolecular structures of the addition of SM to a PC liposome at different ratios.

It is hypothesized that liposome diameter and the packing of adjacent molecules in a PC-SM two component system will be similar to a single component PC liposome system.

4.3 Introduction

Research over the past 20 years has revealed the importance of sphingolipids in cell signaling (Hannun and Obeid 2008). A number of these compounds including, sphingomyelin (SM), ceramides, sphingosine and sphingosine 1-phosphate, (Figure 2.3) have been shown to be of significant importance in cell signaling pathways associated with senescence and apoptosis (Ogretmen and Hannun 2004; Hannun and Obeid 2008). As this class of compounds is capable of inducing apoptosis, this function may be exploited to increase the rate of cell death when used in cancer treatment.

Cancer cells grow rapidly and without regulation of size or shape (Huang, Lee et al. 1992; Yuan, Dellian et al. 1995; McDonald and Baluk 2002). Irregularities in cellular properties propagate into irregularities in the vasculature of the tumor resulting in leaky blood vessels

which allow larger molecules, such as liposomes, to permeate the interior of the tumor (Huang et al. 1992; Yuan et al. 1995; McDonald and Baluk 2002). The nature of tumor structure also results in decreased efficacy of lymph drainage resulting in liposomal retention within the tumor (Huang et al. 1992; Yuan et al. 1995; McDonald and Baluk 2002). The increased blood flow that results from angiogenesis at the tumor site, coupled with long circulating liposomes results in a higher probability of drug-tumor interactions (Huang et al. 1992; Yuan et al. 1995; McDonald and Baluk 2002). These factors are collectively referred to as enhanced permeability and retention effects (Huang et al. 1992; Yuan et al. 1995; McDonald and Baluk 2002).

Research has shown that the application of liposomally encapsulated ceramides can increase the rate of apoptosis in cancer cells *in vivo* (Shabbits and Mayer 2003; Stover and Kester 2003). Stover and Kester (2003) observed the effect of liposomally delivered ceramides on MDA-MB-231 breast adenocarcinoma cells. When compared to free ceramide, ceramides that were delivered as part of a liposome contributed to a larger decrease in cancer cell proliferation (Stover and Kester 2003). Shabbits and Mayer (2003) observed a similar trend with liposomally introduced ceramides in their investigations of MDA435/LCC6 human breast cancer in J774 mouse macrophage cells. Also, increased levels of dietary SM have been shown to decrease cancer lesion size and delay cancer progression in MCF10AT1 breast cancer xenografts in mice (Simon et al. 2010)

Sphingolipids are found in the membranes of cells where they are susceptible to enzymatic degradation. A variety of enzymes have been shown to release these lipids from cellular membranes into the cytoplasm or the extracellular space. Once released these compounds can participate in cell signaling cascades or synthetic pathways (Ogretmen and Hannun 2004; Hannun and Obeid 2008). For example, the action of sphingomyelinase cleaves the phosphatidylcholine head group from SM to produce ceramides. Released ceramides can then stimulate a series of enzymes that carry out their own specific cellular effects leading to senescence or apoptosis (Figure 4.1) (Ogretmen and Hannun 2004; Hannun and Obeid 2008).

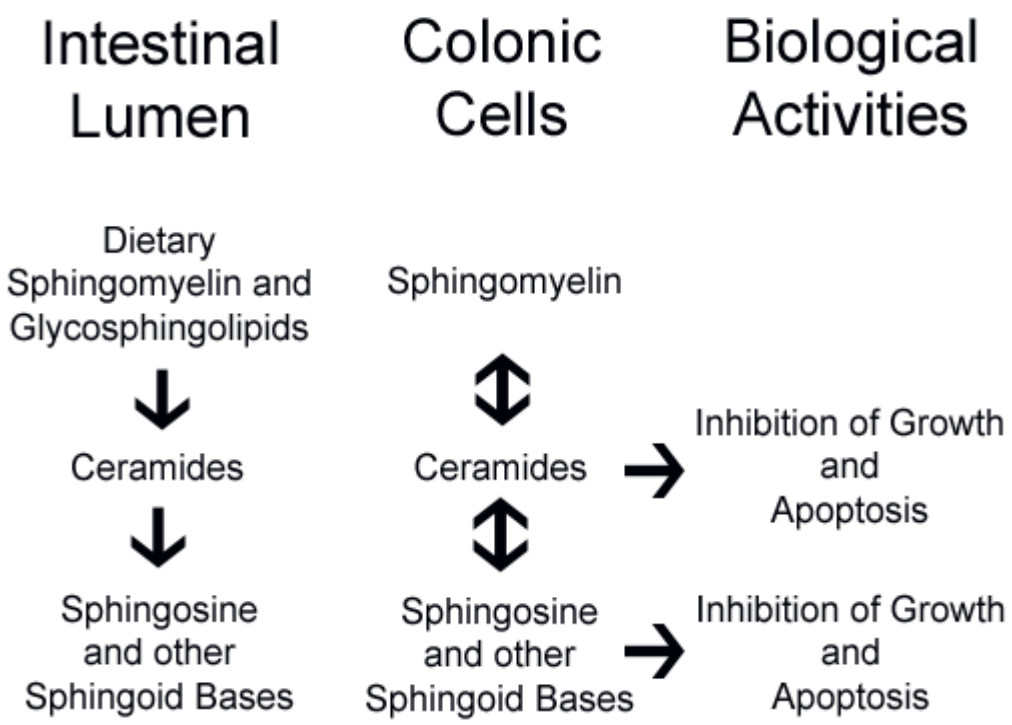


Figure 4.1 Schematic representation of sphingolipid metabolism and their signaling activities, adapted from Vesper et al. (1999).

Previous studies have revealed that the addition of sphingomyelin to a liposome structure increases its stability (Webb et al. 1995; Semple et al. 2005). Increased stability is thought to have been a result of resistance to enzymatic decay provided by the non-ester linkage of one of the hydrocarbon chains to the SM head group as well as the high levels of saturation of the hydrocarbon chains liposome (Webb et al. 1995; Semple et al. 2005). The more stable the liposome structure and the more resistance to enzymatic decay, the longer the liposome can circulate increasing the likelihood of the drug arriving at the disease site (Chonn and Cullis 1995; Drummond et al. 1999).

It is proposed that a drug delivery liposome that contains sphingolipids, namely sphingomyelin, has the potential to create a drug delivery system that can combine the effect of liposomally encapsulated anti-cancer drugs with the anti-cancer effects of the released sphingolipids. To our knowledge, this synergistic concept of the drug and bioactives within the drug delivery system has yet to have been explored. Ideally, sphingomyelinase would act on the

liposome breaking apart the membrane resulting in (1) the release of the drug and (2) the release of apoptosis-inducing ceramides. Sphingomyelinase has been shown to have activity on SM in liposomal membranes, particularly in liposomes with lipid raft structures (i.e. lateral domains composed of SM, PC and cholesterol) designed to mimic those found within mammalian cell membranes (Nurminen et al. 2002). The rationale of conducting this research on sphingomyelin-phosphatidlycholine liposomes is to determine whether or not there are any structural impediments to an optimal liposome structure.

While both PC and SM possess a phosphocholine head group and two hydrocarbon chains, there are differences in their structure that in turn effect their behaviour in phospholipid bilayer systems (Terova et al. 2004). Phosphatidylcholine is synthesized from a glycerol backbone to which the hydrocarbon chains are attached with ester linkages whereas SM is composed of a sphingoid base to which an additional hydrocarbon chain is attached with an amide bond (Terova et al. 2004). The presence of the amine group in the head group structure of SM provides an additional site for hydrogen bonding that is not present on PC molecules (Terova et al. 2004).

Sphingomyelin can be isolated from a variety of plant and animal sources. Depending on the source, the hydrocarbon chain length and their saturation may differ. For example, milk SM is more likely to have longer and less saturated hydrocarbon chains than SM extracted from egg yolk (Astaire et al 2003).

4.4 Materials and Methods

The following were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and used as recieved: 1,2-distearoyl-sn-glycero-3-phosphocholine (PC18) and N-(tricosanoyl)-sphing-4-enine-1-phosphocholine (SM) derived from milk. The predominant hydrocarbon species in milk SM is 23:0 (Avanti Polar Lipids, Alabaster, AL, USA).

Liposomes were formed using a gentle hydration method (Tsumoto et al. 2009). The following were prepared in triplicate: PC18, SM and samples with a molar ratio of 1PC:1SM, 2PC:1SM and 4PC:1SM. The film was prepared by dissolving 0.1 mM of total phospholipids in 2mL of chloroform (Sigma-Aldrich, St. Louis, MO, USA) in a 50 mL round bottom flask. A Buchi rotary evaporator (Flawil, Switzerland) with the water bath at 55^{o}C was used to evaporate the chloroform under reduced pressure leaving a thin dry film of phosphtidylcholine. Two

milliliters of HEPES buffered saline (20 mM HEPES, 150 mM NaCl) (Sigma-Aldrich, St. Louis, MO, USA) was added to the film and was mixed using the rotary action of the Buchi rotary evaporator with the water bath temperature set above the transition temperature of the phopholipid with the high transition temperature. The resulting liposome suspensions were sonicated using a Branson 2510 bath sonicator (Danbury, CT, USA) at room temperature for 10 minutes at 40 kHz (until no large aggregates were visible in the suspension).

A Nikon Eclipse E400 light microscope (Nikon Instruments Inc., Melville, NY, USA) equipped with a Nikon DS-Fi1 color camera (Nikon Instruments Inc., Melville, NY, USA) and a 40x lens and condenser (Nikon Instruments Inc., Melville, NY, USA) were used for light microscopy. Images were captured at a resolution of 1920 by 960 pixels using a Nikon DS-FiL color camera. Images were analyzed using Adobe Photoshop Extended CS4.0 (Adobe Systems Incorporate, San Jose, CA, USA). ANOVA performed with Graphpad Prism 5 (San Diego, California, USA).

Light scattering measurements were performed using a Malvern Hydro 2000S Mastersizer (Malvern, Worcestershire, UK) to determine hydrodynamic diameter.

Fourier transform infrared (FTIR) spectra were collected at the Canadian Light Source (Saskatoon, SK, Canada) at the mid-IR spectromicroscopy beamline (01B1-1). The end station was comprised of a Bruker Optics IFS66v/S interferometer coupled to a Hyperion 2000 IR microscope (Bruker Optics, Billerica, MA, USA). A drop of sample was placed between two CaF_2 optical windows (25 mm diameter, 2 mm thick). Light is focused on the sample using a 15X magnification Schwarzschild condenser, collected by a 15X magnification Schwarzschild objective with the aperture set to a spot size of 40 mm by 40 mm and detected by a liquid nitrogen cooled narrowband MCT detector utilizing a 100 mm sensing element.

A KBr-supported Ge multilayer beamsplitter was used to measure spectra in the mid-infrared spectral region. Measurements were performed using OPUS 6.5 software (Bruker Optics, Billerica, MA). The measured interferograms were an average of 512 scans and were recorded by scanning the moving mirror at 40 kHz (in relation to the reference HeNe laser wavelength of 632.8 nm). The wavelength range collected was 690 to 7899 cm^{-1} with a spectral resolution of 4 cm^{-1}. Single channel traces were obtained using the fast Fourier transform algorithm, without any zero-filling, after applying a Blackman–Harris 3-Term apodization function.

4.5 Results and Discussion

Visualization, using brightfield microscopy, reveals that the single component phospholipid and sphingomylein systems (Figure 4.2) are composed mainly of spherical vesicles with some cylindrical vesicles.

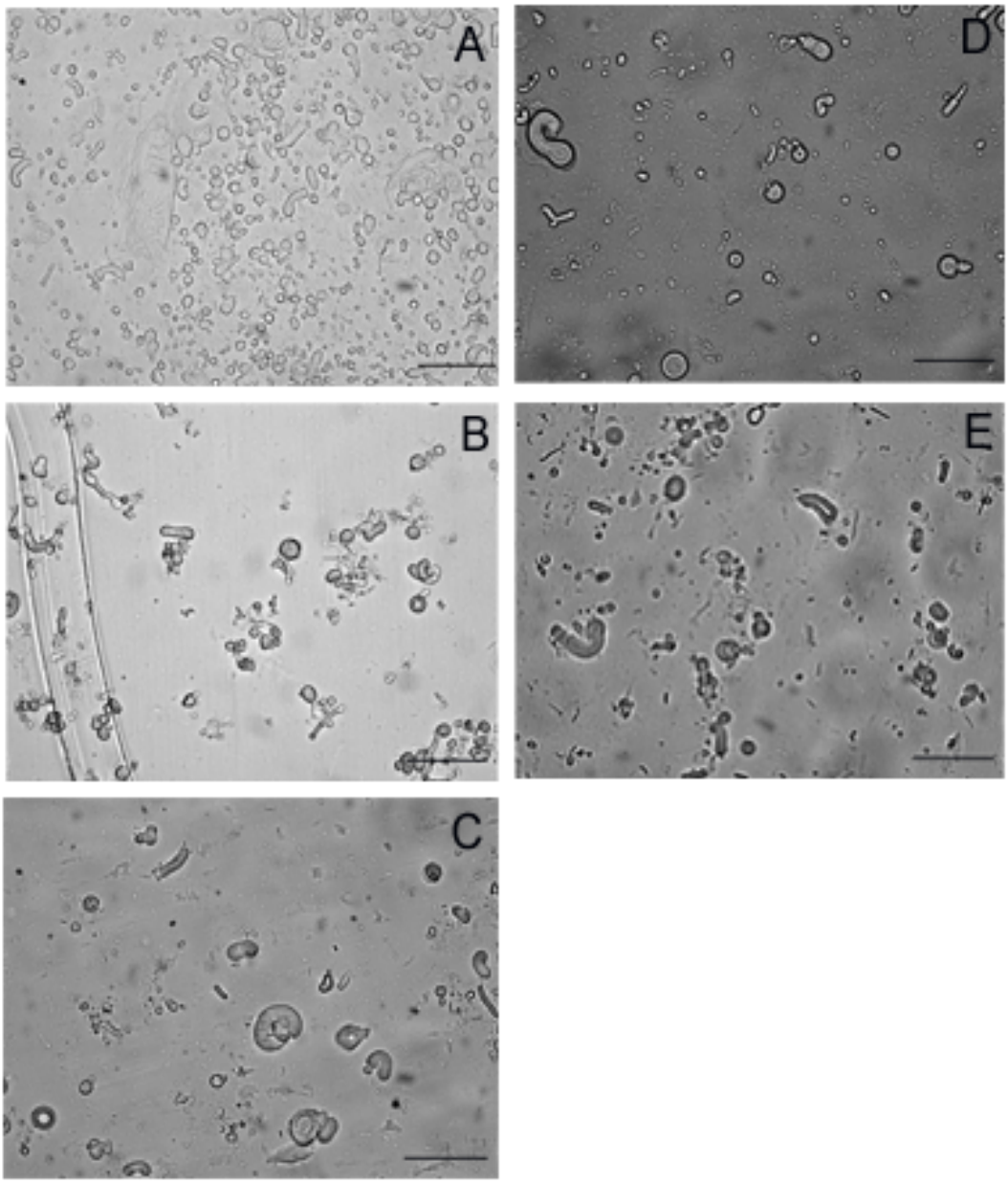


Figure 4.2 Light micrographs of liposomal suspensions composed of PC18 (A), SM (B), 1PC:1SM (C), 2PC:1SM (D) and 4PC:1SM (E) (Scale bar = 5 μm).

Israelachvili (1991) describes three molecular characteristics to calculate a packing parameter that can be used to predict supramolecular structures. These characteristics are the optimal area of the head group (a_o); the volume of the hydrocarbon chain(s) (v), which are fluid and incompressible; and the maximum effective length of the chain (l_c) (Israelachvili 1991). The packing parameter is calculated using $v/a_o l_c$ (Israelachvili 1991). The packing parameter can be approximated by using the head group area for egg lecithin as determined by Israelachvili, $0.717 nm^2$ (1991). Hydrocarbon volume (v) and length (lc) may be approximated using the following equations:

$$v \sim (27.4 + 26.9n) \times 10^{-3}\ nm^3 \quad (4.1)$$

and

$$l_c \sim (0.154 + 0.1265n)\ nm \quad (4.2)$$

where n is the number of carbons in the aliphatic chain (Israelachvili 1991). Thus, the PC18 system has a packing parameter of 0.57 and the SM system, using also the approximate head group area of $0.717 nm^2$ and hydrocarbon chain length of 23:0, has a packing parameter of 0.36. Packing parameters between 1/2 to 1 are expected to form liposomes while cylindrical micelles have an expected packing parameter between 1/3 to 1/2 (Israelachvili 1991). The presence of the cylindrical vesicles in the SM-PC samples is due to both the greater effective headgroup areas and the increase in the hydrocarbon chain length of the SM molecules (Israelachvili 1991). In a one component system, the hydrocarbon chains can pack uniformly which minimizes the effect of hydrocarbon chain length on the supramolecular structure allowing for a more thermodynamically stable structure (spherical vesicle formation) (Israelachvili 1991).

Figures 4.2A and 4.2E appear to have a similar distribution of liposomes and cylindrical micelles. In the 4PC:1SM suspension, the majority of side-by-side interactions in this suspension are PC-PC making it the most similar to the one component PC suspension. By minimizing interactions between SM and PC the most optimal packing conditions of those examined were observed for this two component system. If required, these morphological differences can be readily overcome with physical modifications, such as membrane extrusion, which can be used to prepare homogeneous size and shape distributions of liposomes regardless of phospholipid composition or hydrocarbon chain length (Israelachvili et al. 1977).

Ten randomly selected vesicles from each light micrograph were analyzed using Photoshop; the initial measurement of diameter was taken in pixels and converted to microns

using a calibrated scale bar. This number-mean diameter (D[1,0]) was converted to a volume-moment mean diameter (D[4,3]) (Figure 4.3). The volume-moment mean diameter shows no significant difference ($p < 0.05$) in the single component SM liposomes and the SM-PC liposomes. However, the SM-containing liposomes are smaller than the PC18 liposomes.

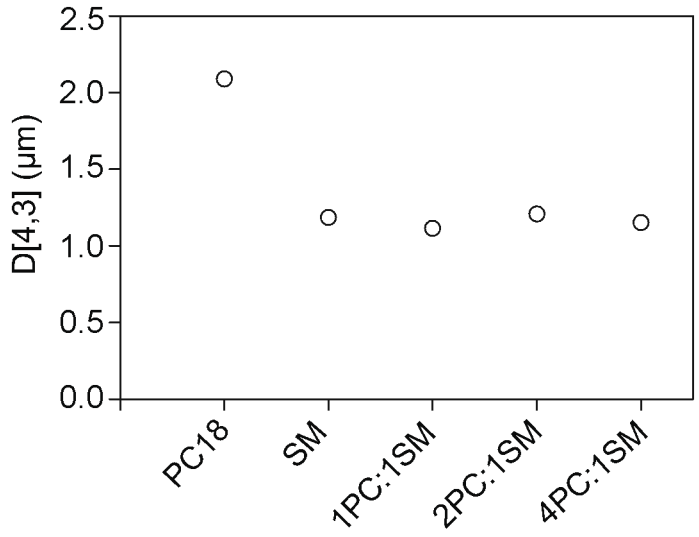


Figure 4.3 Volume-moment mean diameters of liposomal suspensions calculated using pixel measurements obtained from light micrographs.

Light scattering showed a bimodal distribution for each suspensions which, in agreement with the light micrographs, shows that both small vesicles and aggregates of small vesicles were present (Figure 4.4). The size distribution pattern is similar for all suspensions. PC18 (Figure 4.4A) has a greater volume of vesicles between 0.1 and 1 micron and a less aggregates 10 micron in size than the SM and SM containing suspensions (Figures 4.4B-E). The D[4,3] values determined using DLS (Figure 4.5) shows similar trends as the values determined using brightfield microscopy (Figure 4.3) for the SM-PC suspensions.

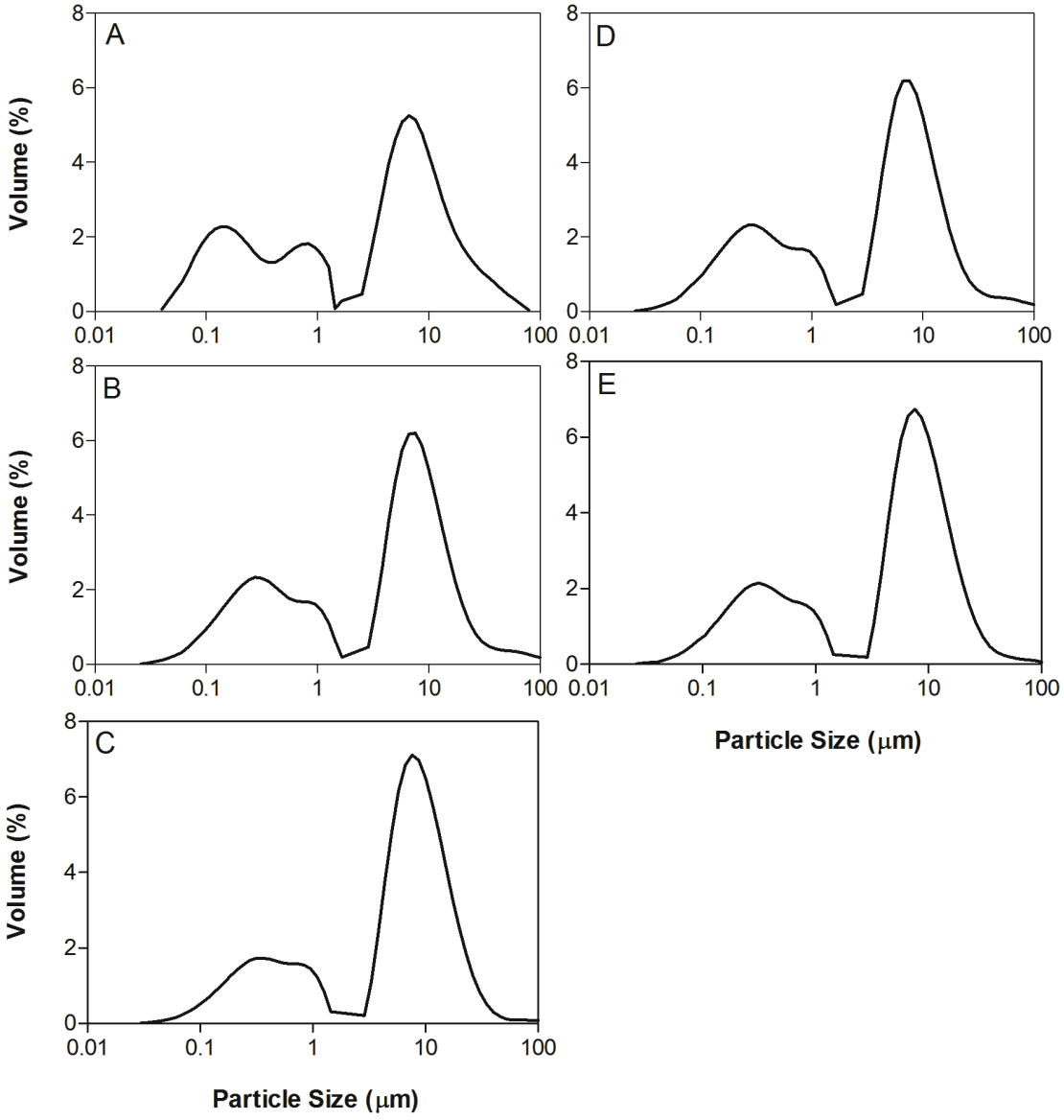


Figure 4.4 Light scattering intensities of liposomal suspensions of PC18 (A), SM (B), 1PC:1SM (C), 2PC:1SM (D) and 4PC:1SM (E).

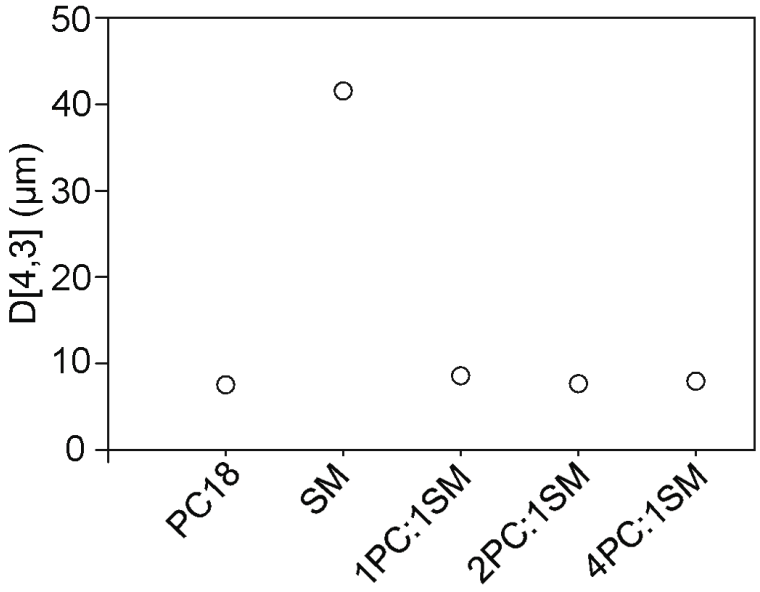


Figure 4.5 Volume-moment mean diameters of liposomal suspensions calculated from dynamic light scattering measurements. Data points represent mean values ± one standard deviation.

The difference in magnitude between the D[4,3] values, determined using microscopy and DLS, is attributed to the light scattering technique detecting aggregates as opposed to single vesicles. Figures 4.3 and 4.5 show opposing trends for the one component liposome systems. By referring to Figure 4.2, however, these trends can be explained by the greater tendency of the SM liposomes to aggregate (Figures 4.2B) due to the increased hydrogen bonding affinity of SM headgroups (Niemela et al. 2004). From Figure 4.2, it is also observed that there are some minor differences in the supramolecular structures in the liposomal suspension leading to some discrepancies in the size measurements obtained from different techniques. The previous analyses have indicated few differences in the supramolecular structures of the prepared suspensions. Further analysis using synchrotron mid-IR microscopy was conducted to assess the impact on the physical chemistry of the systems. The FT-IR data indicates only small differences in both the headgroup and hydrocarbon chain packing for all suspensions (Figure 4.6).

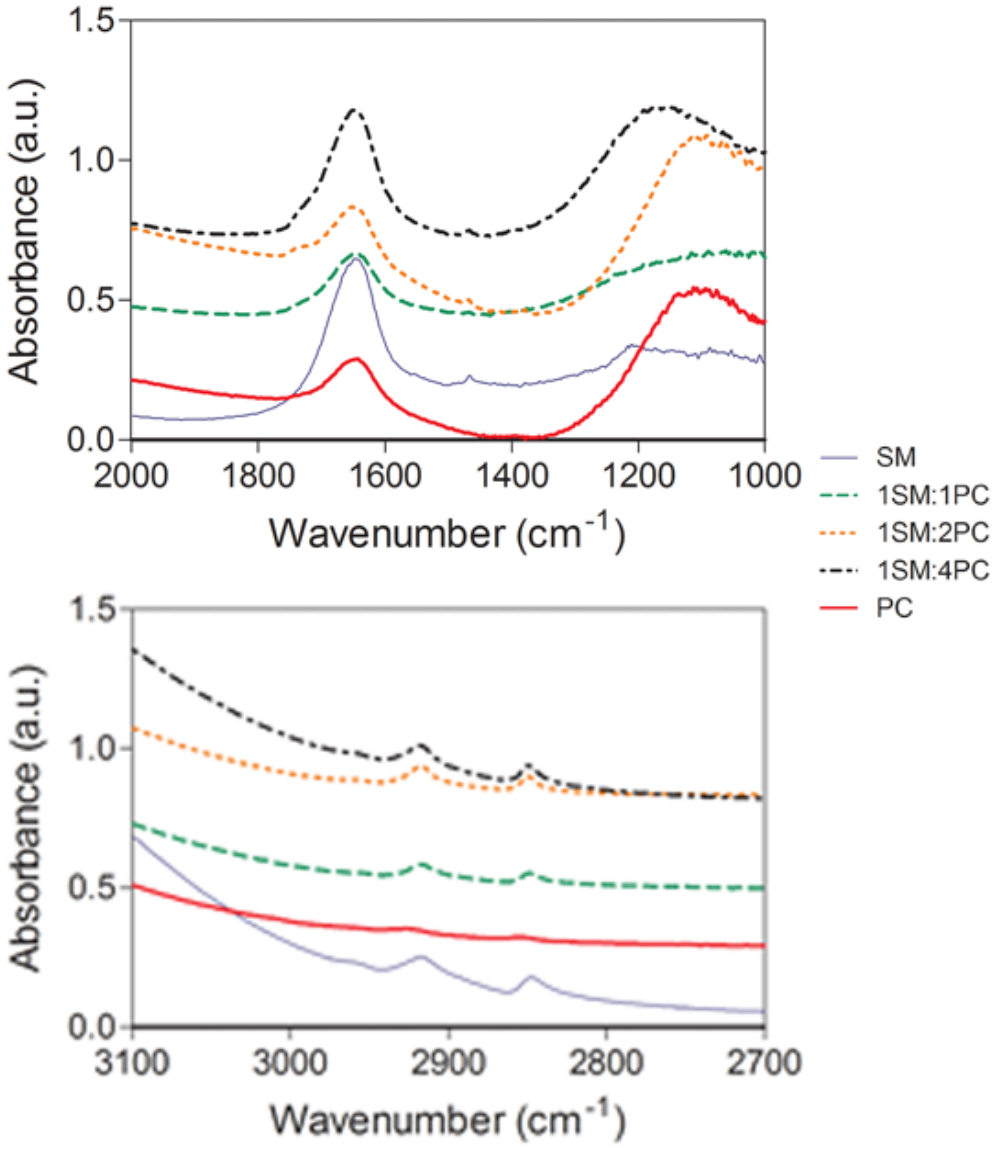


Figure 4.6 FTIR spectra for suspensions composed of PC, SM, 1PC:1SM, 2PC:1SM and 4PC:1SM.

The peak at ~1750 cm^{-1} represents the C=O stretch of the ester bond where the fatty acid chain meets the head group (Jiang, et al. 2005). The single component SM liposomes displayed a greater peak absorbance at this frequency than the single component PC liposomes (Figure 4.6). This result suggests more that the head groups of SM are more highly confined due to their increased capacity for hydrogen bonding.

The CH_2 symmetric stretch (~2850 cm^{-1}) and antisymmetric stretch (~2920 cm^{-1}) and CH_3 antisymmetric stretch (~2957 cm^{-1}) remained similar for all SM-containing liposomal suspensions (Figure 4.6). The SM suspension show a slight increase intensity of this spectral feature due to the introduction of longer, highly saturated hydrocarbon chains.

At a ratio of 1SM:4PC, the head group behaviour is similar to one component SM suspensions while ratios of 1SM:1PC and 1SM:2PC behave in a similar manner to one component PC suspensions (Figure 4.6). Phosphatidylcholine has been used for liposomal drug studies since the advent of research in this area and at present it is incorporated into drugs that are approved for clinical usage making it an important benchmark to measure against.

Of the suspensions investigated, 1SM:4PC, seems to display the most optimal structure of the systems tested. At this ratio, head group confinement is high despite the majority of molecules being PC. Which, as previously described, is likely a result of ordered areas of PC surrounding SM molecules minimizing interactions between unlike adjacent molecules. In all of the SM-PC mixtures the level of head group confinement is never less than pure PC, indicating that these two phospholipids in combination are capable of forming supramolecular structures that are more stable than pure PC.

4.6 Conclusion

Sphingomyelin can be incorporated into phosphatidylcholine vesicles at numerous possible ratios while maintaining size profiles and supramolecular structures similar to those composed of only phosphatidylcholine. This versatility allows for possible modifications to drug delivery systems so as to improve both their function and circulation. The incorporation of sphingomyelin into a liposome that may be loaded with wide spectrum chemotherapy drugs increases the utility of these drug carrier systems. The presence of sphingolipids, like sphingomyelin, in the liposome structure may improve efficacy of current liposomally encapsulated chemotherapeutics due to the possible activation of signalling pathways in which sphingolipids induce apoptosis in cancer cell lines.

5. GENERAL DISCUSSION

Through the use of the gentle hydration method, spherical aggregates formed in all suspensions investigated (Figures 3.1 and 4.2). The presence of particles with diameters exceeding twice the length of the amphiphilic lipids indicated that rather than the formation of a micelle, bilayer vesicles (liposomes) had formed. In order for micelles to form in the aqueous system investigated, the hydrocarbon chains of amphiphilic lipids would be required to associate at the centre of a sphere excluding any portion of the aqueous solution from within the structure. The large aggregate size indicated that the inner vesicle contained aqueous solution. In addition to spherical particles, cylindrical bilayer structures were also observed (Figures 3.1D and 4.2B-D). The coexistance of these supramolecular structures is thermodynamically permissible in suspensions.

Light scattering was used to observe trends in the diameter of the liposomal suspensions. The size distributions of the prepared suspensions can be seen in Figures 3.3 and 4.4. Apart from the suspension of PC 16 (Figure 3.3D), the predominant trend was a bimodal distribution size (Figures 3.3 and 4.4). This bimodal distribution illustrated the capacity of the liposomes investigated to aggregate in suspension. The SM containing suspensions have a distribution that was skewed to higher particle sizes due to the increased hydrogen bonding affinity of SM headgroups (Niemela et al. 2004). To investigate the lower end of the size distribution revealed by light scattering, measurements of individual particles from the light micrographs were examined (Figures 3.2 and 4.3). A comparison of Figures 3.2 and 3.4A which were both PC suspensions, revealed a similar trend. The size of individual liposomes and the size of aggregates of liposomes in the PC suspenions follow the same trend, whereas the SM liposome aggregates are smaller than expected based on the individal SM liposomes. This is due to the enhanced hydrogen bonding ability of SM. The difference in magnitude between the D[4,3] values determined using microscopy and light scattering was attributed to the light scattering technique

used which detects aggregates as opposed to single vesicles, and the increased capacity of SM containing liposomes to aggregate based on their increased hydrogen bonding capabilities.

The hydrocarbon chain length increase of saturated diacyl PC molecules did not result in the expected linear increase in the diameter of the liposomes. The hypothesized outcome was based on previous research showing a linear increase in membrane thickness with increasing hydrocarbon length in PC molecules (Lewis and Engleman 1983). What was observed from both pixel and light scattering measurements was a plateau from PC 10 to 14 and an increase above PC 16. Measurements of surface area showed a parabola-like trend. Since all suspensions were prepared with the same molar ratio these results indicated a change in the packing of PC molecules above a chain length of 14 carbons (Figure 3.4B). The decrease in surface area observed for PC 16-20 indicated a possible transition to multilamellar liposomes. The light scattering data revealed trends that need to be further investigated with techniques appropriate for the elucidation of the molecular structure of the liposomes.

To further investigate the makeup of the bilayers within the liposome, x-ray diffraction was used. The 001 peak at 65.03 Å, which is twice the length of one PC 18 molecule, in Figure 3.5A indicated the presence of a bilayer. The higher order reflections (002-006) present indicated the presence of multiple bilayers in Figure 3.5A and were absent from Figure 3.5B, which represents PC 12. The single long spacing at 29.5 Å, which is twice the length of one PC 12 molecule as seen in Figure 3.5B, represents a suspension with a unilamellar liposome composition. The existence of both unilamellar and multilamellar liposomes within the PC 18 suspension were suggested by the doublet peaks at ~22 Å and ~16 Å indicating that two different structures were present.

Synchrotron mid-IR spectroscopy was used to elucidate the packing of adjacent molecules in all experimental liposomal suspensions. The peak at wavenumber ~1750 cm^{-1} represents the C=O stretching of the ester bond where the hydrocarbon chain meets the head group (Jiang et al. 2005). From Figure 3.6, the shoulder on the 1750 cm^{-1} peak for liposomal suspensions of PC 14 and PC 16, indicated that the head group was more highly confined (greater amplitude of hydrogen bonding) than the other suspensions. This observation of a change in packing supports the x-ray diffraction data which showed a transition from unilamellar to multilamellar lipsomes. In addition, a greater absorbance at this wavenumber was observed for component SM versus single component PC liposomes (Figures 3.6 and 4.6). These results

indicated a higher confinement of SM head group structures due to their higher capacity for hydrogen bonding (Niemela et al. 2004). Most similar to the pure SM suspension was the suspension prepared at a ratio of 1SM:4PC. Ratios of 1SM:1PC and 1SM:2PC behaved in a similar manner to one component PC suspensions (Figures 3.6 and 4.6).

The FTIR spectral features that correspond with the hydrocarbon chains of the amphiphilic lipids are the CH_2 symmetric stretch (~2850 cm^{-1}) and antisymmetric stretch (~2920 cm^{1}) and the CH_3 antisymmetric stretch (~2957 cm^{-1}). Jiang et al. (2005) observed the formation of multiple bilayers of PC on a titanium oxide surface. When PC16 formed a monolayer the CH_2 symmetric stretch was observed at 2851 cm^{-1} and when this phosopholipid layer increased to three the band shifted to 2850 cm^{-1} ,and at five it shifted to 2849 cm^{-1} (Jiang et al. 2005). A similar trend were observed for the CH_2 antisymmetric stretch at 2919 cm^{-1} , 2919 cm^{-1}, and 2918 cm^{-1} for one, three and five layers, respectively (Jiang, Gamarnik et al. 2005). This trend was observed in this research (Figure 3.6B) for suspensions produced with a hydrocarbon chain length of 14 where both the CH_2 antisymmetric and symmetric stretches shifted to lower wavenumbers corresponding to the transition from unilamellar to multilamellar structures. Whereas, the hydrocarbon chain spectral features for SM containing suspensions (Figure 4.6) remained similar. Any slight increase in the sample spectral features at wavenumbers ~2850 cm^{1}, ~2920 cm^{1} and ~2957 cm^{-1} were attributed to the introduction of longer, highly saturated hydrocarbon chains.

Rheological investigations of PC suspensions with differing hydrocarbon chain lengths were conducted to assess the implications of lamellarity on viscoelastic behaviour. From Figure 3.7, suspensions of PC16, PC18 and PC20 showed a crossover of G′ and G″ values at the middle (~10 rad s^{-1}) of the frequency range which indicated more solid-like behaviour at to higher frequencies (Steffe 1996).

6. GENERAL CONCLUSIONS

Phospholipids, as amphiphillic molecules, are capable of associating to form a variety of supramolecular structures in aqueous solutions. Phospholipid vesicles have been used as model systems for biological membranes and have been researched extensively. Phospholipid liposomes were first characterized in the early 1960's by Bangham as bilayer vesicles in aqueous solutions (Bangham et al. 1965; Bangham 1980). It was postulated that the liposomal structure was the most thermodynamically stable state as characterized by the reduction of an interfacial tension between the aqueous medium and the liposome (Bangham, Standish et al. 1965; Bangham 1980). The ability of liposomes to encapsulate both water and lipid soluble compounds made them ideal candidates for drug encapsulation research (Bangham 1980; Gregoriadis 1980; Allen 1996). Medical and pharmaceutical research employs these preparation methods in order to achieve homogenous suspensions of unilamellar liposomes that are best suited to drug encapsulation and delivery research (Gregoriadis 1980; Allen 1996; Drummond et al. 1999).

However, the analysis of phospholipid vesicle structures formed by 'gentle' methods affords the opportunity to observe trends in their assembly that cannot be made when these structures are produced through processing methods (Tsumoto et al. 2009). The value in utilizing a method, such as the gentle film hydration method, is in part its simplicity as well as the ability to relate the results found when using other processing methods to improve the engineering of phospholipid vesicles for a variety of applications in the future (Tsumoto et al. 2009).

An increase in the saturated hydrocarbon chain length of PC molecules from X to Y did not result in a positive linear increase in the size of liposomes which was expected based on previous research that showed a positive linear increase in membrane thickness for the same hydrocarbon chain increase (Lewis and Engelman 1983). Experimental results from this research showed that below a hydrocarbon chain length of 14, PC was observed to associate into unilamellar liposomes, while above a hydrocarbon chain length of 14 multilamellar liposomes formed. FTIR spectra indicated that this was a result of a change in the packing behaviour of the

PC molecules due to the hydrocarbon chain length. Despite the change in liquid crystal morphology, each suspension behaved as a dilute solution. Results from this work showed that hydrocarbon length had an effect on the supramolecular structure of phospholipids which adds to the body of knowledge on the systematic design of liposomes and self assembly of phospholipids.

The addition of SM to a PC liposome at ratios of 1PC:1SM, 2PC:1SM and 4PC:1SM were investigated. At these ratios, the structures of the liposome were not significantly different from that of a single component PC liposome. However, the most optimal packing of adjacent molecules was achieved at a ratio of 4PC:1SM. At this ratio the majority of adjacent molecule interactions are PC-PC while the enhanced hydrogen bonding capabilities of the SM head group allow for high confinement and a more ordered structure. This investigation of PC-SM liposomes showed that this system was comparable to single component PC liposomes in terms of structure. Based on these results, there should not be an impediment to investigating this system as a possible drug delivery system. This versatility in liposome structures composed of two components allows for modifications to a drug delivery system for improved functionality and circulation. Particularly, the incorporation of SM into a liposome that is loaded with wide spectrum chemotherapy drugs is an area that should be investigated for the possible synergistic effects of the apoptitic effect of sphingolipids and cancer treatment drugs.

7. FUTURE DIRECTIONS

Though there are many processing methods that can effectively produce liposomal suspensions for a wide variety of applications, this does not discount the value of investigating 'minimal' liposomal processing methods. Further research into the self assembly of liposomes with different phospholipid characteristics and components may prove that these gentle methods are viable option to current physical processing methods. More likely, however, is the gentle hydration method leading to greater insight into the self assembly of phospholipids.

The inclusion of ceramide, a sphingolipid that plays a role in apoptitic pathways, into liposomal structures has been shown to have anti-cancer activity (Shabbits and Mayer 2003; Stover and Kester 2003). Investigations of sphingomyelin as a liposome component have focused on the creation of lipid raft-like structures, improved stability and in drug delivery systems that include cholesterol (Webb et al. 1995; Semple et al. 2005). It is recommended that future work includes systematic comparisons of systems that utilize phospholipid liposomes and sphingolipid containing liposomes with and without drugs to assess whether a synergistic effect between the anti-cancer drug and bioactive lipids is possible.

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