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The Dependence of Lipid Asymmetry upon Lipid Acyl Chain and Headgroup Structure

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Abstract of the Dissertation

The Dependence of Lipid Asymmetry upon Lipid Acyl Chain and Headgroup Structure

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Membrane lipid species are not identically distributed in the inner and outer leaflet of biological membranes. This metastable asymmetric distribution of lipids is called 'lipid asymmetry', and it is an important feature of many biological membranes. Investigation of various aspects of lipid asymmetry has been limited by a lack of robust methods to prepare stable artificial asymmetric vesicles. We recently developed a method in which methyl-beta-cyclodextrin (MβCD)-induced lipid exchange is used to prepare model membrane vesicles with lipid asymmetry and which, like eukaryotic plasma membranes, have an outer leaflet rich in sphingomyelin (SM) and have an inner leaflet rich in ordinary glycerophospholipids, such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. The range of this method has been extended to generate asymmetric vesicles containing SM in the outer leaflet and phospholipids with different acyl chain and headgroup structures in the inner leaflet. The structure of acyl chains and headgroups was systematically varied, and how this variation affected the capability to exchange lipids as well as the ability to form stable asymmetric vesicles was studied. It was found that the MβCD-induced method efficiently exchanged all the lipids tested. Furthermore, we observed that the formation of stable asymmetry (SM outside/various lipids inside) depended upon the structure of acyl chain and headgroup of lipids. To characterize

the origin of this behavior, the transverse diffusion (flip-flop) rate of lipids in vesicles with various acyl chain and headgroup structures was compared. It was found that there is a correlation between stable asymmetry and transverse diffusion, with transverse diffusion being slower in vesicles containing lipids from which stable asymmetric vesicles could be prepared. These studies show that asymmetric vesicles can be prepared using a wide, but not universal, range of acyl chains and headgroup structures. The presence of lipids that can undergo fast transverse diffusion prevents stable lipid asymmetry. These properties may constrain the species of lipids in natural membranes in which stable asymmetry is an important structural feature.

TO MY FAMILY, WITH LOVE

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16:0-18:2PC, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine

16:0-20:4PC, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine

ATP, adenosine triphosphate

C_6-NBD-PC, 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine

Cardiolipin, 1',3'-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol

di14:1PC, 1,2-dimyristoleoyl-sn-glycero-3-phosphocholine

di16:1PC, 1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine

di18:1PC, 1,2-dioleoyl-sn-glycero-3-phosphocholine

di18:2PC, 1,2-dilinoleoyl-sn-glycero-3-phosphocholine

di18:3PC, 1,2-dilinolenoyl-sn-glycero-3-phosphocholine

di20:1PC, 1,2-dieicosenoyl-sn-glycero-3-phosphocholine

di20:4PC, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine

di22:1PC, 1,2-dierucoyl-sn-glycero-3-phosphocholine

diphyPC, 1,2-diphytanoyl-sn-glycero-3-phosphocholine

DOPA, 1,2-dioleoyl-sn-glycero-3-phosphate

DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine

DOPG, 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)

DOPI, 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol)

DPH, 1,6-diphenyl-1,3,5-hexatriene

DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

HP-TLC, high performance thin layer chromatography

LB technique, Langmuir-Blodgett technique

LB/LS technique, Langmuir-Blodgett/Langmuir-Schafer technique

LB/VF technique, Langmuir-Blodgett/vesicle fusion

Ld, liquid disordered

Lo, liquid ordered

LUVs, large unilamellar vesicles

MLVs, multilamellar vesicles

MβCD, methyl-beta-cyclodextrin

NMR, nuclear magnetic resonance

PA, phosphatidic acid

PC, phosphatidylcholine

PE, phosphatidylethanolamine

PG, phosphatidylglycerol

PI, phosphatidylinositol

POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine

POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)

POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine

PS, phosphatidylserine

SM, sphingomyelin

Soy PI, soy phosphatidylinositol

SUVs, small unilamellar vesicles

Tm, melting temperature

TMADPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate

TOCL, 1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol

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Chapter 1

Introduction and Background

1.1. Lipid asymmetry in cell membranes

1.1.1. Introduction to Asymmetry

Typical biological membranes contain a lipid bilayer. Phospholipids are the fundamental building blocks of the lipid bilayer, which plays a critical role as a barrier that acts as the boundary of the cell, separating the cytosol from the outer environment and setting the boundaries its internal compartments. Membrane lipid molecules exhibit amphipathic properties; the hydrophilic polar head groups are facing outward and exposed to outer aqueous environment, while the hydrophobic non-polar tails aggregate to form the center of the membrane and are kept away from the aqueous environment (Figure 1.1). These two major important regions of lipid molecules determine the structural properties of a lipid bilayer and control its formation and stability.

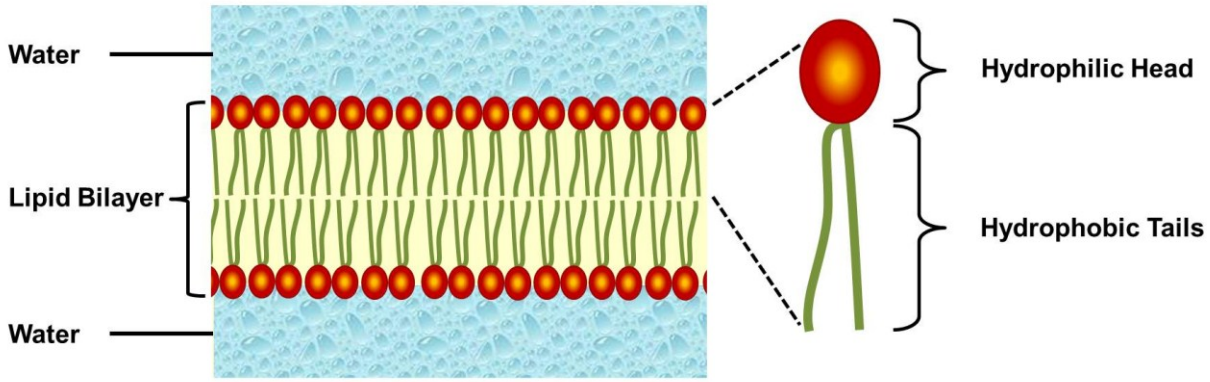


Figure 1.1. A lipid bilayer in an aqueous environment

The formation of a bilayer within an aqueous medium allows the hydrophilic polar headgroups of lipids to be exposed to the water, while the non-polar hydrophobic tail groups to be sheltered inside that is away from the water.

The major lipid components found in eukaryotic plasma membranes are sphingolipids, glycerophospholipids, and sterols. Sphingomyelin (SM) is the most predominant sphingolipid in mammals. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) are the most abundant glycerophospholipids (Figure 1.2 and Figure 1.3) [1-3]. For prokaryotic inner membranes, PE, PI, phosphatidylglycerol (PG) and cardiolipin are often the major lipid species [4].

Membrane lipid species are not identically distributed in the inner and outer leaflet of biological membranes. This metastable asymmetric distribution of phospholipids over the bilayer is called 'lipid asymmetry', and it is a general characteristic of many biological membranes (Figure 1.2). For example, in the eukaryotic plasma membrane, membrane lipids are asymmetrically distributed, such that the outer leaflet is predominantly composed of choline-containing phospholipids such as SM and PC, whereas the inner leaflet generally consists of the amine-containing phospholipids such as PE and PS (Figure 1.3) [1, 5-8]. This membrane lipid asymmetry is widely observed in eukaryotic cells from yeast to human erythrocytes, and it is

important for vital membrane functions [9-12]. Asymmetry is also believed to be present in prokaryotic cells. Although it is difficult to determine lipid distribution in the leaflets of the membrane surrounding the bacterial cytoplasm, it is reported that PI is confined in the inner leaflet, PG is mainly located in the outer leaflet, and cardiolipin is almost symmetrically distributed over both leaflets in gram-positive bacteria *Micrococcus lysodeikticus* [13]. Studies from *Bacillus megaterium* inner membrane suggest PE is concentrated in the inner leaflet [14]. In *Bacillus amyloliquefaciens*, it is found that the majority of cardiolipin is localized in the inner leaflet, while both PE and PG mainly reside in the outer leaflet [15]. In the outer membranes of gram-negative bacteria lipopolysaccharides are predominantly localized in the outer leaflet, while phospholipids are relatively confined to the inner leaflet [16-18].

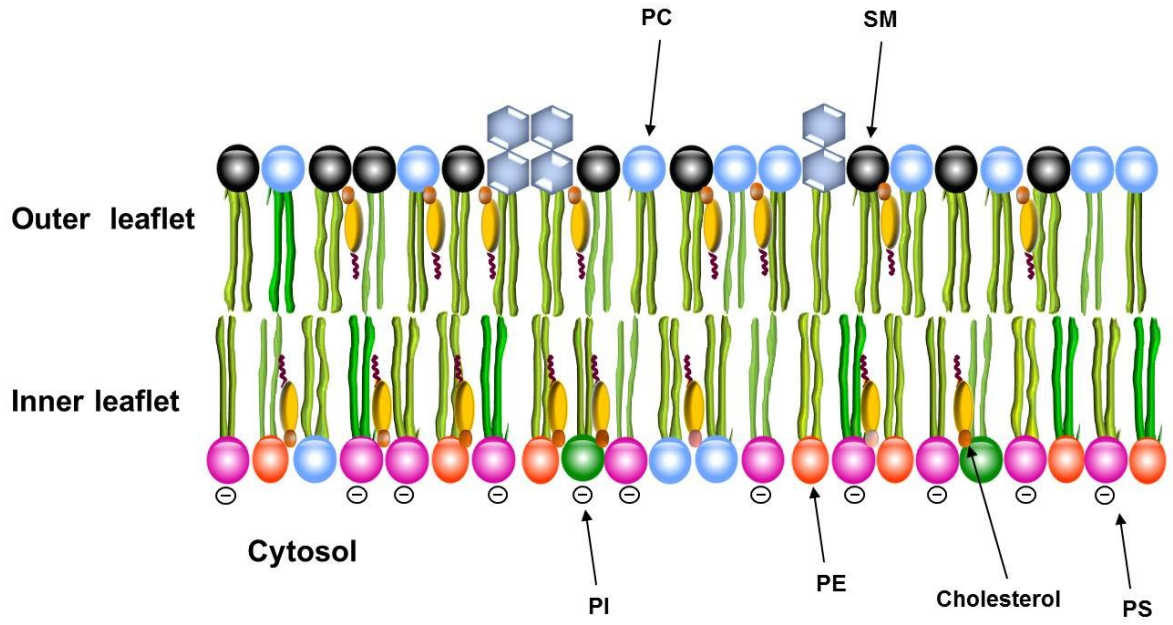


Figure 1.2. Lipid asymmetry in eukaryotic plasma membrane

The distribution of lipids between the lipid bilayer is shown. The outer leaflet is mainly composed of SM and PC, while the inner leaflet is rich in PE and PS.

The asymmetric distribution of lipids in membrane bilayers is initiated primarily during membrane biosynthesis and maintained throughout the life span of the cell. The structural lipids of eukaryotic cells are synthesized in a geographically restricted way. Most of the enzymes responsible for structural lipids synthesis and subsequent turnover are located in the cytosol and on the cytoplasmic surface of the membrane [19]. The major glycerophospholipids (PS, PE, PC, and PI) are synthesized on the cytosolic side of the endoplasmic reticulum (ER), whereas sphingolipids, including SM, galactosylceramide, and complex sugar-containing sphingolipids, are synthesized on the luminal surface of the ER or Golgi, although the initial step takes place on the cytofacial side [19-22].

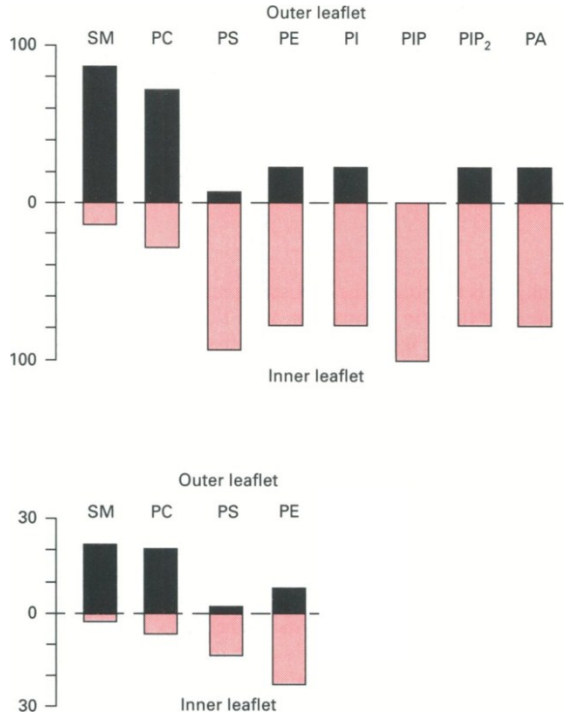


Figure 1.3. Asymmetrical distribution of phospholipids in the human erythrocyte membrane

Top: distribution is given as a percentage of each phospholipid, bottom: distribution is given as a percentage of total phospholipids. This figure is taken and adapted from Zachowski, 1993 [9].

Once lipid asymmetry has been generated, it is preserved by multiple factors, including an extremely slow rate (e.g. PC has typical half-time of several hours to days [8, 9]) of random spontaneous translocation of lipids across the bilayer (Figure 1.4) and the presence of ATP-dependent selective lipid transport proteins (Figure 1.5) [19, 23-26], together with asymmetrical lipid turnover by endogenous phospholipases [1].

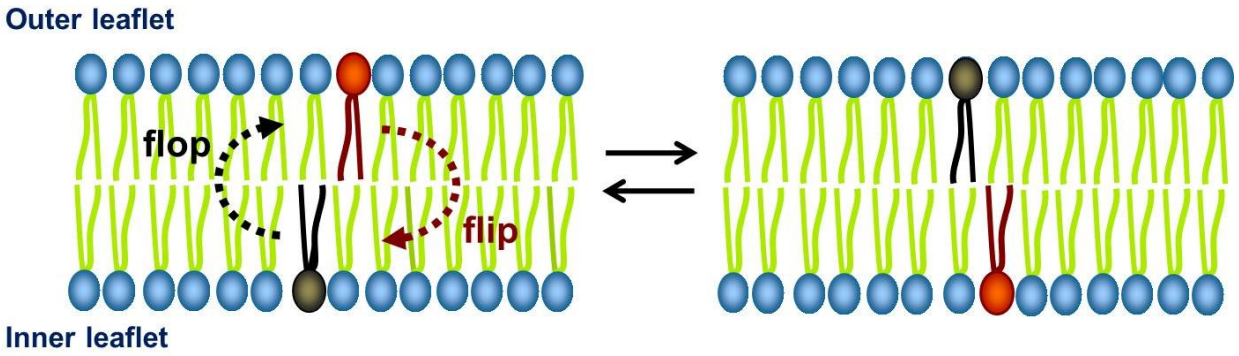


Figure 1.4. Schematic representation of spontaneous transbilayer movement (also known as passive transverse diffusion or flip-flop) across lipid bilayer

1.1.2. Passive Flip-Flop

The movement of hydrophilic polar headgroups across the hydrophobic membrane interior is an energetically unfavorable process. This thermodynamic barrier prevents fast spontaneous transbilayer movement (also known as passive transverse diffusion or flip-flop, Figure 1.4). Another factor that makes the rate of passive transverse diffusion slow is the increased pressure generated by the new phospholipid insertion to only one leaflet of the bilayer

[27]. The uncontrolled process of the lipid removal from one leaflet and insertion into the other leaflet causes lipid quantity imbalance, and would consequently results in abnormally high lateral pressure which is energetically disfavored.

The rate of spontaneous lipid flip-flop is determined by the biophysical properties of both the lipid molecule and the host membrane environment where the lipid molecule exists. For the lipid molecules, features such as the shape, head group polarity, acyl chain length and degree of unsaturation are well-known factors that contribute to the rate of passive transverse diffusion [22, 28-31]. An increase in the size and the number of polar or charged moieties of the headgroup generally reduces the transverse diffusion rate since it raises the free energy barrier for lipid molecule to move across the membrane. However, some weakly acidic phospholipids such as PG and PA can flip flop between leaflets easily when their charged headgroup is neutralized by protonation [32-36]. There is evidence that PG or PA can be effectively trapped in the inner leaflet of artificial vesicles with basic pH interior and acidic pH exterior [33-36]. In a similar fashion, these lipids could be localized in the outer leaflet of vesicles by reversing the transmembrane pH gradient with inside acidic and outside basic [33-36]. In general, host membranes with shorter acyl chains allow a faster rate of flip-flop since they have a lower free energy barrier to across membrane. It is reported that increasing acyl chain length reduces the rate of transverse diffusion for PC analogs, showing a two fold decrease in rate for every two methylene carbons added to the acyl chain at the sn-1 position [31]. Moreover, the host membrane environment where the lipid molecule exists controls the pace of passive transverse diffusion, and the factors include the thickness of the hydrophobic core of the bilayer [37], physical phase [37, 38], degree of lipid packing [39], and the presence of membrane peptides [40-47]. It was reported there is a decrease in the rate of passive transverse diffusion when the phase transition was suppressed by cholesterol, presumably due to reducing molecular packing defects at the phase transition, suggesting that molecular packing defects at the main phase transition may mediate the fast flip-flop [38]. Membrane peptides affecting the rate of passive transverse diffusion include transmembrane peptides [40-43, 47] and pore-forming [44-46]

peptides. To elucidate the mechanism of membrane peptide mediated flip-flop, it was proposed that the incorporation of membrane peptides in a lipid bilayer may lower the thermodynamic barrier to move across the membrane by a lipid bilayer perturbation mechanism in the vicinity of the hydrophobic peptide [41], by disrupting membrane lipid packing [40, 45] or by headgroup insertion into one leaflet of the bilayer, which leads to membrane thinning [42].

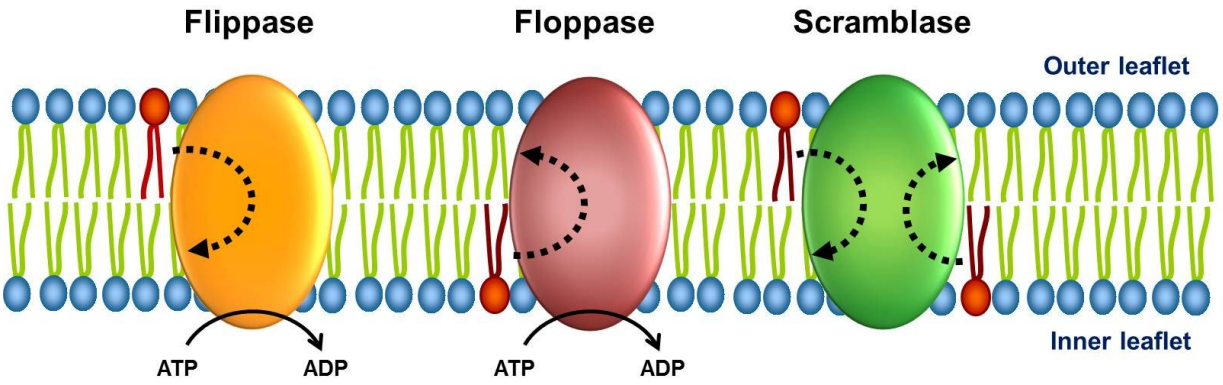


Figure 1.5. Protein-mediated transmembrane movement of lipids between the two leaflets of lipid bilayer

Translocation of lipids in eukaryotic plasma membrane by ATP-dependent monodirectional (inward movement by flippase, and outward movement by floppase), and ATP-independent bidirectional (scramblase) transport proteins is shown.

1.1.3. Active Flip-Flop

The establishment and maintenance of membrane asymmetry cannot be mediated by passive transverse diffusion alone. Numerous proteins have evolved to assist or collapse this lipid asymmetry. Several different lipid transport proteins have been known to regulate asymmetric transbilayer distribution of lipids between two leaflets of the plasma membrane. Lipid transport proteins facilitate the energetically unfavorable transbilayer movement of a lipid's polar headgroup across the hydrophobic membrane interior (Figure 1.5). They can be classified into three groups on the basis of lipid specificity, direction of transport, and energy

requirement; flippases, floppases, and scramblases [25, 27, 48, 49]. These three lipid transport proteins have different functions.

First, flippases (e.g. aminophospholipid translocases) are ATP-dependent transporters which catalyze the movement of substrates such aminophospholipids (PE and PS) from the outer to inner leaflet of eukaryotic plasma membrane against a concentration gradient [50, 51]. It is also reported that even PC can be transported by flippases in mammalian cells [50].

Second, floppases (including some ATP-binding cassette transporters) are ATP-dependent transporters which stimulate the movement of sphingolipid and PC from the inner to outer leaflet of plasma membrane against a concentration gradient. Generally, the rate of outward movement by floppase is slower with a half time on the order of 1.5 h than the rate of inward movement of PE and PS by flippase, with half-times of approximately 5-10 min [49].

Finally, scramblases function as a plasma membrane blender, since they promote bi-directional movement of lipids between two leaflets along a concentration gradient in an ATP-independent fashion. While the cooperative action of flippases and floppases is responsible for generating and maintaining membrane lipid asymmetry, scramblases facilitate the collapse of this asymmetry rather than assist in the maintenance of it. Scramblases do not require energy and are less substrate specific. They are triggered by an elevated cytoplasmic calcium concentration, and it has been shown that removal of intracellular calcium turns off the scrambling process [19, 25, 49, 52, 53]. The persistent and continuous elevation of cytoplasmic calcium is a key regulatory factor for scrambling activity, but the increased cytoplasmic calcium level is not directly associated with enhancement of phospholipid scrambling activity or rate [49, 54, 55]. Scramblases have critical functional roles in the controlled randomization of asymmetric lipid distribution especially during apoptosis [56] and blood coagulation [25, 57].

1.1.4. Functions of Lipid Asymmetry

The asymmetrical distribution of lipids in natural membranes has important functional roles. For example, it has been proposed that the phospholipid asymmetry in the erythrocyte membrane is required to maintain discoid shape [58]. The role of platelets in blood coagulation has been correlated to lipid asymmetry [57, 59-62]. The coagulation cascade is greatly accelerated by a negatively charged phospholipid membrane surface, and normally this is provided by exposure of PS, which is a cofactor for prothrombin to thrombin conversion on the cell surface in the course of platelet-mediated blood coagulation. Scott syndrome, a severe inherited bleeding disorder, is caused by defective Ca^{2+}-induced phospholipid scramblase activity that is not associated with decreased level of coagulation factors [61, 63-65]. The loss of lipid asymmetry, measured as PS externalization on the outer leaflet of membrane, serves as a susceptibility signal for their recognition and engulfment by clearing macrophages, and is the most prominent feature early in the process of apoptosis [66-74]. Both PE and PS appear on the outer surface of the cell during apoptosis, but PS is more highly restrained to the inner leaflet of the membrane in normal cells. PS translocation on the cell surface at the plasma membrane has been also observed during sperm capacitation [75, 76], myotube formation [77], and is considered to be critical for some types of signal transduction [78]. Loss of PS asymmetry in human erythrocytes has been also known to be induced by hyperglycemia [79].

Lipid asymmetry is also thought to be important for membrane fusion [80-82]. For example, studies indicated that loss of lipid asymmetry in human erythrocytes facilitates fusion of vesicular stomatitis virus with erythrocytes [83]. In addition, interactions between PS and membrane skeletal proteins like spectrin serve to localize the spectrin to the cytoplasmic face and modulate the mechanical stability of the membrane [84-86]. Loss of asymmetry by outward migration of PE in the plasma membrane of myocardial cells induces a loss of membrane stability and integrity due to the physicochemical properties of hexagonal II phase preferring PE (Figure 1.6), and eventually leads to irreversible membrane damage [87-90]. PE can be a non-

bilayer-preferring phospholipid, a feature is originating from its cone shape structure (smaller cross sectional area at the headgroup than the tail) of PE molecule [91, 92]. Thus, uncontrolled transbilayer movement of PE from the inner to the outer leaflet of membrane is believed to destabilize the lipid bilayer due to increased pressure [30].

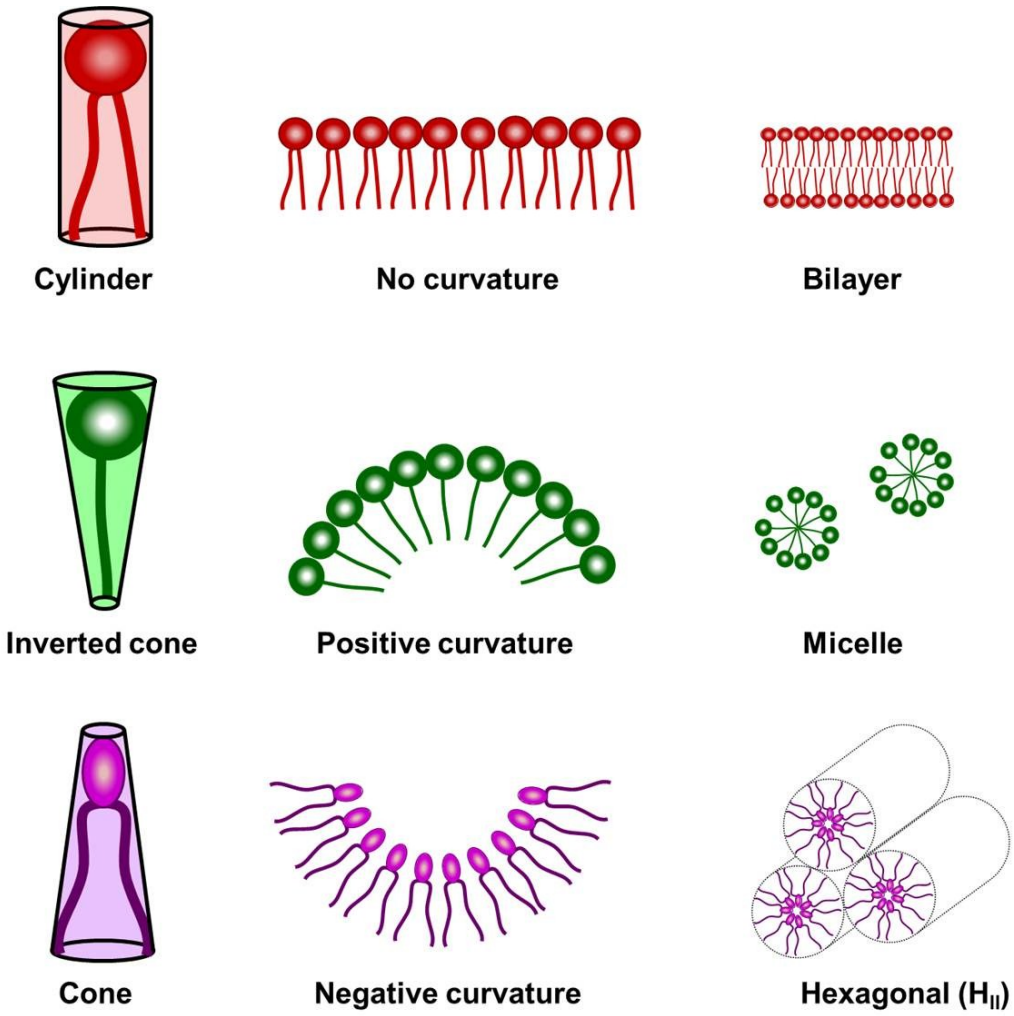


Figure 1.6. Molecular geometry of lipids

The shape of lipids depends on the relative volume of hydrophilic headgroup and hydrophobic tail(s). Using this approach, lipids can be classified as cylinders (e.g., PC) inverted cones (e.g., lysophosphatidylcholine), and cones (e.g., PE). Geometrical models of lipid shapes (left), monolayers formed by lipids with different molecular shapes (middle), and phase preference (right) are shown.

1.2. Lipid phase behavior of membranes

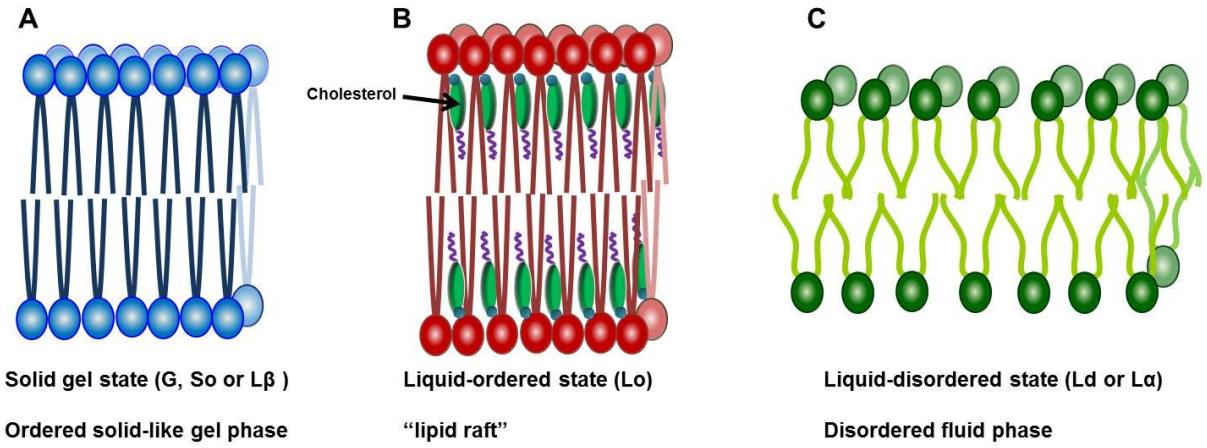


Figure 1.7. Schematic illustration of different physical states in membrane bilayers

(A) The lipids in gel state are tightly packed, and the lateral motion is strongly restricted. **(B)** The lipids in liquid-ordered state are tightly packed like gel state, but have fast lateral motion like Ld state. **(C)** The lipids in Ld state are loosely packed, and have fast lateral diffusion.

One of the major characteristic properties of lipids is their phase behavior. Lipid bilayers can exist either in the liquid state or solid-like gel state depending on the temperature and the structure of the lipid within the bilayer (Figure 1.7) [93]. In the gel state, the lipid molecules are tightly packed and exhibit slow lateral motion. In contrast, the lipid molecules are loosely packed and have fast lateral motion in liquid fluid state which is also called liquid crystalline state (La), or liquid disordered state (Ld). The phase transition from the gel state to fluid state occurs at a specific temperature called the melting temperature (Tm). Tm depends on lipid structure, including both the headgroup and acyl chain structure of lipids, together with the environment in which the lipids exist [93]. Among these properties, acyl chain structure has a

particularly prominent effect on the Tm. Lipids with long, saturated acyl chains, including SM, generally have high Tm, while those with short, unsaturated acyl chains have low Tm [93]. From model membrane studies, it has been determined that cholesterol combined with high Tm lipids such as SM can form the liquid ordered state (Lo), even though cholesterol itself cannot form bilayer phases [24, 93-96]. In the liquid ordered state, which is believed to be that of lipid rafts in cells, the lipid molecules are tightly packed like in gel state, but their lateral motion is almost as fast as in liquid fluid state [93]. It has been known that in model membranes, the lipid mixtures of both saturated and unsaturated lipids can form co-existing gel and Ld domains [97]. Moreover, a high concentration of cholesterol in membranes can promote co-existing Lo and Ld domains as well [96, 98].

1.3. Model membrane systems

Model membrane systems have been developed to obtain insights into membrane structure and function. They have been widely used since model membranes can eliminate the complexity and diversity of biological membranes, so that the physical properties and functional roles of specific components can be easily assessed. Model membrane systems allow us to control systematically individual variables such as lipid composition, protein, or other biological components. There are many different model membrane systems developed so far, but this chapter mainly focuses on vesicles (also known as liposomes, Figure 1.8) which are relevant to the studies undertaken in this thesis. The distinguishing characteristics of different vesicles such as size, the number of layers, method of construction, and function are described below.

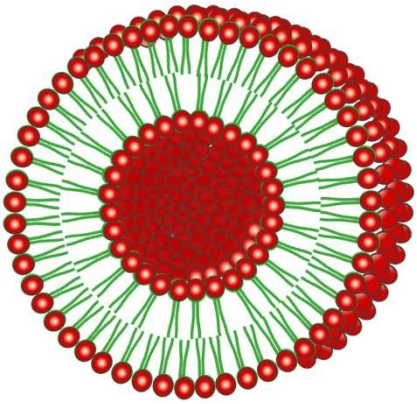


Figure 1.8. Illustration of a vesicle composed of a lipid bilayer

Vesicles are a closed water-filled spherical-shaped model membrane system.

One of the model membrane systems closest to biological membranes in structure are vesicles. Vesicles have a water-filled three dimensional sphere structure consisting of one or more lipid bilayers (Figure 1.8). They are commonly formed when dried lipids are mechanically

dispersed into aqueous solution. Vesicles have been widely used as valuable investigative tools due to their well-characterized membrane features and simple preparation. They have diverse applications in various fields of science and technology such as a delivery system for therapeutic agents, cosmetics, and proteins [99]. In addition, vesicles serve as a good model for complex cell membranes. Membrane peptides and proteins can be easily incorporated into vesicles [100] and phase separation [101] is easily manipulated.

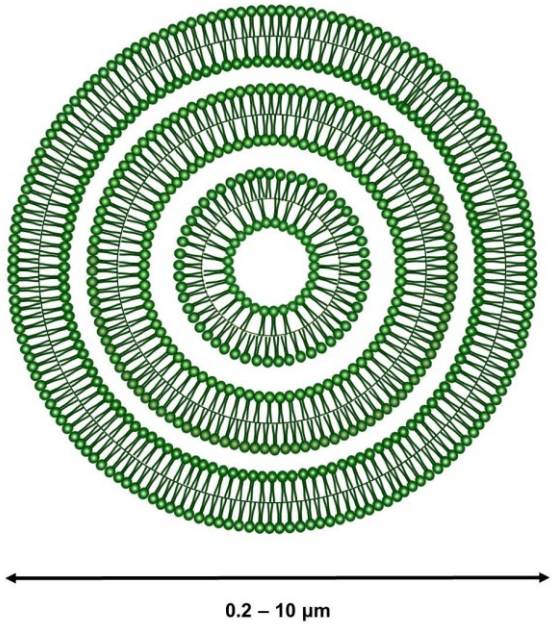


Figure 1.9. Schematic picture of multilamellar vesicles (MLVs)

MLVs exhibit onion-like structure which consists of multiple single bilayers separated by aqueous medium. MLV drawn here is composed of three bilayers, but the typical number of layers ranges from 5 to 20.

Vesicles are classified into two classes by their number of membrane bilayers (lamellae); multilamellar or unilamellar vesicles. Multilamellar vesicles (MLVs) are composed of multiple bilayers separated by narrow aqueous solutions (Figure 1.9). Their diameters range from 0.2 to

10 μm [99, 102]. The number of layers formed depends upon the classes of lipids used and the experimental technique, but the typical layers range from 5 to 20 bilayers. MLVs are the type of vesicles that are prepared most easily just from the process of hydration of thin lipid films by adding aqueous buffer and agitating the dispersion for some time. MLVs have been used extensively to determine the details of bilayer structure due to their wide range of possible lipid compositions and facile preparation. However, MLVs usually exhibit a broad range of size distributions and variations in the number of layers (many internal compartments and lack of well-defined single bilayer). For these reasons, MLVs have limits of their use for studies in which homogenous size distribution or unilamellarity is paramount, for example, studies of membrane properties such as permeability and fusion [103]. Methods have therefore been developed to produce single bilayer vesicles.

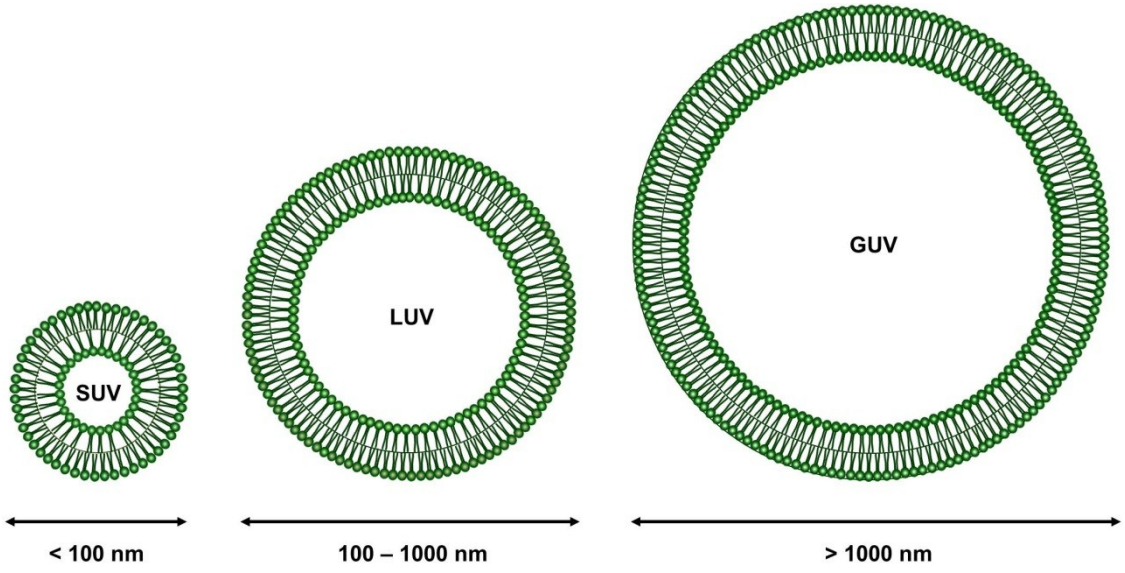


Figure 1.10. Schematic illustration of unilamellar vesicles

Unilamellar vesicles can be further classified by their sizes. SUV (Small Unilamellar Vesicles), LUV (Large Unilamellar Vesicles), and GUV (Giant Unilamellar Vesicles) are shown.

Unilamellar vesicles have only one single bilayer in each vesicle and they are classified into three groups on the basis of their sizes (Figure 1.10); small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), and giant unilamellar vesicles (GUVs). SUVs have diameters below 100 nm, LUVs range from 100 nm to 1000 nm, GUVs above 1000 nm [102, 104]. Different vesicle types are generated by a variety experimental techniques and lipid mixtures [103, 105, 106]. SUVs can be produced by sonication of MLVs [107-109], French press [110], or ethanol (or methanol) injection and dilution methods [111]. LUVs are prepared via freeze/thaw and repeated extrusion of MLVs through polycarbonate filters of defined pore size [105, 112, 113], dilution from certain organic solvents [111, 114-116], or detergent dialysis methods [117-120]. GUVs are typically made by electroformation [121, 122], detergent dialysis [123], or dehydration/rehydration [124]. Unilamellar vesicles are considered biologically more relevant than MLVs due to the presence of only one single bilayer in each vesicle. SUVs and LUVs are preferred model membrane systems for most spectroscopic studies due to their simple preparation method, production in large quantity, and especially for LUVs, high encapsulation efficiency for hydrophilic solutes. SUVs are suitable for the studies that need a high degree of curvature, which arises from their small radius. Due to the high radius of curvature of SUVs, the ratio of lipid in the outer leaflet to that in the inner leaflet of bilayer can be as high as almost 2:1. In a mixture of lipids, the distribution of lipid in the outer and inner leaflet of the bilayer can be asymmetric in SUVs (details are described in below). Therefore, the possibility of asymmetric distribution between inner and outer leaflet should be considered before using SUV. The small size of SUVs is advantageous for certain drug delivery applications. They can be also easily separated from MLVs by simple processes such as centrifugation or size exclusion chromatography. In contrast, LUVs exhibit a significantly higher trapped volume than SUVs and have a relative lack of curvature due to their relatively large size, making them suitable for various applications in membrane studies. The size of GUVs allows straightforward observation under a fluorescent or confocal microscopy, so they provide direct visualization of membranes, including details about large-scale domain structure. GUVs have been extensively used for studying topological and morphological appearance of lipid domains in membrane including

lipid phase separation, lipid raft domain formation and peptide interaction with domain as detected by the partitioning of fluorescent probes into gel, liquid-ordered (Lo), and liquid-disordered (Ld) phases [125-129].

1.4. Artificial asymmetric model membranes

As noted above, it is well known that many biological and physicochemical properties of cellular membranes are influenced by their asymmetric lipid distribution. Investigation of various aspects of lipid asymmetry and related research has been restricted in biological membranes due to factors such as complexity, diversity and rapid redistribution of phospholipid between leaflets under some conditions.

Model membrane systems should be useful tools to study lipid asymmetry since charge/size of lipid headgroup, degree of acyl chain saturation, membrane thickness, membrane physical phase, and reconstitution of membrane peptides can be easily manipulated in a controlled environment. However, they are limited as models of cell membranes since the artificial membranes generally constructed in the lab are symmetric, with identical lipid distribution in the outer and inner leaflet of bilayer, and thus lack the asymmetric lipid distribution of plasma membranes. Developing an asymmetric model membrane system would be ideal for studying the full implications of membrane asymmetry in a system biologically more relevant than symmetric vesicles. Significant progress has been made in developing and producing asymmetric model membrane using various experimental procedures. These are briefly reviewed in the following section.

Asymmetric supported lipid bilayer systems in which the bilayers are supported by solid substrates such as fused silica, glass, quartz, oxidized silicon [130], and mica [131] have been developed using different techniques. For instance, it is reported that asymmetric lipid distribution was formed by adsorption and spontaneous fusion of vesicles from an aqueous solution to a substrate that has a lipid coating surface, a common method to build supported lipid bilayers [131, 132]. Asymmetric supported lipid bilayers were also generated by sequential depositing of lipid monolayers using Langmuir–Blodgett/Langmuir–Schäfer (LB/LS) or the Langmuir–Blodgett/vesicle fusion (LB/VF) methods [133-135]. In LB/LS technique, assembly

of bilayer is prepared using LB deposition via an upstroke of a hydrophilic substrate through the compressed lipid monolayer film at the air-water interface of a Langmuir trough, followed by LS deposition, which involves a dipping the LB monolayer coated substrate by horizontal contact to create second monolayer [130, 136]. In LB/VF technique, a bilayer is formed by incubation of the LB monolayer with vesicles [137].

Asymmetric unsupported planar lipid bilayers which are freestanding membrane systems have been constructed using different approaches. Asymmetric bilayers with lipopolysaccharides (LPS) on one side and phospholipids on the other side which mimic the outer membrane of gram negative bacteria [138-140], were prepared via the Montal-Mueller technique [141] and the Schindler technique [142]. Both the Montal-Mueller technique and the Schindler technique are widely used to produce unsupported planar bilayer systems, in which bilayers are formed over a small hole that connects two aqueous chambers [141, 142]. In the Montal-Mueller technique, the bilayer is prepared from lipid monolayers by spreading non polar solutions of lipids on the surface of each aqueous phase chamber, and the Schindler technique is the essentially same except that the monolayers is formed by adding lipids to the chamber in the form of vesicles [141, 142]. Collins *et al.* generated asymmetric unsupported planar bilayers via Montal-Muller assembly [143].

Generating asymmetric model membrane systems has been further extended by development of droplet interface bilayers, which are the membrane systems that are formed by connecting two lipid monolayer-coated aqueous droplets submerged in an oil-lipid mixture [144]. By extending this method, Hwang *et al*. recently generated asymmetric droplet interface bilayers by joining two aqueous droplets containing vesicles of different lipid compositions [145].

Supported bilayer systems have many advantages, but their use is sometimes limited due to the artifacts induced by supporting materials. Asymmetric unsupported planar bilayers are

free from an underlying support, but also result in lipid bilayers that can be highly mobile, which can lead to unstable asymmetry having a short lifetime. In addition, there is always a concern that there will be an influence of residual organic solvent, since unsupported bilayer systems generally produced incorporating with it.

Although great advances in producing asymmetric membranes have been made, asymmetric lipid vesicles would be the most ideal and effective system for studying various properties of lipid asymmetry. There have been a number of technological developments allowing creation of asymmetric vesicles. One of simple ways to generate some degree of spontaneous asymmetric lipid distribution in vesicles is by a process of sonication which makes vesicle size quite small, and sonicated SUVs have been used to study lipid asymmetry [146-148]. Phospholipids with a large headgroup were found to preferentially orient in the outer leaflet of sonicated SUVs, whereas those with smaller headgroup localize in the inner leaflet. For example, PE which has relatively small headgroup tends to locate in the inner leaflet of the membrane rather than being in the outer leaflet due to its molecular structure (Figure 1.6). This phenomenon presumably results from the different molecular packing arrangement in small vesicles with high curvature like sonicated SUVs. Studies have also shown that a transmembrane pH gradient can be utilized to form an asymmetric redistribution of a small percentage of charged lipids in vesicles [33, 36, 149, 150].

Preparation of asymmetric vesicles with more controlled lipid composition and asymmetry can be possible through a variety of methods. Asymmetric model membranes were produced utilizing enzymes to catalyze chemical reaction of outer leaflet lipids in vesicles. For example, Low *et al.* used phosphatidylinositol-specific phospholipase C to remove phosphatidylinositol selectively in the outer leaflet of SUV, thus producing asymmetric bilayers [151]. In addition, Denkins *et al.* generated asymmetric LUV using phosphatidylserine decarboxylase to convert NBD-PS in the outer leaflet of vesicles to NBD-PE [152]. Methods have been also developed to prepare asymmetric vesicles via layer-by-layer assembly by forming

lipid monolayer at a water-oil interface [153, 154]. Richmond *et al*. reported that asymmetric GUV can be formed by selective incorporation of Ni-chelating lipids into the inner or outer leaflet of the GUV [155]. Pagano *et al.* transferred lipids into the outer leaflet of bilayer via a spontaneous lipid exchange technique to create asymmetric distribution of fluorescent lipid analogues in vesicles [156]. The approach Pagano *et al.* used is based on rapid spontaneous transfer of certain lipid analogues between two vesicle populations. In a similar manner, asymmetric lipid distribution in vesicles was also created with the utilization of a lipid exchange/transfer protein which delivers lipids to the outer leaflet of vesicles [151, 157].

This concept has been further developed in our laboratory to build stable asymmetric vesicles effectively using methyl-beta-cyclodextrin (MβCD) [158-160]. MβCD is a cyclic toroid-shaped oligosaccharide consisting of seven glucopyranose units linked by (1→4) glycosidic bond (Figure 1.11). They feature a hydrophilic outer surface with a hydrophobic internal cylindrical cavity, and this central lipophilic environment enables binding and solubilizing a wide range of hydrophobic molecules. For this reason, MβCD has been used extensively for drug delivery and depletion of sterols from model membranes as well as living cells [161-165]. It has been found that the use of cyclodextrins for extracting lipids is not limited to sterols. Preparation of asymmetric vesicles utilizing cyclodextrins is derived from the observation of the binding of MβCD to phospholipid using their interior cavity center at a high concentration of MβCD [166]. More recently, studies found that MβCD can mediate efficient transfer of 14 carbon chain phospholipids from vesicles to cells without compromising cell growth and viability [167]. It is also reported γ-cyclodextrins, which are composed of eight glucopyranose units, greatly enhanced transfer of hydrophobic fluorescent phospholipids between lipid vesicles or from vesicles to living cells by forming soluble complex of cyclodextrins and various lipids [168, 169].

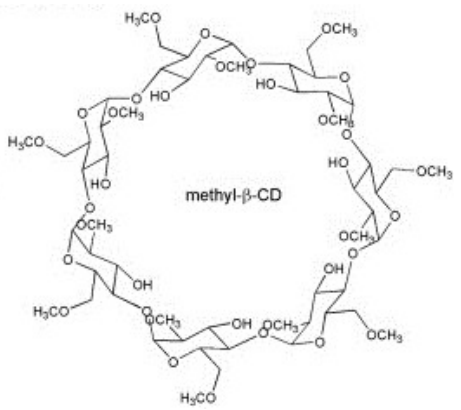


Figure 1.11. Structure of methyl-β-cyclodextrin (MβCD)

This figure is taken and adopted from Peng *et al,* 2012 [170].

With the utilization of MβCD, stable asymmetric vesicles have been created by our group (Figure 1.12) and this system has great potential for various applications since the vesicles can be produced in large quantity, exhibit stable asymmetry, and make it easy to reconstitute membrane peptide with simple methods. One of the primary advantages of MβCD-induced lipid exchange method is it also allows constructing stable asymmetric vesicles of various size, including SUV [158, 171], LUV [159] and GUV [160].

Asymmetric SUVs containing saturated phospholipids (SM and DPPC) in the outer leaflet and unsaturated phospholipids (POPE/POPS, POPS, POPC and DOPC) in the inner leaflet was prepared in our laboratory using MβCD-dependent lipid exchange methods [158]. It was found that the thermal stability of the gel state in a SM-rich outer leaflet was not decreased by contact with an inner leaflet rich in unsaturated lipids in the Ld state. Methods to prepare asymmetric LUVs, which have less membrane curvature than SUVs, were also developed, and indicated that the findings from asymmetric SUVs were not due to their curvature [159]. By creating asymmetric GUVs, we could investigate coupling between the physical properties of the

inner and outer leaflets via microscopy methods [160]. The ability to produce asymmetric vesicles represents an important advance in model membrane studies and should allow a diverse range of applications.

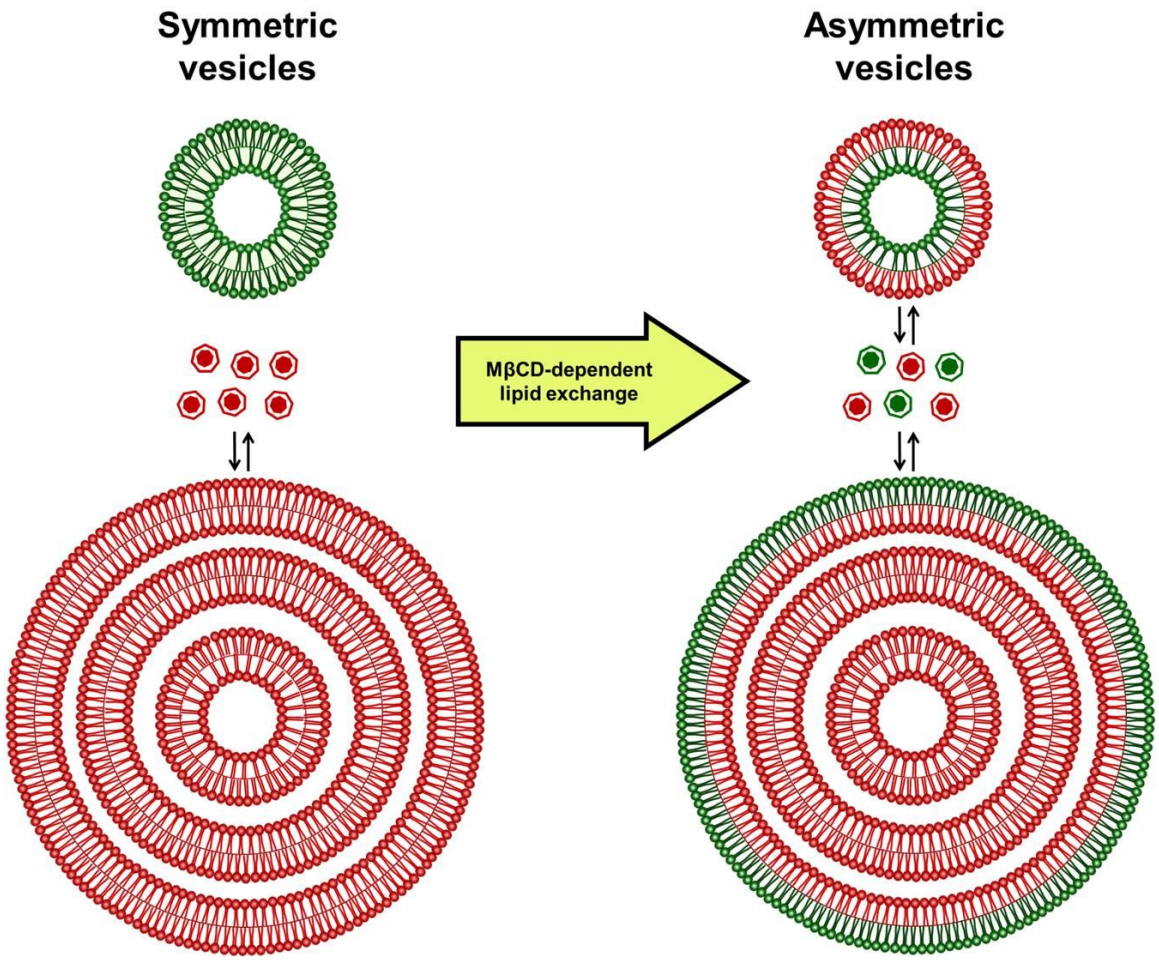


Figure 1.12. Schematic illustration of MβCD-dependent lipid exchange method

MβCD binds phospholipids using their central hydrophobic cavity, and exchanges lipids in the outer leaflet of each vesicle.

1.5. Goal of this work: Prepare asymmetric vesicles using lipids with various phosphatidylcholine acyl chain and headgroup structure

Asymmetric vesicles using MβCD-induced lipid exchange have been prepared, but the range of their inner leaflet lipid composition was limited only to POPE/POPS, POPS, POPC and DOPC in previous studies. In this thesis, I investigated the effect of lipid acyl chain and headgroup structure upon the ability to form asymmetric membranes by constructing vesicles containing SM in the outer leaflet and various species of lipids in the inner leaflet. A clear dependence of the ability to form stable asymmetric vesicle on the structure of lipid used as inner leaflet lipid was observed. I then investigated the origin of this behavior by comparing transverse diffusion of lipids in vesicles with various lipid structures. The results of these studies provide interesting insights into the effects of lipid structure upon lipid function.

Chapter 2

Materials and Methods

2.1. Materials

Porcine brain sphingomyelin (SM), 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (di14:1PC), 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (di16:1PC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC or di18:1PC), 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine (di20:1PC), 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (di22:1PC), 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (diphyPC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0-18:2PC), 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (16:0-20:4PC), 1,2-dilinoleoyl-*sn*-glycero-3-phosphocholine (di18:2PC), 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine (di18:3PC), 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine (di20:4PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DOPG), Soy L-α-phosphatidylinositol (Soy PI), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-myo-inositol) (DOPI), 1',3'-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol (Cardiolipin,

TOCL), 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine (C_6-NBD-PC) were purchased from Avanti Polar Lipids (Alabaster, AL). 1,6-diphenyl-1,3,5-hexatriene (DPH) , methyl-β-cyclodextrin (MβCD), and sodium hydrosulfite (sodium dithionite) were from Sigma-Aldrich (St. Louis, MO). 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMADPH) was from the Molecular Probes division of Invitrogen (Carlsbad, CA). All lipids were stored dissolved in chloroform and stored at –20 °C. DPH and TMADPH were dissolved in ethanol and stored at –20 °C. Concentrations of lipids were measured by dry weight. The concentration of DPH and TMADPH was determined by absorbance using an ε of 84,800 $cm^{-1}M^{-1}$. Methyl-β-cyclodextrin (MβCD) was dissolved in PBS buffer (pH 7.4). Sodium hydrosulfite was freshly prepared in 1.0 M Tris (pH 10). Sepharose CL-4B and Sepharose CL-2B were purchased from Amersham Biosciences (Piscataway, NJ). High performance thin layer chromatography (HP-TLC) plates (Silica Gel 60) were purchased from VWR International (Batavia, IL). The NMR tubes were purchased from Wilmad LabGlass (Wilmad Glass Co, Buena, NJ). The digital thermometer was purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Methods

2.2.1. Formation of Exchange (or Asymmetric) SUVs

Exchange small unilamellar vesicles (SUVs) were formed using a protocol adapted from that described in Cheng *et al*. [158]. First, donor multilamellar vesicles (MLVs), the vesicles from which bSM is exchanged with the outer leaflet of the acceptor SUVs, were prepared. We prepared donor MLVs composed of 16 mM bSM by drying the lipid in a film under a nitrogen gas stream followed by high vacuum for at least 1 h, and then hydrating at 70 °C with 500 µl of PBS (1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4). This sample was vortexed in a multitube vortexer (VWR International) at 55 °C for 15 min and then centrifuged at 11,000 × g for 5 min at room temperature to remove any remaining small vesicles. After removing the supernatant, the pellet was resuspended using 500 µl PBS, and then then 95 µl of 390 mM MβCD dissolved in PBS buffer was added. This SM MLV and MβCD mixture was vortexed in the multitube vortexer at 55 °C for 2 h.

Next, we prepared acceptor vesicles, which provide the inner leaflet lipids of the exchange vesicles. First, 600 µl of 8 mM MLV was prepared as described above. After vortexed for 15 min at 55 °C, the MLV was sonicated to make small vesicles in a bath sonicator (Special Ultrasonic Cleaner Model G1112SP1, Laboratory Supplies Co., Hicksville, NY) at room temperature for at least 15 min until the solution became clearly transparent. This clear sonicated SUV solution was diluted to 4 mM with PBS.

The 500 µl of 4 mM sonicated SUVs were added to the SM MLV-MβCD mixture above and vortexed at 55 °C for 30 min. The samples were cooled down to room temperature and then centrifuged at 49,000 × g for 5 min using an air-driven microultracentrifuge (Beckman Airfuge) to remove MLV. The supernatant was added to the Sepharose CL-2B column (dimensions, 25-cm length and 1-cm diameter), and 1ml fractions collected, with SUVs mainly eluting into

fractions number 16-19. These fractions were combined for further analysis. The lipid concentration in these combined fractions was generally ~150 μM as judged by measuring spot intensity of HP-TLC plate relative to standards with known amounts of lipid after charring plates. We assumed the concentration of exchange vesicle was ~150 μM lipid in all experiments.

2.2.2. High Performance Thin Layer Chromatography (HP-TLC)

Lipids were extracted with chloroform/methanol (1:1 v/v) from ordinary and exchange vesicles as described in [159]. The extracted lipids or pure lipid standards were applied to HP-TLC (Silica Gel 60) plates which was heated at 100 °C for 30 min and cooled down to room temperature. For separating SM and PC, the solvent system 50:38:8:4 chloroform:methanol: water:acetic acid (v/v/v/v) prepared in chamber was used. For separating SM from PG, cardiolipin, PI, or PA, the solvent system 65:25:4 chloroform:methanol:water (v/v/v) was used. When the solvent reached at the upper line of TLC plate, the plate was removed from the chamber and dried in the fume hood. The lipids loaded onto the TLC plate was visualized after spraying 3% (w/v) cupric acetate, 8% (v/v) phosphoric acid solution, followed by drying and charring at 180 °C for 2-10 min. The lipids in the vesicles were quantified by measuring TLC spot intensity vs. a standard curve for each lipid generated on the same TLC plate as described previously [158].

2.2.3. Percent SM in Exchange Vesicles Calculation

For the exchange vesicles of SM and PC with various acyl chain structures, the percent of SM in the vesicle was determined as following. The total % SM in exchange vesicles (both inner leaflet and outer leaflet) was calculated from HP-TLC and, for the inner leaflet, TMADPH anisotropy. TLC data gives total % SM in the bilayer (both leaflets), and % SM derived from the anisotropy of TMADPH incorporated into the outer leaflet, when compared to a standard curve

gives % SM only in the outer leaflet. The percent of SM by TLC was evaluated after calculating the intensity of SM band on the TLC plate. The samples for HP-TLC were from the exchange SUV-containing fractions from the Sepharose CL-2B column. TMADPH fluorescence probe was added into preformed vesicles at a concentration of 0.1 mol% of total lipid concentration.

2.2.4. Fluorescence Measurements

Fluorescence measurements were carried out using a SPEX FluoroLog 3 spectrofluorimeter (Jobin-Yvon, Edison, NJ) with quartz semi-micro cuvettes (path length: excitation 10 mm, emission 4 mm). DPH fluorescence was measured at 358 nm (excitation) and 427 nm (emission). TMADPH fluorescence was measured at 364 nm (excitation) and 426 nm (emission). C_6-NBD-PC fluorescence was measured at 465 nm (excitation) and 534 nm (emission). The bandwidths of slit for C_6-NBD-PC fluorescence measurements were set to 4.2 nm for both excitation and emission. The backgrounds were less than 1% of fluorescence containing samples, and so were not subtracted from the sample signals.

2.2.5. Steady-state Fluorescence Anisotropy Measurements

Fluorescence anisotropy measurements were carried out using a SPEX automated Glan-Thompson polarizer accessory. DPH or TMADPH dissolved in ethanol (stock solutions 20 μM) were added to preformed vesicles to a concentration of 0.1 mol% of total lipid. For DPH, the bandwidths of slit for anisotropy were set to 4.2 nm (2 mm physical size) for both excitation and emission. For TMADPH, the bandwidths of slit for anisotropy were set to 4.2 nm (2 mm physical size) for excitation and 8.4 nm (4 mm physical size) emission. Anisotropy values were calculated from the fluorescence intensities with polarizing filters set at all combinations of horizontal and vertical orientations. For DPH, the background samples were negligible (less than 1%) and were not subtracted from the sample signals. For TMADPH, the anisotropy value

was determined after subtraction of fluorescence intensity in background samples lacking fluorophore since the background samples were not less than 1%. The anisotropy was determined using the following equation: $A = [((I_{vv} \times I_{hh})/(I_{vh} \times I_{hv})) - 1]/[((I_{vv} \times I_{hh})/(I_{vh} \times I_{hv})) + 2]$, where A represents anisotropy, I represents the fluorescence emission intensity, with the vertical (v) and horizontal (h) orientation of the excitation and emission polarization filters [172]. For the temperature dependent DPH fluorescence anisotropy measurements, DPH dissolved in ethanol was added to the cuvettes containing samples of exchange, scrambled, or ordinary symmetric vesicles (~150μM lipid concentration), followed by 5 min incubation in the dark. The DPH / lipid molar ratio was 0.1:100. Samples were scanned stepwise around by 4 °C between 16 °C and 60 °C. A digital thermometer was placed in the cuvette to measure the temperature and each anisotropy measurement was carried out once temperature stabilized. The DPH anisotropy was graphed as a function of temperature and the melting temperature was defined by the midpoint of a sigmoidal fit using the user-defined curve-fitting option of the SlideWrite Plus software (Advanced Graphics Software, Inc., Encinitas, CA).

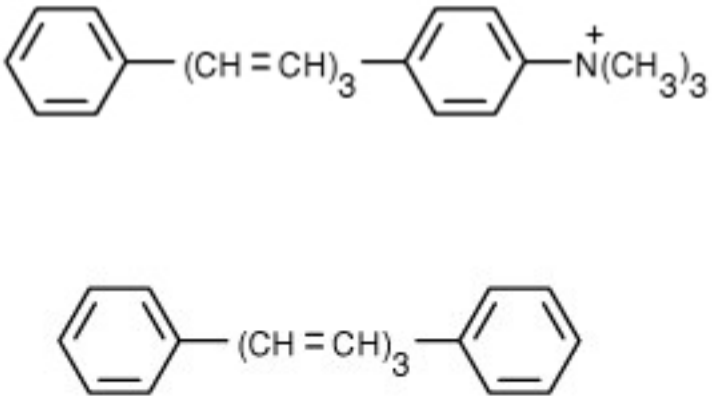


Figure 2.1. Chemical structures of TMADPH and DPH

The structures of TMADPH (top) and DPH (bottom) are shown.

2.2.6. Properties of Scrambled Vesicles

The scrambling process, in which vesicles are destroyed and then reformed as symmetric vesicles, was carried out similarly to previously described protocols [158]. Fractions 16-19 from the Sepharose CL2B column were combined and then divided into four tubes (1 ml each). Two of the samples were subjected to scrambling and two were used without scrambling. The samples to be scrambled were dried by a nitrogen gas stream. The film containing dried lipid and buffer were dissolved in 20 µl of ethanol, and then dispersed at 70 °C in 980 µl of distilled water (which should reconstitute the PBS as well as the lipid vesicles) to create scrambled vesicles. Samples were then cooled to room temperature, DPH or TMADPH, dissolved in ethanol, were added to 0.1 mol% of total lipid concentration.

2.2.7. Measurement of Lipid Transverse Diffusion (Flip-Flop)

The transverse diffusion of C_6-NBD-PC was measured using the dithionite method [173]. The assay was performed by following procedure. First, 1 ml of 100 uM SUVs was prepared by ethanol dilution followed by 1 mol% C_6-NBD-PC dissolved in ethanol was added to these preformed vesicles and then the SUVs were incubated overnight at room temperature. Alternately, large unilamellar vesicles (LUVs) were prepared by lipid extrusion technique using a Mini-Extruder set (Avanti Polar Lipids, Alabaster, AL). A sample containing 1 ml of MLVs (1 mM lipid) was disrupted by 5 times freeze-thaw cycles and then forced through a polycarbonate filter 12 times with a pore size of 100 nm. The sample was then diluted to 100 μM with PBS and C_6-NBD-PC was added (to 1 mol% of lipid). Next, initial NBD fluorescence emission (F_0) was measured. Then, 10 μl of 1.0 M sodium dithionite freshly prepared in 1.0 M Tris pH 10.0 was added, and the fluorescence emission intensity (F_P) was recorded vs. time for up to 1 h. The fraction of C_6-NBD-PC protected from external dithionite due to location in the inner leaflet is given by the ratio F_P/F_0. The fraction of C_6-NBD-PC that had flipped to the inner leaflet was estimated from the F_P/F_0 value at time = 0, determined by extrapolation from fluorescence values between 10 and 60 minutes after dithionite addition, assuming that the leakage of dithionite into the vesicles followed a first order reaction. All calculations were performed by using the Slide-Write Plus software (Advanced Graphics Software, Inc., Encinitas, CA).

2.2.8. Prevention of Lipid Oxidation in Polyunsaturated Lipids

The possibility of lipid oxidation that might occur was determined by the increase in absorption at 233 nm. The polyunsaturated PCs we used in this research, such as 16:0-18:2PC, 16:0-20:4PC, di18:2PC, di18:3PC, and di20:4PC, are highly oxidation-prone especially during the process of sonication. Free radical autoxidation of polyunsaturated lipids is accompanied at an early stage by diene conjugation which result in significant absorbance at 233 nm [174]. To minimize oxidation during vesicle preparation, the test tubes containing polyunsaturated PCs

MLVs were tightly sealed with teflon tape and then nitrogen gas stream was directed down into the test tube before sonication. For all of our lipids, we estimated at most a few % conjugated diene was present, indicating little oxidation. We also carried out the all experiments immediately after the formation of the exchange vesicles, such that all experiments on the exchange vesicles were completed within hours of their preparation.

2.2.9. Proton NMR Spectroscopy

The asymmetry of exchange SUVs with SM containing vesicles was studied using a Bruker Avance 700 MHz proton NMR spectrometer (Bruker Biospin, Billerica, MA). The NMR sample tubes used were Wilmad LabGlass (WG-5MM Economy, 7" length) tubes (Wilmad Glass Co, Buena, NJ). We used the choline N-methyl groups of SM in vesicles as the indicator of lipid asymmetry. Two ^{1}H resonances were observed in SUVs at 55 °C: they are attributed to the choline N-methyl groups, corresponding to the outer membrane leaflet at a chemical shift of 3.47-3.49 ppm and the inner membrane leaflet at a chemical shift about 0.04 smaller. For exchange vesicles, fractions from 16 to 19 eluting from the Sepharose CL2B column were combined, and then 500 µl were taken from this mixture and transferred to the NMR tube before 100 µl D_2O addition. For ordinary, symmetric vesicles, 4ml of 200 µM SUVs were prepared by ethanol dilution. Dialysis was carried out at room temperature for 24 h to remove ethanol. The dialysis buffer (PBS, pH 7.4) was mixed by a magnetic stirrer. The 500 µl of ordinary, symmetric vesicles were taken from the dialysis bag and transferred to the NMR tube. Next, 100 µl of D_2O was added to the ordinary, symmetric vesicles in the NMR tube. The 600 µl samples were incubated in the 55 °C water bath for 10 min before the proton NMR spectrum was recorded. All spectra were acquired at 55 °C and pH 7.4. 128 scans were acquired for each sample, with an acquisition time of 2.297 s (digitized into 64K data points) and a relaxation delay of 2 s between scans. Each acquisition required around 9 min per sample. Water suppression was achieved using a standard presaturation sequence.

2.2.10. Dynamic Light Scattering

The measurement of SUV size was carried out at 20 °C using a Protein Solutions DynaPro 99 dynamic light scattering instrument (Wyatt Technology, Santa Barbara, CA). Fractions 16-19 from the Sepharose CL2B column were applied to dynamic light scattering experiment 5 minutes and 1 day after eluting from the column. Data were analyzed with the software Dynamics version 5.25.44 program (Protein Solutions, Inc. Charlottesville, VA).

Chapter 3

The Dependence of Lipid Asymmetry upon Phosphatidylcholine Acyl Chain Structure

3.1. Introduction

The eukaryotic plasma membrane exhibits asymmetry with respect to its lipid distribution across the bilayer; the majority of sphingomyelin (SM) and phosphatidylcholine (PC) is in the outer leaflet, while the aminophospholipids phosphatidylethanolamine (PE) or phosphatidylserine (PS) are in the cytoplasmic leaflet [1, 5, 6]. It is known that the asymmetric distribution of lipids in the bilayer affects various biological properties such as membrane permeability, membrane potential, surface charge, the mechanical stability of membranes, and membrane shape [85, 175-180]. Therefore, biologically realistic asymmetric vesicles could be widely useful in model membrane studies. However, artificial membranes produced in the laboratory usually have a symmetric distribution of lipids in the inner and outer leaflets of the bilayer. Asymmetric artificial membranes have been difficult to produce, although some

important progress has been made [33, 134, 156, 181-183]. We recently developed a robust method in which methyl-beta-cyclodextrin (MβCD)-induced lipid exchange is used to prepare model membrane vesicles with lipid asymmetry, and have applied the method to produce asymmetric small, large and giant unilamellar vesicles [158-160].

In the present study, we extended the range of this method by using it to construct asymmetric vesicles containing SM in the outer leaflet and various species of PCs in the inner leaflet. PC acyl chain structure was systematically varied, and how this variation affects the ability to form asymmetric membranes was assayed. We found that whether the resulting vesicles were asymmetric (SM outside/PC inside = SMo/PCi) depended upon PC acyl chain structure. Despite this limitation, asymmetric vesicles with a wide range of acyl chain structures could be produced. This is an important advance that should allow new applications of asymmetric vesicles. These studies also allowed analysis of the effect of lipid type upon membrane structure and behavior. We investigated the origin of the acyl chain dependence of asymmetry by measuring the transverse diffusion rate of the various PCs used. There was a correlation between asymmetry and transverse diffusion. Asymmetric vesicles could only be prepared when PCs with slow transverse diffusion were used. It was found that the presence of two polyunsaturated acyl chains or overly short acyl chains prevented the maintenance of asymmetry. This may explain why such lipids are not generally abundant in biological membranes.

3.2. Results

3.2.1. Preparation of Asymmetric (Exchange) SUVs and their SM Content in the Inner and Outer Leaflets

We used MβCD-induced lipid exchange methods to produce asymmetric SUVs with SM in the outer leaflet and PC in the inner leaflet. In this protocol the lipid to be introduced into the outer leaflet is in donor MLV while the lipid to be located in the inner leaflet is in acceptor SUV. One change made from our previous protocol was to fractionate vesicles using Sepharose CL2B, which gave better resolution of vesicles by size than the Sepharose CL4B used previously. Using this approach, the properties of acceptor SUV having PCs with various acyl chains were investigated to determine whether the capability to form asymmetric vesicles is affected by lipid acyl chain structure. The PC tested had monounsaturated acyl chains of different lengths (di14:1PC, di16:1PC, di18:1PC, di20:1PC and di22:1PC), diphytanoyl chains [which have four methyl groups per chain] (diphyPC), one saturated and one poly unsaturated chain (16:0-18:2PC and16:0-20:4PC), or two poly unsaturated chains (di18:2PC, di18:3PC and di20:4PC). HP-TLC analysis of the acceptor vesicles after exchange (= exchange vesicles) revealed that they contained both SM and PC. Based on HP-TLC, the SM content in exchange vesicles was in the range 50-70% (Table 3.1). Because the theoretical value for complete exchange of the entire outer leaflet of small SUV is about 66%, this indicates that the MβCD-induced lipid exchange method is efficient for all PCs tested (Figure 3.1).

To determine the percentage of outer leaflet lipids that were SM after exchange, we examined the steady-state fluorescence anisotropy of TMADPH (Figure 2.1). This probe is localized to the outer leaflet of the bilayer when it is added to the preformed vesicles, due to its charged quaternary amino group. First, a standard curve of anisotropy vs. SM content was generated in ordinary, symmetric vesicles for each of the PC used. Anisotropy increases with SM content because at room temperature SM tends to form the solid-like gel state, in which

TMADPH is highly ordered and has high anisotropy, while by themselves the PCs used are in the disordered Ld state, in which TMADPH exhibits low anisotropy. Next, the anisotropy in the exchange vesicles (Table 3.2) was compared to the standard curves (Figure 3.2) to estimate the % SM in the outer leaflet. This showed the outer leaflet of exchange vesicles was composed of 62-96% SM, depending upon the PC used (Table 3.1 and Figure 3.3).

The percentage of inner leaflet lipids that were SM in the exchange vesicles was then calculated from the total SM in the vesicles (Figure 3.3). The SM content in the inner leaflet of exchange SUVs was found to be highly dependent on the type of PC present. The inner leaflet SM values were close to zero in many cases (di16:1PC, di18:1PC, di20:1PC, di22:1PC, diphyPC and 16:0-18:2PC) indicating that SM in those vesicles was largely restricted to the outer leaflet (i.e. they were SMo/PCi vesicles). In contrast, SM was less asymmetrically distributed (i.e. located to a substantial extent in both inner and outer leaflets of the bilayer) for PC with short acyl chains (di14:1PC), or highly polyunsaturated acyl chains (16:0-20:4PC, di18:2PC, di18:3PC and di20:4PC). In fact, in the case of lipids with two polyunsaturated chains the inner and outer leaflet SM concentrations were almost the same, indicative of no asymmetry.

3.2.2. Assessment of Asymmetry in Exchange Vesicles by Comparison to Scrambled Vesicles with the Same Lipid Composition

It is important to prove exchange vesicles are or are not asymmetric with additional methods. When exchange vesicles are asymmetric, they have different properties than the corresponding symmetric vesicles [158, 159]. The presence or absence of such differences was used to confirm the presence or absence of asymmetry. To prepare ordinary, symmetric vesicles with the same lipid compositions as that in the exchange vesicles, a protocol was used in which the exchange vesicles were dissolved in organic solvent and then reformed by a procedure yielding ordinary, symmetric vesicles ("scrambled vesicles").

The first assay used to compare physical properties of exchange and scrambled vesicles was outer leaflet membrane order measured by TMADPH anisotropy (Table 3.2 and Figure 3.4). As noted above, in asymmetric vesicles containing SM in their outer leaflet, outer leaflet membrane order is especially high at 23^{o}C because the SM is primarily in the solid-like gel state. For the lipid compositions studied, outer leaflet order is lower when vesicles do not form asymmetry due to the introduction of a low-Tm PC into the outer leaflet. As shown in Figure 3.4, relative to exchange vesicles there was no decrease in outer leaflet order for scrambled vesicles containing di18:3PC and di20:4PC. This indicates that the exchange vesicles prepared from these lipids are not asymmetric, in agreement with the estimates above showing that there are similar fractions of SM in the inner and outer leaflets of these vesicles. In addition, there was also only a very small decrease in order for scrambled vesicles vs. exchange vesicles containing di14:1PC, suggesting vesicles with this lipid have very little asymmetry. For the other PCs studied, Figure 3.4 shows there was a significant decrease in order in scrambled vesicles relative to exchange vesicles, indicative of a significant degree of lipid asymmetry in the exchange vesicles, which is in agreement with the conclusions based upon inner and outer leaflet SM content in these vesicles.

However, for vesicles containing 16:0-18:2PC, 16:0-20:4PC, and di18:2PC the decrease in outer leaflet order in scrambled vesicles relative to exchange vesicles was not as large as for the other lipids. By itself changes in overall membrane order are not conclusive, because if SM is very laterally immiscible with PC in symmetric vesicles, the scrambled vesicles appears ordered as asymmetric vesicles. However, in our studies relatively small changes in order are suggestive of incomplete asymmetry in the exchange vesicles, because it is in agreement with conclusions based upon SM content in the inner and outer leaflets.

The second assay used to evaluate the difference between the properties of exchange and scrambled vesicles was the thermal stability of gel state domains in the vesicles. The bilayer of

SM-containing vesicles undergoes a readily detected phase transition from the ordered gel phase to the liquid disordered phase as temperature is increased. The Tm (melting temperature) can be defined as the midpoint of this phase transition [158, 184]. It was previously found that Tm values are higher in asymmetric SMo/PCi vesicles than symmetric vesicles of the same composition [158]. This reflects the fact that the nearly pure SM outer leaflets in asymmetric vesicles melt at a temperature similar to that of SM in pure SM vesicles, while in symmetric vesicles the mixture of SM with low-Tm PC in each leaflet results in a Tm value lower than that of pure SM [158, 159]. Table 3.3 shows Tm values of exchange vesicles both before and after the scrambling process, and the difference between Tm in exchange vesicles and scrambled vesicles prepared from them is shown in Figure 3.5. The results were virtually identical to those obtained from measurements of outer leaflet order in terms of the relationship between PC acyl chain structure and asymmetry at 23^{o}C.

3.2.3. Relationship between PC Acyl Chain Structure and Lipid Transverse Diffusion

By definition, the ability to maintain membrane asymmetry in which the inner and outer leaflets have different lipid compositions requires, in the absence of active lipid transport catalyzed by a protein, that the movement of lipids between inner and outer leaflets (lipid transverse diffusion = flip-flop) is slow. Therefore, we determined if the different extents of asymmetry observed could be explained by differences in lipid transverse diffusion. The transverse diffusion assay used was based on the ability of sodium dithionite to reduce C_6-NBD-PC into a non-fluorescent derivative. C_6-NBD-PC was incorporated into the outer leaflet of preformed vesicles, and translocation of C_6-NBD-PC into the inner leaflet was detected by the amount of NBD fluorescence that was protected from reduction by dithionite added to the external solution. If the extent of transverse diffusion is slow, there should have been very little

protection, but if transverse diffusion is fast, there should have been significant protection from dithionite (Figure 3.6 and Figure 3.7).

To identify the best conditions in which to carry out this assay, the effect of varying time over which transverse diffusion could occur and varying lipid composition was measured (Figure 3.8 and Figure 3.9). Transverse diffusion was both time and composition dependent, and ultimately, mixtures (1:1 mol:mol) of the PC of interest with DOPC or SM were used together with a 24h incubation time for transverse diffusion because these conditions showed the most sensitivity to PC acyl chain structure.

Figure 3.10 shows the degree of protection of C_6-NBD-PC fluorescence in symmetric SUVs containing PCs with different acyl chain structures. Varying PC structure gave an extent of transverse diffusion that closely paralleled the effect of PC structure on asymmetry in exchange vesicles. Transverse diffusion was low for vesicles containing any of the PCs that were able support full asymmetry, and transverse diffusion was high with vesicles containing di14:1PC, di18:3PC and di20:4PC, which do not support asymmetry. The behavior of vesicles containing di18:2PC was surprising in that they seemed to allow only slow C_6-NBD-PC transverse diffusion even though the di18:2PC cannot support the formation of highly asymmetric vesicles. Possible explanations of this are considered in the Discussion.

Alternate explanations of the acyl chain dependence of transverse diffusion were tested. One possible factor would be vesicle size/curvature. However, exchange vesicles are isolated from the same, fixed fractions of a size exclusion chromatography column, so there should be no dependence of exchange vesicle size upon PC acyl chain structure. Nevertheless, transverse diffusion in LUV was compared to that in SUV to investigate the possibility that curvature strongly impacts lipid transverse diffusion (Figure 3.11). The pattern of which lipids supported less or more C_6-NBD-PC transverse diffusion was very similar in SUV and LUV, indicating that the effect of bilayer curvature is not large.

Another possibility is that lipid randomizing during spontaneous vesicle fusion could cause loss of asymmetry. However, using dynamic light scattering, no vesicle fusion was detected over 24h for di18:3PC or di14:1PC, which fail to form asymmetric vesicles (data not shown).

3.2.4. Asymmetric Lipid Distribution is Associated with a Difference between Order in the Inner and Outer Leaflet for SM Outside/PC Inside Vesicles

Previous studies using asymmetric vesicles have shown that when the outer leaflet of a vesicle is SM in the gel state (100% ordered), and the inner leaflet is composed of DOPC or POPC, the inner leaflet remains largely in the Ld state [158, 159]. This means that the physical states of the inner and outer leaflets are not strongly coupled to each other [158, 159]. To see how general this behavior is, we examined the degree of coupling between the inner and outer leaflet physical states for exchange vesicles with a wider variety of PC acyl chains. To do this, the degree of order in the inner leaflet was calculated from the fluorescence anisotropy of DPH (which measures order in both leaflets) and externally added TMADPH (which measures outer leaflet order) [159], as shown in Figure 3.12.

Table 3.2 and Table 3.4 contain the raw anisotropy data for TMADPH and DPH and Table 3.5 shows the calculated % order of the outer and inner leaflets. Figure 3.12 illustrates the difference between the level of order in the inner and outer leaflets. In most cases, the % order in the outer leaflet was higher than that in the inner leaflet, as expected for asymmetric vesicles In the case of almost all of the vesicles that were fully or mostly asymmetric (inside leaflet composed of di16:1PC, di18:1PC, di20:1PC, di22:1PC, 16:0-18:2PC and 16:0-20:4PC), the difference was in the range 50-100%. Because the outer leaflet was almost fully ordered (~90%) in these vesicles (Table 3.5) this means that the inner leaflet was largely disordered (Table 3.5),

and thus that the physical states of the inner and outer leaflets are largely uncoupled. (The experimental error in anisotropy values makes it difficult to make a precise statement concerning low levels of coupling.)

The behavior of diphytanoyl PC was surprising because it is in vesicles that are fully asymmetric, but there did not appear to be any difference in the order of the inner and outer leaflet. This means either that the inner and outer leaflet physical states are coupled in SMo/diphyPCi vesicles, or that DPH does not partition well into the inner leaflet composed of diphytanoyl PC. We suspect the latter possibility is more likely because the methyl groups of diphytanoyl PC would interfere with van der Waals interactions between lipid acyl chains and the flat DPH molecule.

In the cases of di 14:1PC and lipids with two polyunsaturated chains, the % order in the outer leaflet was not significantly higher than in the inner leaflet. This was expected, because it reflects the lack of asymmetry in these vesicles. In the absence of asymmetry, the % order should be almost equal in the inner and outer leaflets. (The observation that the calculated value of order is *higher* for the inner leaflet than the outer leaflet in some of these cases is an artifact of fact that TMADPH does not partition into ordered domains as well as does DPH, and was observed previously [158, 159].) We cannot make any statement about coupling in these vesicles.

3.3. Discussion

3.3.1. The Acyl Chain Dependence of Asymmetric Vesicle Formation is Explained by Lipid Transverse Diffusion (Flip-Flop)

This report shows that asymmetric vesicles can be prepared from lipids having a wide range of acyl chain structures. This ability should be useful in future studies of the effect of acyl chain structure upon the properties and function of asymmetric bilayers. However, the ability to form asymmetric bilayers was not a universal property of lipids. A question that arises from this is, why asymmetric vesicles cannot be formed specifically from lipids with two short acyl chains, or two polyunsaturated acyl chains. Since maintenance of asymmetry requires, by definition, that lipids do not equilibrate between inner and outer leaflets by transverse diffusion across the bilayer, the logical implication is that rapid transverse diffusion for lipids with two short or polyunsaturated acyl chain should explains their inability to form asymmetric vesicles. This was confirmed by the measurements of the extent of transverse diffusion for a probe lipid in vesicles with various lipid compositions. The lipid mixtures that allowed most rapid transverse diffusion of the probe lipid were the same ones in which lipid asymmetry was not present, which means that the lipid compositions were such that they increased the rate of transverse diffusion for both labeled and unlabeled lipids.

A fundamental question that arises from these observations is why lipids with certain acyl chains impart rapid transverse diffusion to all of the lipids in a membrane. Armstrong *et al*. proposed that lipid packing was the important parameter determining transverse diffusion rates [39]. Packing should become looser as the number of double bonds increases. This could increase the spaces in which a molecule crossing the bilayer might dissolve. Another factor arises from the fact that the barrier to transverse diffusion is very likely to be the movement of the polar headgroup of a lipid across the hydrophobic core of the bilayer. In the case of shorter acyl chains, the width of this hydrophobic barrier is small, which should allow more rapid

transverse diffusion. In the case of lipids with polyunsaturated acyl chains, the lesser hydrophobicity of the bilayer core, due to the presence of multiple carbon-carbon double bonds [185], may be a property that allows faster transverse diffusion. In the case of polyunsaturated lipids, the issue of acyl chain oxidation must also be considered. This is important because it has been shown that oxidized lipids exhibit fast transverse diffusion across bilayers [186]. Indeed, when a preparation of polyunsaturated lipid that had been stored for an extended period of time was used, we noted very fast transverse diffusion, suggesting oxidation could have been involved (data not shown). For this reason, we took precautions against oxidation of the lipids with polyunsaturated acyl chains, no or very little acyl chain oxidation was detected (see Methods). Nevertheless, we cannot rule out a small degree of oxidation.

We also observed that mixtures of lipids with slow and fast transverse diffusion tended to show an intermediate extent of transverse diffusion, although generally not showing a linear relationship between lipid composition and extent of transverse diffusion (Figure 3.9). This suggests that transverse diffusion during the lipid exchange process might follow a complex course of events. At the outset of the lipid exchange process, the acceptor vesicles are composed only of the intended inner leaflet lipid, so SM molecules would be expected to flip across the bilayer with a rate reflecting that of the PC present. However, towards the end of the SM exchange process the membrane composition will be very different, and it is likely that transverse diffusion rates will have changed. This may explain why the extent of transverse diffusion observed using symmetric vesicles did not always exactly parallel the loss of asymmetry observed in the exchange vesicles. Another factor could be a difference between the transverse diffusion of the labeled C_6-NBD-PC molecules and that of the unlabeled lipids.

It should be noted that previous studies of the acyl chain dependence of lipid transverse diffusion (in symmetric vesicles) are consistent with our results. In the study of Armstrong *et al.* it was found that increased unsaturation increased transverse diffusion rates for a headgroup

labeled NBD-lipid [39]. The study of Volinsky *et al*. found that oxidized PC transverse diffusion was much more rapid than that of unoxidized PC [186].

Fast lipid transverse diffusion also helps explain the tendency to have the highest extent of SM exchange in cases in which the lipid composition did not result in asymmetric vesicle formation. In vesicles that have low transverse diffusion, once the outer leaflet is nearly 100% SM, there can be no further increase in SM content. However, if transverse diffusion is fast, then SM introduced into the outer leaflet could flip into the inner leaflet, allowing exchange to continue, so that both leaflets would end up with a high SM concentration.

3.3.2. Biological Implications of the Dependence of Asymmetry upon Acyl Chain Structure

Asymmetry is often an important, if poorly understood, aspect of membrane structure and function. Therefore, acyl chains or combinations of acyl chains that do not allow asymmetry to be easily maintained may be disfavored in living organisms. This could be one reason that membrane lipids having short acyl chains, or having two polyunsaturated acyl chains, are rarely found in biological membranes. However, in small amounts these lipids might not greatly destabilize asymmetry, and there are other reasons that might also be important for their absence, including for short acyl chains the inability for form a sufficiently stable bilayer, or for two polyunsaturated acyl chains extreme sensitivity to oxidation. Furthermore, the presence of such lipids, especially in small amounts, might not prevent maintenance of asymmetry because a suitably specific active transport system should be able to counteract the effects of spontaneous diffusion. However, the need for such a transport system might be wasteful of metabolic energy (e.g. ATP). Finally, it is not known if the acyl chains differ for a particular species (e.g. PC) in the inner and outer leaflet. This could also affect the stability of asymmetry.

3.3.3. Potential Application of Asymmetric and Symmetric Exchange Vesicles

One of the most promising potential applications of exchange vesicles is their use in studies of the relationship between lipid composition and membrane protein structure and function. The exchange protocol will allow investigation of lipid asymmetry upon proteins in membranes with a wide range of lipid compositions. However, there are some cases in which it may be desirable to examine the effect of lipid structure upon proteins in symmetric membranes. The observations in this report suggest that this can also be accomplished in an improved fashion using lipid exchange. The approach would be to reconstitute a protein in a vesicle rich in highly polyunsaturated PC, which does not support asymmetry, and then carry out lipid exchange with various lipids. The ability to exchange lipids into preformed vesicles *in situ* in this fashion would avoid many complications that arise when trying to compare samples in which membrane proteins are individually reconstituted into different vesicle populations with different lipid compositions (e.g. different orientations of a membrane protein when reconstituted separately into different lipid vesicles, varying vesicle size in different reconstitution experiments, and different vesicle homogeneity in terms of protein molecules per vesicle).

Table 3.1. Percent SM in exchange vesicles and in the outer leaflet of exchange vesicles

%SM calculated as described in Methods. Average (mean) and S.D for the number of samples shown in parentheses are shown. %SM in exchange vesicles determined by TLC vs. standard curves in which different amounts of lipids were loaded on the TLC plate. %SM in outer leaflet was calculated from the fluorescence anisotropy of TMADPH incorporated into the outer leaflet of the exchange vesicles and standard curves of anisotropy vs. % SM in vesicles as described in Figure 3.2 (see Table 3.3 and Figure 3.4). Measurements made at 23 °C. %SM in vesicle refers to the % of lipids in the vesicle that are SM. %SM in outer leaflet refers to the % of outer leaflet lipids that are SM.

Sample Composition	% SM in vesicle	% SM in outer leaflet
SMo / di 14:1PC in	68 ± 4.5 (3)	83.2 ± 7.9 (7)
SMo / di 16:1PC in	57.2 ± 6.7 (3)	89.8 ± 8.0 (11)
SMo / di 18:1PC in	60 ± 4.2 (3)	95.6 ± 3.7 (5)
SMo / di 20:1PC in	51.6 ± 2.3 (3)	87.7 ± 3.7 (10)
SMo / di 22:1PC in	52.4 ± 1.9 (3)	91.8 ± 7.9 (11)
SMo / diphyPC in	62.8 ± 1.2 (3)	94.5 ± 7.7 (14)
SMo / 16:0-18:2PC in	61.9 ± 7.6 (3)	92.3 ± 8.4 (10)
SMo / 16:0-20:4PC in	68.2 ± 10 (3)	90.6 ± 7.6 (17)
SMo / di 18:2PC in	66.8 ± 6.7 (3)	76.7 ± 4.3 (14)
SMo / di 18:3PC in	65.8 ± 3.1 (3)	62.2 ± 8.2 (18)
SMo / di 20:4PC in	68.6 ± 1.0 (3)	69.6 ± 8.5 (9)

Table 3.2. TMADPH fluorescence anisotropy in ordinary, exchange, and scrambled vesicles at 23 °C

The percent ordered state of outer leaflet was calculated from the following equation: Percent ordered = $(A - A_{100\% \text{ Ld}})/(A_{100\% \text{ ordered}} - A_{100\% \text{ Ld}})$. A is that in an exchange vesicle, $A_{100\% \text{ ordered}}$ is that in SM, and $A_{100\% \text{ Ld}}$ is that in the appropriate unsaturated lipid. This formula assumes that gel (=ordered) domain and Ld domains have A values similar to that in pure gel and Ld state vesicles, respectively. TMADPH was added to preformed vesicles at a concentration of 0.1mol % of total lipid concentration. Ordinary (symmetric) vesicles were prepared by ethanol dilution. Average (mean) values and S.D. are shown. Number of samples shown in parentheses.

Sample composition	TMADPH Anisotropy		% ordered	
	Before scrambling	After scrambling	Before scrambling	After scrambling
SM	0.344 ± 0.010 (12)	0.351 ± 0.014 (12)	≡ 100	≡ 100
di 14:1PC	0.215 ± 0.008 (3)	0.225 ± 0.002 (3)	≡ 0	≡ 0
di 16:1PC	0.242 ± 0.010 (3)	0.250 ± 0.012 (3)	≡ 0	≡ 0
di 18:1PC	0.250 ± 0.008 (3)	0.252 ± 0.005 (3)	≡ 0	≡ 0
di 20:1PC	0.263 ± 0.009 (3)	0.261 ± 0.010 (3)	≡ 0	≡ 0
di 22:1PC	0.267 ± 0.012 (3)	0.255 ± 0.005 (3)	≡ 0	≡ 0
DiphyPC	0.284 ± 0.006 (3)	0.282 ± 0.001 (3)	≡ 0	≡ 0
16:0-18:2 PC	0.245 ± 0.014 (3)	0.235 ± 0.010 (3)	≡ 0	≡ 0
16:0-20:4 PC	0.223 ± 0.007 (3)	0.222 ± 0.006 (3)	≡ 0	≡ 0
di 18:2PC	0.252 ± 0.010 (3)	0.251 ± 0.009 (3)	≡ 0	≡ 0
di 18:3PC	0.219 ± 0.006 (3)	0.220 ± 0.006 (3)	≡ 0	≡ 0
di 20:4PC	0.223 ± 0.001 (3)	0.222 ± 0.012 (3)	≡ 0	≡ 0
SMo / di 14:1PC in	0.318 ± 0.013 (7)	0.302 ± 0.008 (7)	81.4 ± 10.4	58.9 ± 6.1
SMo / di 16:1PC in	0.329 ± 0.015 (11)	0.288 ± 0.012 (11)	87.4 ± 14.7	36.4 ± 11.8
SMo / di 18:1PC in	0.339 ± 0.007 (5)	0.281 ± 0.011 (3)	97.8 ± 7.7	28.3 ± 10.7
SMo / di 20:1PC in	0.327 ± 0.005(10)	0.281 ± 0.011 (10)	82.6 ± 6.9	21.7 ± 11.8
SMo / di 22:1PC in	0.333 ± 0.016 (11)	0.281 ± 0.013 (11)	89.1 ± 17.5	25.7 ± 13.2
SMo / DiphyPC in	0.333 ± 0.013 (14)	0.298 ± 0.013 (14)	86.5 ± 22.9	22.4 ± 18.0
SMo / 16:0-18:2PC in	0.333 ± 0.016 (10)	0.305 ± 0.009 (10)	91.6 ± 16.5	58.4 ± 7.9
SMo / 16:0-20:4PC in	0.333 ± 0.016 (17)	0.302 ± 0.014 (17)	92.4 ± 14.7	59.0 ± 10.9
SMo / di 18:2PC in	0.308 ± 0.006 (14)	0.284 ± 0.011 (14)	62.8 ± 7.2	31.9 ± 10.3
SMo / di 18:3PC in	0.293 ± 0.011 (18)	0.298 ± 0.010 (18)	60.9 ± 8.6	57.4 ± 7.4
SMo / di 20:4PC in	0.303 ± 0.011 (9)	0.309 ± 0.011 (9)	67.6 ± 9.7	65.1 ± 8.5

Table 3.3. Melting temperature of exchange and scrambled vesicles

The melting temperature was estimated from the midpoint of DPH anisotropy vs. temperature curves [158, 159]. DPH was added to preformed vesicles at a concentration of 0.1mol % of total lipid concentration. Average (mean value) and S.D. are shown, with number of samples shown in parentheses.

Sample composition	Melting Temperature (˚C)	
	Before scrambling	After scrambling
SMo / di 14:1PC in	37.7 ± 1.0 (6)	35.6 ± 0.8 (6)
SMo / di 16:1PC in	37.9 ± 1.5 (7)	31.4 ± 0.7 (7)
SMo / di 18:1PC in	36.9 ± 1.0 (4)	28.7 ± 0.4 (4)
SMo / di 20:1PC in	35.2 ± 1.5 (6)	28.4 ± 1.1 (6)
SMo / di 22:1PC in	34.7 ± 1.8 (6)	28.3 ± 2.2 (6)
SMo / diphyPC in	41.8 ± 0.6 (7)	33.0 ± 1.8 (7)
SMo / 16:0-18:2PC in	36.1 ± 1.2 (10)	31.1 ± 1.6 (10)
SMo / 16:0-20:4PC in	37.7 ± 1.4 (10)	32.8 ± 1.6 (10)
SMo / di 18:2PC in	33.5 ± 0.6 (7)	30.0 ± 1.7 (7)
SMo / di 18:3PC in	36.0 ± 1.1 (6)	35.9 ± 0.8 (6)
SMo / di 20:4PC in	36.9 ± 1.0 (11)	37.3 ± 1.2 (11)

Table 3.4. DPH fluorescence anisotropy in symmetric and exchange vesicles at 23°C

The percent ordered state of outer leaflet was estimated from the following equation: Percent ordered = $(A - A_{100\% \text{ Ld}})/(A_{100\% \text{ ordered}} - A_{100\% \text{ Ld}})$. A is that in an exchange vesicle, $A_{100\% \text{ ordered}}$ is that in SM, and $A_{100\% \text{ Ld}}$ is that in the appropriate unsaturated lipid. This formula assumes that gel (=ordered) domain and Ld domains have A values similar to that in pure gel and Ld state vesicles, respectively. DPH was added to preformed vesicles at a concentration of 0.1mol % of total lipid concentration. Ordinary (symmetric) vesicles were prepared by ethanol dilution. Average (mean) values and S.D. are shown. Number of samples shown in parentheses.

Samples	DPH Anisotropy	% ordered
SM	0.311 ± 0.002 (5)	≡ 100
di 14:1PC	0.088 ± 0.001 (3)	≡ 0
di 16:1PC	0.094 ± 0.008 (3)	≡ 0
di 18:1PC	0.107 ± 0.005 (3)	≡ 0
di 20:1PC	0.107 ± 0.004 (3)	≡ 0
di 22:1PC	0.121 ± 0.002 (3)	≡ 0
diphyPC	0.150 ± 0.003 (3)	≡ 0
16:0-18:2 PC	0.096 ± 0.004 (3)	≡ 0
16:0-20:4 PC	0.085 ± 0.002 (3)	≡ 0
di 18:2PC	0.086 ± 0.002 (3)	≡ 0
di 18:3PC	0.063 ± 0.002 (3)	≡ 0
di 20:4PC	0.058 ± 0.006 (3)	≡ 0
SMo / di 14:1PC in	0.278 ± 0.007 (6)	85.3 ± 4.9
SMo / di 16:1PC in	0.246 ± 0.008 (7)	70.1 ± 6.6
SMo / di 18:1PC in	0.206 ± 0.000 (4)	48.5 ± 3.6
SMo / di 20:1PC in	0.214 ± 0.007 (6)	52.5 ± 4.7
SMo / di 22:1PC in	0.236 ± 0.002 (6)	60.5 ± 3.5
SMo / diphyPC in	0.294 ± 0.013 (7)	89.6 ± 10.1
SMo / 16:0-18:2PC in	0.231 ± 0.025 (10)	62.7 ± 12.1
SMo / 16:0-20:4PC in	0.248 ± 0.021 (10)	71.9 ± 9.8
SMo / di 18:2PC in	0.209 ± 0.004 (7)	54.5 ± 3.0
SMo / di 18:3PC in	0.247 ± 0.0010 (7)	74.2 ± 5.2
SMo / di 20:4PC in	0.258 ± 0.009 (11)	79.2 ± 5.6

Table 3.5. The percent ordered state of leaflets in the exchange vesicles

Values of percent of the outer leaflet in an ordered state, and the per cent of the entire bilayer in an ordered state were from Table 3.2 and Table 3.4. The % of the inner leaflet in an ordered state was calculated using the following equation: Inner leaflet % ordered = (% total bilayer ordered – % outer leaflet ordered*0.67) / 0.33. This uses the estimate that the % of total vesicle surface area (and thus lipid) is ~67% in the outer leaflet ~33% in the inner leaflet. Average (mean) values and S.D. are shown.

Sample composition	% Ordered		
	Both leaflets	**Outer leaflet**	**Inner leaflet**
SMo / di 14:1PC in	85.3 ± 4.9	81.4 ± 10.4	93.4 ± 25.5
SMo / di 16:1PC in	70.1 ± 6.6	87.4 ± 14.7	35.7 ± 35.5
SMo / di 18:1PC in	48.5 ± 3.6	97.8 ± 7.7	-50.0 ± 18.8
SMo / di 20:1PC in	52.5 ± 4.7	82.6 ± 6.9	-7.5 ± 19.7
SMo / di 22:1PC in	60.5 ± 3.5	89.1 ± 17.5	3.5 ± 36.5
SMo / diphyPC in	89.6 ± 10.1	86.5 ± 22.9	96.1 ± 54.9
SMo / 16:0-18:2PC in	62.7 ± 12.1	91.6 ± 16.5	5.1 ± 49.1
SMo / 16:0-20:4PC in	71.9 ± 9.8	92.4 ± 14.7	31.1 ± 41.6
SMo / di 18:2PC in	54.5 ± 3.0	62.8 ± 7.2	38.1 ± 17.0
SMo / di 18:3PC in	74.2 ± 5.2	60.9 ± 8.6	101.0 ± 23.2
SMo / di 20:4PC in	79.2 ± 5.6	67.6 ± 9.7	102.6 ± 25.7

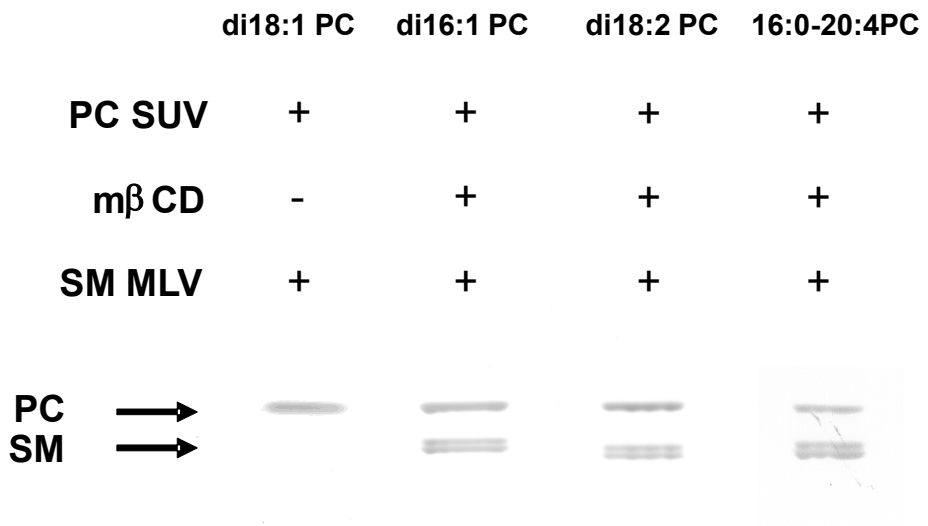


Figure 3.1. Representative HP-TLC profile of SUV fraction from Sepharose CL-2B chromatography after lipid exchange

Note that SM runs as two closely spaced bands.

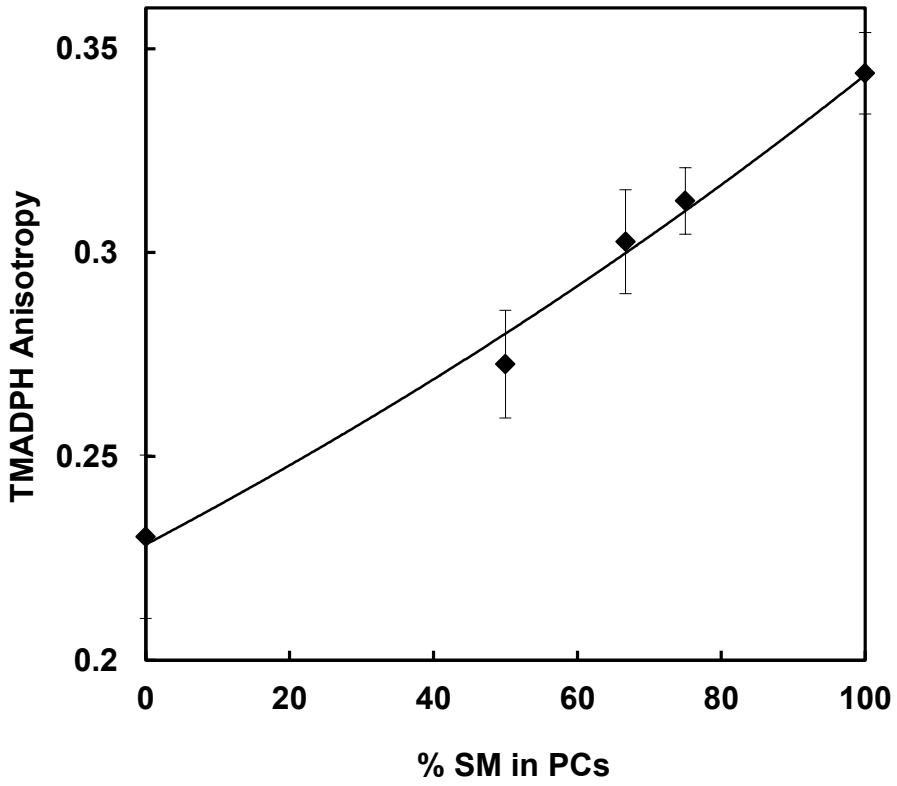


Figure 3.2. Standard curve to estimate the % SM in exchange vesicle outer leaflets at 23 ^{o}C

This curve was generated by adding TMADPH (0.1 mol%) to preformed symmetric vesicles composed of SM/16:0-20:4PC, di18:3PC, or di20:4PC mixtures at varying proportions. These vesicles all gave very similar curves, and the average of the values for these three lipids and S.D. is shown. Similar curves were generated for other binary mixtures of SM with various PC. The symmetric SUVs were made by ethanol dilution. The percent of SM in the outer leaflet was estimated using standard curve fitting (SlideWrite Plus software, Advanced Graphics Software, Inc., Encinitas, CA).

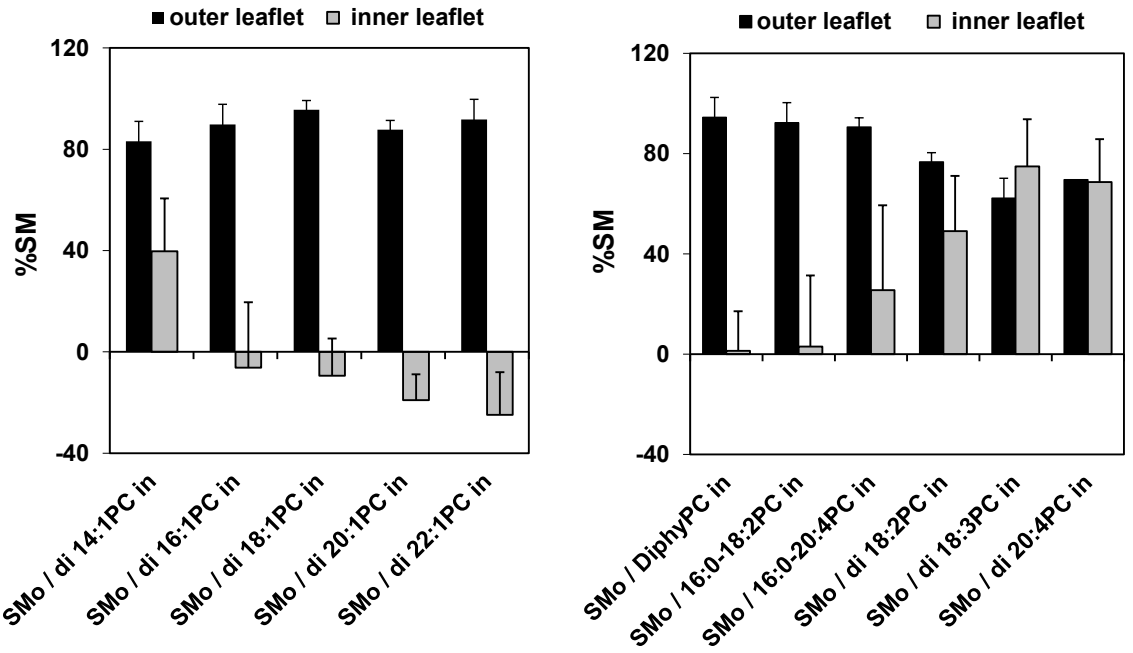


Figure 3.3. Percent SM in the outer leaflet and inner leaflet of exchange vesicles

The average (mean) % of the outer leaflet lipids that are SM (black bars) and the percent of inner leaflet lipids that are SM (gray bars), and S.D. are shown. Notice that this is **not** the same as the % of SM in a vesicle that is in the outer leaflet or inner leaflet, respectively. Values for the outer leaflet are from Table 3.1 and for the inner leaflet calculated from data in Table 3.1 using the equation: %SM in inner leaflet = (%SM total – 0.67*%SM outer leaflet) / 0.33. This assumes the % of total surface area in SUV is ~67% outer leaflet and ~33% inner leaflet. Left panel, PC with different acyl chain lengths. Right panel, lipids with different number and/or position of double bonds. Measurements made at 23^{o}C. In this and all following figures, vesicles were dispersed in PBS, pH 7.4.

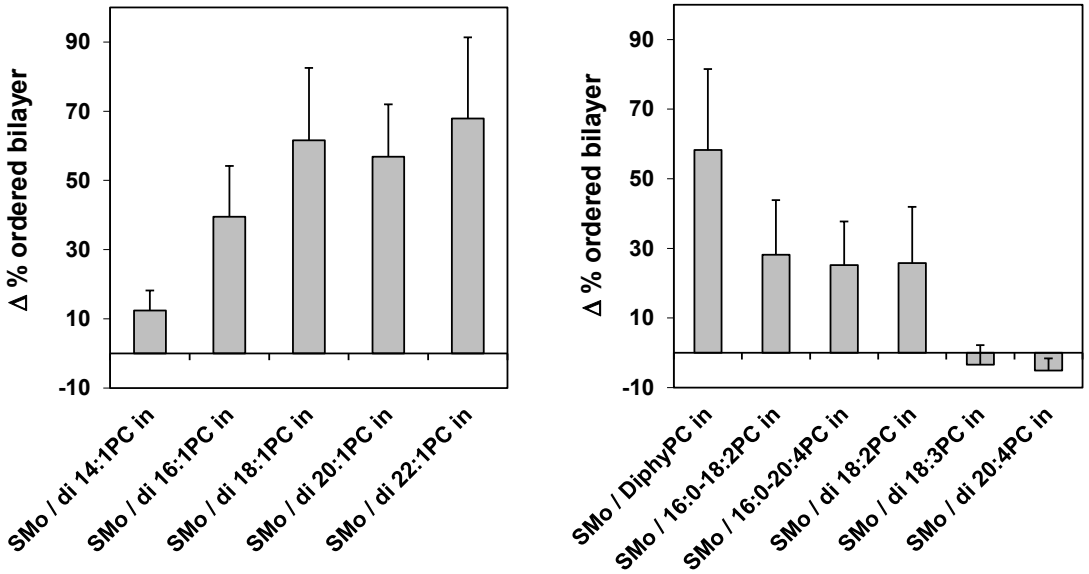


Figure 3.4. Difference between TMADPH fluorescence anisotropy in exchange and scrambled vesicles of identical composition

The average (mean) difference in % ordered bilayer and S.D. between exchange vesicles and scrambled vesicles formed from the exchange vesicles by the scrambling process is shown. Positive values indicate a higher % ordered in the exchange vesicles. Calculated from values in Table 3.5. Measurements made at 23^{o}C. Left panel, PC with different acyl chain lengths. Right panel, lipids with different number and/or position of double bonds.

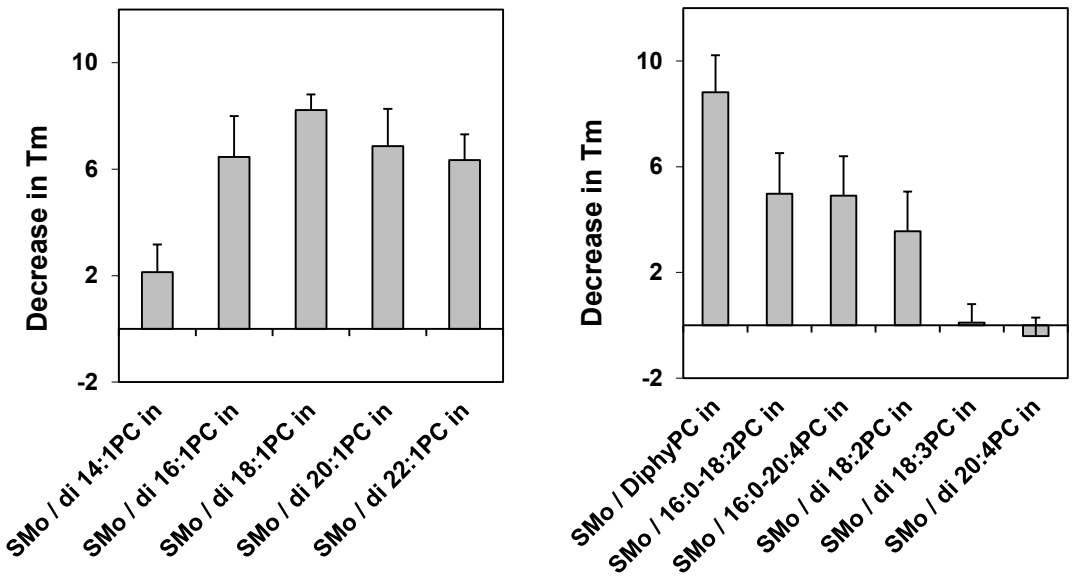


Figure 3.5. Difference between bilayer melting point in exchange and scrambled vesicles of identical composition

The average (mean) difference and S.D. between bilayer melting point (Tm) in exchange vesicles and scrambled vesicles formed from the exchange vesicles by the scrambling process is shown. Positive values indicate a higher Tm in the exchange vesicles. Calculated from values in Table 3.3. Left panel, PC with different acyl chain lengths. Right panel, lipids with different number and/or position of double bonds.

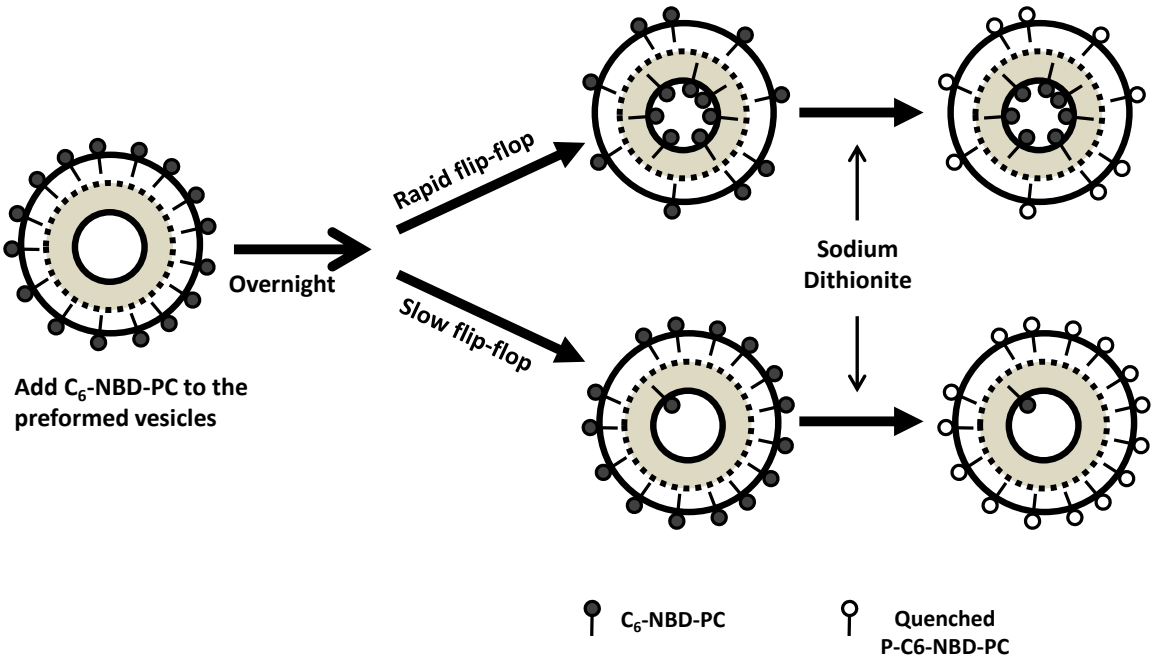


Figure 3.6. Schematic representation of transverse diffusion/flip-flop assay

NBD lipids indicated by circles with line attached. Intact (fluorescent) NBD lipids have filled headgroups, chemically quenching (dithionite reduced) NBD lipids shown with unfilled headgroups. Not shown is the case in which flip-flop is so fast that the NBD lipids in the inner leaflet flip to the outer leaflet after dithionite is added. This case was ruled out by preparing vesicles with NBD lipids in both leaflets and showing that the inner leaflet NBD lipid was inaccessible to dithionite (data not shown).

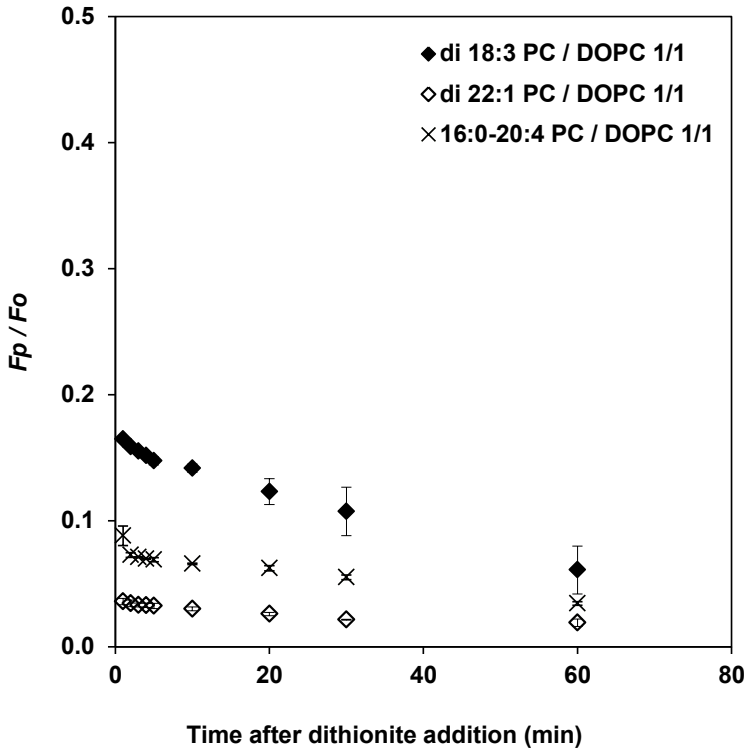


Figure 3.7. Examples of lipid transverse diffusion measurement

The fraction of C_6-NBD-PC protected (*Fp/Fo)* is the ratio the C_6-NBD-PC fluorescence protected (*Fp*) from external dithionite to initial NBD fluorescence emission (*Fo*) before sodium dithionite addition. The fluorescence emission intensity was recorded for 60 minutes after dithionite addition. Average (mean) values and range derived from duplicate samples are shown. Similar curves were generated for other lipid mixtures**.** Experimental protocol is given in the Methods section. *Fp/Fo*=1 at time zero (not shown due to being off-scale).

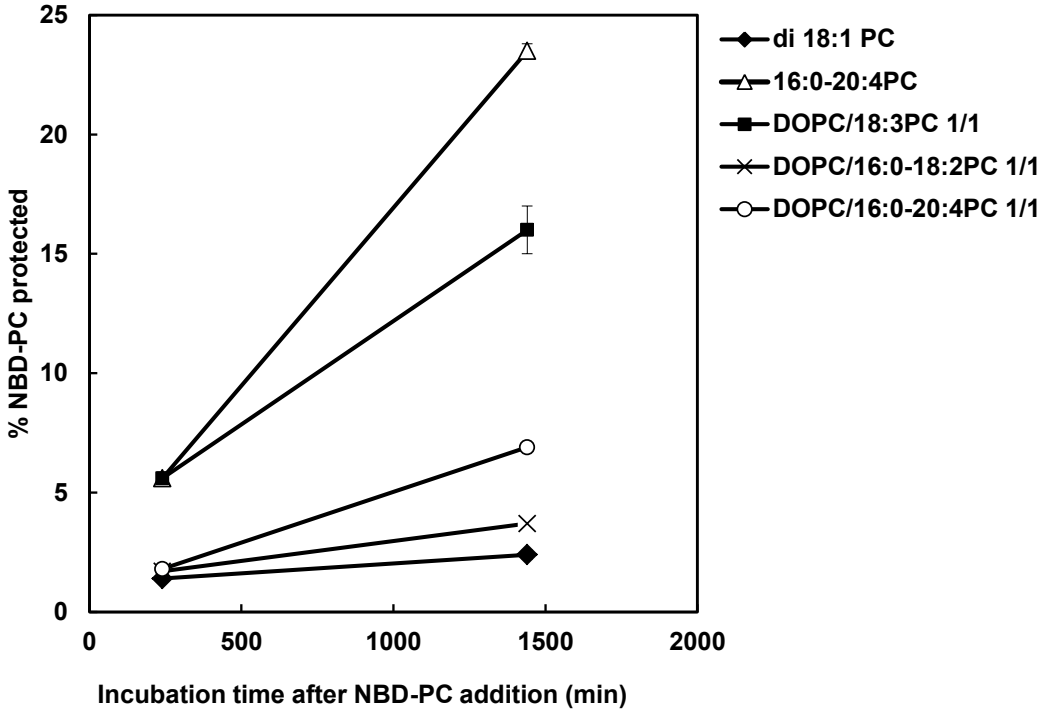


Figure 3.8. Effect of incubation time on protection of C_6-NBD-PC from dithionite in symmetric SUVs

The % NBD protection (which is equal to the % NBD lipid in the inner leaflet) after a 4 h or ~24 h incubation time is shown. Average values and range derived from duplicate samples are shown.

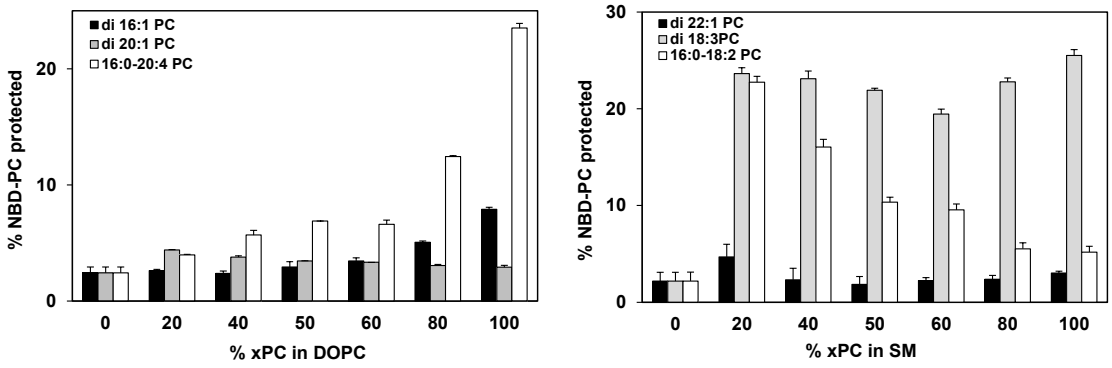


Figure 3.9. Transverse diffusion of C_6-NBD-PC in symmetric SUVs containing different mixtures of PC and SM

Symmetric SUVs composed of di16:1PC, di20:1PC, di22:1PC, di18:3PC, 16:0-18:2PC, or 16:0-20:4PC mixed with DOPC or SM were used to examine the extent of transverse diffusion as a function of lipid composition. Analogous curves were generated for the other PC combinations with DOPC and SM. Average values and range derived from duplicate samples are shown.

Notice that in mixtures of DOPC (which allows only slow transverse diffusion) with different PC species that showed faster transverse diffusion, the extent of transverse diffusion tended to increase as the fraction of DOPC decreased. However, the effect was not linear in the fraction of DOPC in the membrane. The effect of SM concentration upon transverse diffusion in mixtures of SM with PC was more complex, with maximal C_6-NBD-PC transverse diffusion often occurring at intermediate SM contents. This probably reflects de-mixing of unlabeled PC and SM into co-existing SM-rich gel and PC-rich liquid disordered domains in these vesicles. C_6-NBD-PC would locate in the PC-rich domains, which would result in it having significant transverse diffusion if the unlabeled PC species present tends to support fast transverse diffusion. Fast transverse diffusion may also occur in such mixtures at the boundaries between the gel and Ld domains.

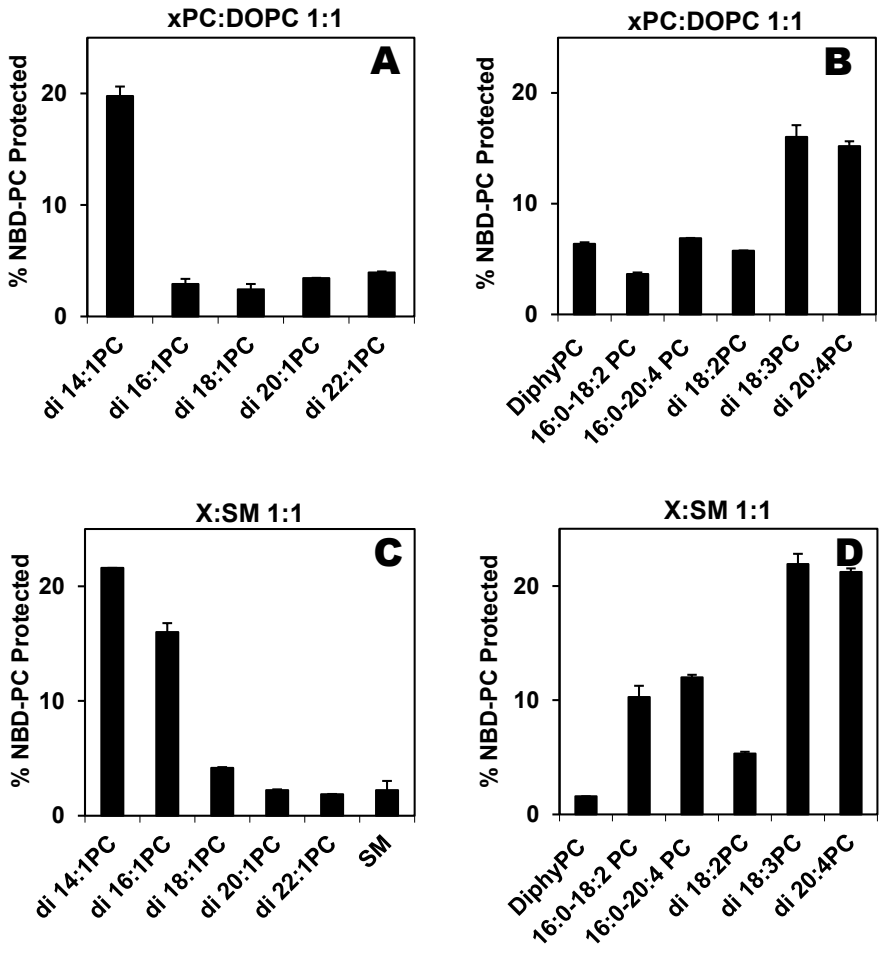


Figure 3.10. Different levels of transverse lipid diffusion in symmetric vesicles of different compositions

The effect of PC acyl chain structure on transverse diffusion was determined from the amount of C_6-NBD-PC (1 mol% of vesicles) that moved overnight into the inner leaflet, as judged by protection from reduction by dithionite. See methods for details. Vesicles were prepared by ethanol dilution. Unlabeled lipids were mixed 1:1 with DOPC (panels A. and B.) or SM (panels C. and D.) A. and C. Lipids with different acyl chain lengths. B. and D. lipids with different number and/or position of double bonds. Value for vesicles in which the unlabeled lipid was 100% SM is also shown. Average values and range derived from duplicate samples are shown. Other details given in Chapter 2 (Methods).

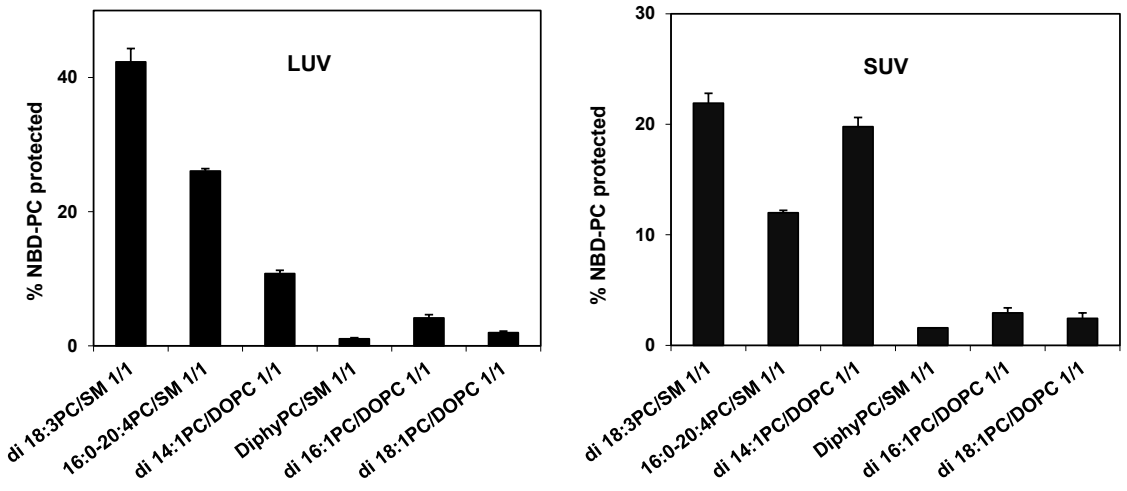


Figure 3.11. Transverse diffusion rates of C_6-NBD-PC in symmetric LUVs and SUVs

The effect of curvature on transverse diffusion as estimated from protection from was dithionite was assayed as described above. Left: Large unilamellar vesicles (LUV). Right: Small unilamellar vesicles (SUV). Symmetric LUVs were prepared by freeze/thaw and extrusion of MLVs through polycarbonate filters with a pore size of 100 nm as described in Methods. Symmetric SUVs were prepared by ethanol dilution. Average values and range derived from duplicate samples are shown.

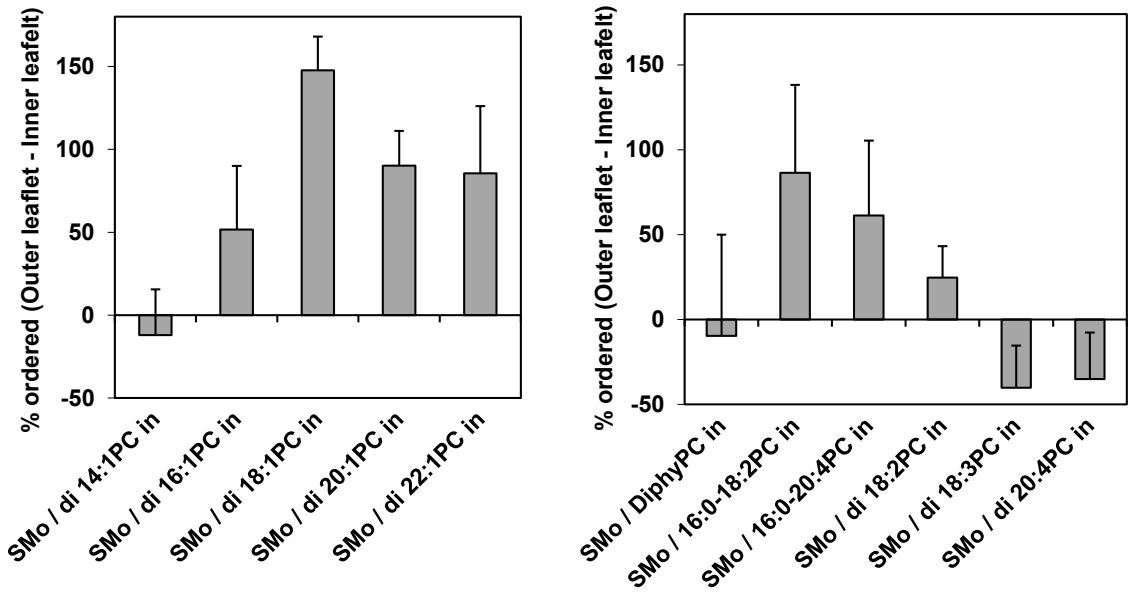


Figure 3.12. Coupling between inner and outer leaflet physical states at 23 ^{o}C estimated from inner and outer leaflet bilayer order

Outer leaflet % ordered lipid and total (both leaflet) % ordered lipid were calculated from TMADPH and DPH anisotropy, respectively, as described in Table 3.2 and Table 3.4. Inner leaflet % ordered lipid was calculated using the following equation: Inner leaflet % ordered = (total % ordered lipid – outer leaflet % ordered lipid*0.67) / 0.33. This assumes the % of total surface area in SUV is ~67% outer leaflet and ~33% inner leaflet.

If inner and outer leaflets have a symmetric composition or have coupled physical states, the difference between the outer and inner leaflet order should be zero. If the vesicles are fully asymmetric and have uncoupled inner and outer leaflet physical states (i.e. outer leaflet SM in gel state, inner leaflet PC in Ld state) the difference between order in the inner and outer leaflet should be 100%. Average (mean) values and S.D. shown for the number of samples given in Table 3.2 and Table 3.4.

Chapter 4

The Dependence of Lipid Asymmetry upon the Structure of Lipid Polar Headgroups

4.1. Introduction

The inner and outer leaflet of many cellular membranes exhibit differences in lipid composition. This difference is called lipid asymmetry. For example, in eukaryotic cells, the choline-containing lipids, sphingomyelin (SM) and phosphatidylcholine (PC), are predominantly located in the outer leaflet of the plasma membrane, while the amine-containing phospholipids, such as phosphatidylethanolamine (PE) and phosphatidylserine (PS), are largely or fully confined to the plasma membrane inner leaflet [1, 5-7]. Many important functional roles of cellular membranes are closely associated with an asymmetrical lipid distribution. For example, dissipation of membrane lipid asymmetry, resulting in PS externalization on the outer leaflet of membrane, facilitates cell recognition and phagocytosis by macrophages during apoptosis [66-

74]. Exposure of PS on the outer cell surface has been also known to be involved in various physiologically important phenomena including blood coagulation [187, 188], cell adhesion [189-191], and myotube formation [77].

To emulate cell membranes more closely, a number of methods have been developed to obtain artificial lipid bilayers (model membranes) that mimic various aspects of the asymmetric lipid distribution of biological membranes [131-134, 138-140, 145, 149, 151-154, 156-160, 182, 183]. However, methods to form asymmetric vesicles with a wide variety of highly controlled lipid components have been lacking. To tackle this problem, our laboratory has developed a method to prepare asymmetric vesicles using MβCD-induced lipid exchange [158-160]. Vesicles prepared by MβCD-induced exchange have great potential for various applications since the procedure is not unduly complicated, they can be prepared with a variety of lipid compositions, and they can be used to create asymmetric vesicles with various size, and reconstituted vesicles with membrane inserted peptides. We recently extended this method by generating asymmetric vesicles containing SM as outer leaflet lipids and a large variety of PC with different acyl chain structures as inner leaflet lipids [171]. It was found that MβCD-induced lipid exchange was always efficient. However, the stability of asymmetry in the vesicles prepared by lipid exchange was dependent on the acyl chain structure, and this was found to be due to the influence of acyl chain structure upon transverse diffusion (flip-flop). In this chapter, the exchange method has been extended to investigate the effect of lipid headgroup structure upon the efficiency of lipid exchange and the ability to form asymmetric vesicles. Lipid exchange was carried out by substituting SM into the outer leaflet of vesicles containing PE, PS, PG, PI, phosphatidic acid or cardiolipin. The results show that efficient exchange is always possible, but that headgroup structure can markedly influence whether the resulting vesicles are fully or only partially asymmetric.

4.2. Results

4.2.1. Preparation of Exchange (Asymmetric) SUVs by the MβCD-induced Lipid Exchange Method

In the lipid exchange method, the lipids in the outer leaflet of an excess of donor vesicles are transferred to the outer leaflet of acceptor vesicles using MβCD, which functions as a carrier that exchanges lipids between two different vesicle populations (MLVs and SUVs) [158-160, 171]. After isolation of acceptor vesicles, this should allow preparation of acceptor vesicles that have a different lipid composition in their inner and outer leaflets. The dependence of lipid asymmetry in isolated acceptor vesicles after lipid exchange (=exchange vesicles) upon exchange vesicle lipid composition is an important topic to study both to define what types of asymmetric vesicles can be prepared for experimental studies and in order to define how lipid structure influences that ability to maintain lipid asymmetry, and important parameter for understanding the origin and maintenance of lipid asymmetry in nature membranes. Previously, we found that efficient lipid exchange is not affected by PC acyl chain structure, but the ability to maintain asymmetry after exchange is highly dependent upon acyl chain structure [171].

In this study, the method has been further extended to determine whether the headgroup structure of lipids has an effect on the efficiency of exchange and ability to generate asymmetric SUVs containing SM in the outer leaflet and lipids with various headgroup structures in the inner leaflet. The headgroup structures we varied as acceptor vesicles included a variety of anionic lipids: phosphatidylglycerol (POPG and DOPG), phosphatidylinositol (soy PI and DOPI), phosphatidic acid (PA), cardiolipin (TOCL), phosphatidylserine (PS), as well as mixtures of anionic lipids and the zwitterionic lipid phosphatidylethanolamine (PE). PA was only studied in a mixed composition with POPC, which was found to be necessary to prepare PA-containing SUVs.

First, the amount of SM exchanged into acceptor vesicles was assayed as a function of acceptor lipid structure. HP-TLC analysis of acceptor vesicles after exchange showed that a significant amount of SM in the donor vesicles was introduced to the acceptor vesicles, showing that the lipids with different headgroup structure listed above did not affect to the capability of lipid exchange induced by MβCD. Table 4.1 shows the average SM content in exchange vesicles was around 60% of total lipids. Since the outer leaflet percent of total surface area in SUV is around 67%, this indicates that MβCD-induced lipid exchange is efficient, corresponding to roughly 90% exchange of outer leaflet lipids, consistent with our prior studies [171], and demonstrates that the exchange method can be successfully used with all lipids studied.

4.2.2. Determination of Asymmetry by Comparison of Ordered (gel) Phase Thermal Stability in Exchange Vesicles and Scrambled Vesicles

To determine whether SM is asymmetrically distributed over the bilayer of the exchange SUVs (SM in the outer leaflet and lipids with other headgroup structures in the inner leaflet), the thermal stability of SM-rich ordered domains in the exchange vesicles was compared with that in symmetric vesicles. To do this, such that the symmetric vesicles had identical lipid compositions as the exchange vesicles, we used prepared "scrambled vesicles" in which the lipids from exchange vesicle preparations were dissolved in organic solvents and then reconstituted to produce ordinary vesicles.

We previously observed exchange vesicles have a higher Tm than scrambled vesicles of the same composition when SM is asymmetrically distributed over the bilayers, i.e., gel state lipid SM in the outer leaflet and fluid state unsaturated lipids in the inner leaflet [158, 159, 171]. The melting transition of the SM-rich outer leaflet in fully asymmetric vesicles occurs at a similar temperature as the pure SM vesicles, evidently because the thermal stability of the ordered gel domain in the SM-rich outer leaflet is not affected by the presence of unsaturated

fluid phase lipids that are in the inner leaflet [158, 159, 171]. In contrast, symmetric vesicles containing mixtures of SM with other lipids exhibit a lower Tm than pure SM [158, 159, 171]. Thus, a decrease of Tm upon lipid scrambling is indicative of asymmetry. When exchange vesicles are not asymmetric, the Tm before and after scrambling is identical [171].

The thermal stability of the ordered domain in both exchange and scrambled vesicles was determined from the steady-state DPH anisotropy measurement as a function of temperature. DPH (Figure 2.1) is a hydrophobic fluorescent molecule that is distributed throughout the hydrocarbon regions of lipid bilayer when it is incorporated into membranes [192]. Since the motion of DPH is sensitive to the packing properties and the degree of lipid ordering environment in the bilayer membrane [193, 194], DPH anisotropy is high in tightly packed gel state ordered domains and low in loosely packed liquid crystalline state disordered domains [195]. The phase transition of the lipid physical state from the ordered gel phase to the disordered liquid crystalline phase is accompanied by a sigmoidal curve of decreasing DPH anisotropy as the temperature is increased. The midpoint of this phase transition is referred as the melting temperature (Tm), a parameter that reflects the thermal stability of the ordered state/ordered domains [196, 197].

Figure 4.1 shows the representative melting curves defined from DPH anisotropy versus temperature in SUVs. In the ordinary symmetric SUVs, the highest Tm values are observed for pure SM vesicles (36.5 °C), with intermediate Tm values for vesicles composed a mixture of 6:2:1 (mol:mol) SM:POPE:POPG (32.2 °C), and lowest Tm (16.3 °C) for 2:1 POPE:POPG. The exchange vesicles, which have an overall lipid composition (with 63.2 ± 3.8% SM) similar to that of the symmetric 6:2:1 SM:POPE:POPG vesicles mixtures, had a Tm (37.1 °C), about as high as pure SM vesicles. Notice that the anisotropy in the exchange vesicles is lower than that of SM vesicles at low temperature. This is as expected from previous vesicles for asymmetric exchange vesicles, because the inner leaflet lipids remain in the fluid liquid disordered state after exchange (see below).

This result (Figure 4.1) shows that the thermal stability of ordered domain in the exchange SUVs was not destabilized by the presence of unsaturated POPE/POPG lipids. However, the Tm value for these exchange SUVs dropped to 31.9 °C in scrambled SUVs, which have the identical lipid compositions with the exchange vesicles. The Tm value for the scrambled vesicles was about the same as in the symmetric 6:2:1 SM:PE:PG vesicles, as expected if exchange vesicles have a symmetric lipid composition after scrambling.

These experiments were repeated for all of the lipids studied. Figure 4.2 shows the melting temperature of exchange vesicles (black bar) and their scrambled vesicles (white bar). (Notice that for the exchange vesicles we are using the nomenclature previous established for asymmetric vesicles, i.e. in this case SMo/Xi where X is the name of the lipid(s) in the acceptor vesicles, o=outer leaflet lipid after exchange, and i= inner leaflet lipid after exchange, if asymmetry is achieved. In this chapter, this nomenclature if used both when exchange vesicles are completely asymmetric and when they are just partly asymmetric.)

A pattern showing a substantial Tm decrease after lipid scrambling was observed for all the different headgroup lipids tested, with a Tm decrease of up to 6 °C upon scrambling. Thus, lipids with different headgroup structures do not affect to the ability to carry out lipid exchange or form vesicles with at least some degree of lipid asymmetry.

4.2.3. Estimating the Stability of Asymmetry from the Time Dependence of Tm Values

Previously we found that the extent of lipid asymmetry in exchange vesicles was linked to the rate of spontaneous lipid movement across the bilayer (= lipid transverse diffusion = flip-flop) [171], such that the formation of vesicles with lipid asymmetry was observed when there

was a slow rate of lipid flip-flop. This would suggest that the extent of lipid asymmetry in exchange vesicles upon headgroup structure also reflected headgroup-dependent differences in flip-flop. To see if the vesicles with different headgroups had a stable level of asymmetry, the Tm value for exchange vesicles immediately after vesicle preparation (Figure 4.2, black bars) was compared to that 1 day after vesicle preparation (Figure 4.2, grey bars). If asymmetry was not stable, then Tm values should decrease and approach that in scrambled vesicles (Figure 4.2, white bars).

Figure 4.2 shows there was a small, but measurable decrease of Tm 1 day after vesicle preparation for exchange vesicles lacking POPE, for exchange vesicles containing PG, TOCL, Soy PI or PA. However, when these lipids were mixed with POPE, the Tm of exchange vesicle did not decrease 1 day after vesicle preparation. In the case of POPS, the Tm of exchange vesicles was stable after 1 day, both with or without POPE in the exchange vesicles.

To see if the behavior of soy PI (one saturated and one poly unsaturated chain [16:0-18:2] as dominant acyl chain species) reflected a difference in acyl chain structure, we also carried a few experiments using DOPI. Exchange vesicles prepared with DOPI, exhibited a higher Tm than after vesicle scrambling and a Tm value that decreased significantly 1 day after preparation (Figure 4.3).

4.2.4. Asymmetry of Exchange Vesicles Assayed by Proton NMR Spectroscopy

The studies above could not distinguish between the case in which freshly prepared exchange vesicles are partly asymmetric and that in which they are fully asymmetric. It was especially important to examine the possibility that the lipid compositions that were found above

to result in unstable lipid asymmetry were only partly asymmetric even right after vesicle preparation.

We previously were able to detect the extent of lipid asymmetry by assaying the SM content in the outer leaflet from the anisotropy of TMADPH incorporated selectively into the outer leaflet [171] and combining that information with total amount of SM in the vesicles to calculate the amount of SM in the inner leaflet [171]. This method could not be used with the vesicles in this report because we found that the cationic TMADPH molecules (Figure 2.1) seemed to associate with residual anionic lipids in the outer leaflet better than SM rich outer leaflet domains, resulting in anomalously low anisotropy values.

Therefore, we further assayed the asymmetry of exchange vesicles by an alternative approach using proton NMR spectroscopy. The small radius and thus high degree of curvature in SUVs results in different headgroup packing in the outer and inner leaflet of bilayer. This difference in packing evidently influences the chemical shift of the choline N-methyl groups on lipids such as SM and PC. The methyl signal, which splits into separate inside methyl and outside methyl peaks, is readily distinguishable by proton NMR, with the outer leaflet methyls having a downfield chemical shift in the inner leaflet methyls having an upfield chemical shift [198, 199]. Thus, it was possible to use the relative intensity of the choline methyl groups of SM to estimate the outer/inner leaflet distribution of SM in SUVs. In order to eliminate complications due to a difference in the physical state of lipids in the inner and outer leaflet, all the proton NMR experiments were carried out at 55 °C, which is well above the melting points of SM and the other lipids tested.

Figure 4.4 shows the chemical shift of the choline N-methyl groups of SM in ordinary and exchange SUVs. For symmetric SUVs composed of pure SM or SM/different headgroup lipids mixture, two split ^{1}H resonances were observed, as expected, corresponding to the outer membrane leaflet at a chemical shift of 3.47-3.49 ppm and the inner membrane leaflet at a

chemical shift about 0.04 lower, with a somewhat smaller difference in shift in some cases, indicating that SM is localized both in the outer and inner leaflet of bilayers, as expected (Figure 4.4). For exchange SUVs, when lipids such as POPG, DOPG, TOCL, or soy PI were used alone as the inner leaflet lipids, freshly prepared exchange vesicles also showed two ^{1}H resonances of SM choline N-methyl groups located in both outer leaflet and inner leaflet of exchange vesicles. This confirmed that these vesicles did not form fully asymmetric vesicles. However, the intensity of the inner peak relative to the outer leaflet peak was significantly less than in the corresponding symmetric vesicles, indicative of some degree of asymmetry. Vesicles containing POPS exhibited only very weak inner leaflet SM peaks, suggesting they were more asymmetric than was the case for the other anionic lipids.

Figure 4.4 also shows that when the various anionic lipids were mixed with POPE, only a very small upfield choline methyl group signal was detected. This indicated that in the presence of POPE the vesicles were very highly asymmetric. We conclude there is partial lipid asymmetry in the exchange vesicles when POPG, DOPG, TOCL, and soy PI were used alone as the inner leaflet lipids, but the vesicles were nearly fully asymmetric with the addition of POPE to the anionic lipids. It appears that POPS can form largely asymmetric vesicles even in the absence of POPE.

It is noteworthy that the pattern of the extent of lipid asymmetry defined by NMR matched the pattern of asymmetry stability defined by the decrease in Tm values 1 day after vesicle preparation. The exchange vesicles that appeared to show a decrease in Tm after 1 day of incubation were the same vesicles that exhibited incomplete asymmetry even when freshly prepared.

4.2.5. Fully Asymmetric Vesicles are not in a Fully Ordered State

Table 4.2 shows that as estimated from the anisotropy of DPH, at 23 °C the level of membrane order in exchange vesicles after introduction of SM is lower than in pure SM vesicles, while, at least in all the exchange vesicles that had full asymmetry, Tm values were similar to those in pure SM vesicles. Our previous studies have shown that in SMo/PCi asymmetric vesicles [171], in which the PC has at least one unsaturated acyl chain, this reflects a lack of coupling between the physical state in the outer leaflet, which is nearly pure SM, and thus is in the gel state at 23 °C, and that of the inner leaflet, which remains in the liquid disordered state. Based on these observations it appears that the lack of coupling between inner and outer leaflet physical states is also true for the asymmetric vesicles with SM outside and lipids other than PC inside, at least when the inside lipids have at least one unsaturated acyl chains.

4.3 Discussion

4.3.1. Effect of Headgroup Lipid upon Asymmetric Vesicle Formation and the Stability of Asymmetry

This study has shown that it is possible to use MβCD-induced lipid exchange form asymmetric vesicles with vesicles having a wide variety of lipid headgroups. However, the stability of asymmetry was strongly affected by lipid headgroup structure. In some cases fully asymmetric vesicles with outer leaflets composed of SM were obtained. For example, fully asymmetric vesicles that remained stably asymmetric over 1 day were obtained when the inner leaflet of the vesicles contained PS, in agreement with our prior studies [158]. Our prior studies with PC having the same acyl chains as the lipids used in this study (i.e. DOPC and POPC) also found that fully asymmetric vesicles were obtained [158]. In contrast, only partial asymmetry that decreased during 1 day of incubation was obtained with the other anionic lipids tested (PG, PI, and TOCL). However, mixing these lipids with PE allowed formation of fully asymmetric vesicles that appeared to remain fully asymmetric after 1 day of incubation.

These observations raise the question of why PS, PE and PC allow or promote the formation of stable asymmetry. One likely factor is that these lipids have multiple charged groups, both anionic and cationic, and rarely (or in the case of PC never) exist in an uncharged state. In contrast, PG, PI and TOCL have only one anionic charge on the phosphate (which in the case of TOCL means one anionic group per two acyl chains). Even a few pH units above their pKa, a small but significant fraction of these lipids will be in an uncharged state, which should have greatly increased hydrophobicity relative to the charged form, and thus more readily penetrate into and cross the hydrophobic core of the bilayer. This tendency would be exacerbated by repulsions between the anionic groups of neighboring lipids, which would raise the free energy of the lipids in the usual non-translocating conformation relative to the transition state expected to form when the lipid are crossing the most hydrophobic part of the bilayer.

The repulsion between anionic lipids in their normal, non-translocating state should be lessened significantly by the presence of zwitterionic PE molecules. This may explain why asymmetry is more stable in the presence of PE. Another factor may be the small headgroup size of PE, which would lessen steric clashes between headgroups. Since the headgroup of PC is larger than PE, this factor might explain why mixtures of PC and PA did not form exchange vesicles with very stable asymmetry. Alternatively, PA has such a small headgroup, it may cross membranes faster than other lipids. Another possibility is that the lipid flip-flop that destroys asymmetry involves more than one lipid in a leaflet flipping at the same time. Dimers of protonated anionic lipids might flip faster than dimers of PE and an anionic lipid.

4.3.2. Potential Biological Implications of the Dependence of Asymmetry upon Lipid Headgroup Composition

The effect of lipid headgroup structure upon lipid asymmetry may help explain some of the functions of different lipids. The observation that mammalian membranes contain considerable PE mixed with anionic lipids in the inner leaflet may reflect the necessity to maintain lipid asymmetry with wasteful expenditure of energy on ATP-consuming lipid translocases/flippases [180]. The case of PS is particularly interesting as its appearance on the outer leaflet of the plasma membrane is a signal that apoptosis is occurring and an external signal to macrophage that the damaged, apoptotic cell needs to be engulfed and consumed [66-74]. We speculate that PS is a particularly good choice for this functional role relative to other anionic lipids because its tendency to spontaneously flip across the lipid bilayers is especially low, minimizing the chance that healthy cell could be accidentally destroyed.

Table 4.1. Percent SM in exchange vesicles

%SM in exchange vesicles was calculated from HP-TLC analysis of Sepharose CL2B SUV fractions (details in Methods). Average (mean) and range derived from duplicate experiments or S.D when n>2 is shown in parentheses are shown. The number of experiments is shown in parenthesis.

Sample composition	SM % by TLC
SMo / POPG in	57.9 ± 4.3 (4)
SMo / DOPG in	57.5 ± 2.1 (2)
SMo / Soy PI in	59.9 ± 1.7 (5)
SMo / TOCL in	67.8 ± 4.1 (2)
SMo / POPC:DOPA 2:1 in	62.2 ± 2.1 (2)
SMo / POPS in	59.1 ± 4.5 (2)
SMo / POPE:POPG 2:1 in	63.2 ± 3.8 (2)
SMo / POPE:DOPG 2:1 in	59.1 ± 2.4 (2)
SMo / POPE:Soy PI 2:1 in	60.5 ± 2.6 (2)
SMo / POPE:TOCL 2:1 in	58.6 ± 3.5 (2)
SMo / POPE:POPS 2:1 in	57.6 ± 2.6 (2)

Table 4.2. DPH fluorescence anisotropy in symmetric and exchange vesicles at 23 ^{o}C

The percent ordered state of outer leaflet was estimated from the following equation: Percent ordered = $(A - A_{100\% \text{ Ld}})/(A_{100\% \text{ ordered}} - A_{100\% \text{ Ld}})$. A is that in an exchange vesicle, $A_{100\% \text{ ordered}}$ is that in SM, and $A_{100\% \text{ Ld}}$ is that in the appropriate unsaturated lipid. This formula assumes that gel (=ordered) domain and Ld domains have A values similar to that in pure gel and Ld state vesicles, respectively. DPH was added to preformed vesicles at a concentration of 0.1 mol% of total lipid concentration. Ordinary (symmetric) vesicles were prepared by ethanol dilution. Average (mean) and range derived from duplicate experiments or S.D when n>2 is shown in parentheses are shown. Number of samples shown in parentheses. * These lipid mixtures may not be totally in Ld state

Sample Composition	DPH Anisotropy	% ordered
SM	0.311 ± 0.002 (5)	≡ 100
POPG	0.115 ± 0.001 (2)	≡ 0
DOPG	0.098 ± 0.002 (2)	≡ 0
Soy PI	0.108 ± 0.002 (2)	≡ 0
TOCL	0.099 ± 0.002 (2)	≡ 0
2:1 POPC:DOPA	0.114 ± 0.004 (2)	≡ 0
POPS	0.105 ± 0.003 (2)	≡ 0
2:1 POPE:POPG	0.158 ± 0.005 (2)	≡ 0 *
2:1 POPE:DOPG	0.129 ± 0.003 (2)	≡ 0 *
2:1 POPE:Soy PI	0.144 ± 0.002 (2)	≡ 0 *
2:1 POPE:TOCL	0.132 ± 0.002 (2)	≡ 0 *
2:1 POPE:POPS	0.143 ± 0.002 (2)	≡ 0 *
SMo / POPG in	0.239 ± 0.008 (7)	63.3 ± 5.2
SMo / DOPG in	0.223 ± 0.012 (2)	58.7 ± 6.4
SMo / Soy PI in	0.224 ± 0.005 (8)	57.1 ± 3.9
SMo / TOCL in	0.210 ± 0.007 (10)	52.4 ± 4.3
SMo / POPC:DOPA 2:1 in	0.237 ± 0.004 (6)	62.4 ± 4.5
SMo / POPS in	0.252 ± 0.004 (6)	71.4 ± 4.4
SMo / POPE:POPG 2:1 in	0.274 ± 0.003 (4)	75.8 ± 6.7
SMo / POPE:DOPG 2:1 in	0.243 ± 0.004 (4)	62.6 ± 4.5
SMo / POPE:Soy PI 2:1 in	0.281 ± 0.003 (4)	82.0 ± 5.5
SMo / POPE:TOCL 2:1 in	0.231 ± 0.005 (4)	55.3 ± 4.4
SMo / POPE:POPS 2:1 in	0.272 ± 0.016 (5)	76.8 ± 10.7

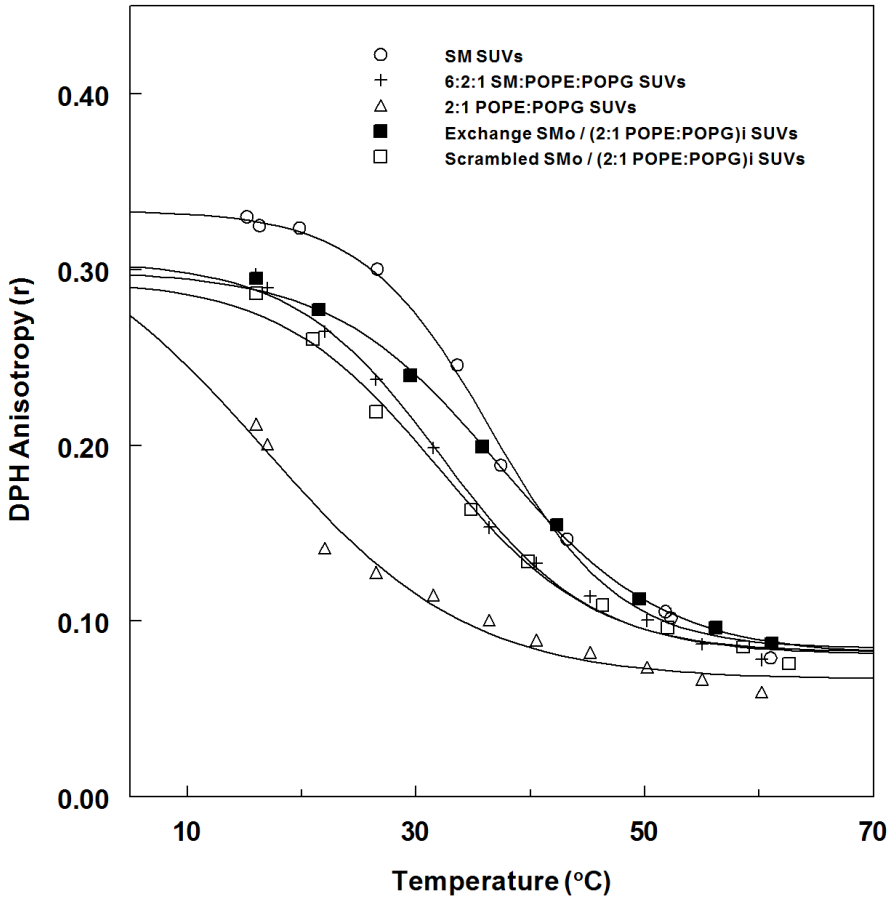


Figure 4.1. Representative steady-state anisotropy of DPH as a function of temperature in ordinary & symmetric, and exchange SUVs

The phase transition from the gel to liquid disordered is observed as a decrease in the steady-state DPH anisotropy. The mid-point of this transition is defined as the melting temperature (Tm). The Tm estimated was 36.5 °C for pure SM vesicles, 32.2 °C for 6:2:1 SM:POPE:POPG vesicles, 16.3 °C for 2:1 POPE:POPG vesicles, 37.1 °C for exchange SMo/(2:1 POPE:POPG)i vesicles, and 31.9 °C for scrambled SMo/(2:1 POPE:POPG)i vesicles.

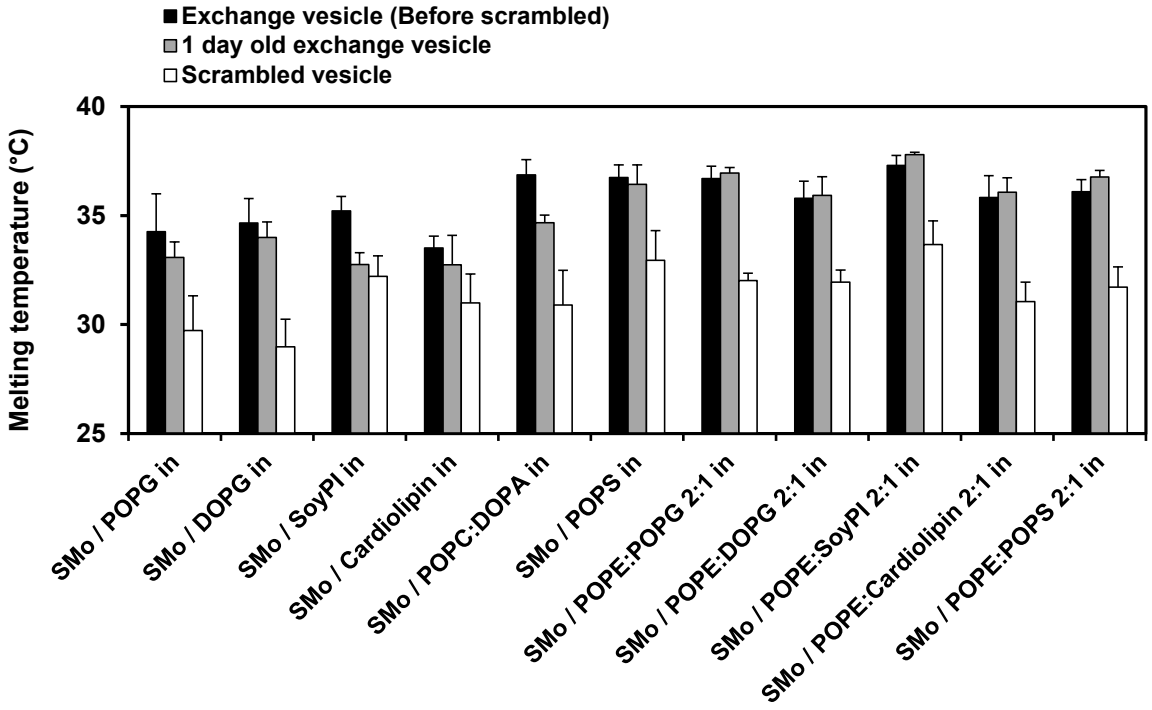


Figure 4.2. The melting point of exchange, 1day old, and scrambled SUVs

The average (mean) and S.D. are shown. Number of samples are same as shown in Table 4.2. DPH was added to preformed vesicles at a concentration of 0.1mol % of total lipid concentration. The steady-state DPH anisotropy measurements as a function of temperature were made immediately after exchange vesicle preparation for samples of exchange vesicles, and 1 day after at room temperature incubation for samples of 1 day old exchange vesicles.

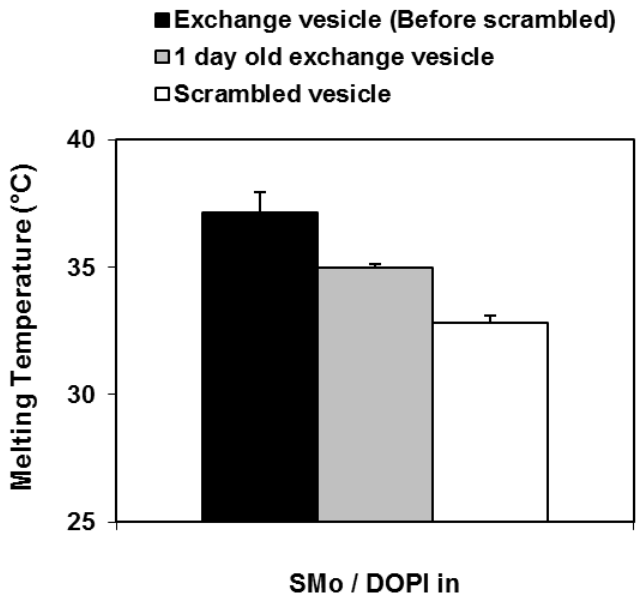
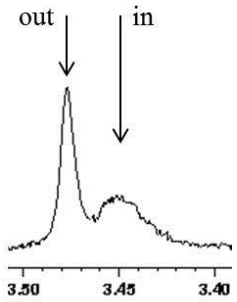


Figure 4.3 The melting point of exchange, 1day old, and scrambled SUVs prepared with DOPI

The average (mean) and S.D. are shown. DPH was added to preformed vesicles at a concentration of 0.1 mol% of total lipid concentration. The steady-state DPH anisotropy measurements as a function of temperature were made immediately after exchange vesicle preparation for samples of exchange vesicles, and 1 day after at room temperature incubation for samples of 1 day old exchange vesicles.

A



B

	Ordinary, symmetric vesicle		Lipid exchange vesicle	
	SM-X	**SM-PE-X**	**SM-X**	**SM-PE-X**
PG				
TOCL				
PI				
PS				

Figure 4.4. Proton NMR spectra at 55 °C of SM choline N-CH_3 in SUVs

The chemical shift of choline N-methyl groups of SM is shown with the outer leaflet at δ 3.47-3.49 ppm and the inner leaflet at a lower δ by about 0.04 ppm. Experimental errors of the chemical shift were ± 0.02 ppm. **A**. Chemical shift in symmetric pure SM SUVs. The arrows denote the SM distributed in the outer and inner leaflet of membrane. **B.** Ordinary, symmetric vesicles of SM/X 2:1 and SM/PE/X 6:2:1 are shown left. Exchange vesicles composed of SM, POPG, cardiolipin, Soy PI, and POPS mixed with or without POPE are shown right.

Chapter 5

Summary and Future Directions

5.1. Summary

The MβCD-induced lipid exchange method developed in our laboratory has previously been used to generate stable asymmetric vesicles which mimic the eukaryotic plasma membrane with SM as the predominant lipid in the outer leaflet and POPE/POPS, POPS, POPC or DOPC in the inner leaflet. In the research project of this thesis, I examined for a much wider range of lipids to see how the structure of lipids influences the capability to exchange them using MβCD as well as impacts the ability to form stable asymmetric vesicles. To do this, I prepared exchange/asymmetric SUVs composed of SM as outer leaflet lipid and various types of lipids as the inner leaflet lipids. The variations of inner leaflet lipid structures investigated can be divided into two categories; 1) acyl chain structure, and 2) headgroup structure. A more detailed description of the lipids studied is given below.

First, I exchanged SM into the outer leaflet of SUVs composed of PCs with different acyl chain structures. Acyl chains of PCs with different lengths (di14:1PC, di16:1PC, di18:1PC, di20:1PC, and di22:1PC), fatty acid branching (diphytanoylPC; four methyl group per chain), unsaturation levels in one chain (16:0-18:2PC and 16:0-20:4PC), or unsaturation levels in both chains (di18:2PC, di18:3PC, and di20:4PC) were studied. HP-TLC analysis showed efficient SM exchange into SUV for all of the cases I tried. DPH and TMA-DPH fluorescence anisotropy indicated that ordered domains appeared in the exchange vesicles, and the thermal stability of ordered domains was determined via the temperature dependence of DPH fluorescence anisotropy. We also found that in cases where the exchange vesicles were asymmetric, they had a melting temperature (Tm) similar to pure SM vesicles, showing that the thermal stability of ordered domains in the outer leaflet is not decreased by the presence of a wide variety of unsaturated lipid-rich inner leaflets. Vesicle scrambling experiments were done to confirm lipid asymmetry. Scrambled vesicle samples having the identical lipid composition as the original samples, in most cases had lower Tm than original vesicles, confirming asymmetry. Exchange vesicles with two 14 carbon monounsaturated chains or two polyunsaturated chains, however, had a similar Tm and percent order after the scrambling process. This suggests that PC with two 14 carbon monounsaturated chains or two polyunsaturated chains may not form asymmetric vesicles, although they do not affect the ability to exchange lipids. I investigated the origin of these patterns by comparing transverse diffusion (flip-flop) of lipids in vesicles with various acyl chain structures. A correlation between asymmetry and transverse diffusion was observed, with transverse diffusion slower in vesicles containing PCs from which asymmetric vesicles could be prepared.

In a second study, lipids with headgroups other than PC were investigated. In this study, we exchanged SM into vesicles containing POPG, DOPG, dioleoyl cardiolipin, Soy PI, DOPA, and POPS alone or mixed with POPE or (in the case of DOPA) with POPC. We used time dependent changes in the thermal stability of ordered domains and proton NMR experiments to study the asymmetry of vesicles. These lipids also did not affect the ability to exchange SM with

MβCD, but, in many cases, did impact the stability of asymmetry. However, when these lipids were mixed with POPE, they formed vesicles with stable asymmetry. Interestingly, POPS allowed formation of vesicles with stable asymmetry even when not mixed with POPE.

These studies indicate that MβCD-dependent lipid exchange method can be applied to generate asymmetric vesicles using a wide, but not universal, range of acyl chains and headgroup structures. This ability will be useful in future studies of the effect of acyl chain and headgroup structure upon the properties and function of asymmetric bilayers. The presence of lipids that can undergo fast transverse diffusion prevents the formation of stable lipid asymmetry in exchange vesicles. These properties may explain why such lipids are not generally abundant in natural membranes in which stable asymmetry is a critical structural feature.

5.2. Future Directions

5.2.1. Preparation of Exchange Vesicles with Cholesterol

Cholesterol is one of the major classes of structural lipids in eukaryotic plasma membrane. Numerous experiments have shown that cholesterol affects the physical properties of the plasma membrane and model membranes. Investigation of the effect of cholesterol introduction on the ability to lipid exchange and form asymmetric vesicles using various lipids studied in this thesis would be an impact area for future research. In our laboratory, we have already generated SMo/DOPCi and SMo/POPE:POPSi asymmetric vesicles containing about 25 mol% cholesterol by incubating asymmetric vesicles with cholesterol-loaded MβCD [158]. This protocol allows preparation of cholesterol containing exchange/asymmetric vesicles that contain SM outside and lipids with various acyl chain and headgroup structure inside. Using these vesicles, an analysis can be carried out to evaluate the impact of cholesterol on the rate of transverse diffusion in exchange vesicles.

5.2.2. Varying Outer Leaflet Lipids

The exchange/asymmetric vesicles I generated have sphingomyelin as the predominant lipid in the outer leaflet. The next set of experiments can be to observe the effect of lipid structure on the formation of asymmetric vesicles by using additional types of lipid mixtures other than SM as outer leaflet lipids. To mimic asymmetric lipid distribution in eukaryotic subcellular membranes such as endoplasmic reticulum which contains only a small percent of SM (~ 3.7% of total lipids) [200], exchange vesicles composed of lipids other than SM will be ideal. The possible examples for the outer leaflet lipids are low Tm lipids such as POPC, POPS, POPG, and POPA. In addition, DPPC, DPPE, DPPS, DPPG, DPPA, and SM with different acyl

chain lengths can be also tried as additional types of high Tm lipids. By generating exchange/asymmetric vesicles composed of various lipids as the outer leaflet lipids, we can also learn how these lipids affect the stability of asymmetry and coupling between the physical state of the inner and outer leaflet lipids.

5.2.3. Preparation of "Reverse" Asymmetric Vesicles

The asymmetric vesicles generated in this thesis have SM as the outer leaflet lipid and unsaturated phospholipids as the inner leaflet lipids. This was chosen originally in an effort to mimic the natural eukaryotic plasma membrane. An important goal would be to prepare vesicles with reversed asymmetry, in which outer leaflets contain unsaturated lipids and the inner leaflets contain SM. These vesicles will be useful to study the membrane association of proteins that reside in the cytosol and interact with lipids in the inner leaflet of bilayers.

5.2.4. Studying the Relationship between Lipid Composition and Membrane Protein Structure and Function

Exchange vesicles have potential applications in the study of the relationship between lipid composition and membrane protein structure and function. One of great advantages using MβCD-dependent lipid exchange method is that it could allow easy reconstitution with membrane proteins in asymmetric membranes. The lipid exchange can be accomplished in the presence of membrane protein using a wide range of lipid compositions and would allow us to compare the function of protein in symmetric and asymmetric vesicles. However, it will be challenging to control the orientation of membrane proteins in these experiments. Controlled protein orientation will be necessary to allow interpretation of data from asymmetric vesicles.

This would also allow studies of whether the ability to form asymmetric vesicle is influenced by in the presence of membrane protein.

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