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Lipid phase separation in phospholipid bilayers and monolayers modeling the plasma membrane

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Abstract

It is postulated that biological membrane lipids are heterogeneously distributed into lipid microdomains. Recent evidence indicates that docosahexaenoic acid-containing phospholipids may be involved in biologically important lipid phase separations. Here we investigate the elastic and thermal properties of a model plasma membrane composed of egg sphingomyelin (SM), cholesterol and 1-stearoyl-2-docosahexaenoyl-*sn*-glycerophosphoethanolamine (SDPE). Two techniques are employed, pressure–area isotherms on monolayers to examine condensation and interfacial elasticity behavior, and differential scanning calorimetry (DSC) on bilayers to evaluate phase separations. Significant levels of condensation are observed for mixtures of SM and cholesterol. Surface elasticity measurements indicate that cholesterol decreases and SDPE increases the in-plane elasticity of SM monolayers. At $X_{SDPE} \geq 0.15$ in SM, a more horizontal region emerges in the pressure–area isotherms indicating 'squeeze out' of SDPE from the monolayers. Addition of cholesterol to equimolar amounts of SM and SDPE further increases the amount of 'squeeze out', supporting the concept of phase separation into a cholesterol- and SM-rich liquid ordered phase and a SDPE-rich liquid disordered phase. This conclusion is corroborated by DSC studies where as little as $X_{Chol} = 0.0025$ induces a phase separation between the two lipids.

Keywords: Lipid microdomain; Phase separation; Squeeze out; Plateau region; Docosahexaenoic acid

1. Introduction

Biological membranes appear to consist of heterogeneous dispersions of phospholipids and proteins existing in dissimilar regions of the membrane referred to as domains [1,2]. Domains have been shown to exist as macrodomains that are protein-driven and microdomains that are to a large extent lipid-driven. Protein-based macrodomains are known to be stable and are often well characterized entities while little is known about lipid microdomain composition, stability and size. Much of the evidence for the existence of lipid microdomains comes mainly from indirect biophysical measurements and from membrane fractions that are insoluble in cold Triton X-100 (detergent resistant membranes, DRMs) [3]. DRMs are composed primarily of sphingolipids and cholesterol

Abbreviations: Chol, cholesterol; C_s^{-1}, surface elasticity moduli; DHA, docosahexaenoic acid; DPPC, dipalmitoylphosphatidylcholine; DRM, detergent resistant membrane; DSC, differential scanning calorimetry; DSM, detergent soluble membrane; MLV, multilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SDPC, 1-stearoyl-2-docosahexaenoyl-*sn*-glycerophosphocholine; SDPE, 1-stearoyl-2-docosahexaenoyl-*sn*-glycerophosphoethanolamine; SM, sphingomyelin; T_m, transition temperature

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in a liquid ordered state. The DRM microdomains are believed to affect the function of some proteins, thereby influencing signaling within the cell [3]. Historically the best documented examples of lipid microdomains come from phase separations in liquid crystalline and gel state bilayers in phospholipid model membranes [1]. Although there have been numerous reports of liquid crystalline and gel state phase separations, they have limited biological relevance since few gel state lipids actually exist in significant quantities in most biological membranes.

It has been suggested that lipid microdomains may range from as little as 20 molecules up to tens of square microns in size and range in stability from nanoseconds up to the lifetime of a cell [4,5]. The effect of lipids on microdomain size and stability must reflect both phospholipid head group structure, size and charge as well as the composition (chain length and degree of unsaturation) of the acyl chains. Lipid microdomains are therefore largely the result of lipid–lipid interactions. The liquid ordered phase found in DRMs, for example, demonstrates a high affinity between sphingomyelin (SM) and cholesterol (Chol). If there are patches of the membrane that are cholesterol- and SM-rich (i.e. DRMs) then there must also be regions or domains in the membrane that are cholesterol- and SM-poor. It can be predicted that these domains would exist in the liquid disordered phase and would be detergent soluble (detergent soluble membranes, DSMs). DSMs should be enriched in lipid components that have a low affinity for both SM and cholesterol.

It is possible, perhaps likely, that biological membranes are largely composed of a patchwork of lipid microdomains formed as a result of fluid–fluid phase separations. Here we model a mammalian plasma membrane using a three-lipid component monolayer and bilayer system. The lipids chosen are cholesterol, SM and a phosphatidylethanolamine (PE) with stearic acid as the *sn*-1 chain and docosahexaenoic acid (DHA) as the *sn*-2 chain. Lipids comprising the mammalian plasma membrane can be categorized into three principal types: phospholipids, sphingolipids, and cholesterol [6]. Phospholipids are found as the major polar lipid component whereas sphingolipids can comprise 10–20 mol% of the plasma membrane [7]. Cholesterol, the third major component, can comprise up to 50 mol% of the total plasma membrane lipids [8]. DHA, an ω-3 fatty acid, is the longest and most unsaturated fatty acid commonly found in membranes [9]. DHA is found in high quantities in neural membranes, rod outer segments of the eye and sperm plasma membranes [10] and has been linked in a positive way to a wide variety of human afflictions including heart disease, cancer, autoimmune diseases, abnormal brain development and visual problems. Cholesterol exhibits a high affinity for SM but a poor affinity for PE [11,12] and DHA-containing phospholipids [13–15]. Although sphingomyelin is a gel state lipid at room temperature, it exists in a liquid ordered fluid state in the presence of cholesterol [6]. We therefore predict that our three-component model membrane will phase separate into a cholesterol-rich, SM-rich, DHA–PE-poor liquid ordered phase and a cholesterol-poor, SM-poor, DHA–PE-rich liquid disordered phase, mimicking DRM and DSM fractions, respectively. Here we employ pressure–area isotherms and differential scanning calorimetry to follow a biologically relevant phase separation in this model membrane.

2. Materials and methods

2.1. Materials

Egg SM and 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphoethanolamine (SDPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was acquired from Sigma Chemical (St. Louis, MO). Lipid purity was assessed by thin-layer chromatography. Lipid and cholesterol concentrations were quantified using phosphate analysis and dry weight analysis, respectively. Hexane and 2-propanol (HPLC grade) used in the monolayer studies, were purchased from Aldrich Chemical (Milwaukee, WI). Water for the monolayer studies was deionized, glass-distilled and run through a Milli-Q Plus Water Purification System (Millipore, Milford, MA).

2.2. Lipid monolayer studies

Pressure–area isotherms (π–A) were obtained using a Mini LB Trough (KSV Instruments, Helsinki, Finland) and a Wilhelmy plate. All equipment was rinsed with ethanol twice and five times with distilled

water before use. Lipid monolayers composed of phospholipids, SM, cholesterol, or combinations thereof, were spread onto a deionized water subphase using hexane/2-propanol (3:2) at 23°C. The carrying solvent was allowed to evaporate for 5 min and compression rates were set at 1 mN/m per minute. All combinations of lipids/cholesterol were run a minimum of three times to ensure reproducibility.

2.3. Analysis of pressure–area isotherms

Lipid area/molecule was calculated by linear extrapolation from the target pressure of 30 mN/m. The ideal mean molecular area (A_π) of two-component mixed monolayers was calculated at a constant surface pressure (π) by:

$$A_\pi = X_1(A_1)_\pi + (1-X_1)(A_2)_\pi \quad (1)$$

where X_1 is the mol fraction of component 1 and A_1 and A_2 are the mean molecular areas of pure components 1 and 2 at identical surface pressures [16].

The percent condensation was calculated at a constant surface pressure using the following equation [17]:

$$\%\ \text{Condensation} = [(A_{\text{ideal}}-A_{\text{obs}})/A_{\text{ideal}}]\times 100 \quad (2)$$

where A_{ideal} is the ideal mean molecular area and A_{obs} is the experimentally obtained mean molecular area.

The π–A data were also used to determine the surface elasticity moduli C_s^{-1} [18]:

$$C_s^{-1} = (-A)(\mathrm{d}\pi/\mathrm{d}A)_{\Pi} \quad (3)$$

where A is the area per molecule of the lipid component at the indicated surface pressure π. Elasticity values are often fitted to an osmotic-based monolayer equation of state in order to improve data at high surface pressures (>30–35 mN/m) [18]. Since we evaluated our data at a surface pressure of 30 mN/m, we did not fit our data to such an equation. The π–A data allowed the estimation of the number of molecules lost (percent 'squeeze out') in the flat portions of the π–A isotherms [19]:

$$L = 1-(A_e/A_b)\times 100 \quad (4)$$

where L is the percentage of molecules lost, A_b and A_e are the beginning and end, respectively, of the surface area of the near-horizontal ('plateau') regions [19]. The values for A_b and A_e were determined by drawing tangents on each side of the plateau region. The tangents were then extrapolated and points of intersection determined the plateau region. Estimation of A_b and A_e had some uncertainty and thus percentage of molecular loss is simply an estimate.

2.4. Differential scanning calorimetry (DSC) studies

Lipids dissolved in chloroform were dried under nitrogen for 30 min followed by 12 h under vacuum to remove traces of organic solvents. Multilamellar vesicles (MLVs) were made by hydrating overnight the appropriate phospholipids at 10 mg/ml in 10 mM sodium phosphate, pH 7.0 and then oxygen was removed by purging the solution with nitrogen. MLVs were frozen in liquid nitrogen and thawed three times in a 50°C water bath. The MLV solutions (500 μl) were added to each of the three chambers of a Hart Scientific differential scanning calorimeter (Provo, UT) with the 4th chamber containing 500 μl of the buffer. Heating and cooling scans were made at 5°C/h. Only the cooling scans are presented although both scans appeared nearly identical.

3. Results

3.1. Do SM and cholesterol have an 'affinity' for one another?

In order to understand phase separation in a three-component system, we first assessed the affinity between two of the components, SM and cholesterol. Fig. 1 shows the π–A isotherms for SM with increasing mol fractions of cholesterol (X_{Chol}). SM, by itself (curve a), exists in the liquid-expanded state at low surface pressures and undergoes a transition to a more condensed state at about 15 mN/m, similar to values reported by others [16,20]. Increasing the amount of cholesterol obliterates the liquid-expanded state at low surface pressures and lowers the area per molecule of SM. At 30 mN/m, the area per molecule of SM was found to be 50.6 A^2 and 35.2 A^2 for pure cholesterol (Table 1). Cholesterol, with its series of rigid planar rings, occupies almost the same area per molecule at both low and high lateral pressures with less than a 2% change observed from 5 mN/m to

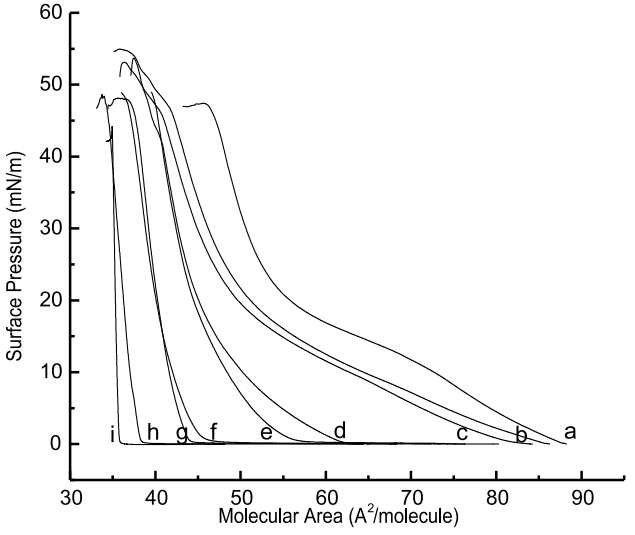

Fig. 1. Pressure–area isotherms for SM/cholesterol mixed monolayers. Mol fraction of cholesterol in SM: (a) 0; (b) 0.05; (c) 0.10; (d) 0.20; (e) 0.30; (f) 0.40; (g) 0.50; (h) 0.60; (i) 1.0.

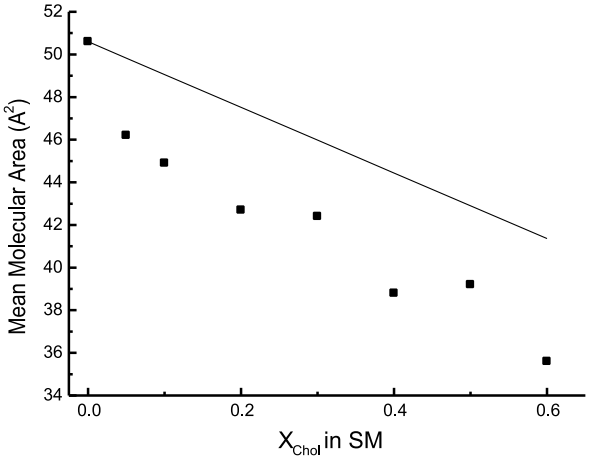

Fig. 2. Mean molecular area as a function of mol fraction cholesterol (X_{Chol}). Solid squares represent experimental results and ideal additivity is represented by the solid line. Data were obtained from the pressure–area isotherms of SM and cholesterol at 30 mN/m reported in Fig. 1.

30 mN/m (Fig. 1). Thus, it is concluded that any condensation found between SM and cholesterol must be conferred by SM.

Condensation values for $X_{Chol} = 0.05$ and 0.60 range from 7.3% to 14.0%, respectively (Table 1). Condensation values varied inversely as a function of surface pressure (lower surface pressures demonstrated higher condensations, data not shown). Fig. 2 presents the mean molecular area of egg SM/cholesterol as a function of X_{Chol}. The solid line indicates ideal additivity (Eq. 1), while the closed squares denote the experimentally obtained values.

For these same monolayers, upon the addition of cholesterol to SM we observed an increase in the surface elasticity moduli (a decrease in elasticity) (Table 1). The elasticity value at 30 mN/m for pure SM was 154.4 mN/m and for pure cholesterol was 2484 mN/m, a value indicating little or no compressibility. This again demonstrates that any measured change in area per molecule in the mixed lipid monolayers must be conferred by the compressibility of SM and not cholesterol.

To demonstrate an 'affinity' between SM and cholesterol in bilayers, we next examined the change in phase behavior of SM upon the addition of cholesterol using DSC (Fig. 3). As little as $X_{Chol} = 0.01$ significantly lowers the SM phase transition and by $X_{Chol} = 0.1$ the SM phase transition is shifted to a lower melting temperature and is nearly obliterated. The π–A isotherm and DSC data both indicate that

Table 1
Area per molecule, condensation, and interfacial surface elasticity modulus values for varying mol fractions of cholesterol with SM at a surface pressure of 30 mN/m

Mol fraction cholesterol	Area/molecule (A^2) (experimental)	Percent condensation	C_s^{-1} (mN/m)
0	50.6	–	154.4
0.05	46.2	7.3	137.7
0.10	44.9	8.5	129.4
0.20	42.7	10.1	242.6
0.30	42.4	7.8	234.9
0.40	38.8	12.7	365.6
0.50	39.2	8.6	457.3
0.60	35.6	14.0	416.8
1.0	35.2	–	2484.1

Values were obtained from pressure–area isotherms in Fig. 1.

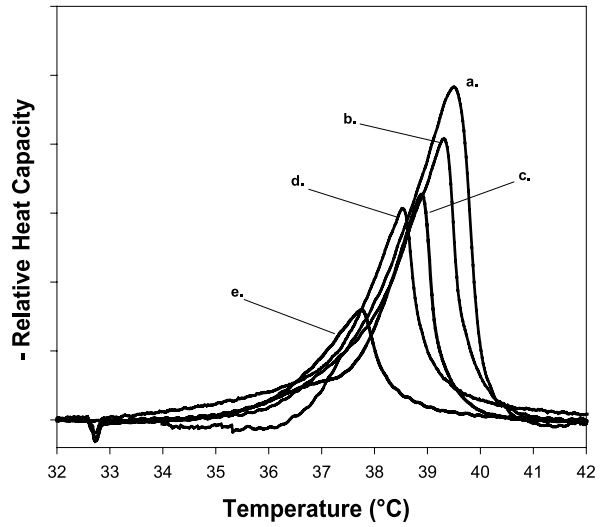

Fig. 3. DSC cooling scans of MLVs composed of (a) 0, (b) 0.01, (c) 0.03, (d) 0.05 and (e) 0.1 mol fraction cholesterol (X_{Chol}) in SM. (Note cooling scans are exothermic and so the values are plotted as a negative excess heat capacity.)

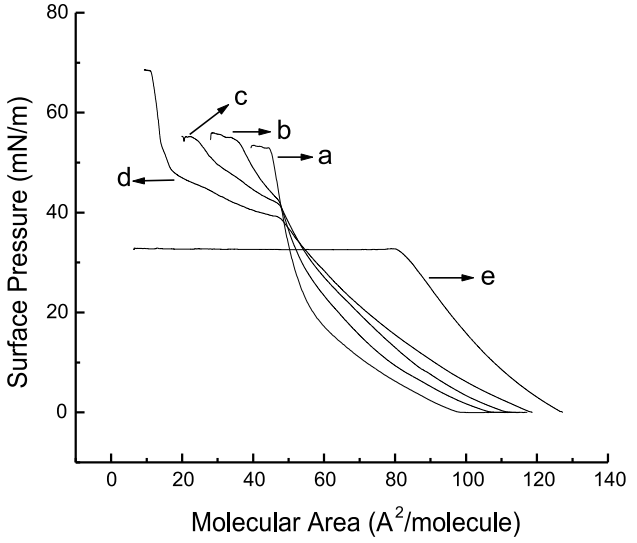

Fig. 4. Pressure–area isotherms of SM/SDPE mixed monolayers. Mol fraction of SDPE (X_{SDPE}) in SM: (a) 0; (b) 0.15; (c) 0.30; (d) 0.40; (e) 1.0.

SM and cholesterol have a high 'affinity' for one another, a finding consistent with the reports of others [16,20,21].

3.2. Do SM and SDPE have an 'affinity' for one another?

Interactions between SM and SDPE were assessed by evaluation of π–A isotherms as seen in Fig. 4. For simplicity, only a few of the π–A isotherms obtained are shown. At surface pressures of approx. 35 mN/m and below, increasing mol fractions of SDPE (X_{SDPE}) in SM results in an increase in the combined area per molecule of SM/SDPE. At higher surface pressures (>35 mN/m), increasing X_{SDPE} in SM decreases the combined area per molecule of SM/SDPE. The π–A isotherm for pure SDPE interestingly shows a very large plateau region at 32 mN/m (Fig. 4) that continues until the barriers touch. The distinct near horizontal region of the π–A isotherms emerges at $X_{SDPE} = 0.15$ and increases with increasing amounts of SDPE.

Table 2 reports the area/molecule, percent condensation, percent 'squeeze out' and the elasticity modulus at a surface pressure of 30 mN/m for SM/SDPE mixed monolayers. The values were obtained from the π–A isotherms, some of which are reported in

Table 2
Area/molecule, percent condensation, percent 'squeeze out', and interfacial elasticity modulus values for varying mol fractions SDPE in SM at a surface pressure of 30 mN/m

Mol fraction SDPE in SM	Area/molecule (A^2) experimental	Percent condensation	Percent 'squeeze out'	C_s^{-1} (mN/m)
0.10	54.0	9.4	0.0	79.6
0.15	53.6	4.1	0.0	76.0
0.20	60.6	–[a]	5.1	86.8
0.30	55.9	8.6	31.4	52.9
0.40	58.1	10.2	48.9	45.8
0.50	63.6	6.0	62.2	49.8
0.60	77.7	–[a]	81.3	54.3
1.0	84.6	–	92.6	66.4

Values were calculated from pressure–area isotherms shown in Fig. 4.
[a]These values are not indicated because no percent condensation was found.

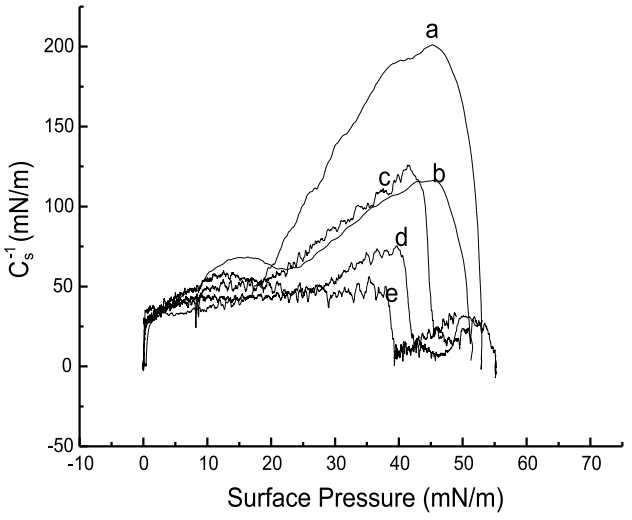

Fig. 5. Surface elasticity moduli (C_s^{-1}) versus lateral surface pressure (mN/m) of mixed SM/SDPE monolayers. C_s^{-1} (mN/m) was calculated using data obtained from pressure–area isotherms in Fig. 4. Mol fraction of SDPE (X_{SDPE}) in SM: (a) 0; (b) 0.10; (c) 0.20; (d) 0.30; (e) 0.40.

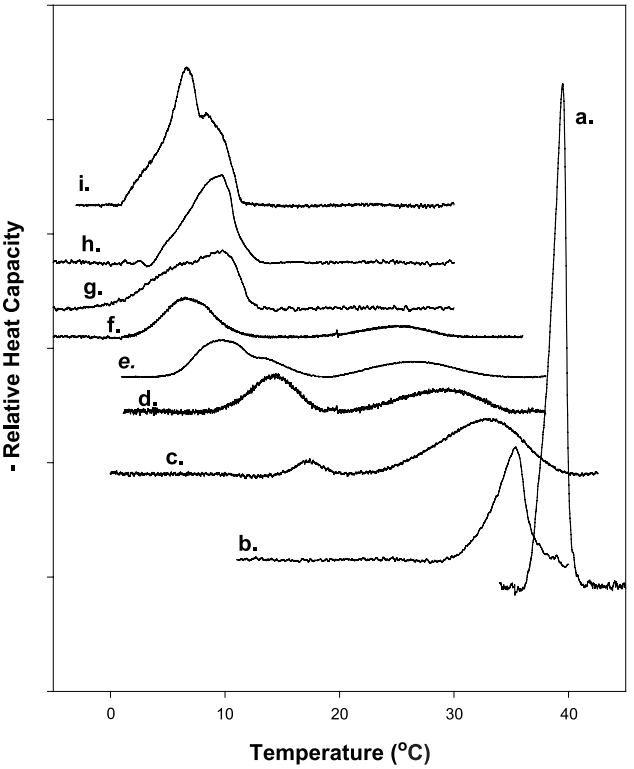

Fig. 6. DSC cooling scans of MLVs made from mixtures of SM and SDPE. Mol fraction of SDPE (X_{SDPE}) in SM: (a) 0; (b) 0.10; (c) 0.25; (d) 0.40; (e) 0.50; (f) 0.60; (g) 0.75; (h) 0.90; (i) 1.0. (Note cooling scans are exothermic and so the values are plotted as a negative excess heat capacity.)

Fig. 4. Percent condensation varies from 4.1% to 10.2% for $X_{SDPE} = 0.15$ and 0.40, respectively. 'Squeeze out' was calculated by measuring the percentage of molecular loss (Eq. 4). At biologically significant high lateral pressures (e.g. $\pi = 30$ mN/m) the elasticity modulus decreases upon addition of SDPE to SM (Fig. 5), while at a low pressures (e.g. $\pi = 10$ mN/m), the elasticity modulus remains relatively constant.

The DSC scans for MLVs made from SM and SDPE are presented in Fig. 6. In the absence of SDPE, SM undergoes a phase transition at 39°C. With $X_{SDPE} = 0.1$ mixed with SM, the melting temperature of SM is lowered and the transition enthalpy, ΔH, is also decreased (Table 3). With the addition of $X_{SDPE} = 0.25$, phase separation is observed with the observation of SM-dependent and SDPE-dependent peaks. By $X_{SDPE} = 0.60$, the phase transition of the SM-dependent peak is nearly obliterated.

3.3. *Do cholesterol and SDPE have an 'affinity' for one another?*

There appears to be little or no 'affinity' between SDPE and cholesterol. With $X_{Chol} \geq 0.10$ in SDPE, π–A isotherms of the two components increase with lateral pressure up to 10 mN/m and then collapse (data not shown), indicating the formation of an

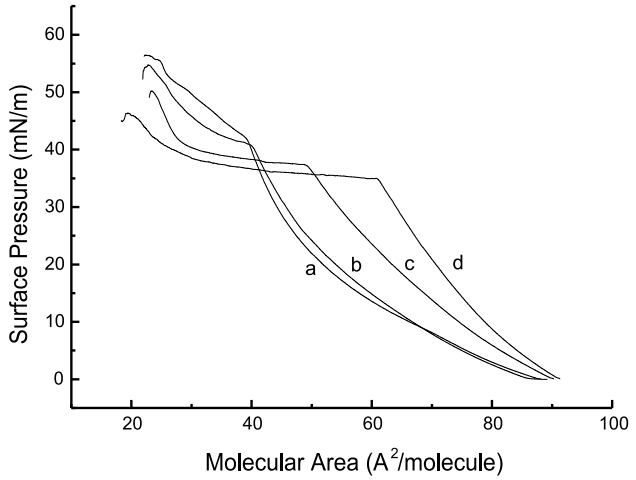

Fig. 7. Pressure–area isotherms for SM/SDPE/cholesterol monolayers. Monolayers were composed of SM/SDPE to which an additional 0.05 mol fraction cholesterol was added. Mol fraction SDPE (X_{SDPE}) in SM are: (a) 0.10; (b) 0.20; (c) 0.30; (d) 0.40.

Table 3
Enthalpy values (cal/mol) for varying mol fractions of SDPE with SM and SDPE

Mol Fraction SDPE	SDPE low melting component		SM high melting component	
	T_m (°C)	ΔH (cal/mol)	T_m (°C)	ΔH (cal/mol)
0.00	nd	nd	39.4	4068
0.10	nd	nd	35.3	3413
0.25	17.2	212	33.1	2778
0.40	14.4	1034	29.1	1029
0.50	9.8	1485	26.6	786
0.60	6.5	1262	25.2	415
0.75	9.8	2418	nd	nd
0.90	9.5	2777	nd	nd
1.00	6.6	4513	nd	nd

Data were obtained by integrating the area under the DSC curves from Fig. 6. nd, not determined.

unstable monolayer. Many years ago Demel et al. [13] reported very little cholesterol-induced condensation with docosahexaenoic acid-containing phosphatidylcholines (PCs), even though PC has a higher affinity for cholesterol than does PE [22]. Thus, our results with SDPE and cholesterol were not surprising.

3.4. Phase separation with the three-component system

We next obtained π–A isotherms for mixtures of SM and SDPE in varying mol fractions with increasing amounts of cholesterol. For clarity, only a few of the π–A isotherms obtained (those with $X_{Chol} = 0.05$) are presented (Fig. 7). Once again, a near horizontal region was observed in the π–A isotherms, indicating molecular loss or 'squeeze out'. In Fig. 8 we present the percent 'squeeze out' at 30 mN/m as a function of X_{SDPE} in SM with either zero or 0.20 mol fraction additional cholesterol. Although only the 0 and 0.20 mol fraction cholesterol curves are presented in Fig. 8, several other cholesterol concentrations were tested and for each mol fraction of SDPE in SM, the percent 'squeeze out' from the 'plateau' region increased with the mol fraction of cholesterol.

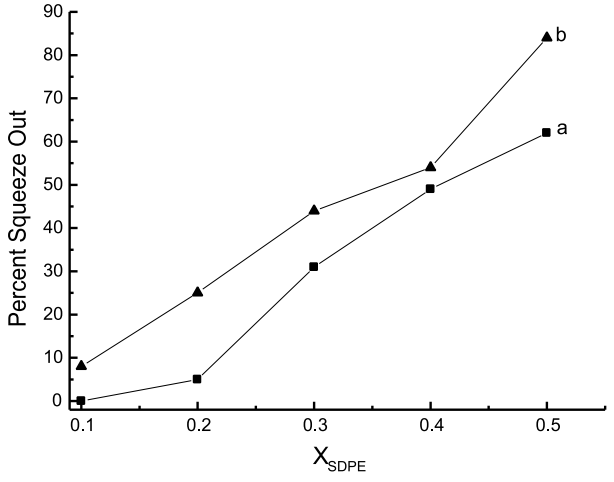

Fig. 8. Percent 'squeeze out' of SM/SDPE monolayers with either 0 mol fraction (curve a) or 0.2 mol fraction (curve b) additional cholesterol. Values were calculated from pressure–area isotherms like those shown in Fig. 7.

To corroborate our monolayer results using bilayers, we next ran DSC cooling scans on MLVs made from SM/SDPE (1:1, mol/mol) to which increasing mol fractions of cholesterol were added (Fig. 9). Equimolar mixtures of SM and SDPE result in a low melting, SDPE-dependent peak and a high melting SM-dependent peak with transition temperature (T_m) values of 9.8°C and 26.6°C, respectively. Upon the addition of as little as $X_{Chol} = 0.0025$–0.01, a decrease in the enthalpy of SM is observed (Fig. 9, upper panel), while at these same levels of cholesterol no significant effect on the phase transition of SDPE is seen. The enthalpy values of the SDPE low melting component remain relatively constant at $0.00 \leq X_{Chol} \geq 0.0075$, ranging from 1485 to 2094 cal/mol, respectively; in addition, the transition temperature also remains constant for X_{Chol} from 0.00 to 0.0075 (Table 4). It is not until the X_{Chol} levels reach 0.03–0.05 that large alterations in the transition temperature and enthalpy of the SDPE transition can be noticed (Fig. 9, lower panel, Table 4). Thus, in the

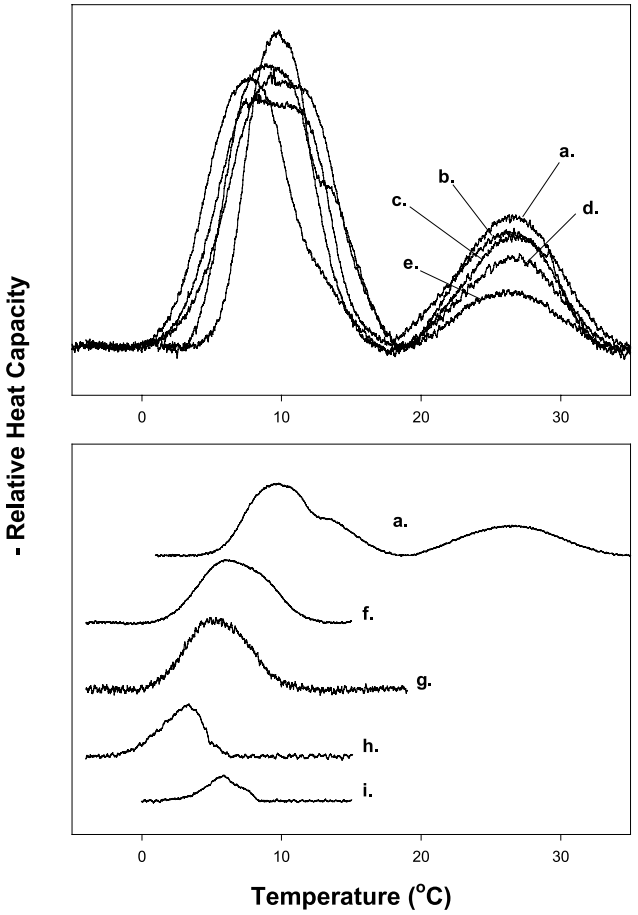

Fig. 9. (Top panel) DSC cooling scans of MLVs made from equimolar mixtures of SM/SDPE with (a) 0, (b) 0.0025, (c) 0.0050, (d) 0.0075 and (e) 0.01 mol fraction additional cholesterol. (Bottom panel) DSC cooling scans of MLVs made from equimolar mixtures of SM/SDPE with (a) 0, (f) 0.03, (g) 0.05, (h) 0.10, and (i) 0.20 mol fraction additional cholesterol. (Note cooling scans are exothermic and so the values are plotted as a negative excess heat capacity.)

presence of SM, there appears to be little or no interaction between SDPE and cholesterol at low levels of cholesterol. Only at much higher amounts of cholesterol is the phase transition of SDPE affected. In agreement with the DSC data, the π–A experiments support the notion that the SDPE-rich phase is separated from the SM/cholesterol-rich phase.

4. Discussion

Lipid domain formation in the plasma membrane is postulated to be a result of interactions between lipids as well as between lipids and proteins. The best proof of the existence of lipid microdomains has come from studies of DRMs or lipid rafts in biological membranes as well as from model membranes with co-existing liquid crystalline and gel state lipids. In the present study, we investigate a three-component model membrane system that accurately parallels the composition of mammalian plasma membranes. The model membrane is composed of a phospholipid (SDPE), a sphingolipid (egg SM) and cholesterol. In addition we have included in the phospholipid DHA, the longest and most unsaturated fatty acid commonly found in the plasma membrane. We [23] and others [15,24] have suggested that an important part of DHA's function in membranes is in affecting lipid microdomain formation. Specifically, it appears that DHA can induce phase separations in some model membranes [23] and possibly in vivo [24].

We chose to emphasize our π–A isotherm data at a

Table 4
Enthalpy values (cal/mol) for varying mol fractions of cholesterol with SM and SDPE (1:1 mol/mol)

Mol fraction cholesterol	SDPE low melting component		SM high melting component	
	T_m (°C)	ΔH (cal/mol)	T_m (°C)	ΔH (cal/mol)
0.00	9.8	1485	26.6	786
0.0025	9.2	2010	26.5	692
0.0050	9.0	1484	26.6	654
0.0075	9.4	2094	26.6	501
0.01	7.8	1712	26.4	316
0.03	6.2	1216	nd	nd
0.05	5.2	1163	nd	nd
0.1	3.4	591	nd	nd
0.2	5.7	209	nd	nd

Data were obtained by integrating the area under the DSC curves from Fig. 9. nd, not determined.

lateral pressure of 30 mN/m, a pressure believed to be similar to that of biological membranes [25]. The condensation and elasticity data indicate an 'affinity' between SM and cholesterol, a finding that would be consistent with the existence of liquid ordered phase found in DRMs [3]. The extent of condensation at 30 mN/m of surface pressure can be seen with the mean molecular area versus composition plot (Fig. 2). Condensation can be attributed to area changes within SM when this lipid is mixed with cholesterol, since cholesterol is a rigid molecule whose area does not significantly change as a function of surface pressure (Fig. 1). The 'affinity' is probably due primarily to van der Waals forces between the rigid rings of cholesterol and the saturated hydrocarbon chains of SM [26]. Also contributing to the interaction is hydrogen bonding between the 3-OH of cholesterol and the amide bond of SM [3,26].

Using condensation or elasticity data in monolayers alone as a means of estimating lipid–lipid 'affinity' has been questioned [21]. Therefore, supporting DSC experiments with bilayers were run (Fig. 3). Addition of cholesterol resulted in a decrease in enthalpy and a shift in the phase transition temperature of the SM peak, reflecting a high amount of miscibility between these two lipids.

Dipalmitoylphosphatidylcholine (DPPC) has sometimes been used to replace SM in model membrane studies in evaluating interactions with cholesterol. Similar results have been reported for both lipids [27]. DPPC has a similar phase transition to SM ($T_m = 41$°C for DPPC and 40°C for SM), its gel state resembles the SM liquid ordered state, it phase separates from 1-stearoyl-2-docosahexaenoyl-*sn*-glycerophosphocholine (SDPC) [23] and its phase separation resembles that of SM [3]. The head group of SM is structurally analogous to that of DPPC. DPPC has both acyl chains saturated while the egg SM used in these studies have predominantly saturated acyl chains as well (77.7% palmitate (16:0), 7.4% stearate (18:0), 1.9% arachidate (20:0), 4.0% behenate (22:0), 2.8% docosahexanoate (22:6) and 4.3% nervonate (24:1)) [28]. With DPPC it has been shown that below the T_m, there appears to be phase separation between solid ordered and liquid ordered phases at X_{Chol} from 0.08 to 0.3. Above $X_{Chol} = 0.3$, there is formation of a liquid ordered phase alone. In our π–A isotherms, the experimental temperature (23°C) was below that of the SM T_m and thus, it can be proposed that the monolayers may exhibit coexisting solid and liquid ordered phases at cholesterol concentrations similar to those reported for DPPC [3].

We also evaluated the role of cholesterol with our phospholipid component SDPE (data not shown). Although we were not able to run successful π–A isotherms of SDPE/cholesterol, much is known about the interactions between PEs and cholesterol. Earlier studies indicate that cholesterol has a destabilizing effect upon the structure of PE model membranes [29]. It has also been reported numerous times that cholesterol has little affinity for PEs when compared to other phospholipids including PCs and SMs [11,12]. Still, recently it has been demonstrated with NMR and IR techniques that cholesterol and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine can form a liquid ordered phase [30]. Cooling scans from high sensitivity DSC studies of dipalmitoylphosphatidylethanolamine alone show two peaks that may indicate a mixture of domains that differ in their thermal behavior [31]. The effect of cholesterol on these domains remains to be studied. Simultaneous X-ray diffraction and DSC studies of dielaidoylphosphatidylethanolamine (DEPE)/cholesterol mixtures point to the formation of DEPE-rich and cholesterol-rich domains [32]. It is possible then that in the absence of SMs or PCs there may even be PE or PE/cholesterol domains, although the evidence remains inconclusive [31,32].

In order to investigate the ability of DHA to induce phase separations, we evaluated the interactions between SM/SDPE and between SM/SDPE/Chol. Pressure–area isotherms for SM/SDPE monolayers show that mixtures containing $X_{SDPE} \geq 0.15$ exhibit near horizontal plateau regions in the curves (Fig. 4). We previously observed with DPPC/SDPC monolayers the onset of a similar plateau region at $X_{SDPC} \geq 0.20$ [23]. The SM/SDPE data were first analyzed as mean molecular area as a function of X_{SDPE} as described by Smaby et al. [16]. This analysis did not result in any clear indication of miscibility. We then quantified the number of molecules lost in the plateau regions (Table 2). The mol fraction of SDPE 'squeezed out' above $X_{SDPE} = 0.30$ in these plateau regions is greater than the X_{SDPE} that formed the initial monolayer. Clearly, the lipids being elim-

inated from the monolayer ($X_{SDPE} \geq 0.30$) are predominantly SDPE with a lesser amount of SM. It can bee seen (Fig. 4) that for the mixtures of SM/SDPE (except for the pure SDPE component), after the SDPE is 'squeezed out', the pressure continues to rise indicating a monolayer that is primarily SM. Thus, it can be concluded that the plateau regions of the π–A isotherms are not simple monolayer collapse, but rather a preferential 'squeeze out' of SDPE or SDPE/SM. The concept of 'squeeze out' has been reported by others [19,33]. Bangham et al. [33] have reported 'squeeze out' of egg phosphatidylglycerol (egg PG) from mixtures of egg PG/DPPC. 'Squeeze out' has also been reported with the lung surfactant proteins (SP-B and SP-C) from monolayers composed of DPPC/dipalmitoylphosphatidylglycerol [34].

In Fig. 5 it is demonstrated that increasing the X_{SDPE} in SM lowers the C_s^{-1}, therefore increasing the in-plane elasticity. Changes in the elasticity curves are indicative of changes in slope of the π–A isotherms (Fig. 4) and may reflect a redistribution of lipids within the monolayer [35]. The curves with SM/SDPE are similar to what has been observed with DPPC/SDPC monolayers [23]. The C_s^{-1} value for SM alone (154.4 mN/m) is higher than that of SDPE (66.6 mN/m). Thus, the changes in elasticity observed with SM/SDPE mixtures can be attributed primarily to the PE component, but further studies are needed to evaluate whether the changes in elasticity are conferred by the head group or hydrocarbon structure. It is well established that acyl chain unsaturation plays a role in affecting interfacial elasticity. Smaby et al. [18] demonstrated that the in-plane elasticity of PCs is dependent upon the hydrocarbon structure in mixtures of PC/cholesterol. These workers reported that in-plane elasticity decreases due to cholesterol in their PC/cholesterol mixtures when both of the PC acyl chains were saturated. However, a much smaller effect was noted when the *sn*-1 chain was saturated and the *sn*-2 chain was polyunsaturated [18]. In the DSC studies of SM/SDPE (Fig. 6) as little as $X_{SDPE} = 0.25$ in SM shows phase separation (Table 3), consistent with the conclusions made from the π–A isotherms (Fig. 4). Still, the two peaks observed with DSC are not pure SM and SDPE; rather, there appears to be some miscibility between the two lipids suggesting the formation of SM-rich, SDPE-poor and SM-poor, SDPE-rich domains.

We attempted to assess miscibility from our π–A data using Crisp's approach [36] of collapse pressure versus composition (data not shown). The collapse pressures of the SM/Chol mixtures (Fig. 1) or SM/SDPE mixtures (Fig. 4) did not provide a clear indication of miscibility. Hence, utilization of the collapse pressure versus composition as a means of assessing miscibility appeared very limited in our model system. Perhaps this is the result of kinetic limitations on mixing between SM and SDPE or cholesterol. Similar problems have been reported with galactosylceramides and cholesterol [17] and with sphingomyelins or phosphatidylcholines and cholesterol [16]. In addition, Crisp's classical approach of assessing miscibility by collapse pressure was intended for binary lipid systems and apparently could not readily be used for analysis of our three-component model [36].

Our results indicate that some lateral phase separation occurs between SM and SDPE and is enhanced upon the addition of cholesterol. With increasing cholesterol, the plateau regions indicate a greater 'squeeze out' as seen in Fig. 8. We attempted to assess miscibility in our three-component system using mean molecular area versus composition diagrams. Results from these plots were again inconclusive. Phase separation of the three components is more clearly evident in the DSC curves. At $X_{Chol} \leq 0.01$, mixed with equimolar SM and SDPE, the enthalpy of the higher melting, SM-rich peak decreases whereas the lower melting, SDPE-rich peak is almost unaffected (Fig. 9, top panel, Table 4). Cholesterol therefore first reduces and eventually obliterates the SM-rich peak before affecting the SDPE-rich peak. It appears that at $X_{Chol} \leq 0.03$, cholesterol interacts preferentially with SM reflecting the strong association between these two lipids, and at higher levels also interacts with SDPE. These results are in agreement with prior reports indicating that cholesterol prefers PCs over PEs [11,29]. It has also been shown that cholesterol prefers SMs over PCs [12]. Using π–A isotherms as well as fluorescence microscopy of monolayers, Mattjus et al. [37] showed that cholesterol interacts more favorably with SMs than PCs.

In summary, SM and cholesterol have a strong

affinity for one another, consistent with these lipids forming the core of liquid ordered phase DRMs. In addition, the liquid ordered phase separates from the liquid disordered SDPE phase that would be a component of the detergent soluble membrane fraction. Since bilayers made from these three lipids are a good approximation of the plasma membrane [6], it can be concluded that DHA may be involved in affecting the formation of lipid microdomains within the plasma membrane. Saturated acyl chain-rich DRMs are known to be involved in accumulation of GPI-anchored and other cell signaling proteins [38]. No related set of activities have yet been proposed for the DHA-rich, detergent soluble membrane fraction, however.

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