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The Interaction Energies of Cholesterol and 1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine in Spread Mixed Monolayers at the Air-Water Interface

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Abstract

The interaction of cholesterol with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was investigated in insoluble miscible mixed monolayers at the air-water interface using a Langmuir balance technique. The strong condensation effects observed at all compositions were quantified on the basis of excess thermodynamic properties of the system. It was found that partial molar areas and work of compression of cholesterol in the mixed monolayers were greatly reduced and increased, respectively, at x_{DOPE} of 0.8, while in accord to the “umbrella model” the character of cholesterol monolayers was drastically affected even at mole fractions of DOPE as low as 0.2. Calculated Gibbs free energies of mixing were shown to be symmetric about equimolar lipid quantities, and considerably decreased at high surface pressures. Interaction energy parameters calculated from values of excess Gibbs energy are found to decrease linearly with surface pressure at a rate of 100 k T m N^{-1} , irregardless of composition. All evidence, points out that cholesterol-DOPE molecular interactions can be adequately simulated using a simple regular mixture model.

Keywords

Cholesterol; DOPE; Monolayer; Miscibility; Surface pressure; Excess area; Excess Gibbs energy; Activity coefficient; Interaction energy parameter

Introduction

In our recent comparative studies we have shown that unlike the commercially available cationic lipid 3β -[N-(N',N'-dimethylaminoethane)-carbamoyl]-cholesterol (DC-Chol), the corresponding primary and secondary amine analogs can mediate potent transfection activity in the absence of the “helper” lipid DOPE.¹ One of our primary concerns was to first develop a sensitive method that can quantify concentrations of these cationic lipids in aqueous phases, which lack strong UV activity, below their critical micelle concentration.² Since these cationic lipids are cholesterol derivatives and cholesterol is abundant in mammalian cell plasma membrane and organelles, as part of our priorities, we set to investigate the cholesterol-based cationic lipid-mediated transfection pathways, by first developing our understanding of cholesterol-DOPE intermolecular forces within well-defined miscible monolayers at the air-water interface. Phosphatidylethanolamines, on the other hand, particularly enrich the cytofacial leaflet of plasma membranes and are also present in various organelles such as endosome and lysosome membranes. Thus, although

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DOPE is of a purely synthetic origin, the fact that it is the exclusively universal helper lipid used to improve transfection activity of non-viral delivery systems, renders such studies biologically relevant.

Few articles discussed the interaction of cholesterol with phospholipids in Langmuir monolayers^{3–11}, but notably, there are no published reports, in general, about cholesterol-DOPE interaction in binary mixtures.

In this study, cholesterol-DOPE molecular interactions were evaluated under isothermal conditions in mixed monolayers formed by spreading at the air-water interface. Langmuir films appeared to be an attractive choice as firstly, neither of these lipids undergoes phase transitions in useful temperature range and secondly, their three-dimensional assemblies at physiological pH would most probably be reverse hexagonal aggregate structures, difficult to isolate and study. Measured excess molecular areas were used to calculate interaction energy parameters of cholesterol with DOPE as a function of composition and surface pressure, at physiological pH. The interaction of cholesterol with DOPE appeared to be symmetric in mixed monolayers exhibiting maximum stability at equimolar amounts of the two lipids. The assessed thermodynamic properties of the miscible monolayers conformed satisfactorily with the simple regular solution theory.

Theoretical

Unlike ideal gases, cohesive and adhesive forces within an ideal binary condensed phase are identical, thus, monolayer properties such as mixed molecular areas depend linearly on composition (eq 1).

$$A_{12}=x_1A_1+x_2A_2 \quad \text{or} \quad a_{12}=x_1a_1+x_2a_2 \quad (1)$$

A_{12} is the trough or interfacial area occupied by the total moles of an ideal binary mixture at constant temperature and surface pressure Π . A_1 and A_2 , are the trough or interfacial areas occupied by pure component 1 and pure component 2, respectively, in isolation, whereas, x_1 and x_2 are the mole fractions of components 1 and 2, respectively. Similarly, a_{12} , a_1 and a_2 are the molecular areas of the mixed system, component 1 and component 2, respectively. Molecular areas are not measured. Interfacial trough areas A_1 and A_2 are the raw measured data and their magnitude is directly reflective of the number of molecules accumulated at the air-water interface, while the mean molecular areas are calculated from the measured interfacial areas, assuming that all the spread molecules are present on the surface.² It is also important to emphasize, that while A_1 and A_2 are measured experimentally, A_{12} is calculated using eq 1. Furthermore, in order to estimate deviations of the ideal additivity rule in the binary mixtures from the measured interfacial areas (and not the normalized molecular areas), the total number of moles of lipid (n_o) spread on the interface was kept constant at 30 nmoles for pure and mixed films.

In our first approach, interfacial areas were determined experimentally as a function of composition. Attractive or repulsive interactions in binary mixed Langmuir films were evaluated on the basis of negative or positive deviations, respectively, from the additivity rule bona fide in ideal systems. That is, the value of the mean molar area, A_m , which is the experimentally determined trough or interfacial area in mixed monolayers at constant T and Π , is compared to the ideal interfacial area, A_{12} . The difference between these two areas is called the excess surface area, A^E (see below).

To further identify preferential condensation or expansion of component 1 versus component 2 and vice versa in mixed monolayers, partial molar areas $\bar{A}_i$ or partial molecular areas $\bar{a}_i$, were estimated and compared to the measured corresponding values of pure components A_i^o . It should be recognized that A is an extensive property related to mean molar area via eq 4.

$$A_m = x_1 \bar{A}_1 + x_2 \bar{A}_2 \quad \text{or} \quad a_m = x_1 \bar{a}_1 + x_2 \bar{a}_2 \quad (2)$$

$$\bar{A}_i = \left(\frac{\partial A}{\partial n_i} \right)_{T, \Pi, n_{j \neq i}} \quad (3)$$

$$A = A_m \cdot \sum_i n_i \quad (4)$$

It can also be shown that partial molar areas in binary mixtures are related to mean molar areas by eq 5. Consequently, partial molar areas $\bar{A}_i$, were evaluated graphically from the slope and intercepts of mean molar area-composition isotherms at discrete surface pressures and mole fractions, x . By systematically varying the composition of non ideal binary systems, we recovered various partial molar areas that were in turn weighed against the experimental area values of the corresponding pure Langmuir films. The molecular dimensions a_m , were calculated from A_m , after appropriately normalizing the units and using the Avogadro number, as described above.

$$\begin{aligned} \bar{A}_1 &= A_m - x_2 \cdot \left(\frac{\partial A_m}{\partial x_2} \right)_{T, \Pi} \\ \bar{A}_2 &= A_m - x_1 \cdot \left(\frac{\partial A_m}{\partial x_1} \right)_{T, \Pi} \end{aligned} \quad (5)$$

The work of compression w_r , for cholesterol and DOPE was determined in mixed monolayers using,

$$w_{r,i} = -k \cdot T \cdot \int_{a_i^o}^{\bar{a}_i} \frac{da}{a} \quad (6)$$

where k is the Boltzmann constant and $i = 1, 2$ denote cholesterol and DOPE, respectively. All experiments were carried out under changing surface pressure, that is, mixed monolayers were kept at a constant temperature while the trough barriers were closed symmetrically at a constant rate of 9.99 mm min^{-1} . Eq 6 applies to isothermal reversible processes and provides the upper limit of the work of compression. The negative sign in the formula was chosen to denote work lost to the surroundings when the external force is applied in the opposite direction of the displacement ($\angle \theta = \pi$).

The work of compression for cholesterol and DOPE in pure monolayers was determined under conditions of constant temperature and surface area by performing multiple

compression experiments with various amounts of lipids applied on the surface. Properties of internal energy U , and Helmholtz free energy F , were employed to calculate work of compression, since both are expressed as a function of temperature and volume. It can easily be shown that for a reversible isothermal process, work of compression equals the Helmholtz free energy.

$$dF = dU - TdS = \delta q_{rev} + \delta w_r - \delta q_{rev} = \delta w_r$$

Where work of compression,

$$w_r = -\Pi \cdot \int_{A_i}^{A_f} dA \quad (7)$$

The surface pressure Π is the one corresponding to the final interfacial area A_f , while the initial area A_i was taken as the limiting area.

The solubility of cholesterol and DOPE was determined in pure monolayers as described elsewhere², and/or from the $w_r - n_o$ plots, where n_o is the amount of lipid spread on the surface.¹² The solubility of cholesterol was also measured in the presence of DOPE, at discrete mole fractions of cholesterol.¹³

In our second approach, molecular interactions were evaluated quantitatively by treating mixed monolayer thermodynamic properties in terms of excess functions, commonly defined as the difference between the measured thermodynamic function of mixing and the corresponding function of an ideal mixture. The criterion for the thermodynamically most stable membrane composition is the one that yields the most negative G^E or $\Delta_{mix} G$.

$$G^E = \Delta_{mix} G - \Delta_{id} G \quad (8)$$

$$\Delta_{id} G = RT(n_1 \ln x_1 + n_2 \ln x_2) \quad (9)$$

The excess Gibbs energy was calculated from direct measurements of interfacial areas A_m as a function of composition, at individually distinct surface pressures using the two-dimensional relationship, shown below:

$$G^E = \int_0^\Pi A^E d\Pi = \int_0^\Pi (A_m - A_{12}) d\Pi \quad (10)$$

Further thermodynamic analysis was applied to binary monolayer mixtures using the regular solution model, which entails entropy of mixing equal to that of an ideal solution and excess chemical potentials that are composition-dependent and proportional to an interaction energy parameter I .¹⁴

$$\begin{aligned}\mu_1^E &= I \cdot x_2^2 \\ \mu_2^E &= I \cdot x_1^2\end{aligned}\quad (11)$$

The energy parameter I , depends on components molecular structure and surroundings, and it is independent of T , P and composition. Substituting eqs 11 into the Gibbs-Helmholtz relationship yields the enthalpy of mixing for a regular mixture.

$$\sum_i n_i \cdot \frac{\partial}{\partial T} \left(\frac{\mu_i^E}{T} \right)_{P, n_j \neq i} = - \frac{\Delta_{mix} H}{T^2} \quad (12)$$

$$\Rightarrow \Delta_{mix} H = I x_1 x_2 \quad (13)$$

In general, for a non-ideal binary mixture,

$$\Delta_{mix} G = RT(x_1 \ln \alpha_1 + x_2 \ln \alpha_2) \quad (14)$$

Where α_i represent activities related to mole fractions and activity coefficients, $\alpha_i = x_i \gamma_i$
Combining eqs 8, 9 and 14,

$$G^E = RT(x_1 \ln \gamma_1 + x_2 \ln \gamma_2) \quad (15)$$

Since, $S^E = 0$,

$$\Rightarrow x_1 \ln \gamma_1 + x_2 \ln \gamma_2 = \frac{I}{RT} x_1 x_2 = \xi x_1 x_2 \quad (16)$$

Multiplying the right side with $(x_1 + x_2)$ and solving,

$$\begin{aligned}\ln \gamma_1 &= \xi x_2^2 \\ \ln \gamma_2 &= \xi x_1^2\end{aligned}\quad (17)$$

Substituting the Margules equations (eq 17) into eq 14 yields,

$$\Delta_{mix} G = RT [x_1 \ln x_1 + x_2 \ln x_2 + \xi x_1 x_2] \quad (18)$$

The unitless interaction energy parameter ξ , is a measure of adhesive interactions relative to cohesive interactions, and a phase separation is anticipated in regular mixtures for large enough ξ values. Given that in a two-dimensional interfacial monolayer only pair wise interactions are important in determining mixing properties, the interaction parameter can be

expressed in terms of intermolecular energies u_{ij} , as shown in eq 19. Negative values of the interaction parameters denote attractive interaction among the molecules.

$$\xi = z \cdot \frac{1}{kT} \cdot \Delta\epsilon \quad (19)$$

z = is the number of nearest neighbors equal to 6 in closely packed monolayer, and the interaction energy $\Delta\epsilon = \left(u_{12} - \frac{u_{11} + u_{22}}{2}\right)$.

Lastly, we have used the Joos theory to obtain valuable information about lipid-lipid molecular interaction at the experimentally observed monolayer collapse surface pressure, as a function of composition.

In thermodynamic contexts, migration of molecules from the subphase or bulk to the interface takes place because of chemical potential difference of the molecule in the two phases, and it will not cease until the two chemical potentials μ_i^B and μ_i^S , become equal. In a manner similar to the hydrostatic pressure build up (called osmotic pressure when equilibrium is reached) opposing the diffusion of solvent molecules into the solution compartment through a molecular sieve, the surface pressure Π , is a property that contributes to the magnitude of the chemical potential of a monolayer and opposes diffusion of the molecules from the bulk to the interface. Similarly, compressing the monolayers at surface pressures higher than the equilibrium one will result into a diffusion of molecules into the subphase, until equilibrium is reestablished.¹⁵ At equilibrium,

$$\mu_i^{S,o} = kT \ln x_i^S \gamma_i + \Pi \cdot a_i = \mu_i^{B,o} = kT \ln x_i^B f_i \quad (20)$$

The left and right hand side of the equation represent the chemical potential of molecules at the surface, μ_i^S , and in the bulk, μ_i^B , respectively, k is the Boltzmann constant, while the terms γ_i and f_i denote the activity coefficients of the molecule in the surface and bulk phase, respectively.

Joos was the first one to use this precondition to deduce relationships that could predict properties and other useful parameters of mixed monolayers at collapse surface pressure.¹⁶ Approximating the standard chemical potential difference between the interface and bulk phase of i th-component in mixed films at monolayer collapse, as being equal to $-\Pi_{c,i} a_i$, he derived the following equation for a 2-component miscible film.

$$1 = x_1^S \gamma_{1,m} e^{\left(\frac{\Pi_{c,m} - \Pi_{c,1}}{kT} a_1\right)} + x_2^S \gamma_{2,m} e^{\left(\frac{\Pi_{c,m} - \Pi_{c,2}}{kT} a_2\right)} \quad (21)$$

The properties $\Pi_{c,i}$, $\Pi_{c,m}$ and $\gamma_{i,m}$ represent collapse surface pressure of pure monolayers composed only of the i th-compound, collapse surface pressure of mixed monolayers and surface activity coefficient of the i th-component in mixed monolayers calculated at monolayer collapse, respectively. For miscible monolayers that obey the regular solution theory, the activity coefficients can be replaced appropriately by eq 17,

$$1 = x_1^s e^{\left(\frac{\Pi_{c,m} - \Pi_{c,1}}{kT} a_1\right)} e^{[\xi(x_2^s)^2]} + x_2^s e^{\left(\frac{\Pi_{c,m} - \Pi_{c,2}}{kT} a_2\right)} e^{[\xi(x_1^s)^2]} \quad (22)$$

When ideal mixing or complete immiscibility is observed in mixed monolayers, the surface activity coefficients of components 1 and 2 become equal to unity and eq 21 is simplified.

$$1 = x_1^s e^{\left(\frac{\Pi_{c,m} - \Pi_{c,1}}{kT} a_1\right)} + x_2^s e^{\left(\frac{\Pi_{c,m} - \Pi_{c,2}}{kT} a_2\right)} \quad (23)$$

More importantly eq 13, which is used to calculate interaction parameters from measured A^E cannot be applied at collapse surface pressures, because at and during monolayer collapse, reduction in interfacial areas would surely be due to the passage of molecules into the bulk phase and not due to attractive intermolecular forces. Furthermore, solving eq 21 could be quite challenging, particularly due to possible composition changes at monolayer collapse. Joos has instead derived a relationship to calculate the composition of an azeotropic phase, formed when adhesive forces differ significantly from cohesive ones, at equilibrium with the surface. These values could be in turn used to determine the interaction energy in the azeotrope.¹⁶

$$\xi = - \left[\frac{\Pi_{c,m} - \Pi_{c,1}}{kT(x_2^s)^2} a_1 \right]_{az} = - \left[\frac{\Pi_{c,m} - \Pi_{c,2}}{kT(x_1^s)^2} a_2 \right]_{az} \quad (24)$$

$\Pi_{c,m}$ denotes the maximum or minimum offset collapse surface pressure at all compositions.

A great concern is the accurate determination of isothermal collapse surface pressures. We routinely determine phase transitions and collapse surface pressures from values of the compressibility moduli as a function of surface area. Phase transition peaks and onsets are identified from local maximum compressibility modulus, whereas maximum compressibility modulus (minimum monolayer compressibility) corresponds to the onset of monolayer collapse and approach zero values at the conclusion of the collapse.^{11,17-18} Due to the exponential growth of surface pressure as a function of surface area, onset-offset collapse pressures can differ greatly. Specifically, offset-onset collapse pressure differences, particularly in mixed monolayers described herein, were as high as 16 mN m⁻¹. We have determined onset, midpoint and offset collapse surface pressure, but only used offset and midpoint collapse pressures in Joos' equations since the creation of a new bulk phase in equilibrium with the surface is implied. Differences between the midpoint and offset values of collapse surface pressures were relatively small and did not lead to significant changes in the two-dimensional phase diagram. Interaction parameters in mixed monolayers determined from excess areas, were calculated only for surface pressures well below the onset of monolayer collapse.

Experimental Methods

Stock solutions of cholesterol (CASRN: 57-88-5, purity 99+ %, Sigma-Aldrich Inc., St. Louis, MO), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE; CASRN: 4004-05-1, purity > 99 %, Avanti Polar Lipids, AL) and binary mixtures were prepared in chloroform (2 mM). For a typical experiment, 15 μ L from stock solution equivalent to 30 nmoles of pure lipid or lipid mixture in chloroform were drop wise applied on the surface of 150 mL tris

buffer 40 mM pH 7.2, contained within a thermostated trough at 295.15 K. Each run was repeated at least three times to ensure Π -A isotherm reproducibility. Due to the large volume of data and the sequential analysis by multiple equations, raw data were extracted from LB software into a Microsoft Excel spreadsheet, appropriately “coded” with all relevant formulas for automated calculation of properties and spontaneous plotting. To further prevent inadvertent errors, calculations were performed for two separate sets of data, that is, Trough area (A) and Mean molecular Area (a), by the principal investigator and the graduate student. The instrument, experimental set up and other data analysis were described elsewhere.²

Results and Discussion

The chemical structures and anticipated ionic state of compounds 1 and 2 at physiological pH, is shown in Figure 1. DOPE is a synthetic anionic amphiphil known to assemble into stable reverse hexagonal structures (H_{II}) at physiological pH and temperature, due to a reduced polar head area as compared to its expanded, “fluid” unsaturated acyl chains.^{19–21} As shown in Figure 2, surface pressure at collapse is similar for the two lipids in pure monolayers, but molecular dimensions and molecular elasticity at monolayer collapse, as well as, the limiting areas, are all smaller for cholesterol (Table 1). Careful inspection of Figure 2A signifies that the presence of cholesterol in DOPE-rich phases profoundly affected the average molecular dimensions of the mixed monolayer, while the presence of DOPE in cholesterol-rich phases appeared to condense the mixed monolayers beyond the molecular dimensions of cholesterol.

To gain more insight about the actual molecular dimensions of the two individual substances in the mixed monolayers, the partial molar areas of DOPE and cholesterol were determined at various Π and mole fractions of DOPE and plotted as a function of composition (Figure 3). In general, maximum reduction in DOPE molecular dimensions was observed at x_1 of 0.8, irregardless of surface pressure, while the partial molar area of DOPE $\bar{a}_2(x_1 = 0.2)$ was practically unaffected by the presence of cholesterol and remained essentially equal to a_2^0 . Greatest reduction in cholesterol molecular dimensions was observed at x_2 of 0.8, reaching a 70 % contraction from its actual molecular dimensions at surface pressure 25 to 30 mN m^{-1} , but unlike DOPE, surface pressure-dependent moderate reduction in the partial molar area of cholesterol $\bar{a}_1$, was observed at x_2 of 0.2.

Similarly to the partial molar area plots, work of compression of cholesterol in binary mixtures was highest at x_2 of 0.8 and lowest at x_2 of 0.2, ranging from 100 to 3200 J mol^{-1} (Figure 4A). Average values of work of compression for DOPE in mixed monolayers were lowest and highest, at highest and lowest DOPE mole fractions, respectively, ranging from 0 to 1000 J mol^{-1} . The higher work of compression determined for cholesterol as compared to DOPE, indicated that at $x_2 = 0.8$ and all surface pressures, cholesterol is by far more compressible than DOPE in mixed monolayers. As discussed in the methodology section, these calculations were based on an isothermal thermodynamic process that can be reversed by means of infinitesimal changes in surface pressure without loss of energy. This was necessary because the total number of moles spread on the interface was kept constant and thus, different interfacial areas can only be achieved at different surface pressures. For comparative purposes, the work of compression for cholesterol and DOPE was also calculated in pure monolayers, but contrary to the mixed monolayers, force-area isotherms of pure monolayers were collected with various amounts of lipid applied on air-water interface at constant temperature and surface area. In the case of cholesterol we have used previously published data collected with water as the subphase², whereas work of compression for DOPE was determined with subphase as 40 mM Tris buffer pH 7.2. As shown in Figure 4B, w_r is dependent not only on surface pressure but also on the amount of

lipid used to form the monolayer. For cholesterol w_r in J mol^{-1} varied from 20 to 30, 35 to 56 and 73 to 111 at surface pressures 16, 20 and 30 mN m^{-1} , with amounts of lipid spread on the interface ranging from 10 to 29 nmoles. The w_r for DOPE at 20 mN m^{-1} varied from 2000–2500 J mol^{-1} , with amounts spread on the air-water interface ranging from 10–36 nmoles. Therefore, DOPE is by far more compressible than cholesterol in pure monolayers.

Taken together, that is, the tremendous reduction of $\bar{a}_1$ as compared to $\bar{a}_1^0$ and tremendous increase in $w_{r,1}$, accompanied by invariable $\bar{a}_2$ (Figures 3 and 4) observed at $x_1 = 0.2$, combined with practically invariable monolayer elasticity (Table 1), suggest that DOPE molecules can nicely accommodate cholesterol in DOPE-rich phases within the unsaturated acyl chains, shielding them from exposure to the aqueous environment. On the other hand, the slight reduction of $\bar{a}_1$, accompanied by a reduced $\bar{a}_2$ and reduced work of compression at $x_2 = 0.2$ (Figures 3 and 4), combined with drastic decrease of the compressibility modulus values (Table 1), suggest that DOPE present in cholesterol-rich phases is spontaneously oriented with the polar head toward the aqueous subphase, displacing a bit of cholesterol molecules into the hydrophobic environment of the unsaturated acyl chains. These results are in accordance with the “umbrella model” first proposed by Huang and Feigenson to explain equilibrium maximum solubility of cholesterol in various phosphocholines and POPE bilayers.²²

Importantly, although the solubility of cholesterol in pure water was estimated to be 6.52 nM, its solubility in the presence of DOPE in mixed monolayers was below the nanomolar range, which was the lower level of detection of our method.² The solubility of DOPE in pure monolayers was also practically zero (Figure 5). It is therefore obvious, that the aqueous solubility of cholesterol and DOPE in mixed monolayers is not a concern that can affect the accuracy of the data. The slightly negative intercept can be attributed to experimental uncertainties given the extremely low concentrations used in the method. As discussed elsewhere, we have observed surface pressure dependence of the slope and intercept of these isotherms.² The increase in the slope as a function of surface pressure is just natural as the surface density increases with compression. In this case, the slope of cholesterol-DOPE mixed monolayer is steeper because the average molecular dimensions of the cholesterol-DOPE dimer is smaller than the molecular dimensions of a DOPE-DOPE dimer. The slight increase (smaller negative values) of the intercept observed with increasing surface pressures is attributed to the increased desorption of molecules into the subphase (maximum desorption was $\sim 4.5\%$ of total lipid; not shown).

To quantitatively evaluate lipid interactions, molecular areas in mixed monolayers were measured as a function of surface pressure and used to calculate the excess areas shown in Table 2. Negative values of A^E obviously denote contraction of the interfacial areas as compared to those of the pure components. The stability of the mixed monolayers was evaluated by the calculated magnitude of the Gibbs free energy of mixing. As shown in Figure 6A, the interaction of cholesterol with DOPE appears to be symmetric about 0.5 mole fraction. Maximum stability of the mixed monolayers exhibited an average $\Delta_{mix} G$ of about -3.5 kJ mol^{-1} and was demonstrated at x_1 of 0.4 to 0.6 and 0.4 to 0.5, at low and high surface pressures, respectively. Furthermore, as depicted in Figure 6B, the Gibbs energy of mixing is linearly or almost linearly decreased with surface pressure in cholesterol-DOPE mixed monolayers, with the only exception at mole fraction of cholesterol $x_1 = 0.6$, which it appears to increase at a slower rate at surface pressures $> 15 \text{ mN m}^{-1}$.

Because of the symmetric values of $\Delta_{mix} G$ about the cholesterol mole fraction 0.50, interaction of lipids in mixed monolayers was investigated in accord with the regular solution theory. Interaction parameters were calculated as a function of composition and surface pressure using eq 12 (Table 3), and subsequently used to calculate activity coefficients of cholesterol and DOPE using eq 17. The simple regular solution model

predicts that a log plot of activity coefficient as a function of mole fraction square, should yield a straight line with the slope being equal to the unitless interaction parameter ξ . As Figure 7-top panel- indicates, the regular solution theory could be used to adequately characterize the interaction of cholesterol with DOPE in mixed monolayers. Interaction parameters (absolute value) were found to be independent of composition and to increase linearly with surface pressure, at a rate of $-0.1 \text{ k T per mN m}^{-1}$, that is, it reaches -1 k T , -2 k T and -3 k T at surface pressures 10, 20 and 30 mN m^{-1} , respectively (Table 3). Interestingly, the difference of $\Delta_{mix} G$ between cholesterol mole fractions 0.2 (or 0.8) and 0.5, is around 500, 650, 700, 800 and 1000 J mol^{-1} at surface pressures from 5 to 10, 15, 20, 25 and 30 mN m^{-1} , respectively. Thus, as the interaction energy $\Delta\epsilon$ decreases below -0.333 k T , stable monolayer formation at cholesterol mole fraction 0.5 becomes extremely more favorable. In qualitative agreement with our monolayer studies, Huang and Feigenson have determined that pairwise-additive interaction energies $< -2 \text{ k T}$ cause drastic increase in the chemical potential of cholesterol at mole fractions higher than 0.5, in lamellar assemblies of cholesterol-POPE.

To further characterize the cholesterol-DOPE interaction in extremely dilute mixtures in which the surface pressures approach zero, we have deduced the dependency of the rate of change of the interaction parameter with surface pressure changes, as a function of surface pressure $\Delta\Pi$.

$$\left(\frac{\xi}{\partial\Pi}\right)_T = \lim_{\Delta\Pi \rightarrow 0} \left(\frac{\xi}{\Delta\Pi}\right) = \frac{a^E}{k \cdot T \cdot x_1 \cdot x_2} \quad (25)$$

As suggested by eq 25, a plot of the unitless interaction parameter normalized by surface pressure changes versus the surface pressure difference $\Delta\Pi$, with Π_{initial} set to zero, should yield a straight line with the y-intercept being equal to the excess area at $\Delta\Pi = \Pi_{\text{initial}}$. Least squares analysis of the data points shown in Figure 7 (bottom panel) yielded a value of the interaction parameter at surface pressures approaching zero equal to -0.08 . Assuming a hexagonal lattice (Huang et al., 1999 and references within) for the cholesterol-DOPE mixture an interaction $\Delta\epsilon$ equal to 32.7 J mol^{-1} was calculated using eq 19. Factoring in Boltzmann constant and absolute temperature, an excess molecular area a^E , pertinent to the spontaneous interaction of cholesterol with DOPE equal to $-5.2 \text{ \AA}^2$ per molecule was calculated at $x_1 = 0.2$ ($x_2 = 0.8$) or $x_2 = 0.2$, while a maximum excess molecular area a^E , of $-8.1 \text{ \AA}^2$ per molecule was calculated at equimolar quantities of the two lipids.

Lastly, with the aid of individual substance molecular dimensions and surface pressures determined at offset collapse point of single-component monolayers, the collapse pressure of mixed monolayers was calculated according to eq 23, and compared with the experimental points. Contrary to the large negative interaction parameters determined in intact monolayers, the interaction parameters of mixed monolayers were essentially zero at monolayer collapse. As it is evident from Figure 8, no positive or negative azeotrope was formed with any of the lipid mixtures upon collapse of the miscible monolayers.

Conclusions

Table 4 summarizes all available information about cholesterol-phospholipid interaction in Langmuir monolayers formed at air-water interface. The few investigators that have studied interaction of cholesterol with PE have found that there is a predominantly repulsive interaction between cholesterol and double-chained saturated phosphoethanolamines, no

interaction when one of the acyl chains is polyunsaturated and strong attractive interactions when one of the chains is monounsaturated (oleoyl).

In the present investigation, we concluded that the interaction of cholesterol with DOPE in mixed monolayers can be modeled in accord with the simple regular solution theory, although the assumption of maximum randomness contradicts the idea of a “lattice model”. Most probably the excess entropy S_E contribution to the Gibbs free energy of mixing is very small. When Hildebrand first proposed the term “regular solutions” in 1929, it was clear that the absence of any specific interaction is the most important feature of these solutions.²³ Similarly, when Scatchard derived the energy of mixing of two liquids at constant volume in 1931, he assumed that the mutual energy of two molecules is dependent only upon the distance between them and their relative orientation.²⁴ The composition-independent linear increase of the interaction energy with surface pressure deduced in this study strongly supports a cholesterol-DOPE regular mixture. Furthermore, the properties of the cholesterol-DOPE mixed monolayers determined in this study are in absolute agreement with the “umbrella model” proposed by Huang and Feigensohn in cholesterol-phospholipid bilayer mixtures.

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CHOL

DOPE

Figure 1.
Chemical structures of cholesterol and DOPE.

Figure 2.

(A) Representative Surface pressure – Interfacial area isotherms of cholesterol and DOPE mixed monolayers at 295.15 K. Composition of mixed air-water monolayers from right to left x_2 : 1, 0.8, 0.6, 0.5, 0.4 (dotted lines), 0.2 (dashed lines) and 0 (thick lines) (B) Surface pressure and elastic modulus plotted as a function of mean molecular area of pure cholesterol and pure DOPE monolayers at 295.15 K. Empty symbols represent values of elastic modulus ($\diamond$) Cholesterol ($\circ$) DOPE, while thick and thin continues lines represent surface pressures of cholesterol and DOPE, respectively. Cholesterol empty symbols were joined with dotted line to better indicate the transition just prior to monolayer collapse. Values of mean molecular areas were calculated as described in the theory section.

Figure 3.

Average partial molecular areas of cholesterol and DOPE depicted at various surface pressures as a function of mole fractions of DOPE ($n = 3$). Error bars were omitted for the

sake of clarity. $\% \text{ change from } a^\circ = \frac{\bar{a}_i - a_i^\circ}{a_i^\circ} \cdot 100$, where $i = 1$ denotes cholesterol and $i = 2$ denotes DOPE. Solid symbols joined by a line represent % change from a_1° , whereas empty symbols joined by a dotted line represent % change from DOPE (a_2°) molecular dimensions.

Figure 4A

Figure 4B

Figure 4.

(A) Average values of isothermal reversible work of compression for cholesterol (solid bars) and DOPE (empty bars) in mixed monolayers. (B) Work of compression in pure monolayers at 20 mN·m⁻¹ and 295.15 K (◆) DOPE ($n = 3$). Inset: Surface pressure dependence of work of compression of pure cholesterol monolayers at 295.15 K, with water as the subphase. From bottom to top: (◇) 16 (□) 20 (△) 24 and (○) 30 mN·m⁻¹ ($n = 3$).

Figure 5. Mass preservation plot of DOPE (◇) and mixed cholesterol-DOPE (◆) monolayers formed at equimolar amount of cholesterol and DOPE (15 nmoles). Experimental points are the average of three independent measurements with error bars representing standard uncertainties.

Figure 6.

(A) Excess Gibbs free energy of mixing as a function of mole fraction of cholesterol at various constant surface pressures and constant temperature 295.15 K ($n = 3$). (B) Isothermal Gibbs free energy of mixing as a function of surface pressure at various mole fractions of cholesterol ($\circ$) 0.2 ($\blacksquare$) 0.4 ($\triangle$) 0.5 ($\blacktriangle$) 0.6 ($\bullet$) 0.8 ($n = 3$). Average data points were connected with line segments to enhance the visual clarity of the plots. Error bars represent standard uncertainties.

Figure 7.

Top Panel: Log of the activity coefficients of cholesterol (γ_1) and of DOPE (γ_2), plotted as a function of the mole fraction square of lipid in mixed monolayers. Data points are the average of three independent experiments. Solid symbols joined by lines represent γ_1 , whereas empty symbols joined by dotted lines represent γ_2 , at corresponding surface pressures. Bottom Panel: Unitless interaction parameter ξ , plotted as a function of surface pressure change with Π_{initial} equal to zero. The data are the average of three experiments with the error bars representing standard uncertainties.

Figure 8.

Offset cholesterol-DOPE mixed monolayer collapse pressures plotted as a function of composition. Dotted lines represent calculated collapse surface pressures by eq 23, assuming activity coefficients equal to unity. Standard uncertainties shown with error bars were calculated from three independent experiments.

TABLE 1

Mixed Monolayer Properties at Onset of Monolayer Collapse.

x_{Chol}	a ($\text{\AA}^2\text{molecule}^{-1}$)	Π ($\text{mN}\cdot\text{m}^{-1}$)	K ($\text{mN}\cdot\text{m}^{-1}$)
0	55 (2.1)	37.0 (7.3)	86 (6)
0.2	44 (2.9)	36.6 (5.5)	81 (11)
0.4	37 (2.8)	35.6 (7.5)	79 (26)
0.5	34 (3.1)	36.7 (3.1)	71 (22)
0.6	36 (6.9)	39.3 (4.5)	146 (15)
0.8	33 (2.6)	34.2 (4.4)	149 (5.7)
1	35 (0.0)	33.3 (8.5)	306 (14)

Numbers in parentheses are % relative uncertainties calculated using, $\% \text{ relative uncertainty} = \frac{\text{standard uncertainty}}{\text{average value}} \cdot 100$.

TABLE 2

Excess Molecular Areas a^E of Mixed Monolayers as a Function of Mole Fraction of Cholesterol, at Various Surface Pressures and Temperature 295.15 K.*

x_1	$a^E (\text{\AA}^2 \text{molecule}^{-1})$					
	$\Pi = 5 \text{ (mN}\cdot\text{m}^{-1})$	$\Pi = 10 \text{ (mN}\cdot\text{m}^{-1})$	$\Pi = 15 \text{ (mN}\cdot\text{m}^{-1})$	$\Pi = 20 \text{ (mN}\cdot\text{m}^{-1})$	$\Pi = 25 \text{ (mN}\cdot\text{m}^{-1})$	$\Pi = 30 \text{ (mN}\cdot\text{m}^{-1})$
0.2	-5.4 ± 2.0	-5.8 ± 1.6	-6.4 ± 1.1	-6.5 ± 1.0	-6.6 ± 1.1	-6.7 ± 1.3
0.4	-8.8 ± 1.5	-9.0 ± 1.4	-9.0 ± 1.1	-9.2 ± 0.8	-9.5 ± 0.9	-10.0 ± 1.4
0.5	-5.8 ± 2.0	-6.6 ± 2.0	-7.1 ± 1.8	-8.0 ± 1.6	-8.5 ± 1.6	-10.0 ± 1.4
0.6	-9.4 ± 1.9	-8.9 ± 1.7	-8.3 ± 1.9	-7.7 ± 2.2	-7.1 ± 2.5	-6.6 ± 2.7
0.8	-5.5 ± 1.8	-5.9 ± 1.4	-6.2 ± 1.0	-6.4 ± 0.8	-6.4 ± 0.7	-6.4 ± 0.6

* Values tabulated are the average of three independent experiments $\pm$ standard uncertainties.

Unitless Interaction Parameter ξ , in Mixed Monolayers Composed of Cholesterol and DOPE, as a Function of Mole Fraction of Cholesterol, at Various Surface Pressures and Temperature 295.15 K.*

TABLE 3

x_1	ξ					
	$\Pi = 5$ (mN·m ⁻¹)	$\Pi = 10$ (mN·m ⁻¹)	$\Pi = 15$ (mN·m ⁻¹)	$\Pi = 20$ (mN·m ⁻¹)	$\Pi = 25$ (mN·m ⁻¹)	$\Pi = 30$ (mN·m ⁻¹)
0.2	-0.414 ± 0.1	-0.897 ± 0.2	-1.47 ± 0.3	-2.00 ± 0.3	-2.53 ± 0.4	-3.10 ± 0.6
0.4	-0.450 ± 0.1	-0.917 ± 0.1	-1.38 ± 0.2	-1.88 ± 0.2	-2.44 ± 0.2	-3.07 ± 0.4
0.5	-0.284 ± 0.1	-0.648 ± 0.2	-1.05 ± 0.3	-1.56 ± 0.3	-2.10 ± 0.4	-2.84 ± 0.4
0.6	-0.479 ± 0.1	-0.911 ± 0.2	-1.27 ± 0.3	-1.58 ± 0.4	-1.82 ± 0.6	-2.02 ± 0.8
0.8	-0.420 ± 0.1	-0.910 ± 0.2	-1.42 ± 0.2	-1.96 ± 0.2	-2.45 ± 0.3	-2.95 ± 0.2

* Data are the Average of three independent experiments ± standard uncertainties.

TABLE 4

Published Reports of Miscibility Studies of Cholesterol with PC and PE in Spread Mixed Monolayers at Air-Water Interface.*

Lipid	Interaction Forces
DLPC	Attractive at all Π and x . ⁵
DMPC	Attractive at all Π and x . ^{3,5}
DPPC	Pronounced attractive interaction observed at low Π and all x . ^{5-7,10-11} No interaction at high Π (collapse pressure) at all x . ^{5,7}
DSPC	No interaction at all Π and x . ^{3,5} Attractive at all Π and x . ⁸
DOPC	Attractive at all Π and x . ^{3,5,8,11}
Dilinoleoyl-PC	No interaction at low Π at all x . ^{3,5}
SOPC	Attractive at all Π and x . ^{3,5,8}
SLPC	Attractive at all Π and x . ³
1-linoleoyl-2-O-PC	No interaction at low Π at all x . ³
DPPE	No interaction at low Π at all x . ³ Attractive at $x_{\text{CHOL}} = 0.2-0.4$, repulsive or no interaction observed at higher fractions; 11 Repulsive at all Π and x . ¹⁰
DSPE	Repulsive at $x_{\text{DSPE}} = 0.25-0.5$, no interactions (ideal behavior) at $x_{\text{DSPE}} = 0.75$. ⁸
SOPE	Attractive at all Π and x . ³
1-linolenoyl-2-P-PE	No interaction at low Π at all x . ³
1-P-2-linolenoyl-PE	No interaction at low Π at all x . ³
1-P-2-linoleoyl-PE	No interaction at low Π at all x . ³

* All experiments were performed with unbuffered water as subphase. DLPC, DMPC, DPPC, DSPC, DOPC, SOPC, SOPE stand for 1,2-dilauroyl-*sn*-glycero-3-phosphatidylcholine, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, 1,2-distearoyl-*sn*-glycero-3-phosphatidylcholine, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, respectively.