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Complex Formation Equilibria in Two-Component Bilayer Lipid Membrane: Interfacial Tension Method

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Abstract We measured the interfacial tension of lipid membranes composed of ceramide-cholesterol, ceramide-sphingomyelin, and sphingomyelin-cholesterol. The membrane components formed 1:1 complexes. Interfacial tension measurements were used to determine the membrane surface concentration A_3^{-1}, the membrane interfacial tension γ_3, and the stability constant K.

Keywords Interfacial tension · Sphingomyelin · Ceramide-cholesterol · 1:1 complex

The membranes of eukaryotic cells contain three classes of lipids: glycerophospholipids, sphingolipids, and cholesterol (Ch) or a closely related sterol. The plasma membranes of eukaryotic cells are unusual in that they contain higher levels of sphingolipids and Ch (McMullen et al. 2004). Although these molecules are chemically and functionally dissimilar, they appear to colocalize in the same membrane regions and even appear to exhibit mutual attraction (Slotte 1999).

The physical properties of Ch-sphingolipid mixtures in monolayer and bilayer membranes are of particular interest to physical chemists, and a great deal of effort has been devoted to determining their structure. Studies of monolayer and bilayer lipid membranes aid in understanding the physical properties of animal cell membranes (Slotte et al. 1990; Bernholz 2004; Epand and Epand 2004; Halling and Slotte 2004). The interactions between sphingomyelin (SM) and Ch appear to be significant deviations from the additivity rule. Attempts to explain the nonideal behavior of the SM-Ch system have invoked the condensing effect of Ch (increased surface density of the membrane in presence of sterols) or the ordering effect (the presence of sterols increases the packing order of the hydrocarbon chains). Both effects have been observed in model systems and in biological membranes (Hianik et al. 1998; Preston et al. 2003; Rog and Pasenkiewicz-Gierula 2004). Other workers have proposed the formation of complexes. There seems to be little agreement concerning the stoichiometry of these complexes, but the values most commonly claimed for monolayers and bilayers are 1:1, 1:2, and 2:1 (Radhakrishnan et al. 2001; Ohvo-Rekila et al. 2002; McConnell and Radhakrishnan 2003). A variety of other structures has also been proposed for SM-Ch membranes, including microdomains (Wolf and Chachaty 2000; Massey 2001; Kahya et al. 2005), sphingolipid-Ch rafts (Armstrong et al. 2002; Filippov et al. 2006), and Ch-rich domains (Mattjus and Slotte 1996; Waarts et al. 2002; Pandit et al. 2004).

The effects of membrane composition on interfacial tension were previously described in PC (phosphatidylcholine)-Ch, PC-PE (phosphatidylethanolamine), and PE-Ch systems (Petelska et al. 2006a, b). This work examines the physical properties of three other lipid-lipid systems and presents new data pertaining to the effects of sphingolipid-Ch interactions in membranes. We describe the dependence of interfacial tension on membrane composition in ceramide (Cer)-Ch, Cer-SM, and SM-Ch over a wide range of compositions and present a comparison of the stability constants of the complexes and the surface areas occupied by the pure membrane components.

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Theory

In the cases where the membrane components do not form chemical compounds, their interaction can be described by the following set of equations (Petelska and Figaszewski 1998; Petelska et al. 2006a):

$$\begin{aligned} \gamma_1 m_1 A_1 + \gamma_2 m_2 A_2 &= \gamma \\ \frac{m_1}{m_1 + m_2} &= x_1 \\ x_1 + x_2 &= 1 \end{aligned} \tag{1}$$

where A_1^{-1}, A_2^{-1} (mol m^{-2}) are the surface concentration of components 1 and 2; m_1, m_2 (mol m^{-2}) are the quantities of components 1 and 2 per unit area of the membrane; γ_1, γ_2 (N m^{-1}) are the interfacial tensions of membranes assembled from pure components 1 and 2; γ (N m^{-1}) is the measured interfacial tension of the membrane; and x_1, x_2 are the solution mole fractions of components 1 and 2. Elimination of m_1 and m_2 yields the linear equation

$$(\gamma - \gamma_1)x_1 = \frac{A_2}{A_1}(\gamma_2 - \gamma)x_2 \tag{2}$$

Membranes may also be assembled from two components capable of forming a complex. The stoichiometry of the complex may vary, but because the first stability constant in these complexes is usually the largest (Inczedy 1976), we assumed that the complexes are primarily of 1:1 stoichiometry.

In cases where the membrane components form a 1:1 complex, interactions in the membrane may be described by a previously published set of equations (Petelska et al. 2006a). The equilibrium between the individual components and the complex is represented by

$$\underset{\text{(Component1)}}{\text{A}} + \underset{\text{(Component2)}}{\text{B}} \Leftrightarrow \underset{\text{(Complex)}}{\text{AB}}$$

and the basic equation describing the interaction between component 1 and component 2 can be written (Petelska et al. 2006a, b)

$$\begin{aligned} &\left[(\gamma - \gamma_1)B_2 x_1 + (\gamma - \gamma_2)B_1 x_2\right]\left[(\gamma_3 - \gamma_1)B_2 x_1 + (\gamma_3 - \gamma_2)\right. \\ &\quad \left. \times B_1 x_2 + (\gamma_1 - \gamma_2)(x_1 - x_2)\right] = KA_3^{-1}B_1 B_2 \\ &\quad \times \left[(\gamma - \gamma_1)(x_2 - x_1) + (\gamma_3 - \gamma)B_1 x_2\right] \\ &\quad \times \left[(\gamma - \gamma_2)(x_1 - x_2) + (\gamma_3 - \gamma)B_2 x_1\right] \end{aligned} \tag{3}$$

where $B_1 = A_3/A_1$, and $B_2 = A_3/A_2$. Equation (3) may be simplified by taking into account the high stability constant of the complex. Applying this simplification results in linear behavior for small ($x_2 < x_1$) and large ($x_2 > x_1$) x_2 values (Petelska et al. 2006a, b).

$$(\gamma_1 - \gamma)\frac{x_1 - x_2}{x_2} = -B_1\gamma_3 + B_1\gamma \tag{4}$$

$$(\gamma_2 - \gamma)\frac{x_2 - x_1}{x_1} = -B_2\gamma_3 + B_2\gamma \tag{5}$$

When calculating the stability constant for the complex, Eq. (3) can be simplified to $x_1 = x_2$ (Petelska et al. 2006a).

$$\begin{aligned} K\left(A_1^{-1}\right)^2\left(A_2^{-1}\right)^2\left(A_3^{-1}\right)^{-1}(\gamma - \gamma_3)^2 = &\left[\gamma_2 A_1^{-1} + \gamma_1 A_2^{-1}\right. \\ &\left. -\gamma\left(A_1^{-1} + A_2^{-1}\right)\right]\left(\gamma_2 A_1^{-1} + \gamma_1 A_2^{-1}\right) \\ &- \left[\gamma_2 A_1^{-1} + \gamma_1 A_2^{-1} - \gamma\left(A_1^{-1} + A_2^{-1}\right)\right]\left(A_1^{-1} + A_2^{-1}\right)\gamma_3 \end{aligned} \tag{6}$$

The parameters describing the complex may be used to calculate theoretical points using the equation presented below (agreement between theoretical and experimental values implies that the system is well described by the above equations):

$$\begin{aligned} &KA_1^{-1}A_2^{-1}(a_1 + a_2)(a_3 - a_1)\gamma^2 + \\ &\quad + \left[KA_1^{-1}A_2^{-1}(\gamma_1 a_1 - \gamma_3 a_3)(a_1 + a_2) - KA_1^{-1}A_2^{-1}\right. \\ &\quad \left. \times (\gamma_2 a_1 + \gamma_3 a_2)(a_3 - a_1) + a_4 A_3^{-1}(a_3 + a_2)\right]\gamma + \\ &\quad + KA_1^{-1}A_2^{-1}a_3\gamma_3(\gamma_3 a_2 + \gamma_1 a_2) - KA_1^{-1}A_2^{-1}a_1\gamma_1 \\ &\quad \times (a_1\gamma_2 + a_2\gamma_3) - a_4 A_3^{-1}(\gamma_2 a_3 + \gamma_1 a_2) = 0 \end{aligned} \tag{7}$$

where

$$\begin{aligned} a_1 &= A_3^{-1}(x_2 - x_1) \\ a_2 &= A_2^{-1}x_1 \\ a_3 &= A_1^{-1}x_2 \\ a_4 &= \left[A_3^{-1}(\gamma_1 - \gamma_2)(x_2 - x_1) + (\gamma_1 - \gamma_3)x_1 A_2^{-1}\right. \\ &\quad \left. +(\gamma_2 - \gamma_3)x_2 A_1^{-1}\right] \end{aligned}$$

For systems containing two lipid components, 1:1 complex formation was assumed to be the explanation for deviation from the additivity rule. Model curves were constructed using calculated parameters such as equilibrium constants, molecular areas of the complexes, and interfacial tension of molecules and complexes. The accuracy of the models was verified by comparison to experimental results.

Materials and Methods

The interfacial tension method is based on Young and Laplace's equation. The tension in a lipid bilayer sample is determined by measuring the radius of curvature of the convex surface formed when a pressure difference is applied across the bilayer (Adamson 1960). The apparatus and measurement method have been described in previous papers (Petelska and Figaszewski 1998, 2000; Petelska et al. 2006a, b). The measurement system consists of two glass chambers separated by a mount holding a 1.5-mm-

diameter circular Teflon element axially pierced by a small orifice.

Membranes were formed by the Mueller–Rudin method (Mueller et al. 1963) on the flat end of the Teflon element. Both chambers were filled with an electrolyte solution. The membrane-forming solution was introduced to the flat wall of the Teflon element using a micropipette, and pressure was applied to the left chamber using a manometer.

The convexity of the lipid membrane cap was measured to 0.05-mm precision. The radius of curvature was determined using this value and the diameter of the Teflon element, corresponding to the diameter of the lipid cap.

The following reagents were used for preparation of the membrane-forming solution:

(1) SM from chicken egg yolk (98%; Fluka),
(2) Cer from bovine brain (98%; Fluka), and
(3) Ch (99%; Sigma).

The as-received SM and Cer were purified by dissolving them in chloroform and evaporating the solvent under argon. The stock membrane-forming solutions consisted of 20 mg cm^{-3} of the desired lipid (SM, Cer, or Ch) in 20:1 *n*-decane:butanol. The solution containing the membrane components was not saturated and could therefore contain the components in any proportion. During membrane formation, the solvent was removed, leaving a membrane composed of lipids at the same ratio as in the stock solution.

The electrolyte solution contained 0.1 M potassium chloride and was prepared using triple-distilled water and KCl produced by POCh (Poland). The KCl was calcined to remove any organic impurities.

All solvents were chromatographic standard grade. The *n*-decane was purchased from Merck and the chloroform and butanol were obtained from Aldrich.

All experiments were carried out at 293 ± 2 K.

Results and Discussion

Figure shows graphs of $(\gamma - \gamma_1)x_1$ versus $(\gamma_2 - \gamma)x_2$ for the three systems Cer-Ch (Fig. 1a), SM-Cer (Fig. 1b), and SM-Ch (Fig. 1c). According to Eq. (2), when the membrane components do not interact these functions should yield straight lines. This is clearly not the case, which suggests that a complex or other structure exists in Cer-Ch, SM-Cer, and SM-Ch bilayers. Because the use of Eq. (3) presupposes the existence of 1:1 complexes, our initial assumption was that the complexes formed were 1:1. The interfacial tension of the lipid membrane was studied over a wide range of lipid compositions.

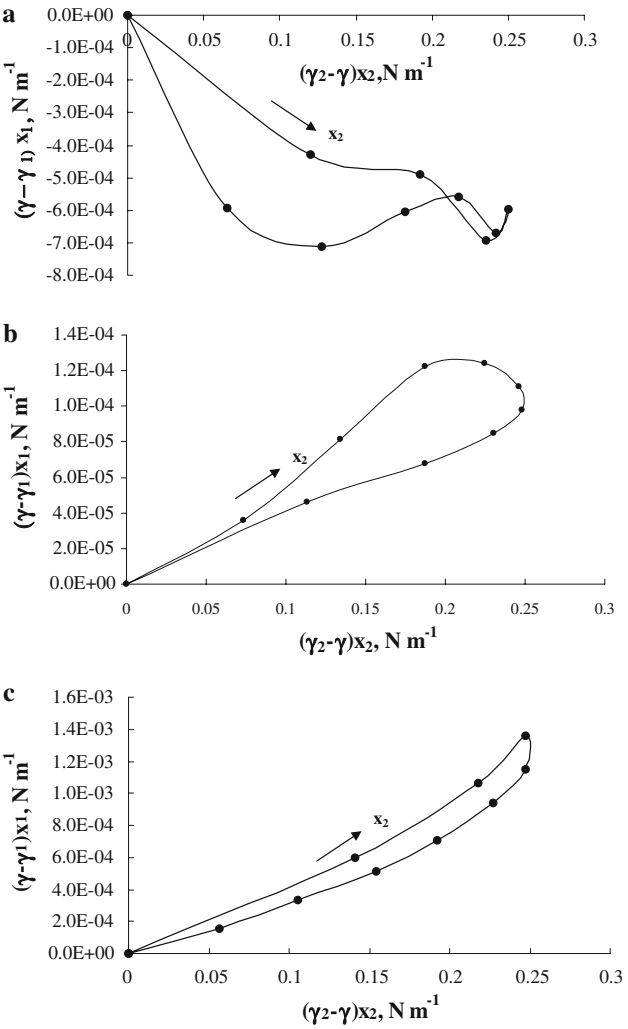

Fig. 1 Graph of Eq. (2) for ceramide-cholestrol (**a**), ceramide-sphingomyelin (**b**), and sphingomyelin-cholesterol (**c**), where x_2 is the mole fraction of component 2 (cholesterol or ceramide)

Ceramide-Cholesterol Complex

Figure 2a depicts the interfacial tension of a Cer-Ch membrane as a function of Ch mole fraction. The interfacial tension values of pure Cer and pure Ch membranes are 1.29×10^{-3} and 7.42×10^{-3} N m^{-1}, respectively.

Equation (2) predicts that in the absence of interactions, the plot in Fig. 1a should yield a straight line. The nonlinear nature of the plot indicates some form of interaction between Ch and Cer. Such interactions in sphingolipid-Ch systems can be explained in terms of complexes (Radhakrishnan et al. 2001; Ohvo-Rekila et al. 2002; McConnell and Radhakrishnan 2003). Radhakrishnan et al. (2001) demonstrated the formation of complexes in the SM-Ch system. Naumowicz and Figaszewski (2007) found that bilayers formed from SM and Ch contained exclusively

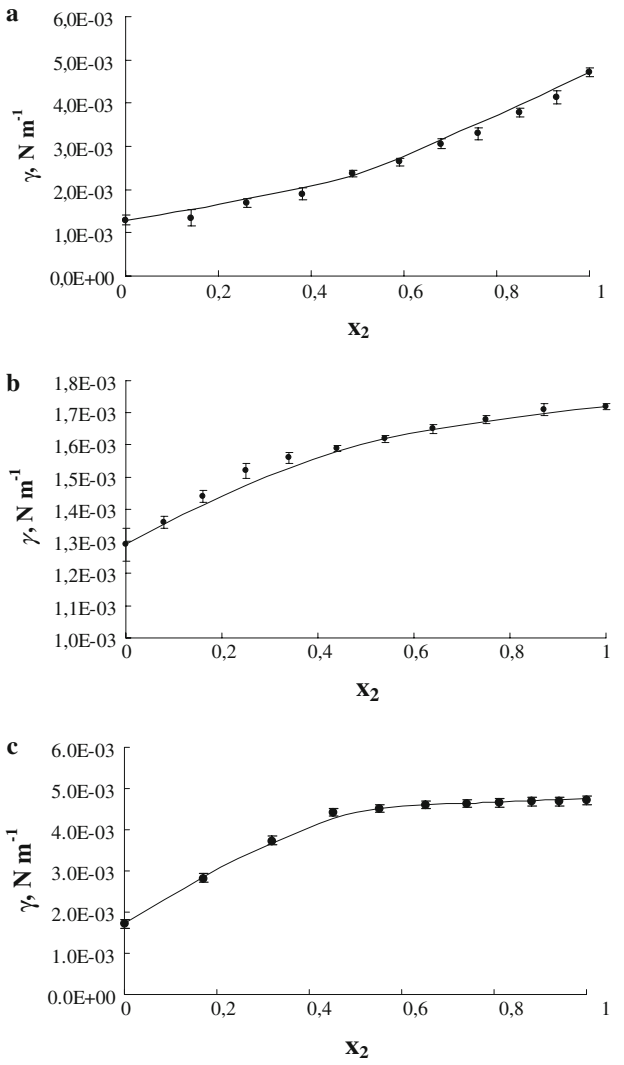

Fig. 2 The interfacial tension γ of ceramide-cholesterol (**a**), ceramide-sphingomyelin (**b**), and sphingomyelin-cholesterol (**c**) as a function of the mole fraction x_2 of component 2. Experimental values are marked by *points* and theoretical values are indicated by the *curve*

complexes of 1:1 stoichiometry. The 1:1 complex is formed in the initial stage of complexation, followed by other compositions in subsequent stages. In our case, an equation derived to describe the equilibrium of 1:1 complex formation was sufficient for the entire concentration range.

Based on the literature, an assumption was made that a 1:1 complex formed the most prevalent structure and was characterized by a maximal stability constant K (Inczedy 1976). Given this assumption, the dependence of interfacial tension on the composition of the membrane-forming solution is described by Eq. (3). The interfacial tensions of membranes formed from the pure components were experimentally determined. The constants B_1, B_2, and γ_3 were determined assuming that the value of the stability constant for the Cer-Ch complex was sufficient with respect to the simplified Eq. (3) to Eqs. (4) and (5).

Graphs of functions (4) and (5) are presented in Fig. 3. The values of B_1 (2.850) and B_2 (1.639) were determined from the slopes of the lines. The intersections of the straight lines with the ordinate provide $-B_1\gamma_3$ and $-B_2\gamma_3$, which can be used to determine γ_3, the interfacial tension of the Cer-Ch complex. The mean value obtained in this way was 2.33×10^{-3} N m^{-1}.

Determining the interfacial tension as a function of composition enabled calculation of surface concentrations for membranes formed of pure components. At least one of these calculations is necessary to determine the value of A_3^{-1}. The surface areas occupied by Cer and Ch are 50 Å^2 molecule^{-1} (Imura et al. 2000) and 38 Å^2 molecule^{-1} (Jain 1972), respectively.

The surface concentrations of Cer and Ch in membranes of pure components are 3.32×10^{-6} and 4.37×10^{-6} mol m^{-2}, respectively. Knowing A_1^{-1} and A_2^{-1} as well as B_1 and B_2, the surface concentration of a membrane composed of Cer-Ch complex can be determined. The

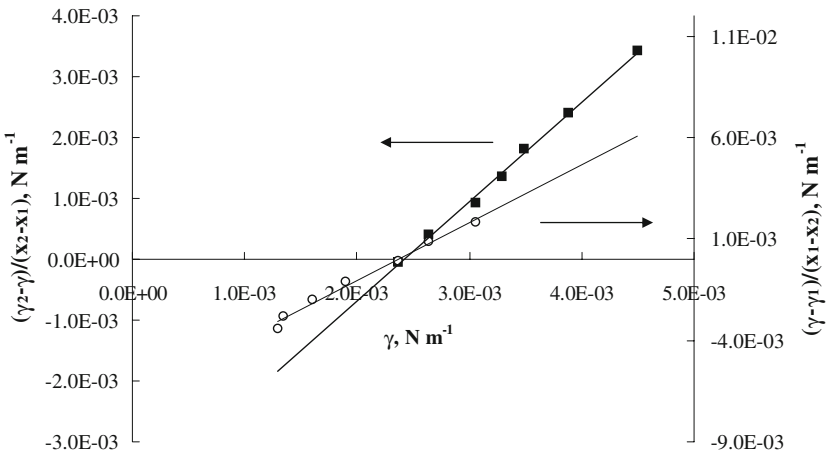

Fig. 3 A plot illustrating Eqs. (4) and (5) for calculation of the parameters B_1, B2, and γ_3 for the ceramide-cholesterol system

resulting surface concentration value A_3^{-1} for the Cer-Ch complex was 2.00×10^{-6} mol m^{-2}. From this it was possible to determine that the area occupied by one Cer-Ch complex is $\sim$ 83 Å^2 molecule^{-1}, smaller than the sum of the areas occupied by individual Cer and Ch molecules. Addition of Ch to membranes constructed from phospholipids resulted in increased stability and reproducibility. Ch condenses some membrane components (the so-called condensation effect), making the membrane structures more rigid (Przestalski 1983). It also improves the packing of membrane lipids as, unlike sphingolipid molecules, it preferentially occupies voids in the hydrophobic layer of the membrane rather than spaces in the polar region.

The stability constant of the Cer-Ch complex was determined from Eq. (6) by setting $x_1 = x_2 = 0.5$. The stability constant was 8.30×10^7 m^2 mol^{-1}.

Sphingomyelin-Ceramide and Sphingomyelin-Cholesterol Complexes

The dependence of lipid membrane interfacial tension on composition for SM-Cer and SM-Ch systems was studied over a wide concentration range. The results are depicted in Fig. 2b and c.

According to Eq. (2), the plots in Fig. 1b as well as Fig. 1c should yield a straight line. It can be seen from Fig. 1b and c that such linear dependences are not obtained. The curves indicate that the SM-Cer and SM-Ch systems both form complexes.

The interfacial tensions of membranes formed using pure components were measured directly. The pure SM membrane exhibited an interfacial tension of 1.72×10^{-3} N m^{-1}.

The values of γ_3 for the SM-Cer (1.62×10^{-3} N m^{-1}) and SM-Ch (4.48×10^{-3} N m^{-1}) complexes were calculated using Eqs. (4) and (5). Equations (4) and (5) were also used to calculate the surface concentrations per unit area of membranes formed entirely from Cer-SM and SM-Ch complexes (the surface area occupied by a SM molecule is 45 Å^2 [Chiu et al. 2003; Petelska et al. 2008], and the surface concentration calculated for a pure SM membrane is 3.69×10^{-6} mol m^{-2}).

The surface concentrations for Cer-SM and SM-Ch complexes are 1.70×10^{-6} and 2.08×10^{-6} mol m^{-2}, respectively. From these values it is possible to determine the areas occupied by Cer-SM and SM-Ch complexes, which are 98 and 80 Å^2 molecule^{-1}, respectively. The stability constants of the Cer-SM and SM-Ch complexes were determined using Eq. (6). The stability constant of Cer-SM is 1.47×10^7 m^2 mol^{-1} and the constant of SM-Ch is 1.60×10^8 m^2 mol^{-1}. The stability constant of the SM-Ch complex is in agreement with the value of 1.18×10^8 m^2 mol^{-1} obtained from impedance spectroscopy data (Naumowicz and Figaszewski 2007).

The experimental values in Fig. 2b and c are marked by points, and the theoretical ones obtained from Eq. (7) by lines. It can be seen from these figures that the agreement between experimental and theoretical points are good, which verifies the assumption of a formation of 1:1 Cer-SM and SM-Ch complexes in the lipid membranes.

Table 1 lists several physicochemical parameters for membranes containing Cer-Ch, Cer-SM, or SM-Ch complexes. Analysis of the results presented in Table 1 leads to the following conclusions.

1. The stability constant of the Cer-Ch complex is 8.30×10^7 m^2 mol^{-1}, whereas the stability constant of the Cer-SM complex is 1.47×10^7 m^2 mol^{-1} and the stability constant of SM-Ch is 1.60×10^8 m^2 mol^{-1}. These values are relatively high, providing additional support for the prevalence of 1:1 complexes in mixed bilayers. These values also confirm that the assumption used to simplify Eq. (1) was correct. This paper contains the first report of stability constants for Cer-Ch, Cer-SM, and SM-Ch complexes.
2. The stability constants of the Ch-containing complexes are higher, indicating that the Cer-Ch and SM-Ch complexes are more stable than the SM-Cer complex.
3. The experimentally obtained value for the area occupied by the Cer-Ch complex is 83 Å^2, the area occupied by Cer-SM is 98 Å^2, and the area occupied by SM-Ch is 80 Å^2. The calculated values for the areas occupied by SM-Ch, SM-Cer, and Cer-Ch complexes are 121, 129, and 83 Å^2, and the areas occupied by PC-Ch, PC-PE, and PE-Ch complexes are 127, 154,

Table 1 Selected physicochemical parameters for three complexes: ceramide-cholesterol (Cer-Ch), ceramide-sphingomyelin (Cer-SM), and sphingomyelin-cholesterol (SM-Ch)

Complex	Interfacial tension (N m^{-1})	Area occupied by 1 molecule (Å^2 molecule^{-1})	Stability constants (m^2 mol^{-1})
Cer-Ch	2.33×10^{-3}	83	8.3×10^7
Cer-SM	1.62×10^{-3}	98	1.47×10^7
SM-Ch	4.48×10^{-3}	80	1.60×10^8

and 127 Å^2, respectively. The areas of complexes containing Ch are lower. Ch condenses some membrane components (the so-called condensation effect), making the membrane structure more rigid. Sterols also improve the packing of membrane lipids, as they are more likely to occupy interstices in the hydrophobic layer of the membrane and less likely to reside near the polar head groups, unlike phospholipid molecules.

4. Good agreement between the experimental and the theoretical points verifies the assumption of a 1:1 complex in the lipid membrane. The lack of variation between theoretical and experimental points indicates that our theoretical model (presented under Theory, above) is sufficient to describe the interaction in sphingolipid-Ch and sphingolipid-phospholipid systems. The agreement between the experimental results and the model predictions for Cer-Ch, SM-Ch, and Cer-SM membranes justifies the statement that other complexes do not represent a significant component of these systems.
5. The mathematically derived and experimentally confirmed results presented here are of great importance for the interpretation of phenomena occurring in lipid bilayers. These results can help lead to a better understanding of the physical properties of biological membranes. The simple and very interesting methods proposed in this paper and in earlier studies (Petelska and Figaszewski 1998; Petelska et al. 2006a, b) may be used with success to determine the equilibrium constant values of 1:1 lipid-lipid systems.

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