Acta Biochimica Polonica

Vol. 55 No. 4/2008, 721–730

on-line at: www.actabp.pl

Regular paper

Physicochemical analysis of phosphatidylcholine-ceramide system in bilayer lipid membranes

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Received: 11 June, 2008; revised: 02 November, 2008; accepted: 03 December, 2008
available on-line: 16 December, 2008

Electrochemical impedance spectroscopy was used for the study of two-component lipid membranes. Phosphatidylcholine and ceramide were to be investigated, since they play an important biochemical role in cell membranes. The research on biolipid interaction was focused on quantitative description of processes that take part in a bilayer. Assumed models of interaction between amphiphilic molecules and the equilibria that take place there were described by mathematical equations for the studied system. The possibility of complex formation for two-component system forming bilayers was assumed that could explain the deviation from additivity rule. Equilibria were described by mathematical equations that were further verified experimentally. The determined values of parameters (stability constant, molecular area of complex, capacitance and conductance of the lipid membranes formed from molecules and complexes) were used for calculation of model curves. The comparison of model curves and experimental points verified the assumed model.

Keywords: bilayer lipid membrane, equilibrium, complex formation, phosphatidylcholine, ceramide, electrochemical impedance spectroscopy

INTRODUCTION

Bilayer lipid membranes are made predominantly from amphiphiles, a special class of surface-active molecules, which are characterized by having a hydrophilic and a hydrophobic group in the same molecule (Przestalski *et al*., 2000). Usually, zwitterionic or non-ionic lipids are used as the basic lipids for the preparation of bilayers. Biolipids can be categorized into three principal types: phospholipids, sphingolipids, and cholesterol. They each play a different role in the membranes. Sphingolipids differ from phospholipids in being based on a lipophilic amino alcohol (sphingosine) rather than glycerol. Ceramides are the biological building blocks of more complex sphingolipids. The cellular concentration of ceramides can be quite high in reaching levels of 1–10 mol% of the total phospholipid concentration (Hannun, 1996), free ceramides are found in large amounts in the skin stratum corneum (Goni & Alonso, 2006). Metabolism of ceramides typically occurs in Golgi and endoplasmic reticulum membranes, and fluorescent ceramide analogs are important probes for measuring the intracellular distribu-

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Abbreviations: ***Names of compounds:*** component 1, phosphatidylcholine; component 2, ceramide; compound 3, phosphatidylcholine-ceramide complex. ***Variables:*** c_i^s, the surface concentration of component "i" and compound 3, in the membrane; C_i, the capacitance of the membrane built by component "i" and compound 3, referred to the unit area of the membrane; C_m, the measured capacitance of the membrane referred to the unit area of the membrane; c_{ti}^s, the total surface concentration of component "i" in the membrane; K_R, the stability constant of compound 3; R_i^{-1}, the conductance of the membrane built by component "i" and compound 3, referred to the unit area of the membrane; R_m^{-1}, the measured conductance of the membrane referred to the unit area of the membrane; R_0, the resistance of the electrolyte; S_i, the surface area, occupied by one mole of component "i" and compound 3; x_i, the molar fraction of component "i".

tion and transport of the labeled molecules in living cells.

The complexity of biological membranes makes it virtually impossible to draw detailed physical conclusions from studies of these membranes and a simplification is therefore required. Since the realization that lipid bilayers comprise the fundamental structure of all biological membranes, they have been the subject of numerous experimental studies. As a result, membrane models of variable complexity and destination have emerged, some aiming at elucidating structural details of the bilayer membrane and others striving to mimic its functions.

Most of the lipid bilayer studies concentrate on the surface potential (Hianik *et al*., 1995; 1998) or surface pressure measurements (Rao & Damodaran, 2004; Zhao & Feng, 2004), spectroscopy (Costa *et al*., 1997; Horváth *et al*., 2003) and microscopic visualisation of lateral domains (London, 2002; Laggner *et al*., 2003). As is well known, biological membranes and black lipid membranes used as model membranes can also be very well characterized by electrochemical impedance spectroscopy (Coster *et al*., 1996; Steinem *et al*., 1996; Ye *et al*., 2001; Boncheva *et al*., 2008). For the past century, the impedance technique has provided a non-invasive means of characterizing the electrical properties of many systems. Even today, it often provides the only non-invasive method for detailed structural-functional studies of these systems (Coster *et al*., 1996). In spite of a wide variety of experimental methods for the study of lipid bilayers, some long-lasting problems remain. One of them is the complex formation between two kinds of lipids in a bilayer or in a monolayer at the air/water interface. Moreover, there is still the lack of the quantitative description of the lipid-lipid systems. It is required for a better understanding of the processes that take place in biological membranes with the aim of forming the artificial membrane that would very closely resemble the properties of the natural membrane. Therefore, the knowledge of molecular structure and organisation of biolipids is necessary.

In this article, we model biomembranes using a two-component bilayer system. The lipids chosen are phosphatidylcholine from egg yolk and sheep brain ceramide. Egg phosphatidylcholine was selected mainly due to its acyl chain composition, which resembles many biological membranes. Ceramide was used because its effect on the organization of membranes is not very clear (Dobrowsky, 2000; Holopainen *et al*., 1997; 1998; Veiga *et al*., 1999; Huang *et al*., 1999; Massey, 2001) in spite of the fact that recently, with the discovery of the sphingolipid signaling pathway, the interest in this lipid was renewed (Goni & Alonso, 2006). We have assumed here that the model membranes, created by us, would contain the 1:1 phosphatidylcholine-ceramide complex. We have utilized electrochemical impedance spectroscopy to study the formation of the complex in lipid bilayers. The determination of the area occupied by one phosphatidylcholine-ceramide complex molecule and the stability constant of the complex is the final research result. We would like to emphasize that the value of the stability constant of phosphatidylcholine-ceramide complex is reported for the first time.

Data presented in this work, obtained as the result of mathematical derivation and confirmed experimentally, are of great importance for the interpretation of the phenomena occurring in lipid membranes. The knowledge of equilibrium presented by the complexing reaction allows us to understand the processes that take place on the bilayer surface. The obtained results can be used in quantitative description of physical and chemical properties of biological membranes and, in our opinion, can help in a better understanding of biological membranes and in their biophysical studies.

THEORY

A two-component forming solution can be used to obtain a lipid membrane. The components may or may not form another compound.

The model, which has been presented in full detail previously (Petelska *et al*., 2006; Naumowicz *et al*., 2006; Naumowicz & Figaszewski, 2007) assumes that in the case where the membrane components do not form chemical compounds, any two-component system, regardless of whether it is a monolayer or a bilayer, can be described by the equation expressing additivity of the capacitance:

$$C_m = C_1 c_1^s S_1 + C_2 c_2^s S_2 \quad (1a)$$

and

$$R_m^{-1} = R_1^{-1} c_1^s S_1 + R_2^{-1} c_2^s S_2 \quad (1b)$$

here:

$$x_1 = c_1^s/(c_1^s + c_2^s) \quad (2)$$

$$x_1 + x_2 = 1 \quad (3)$$

where:

C_m [μF cm^{-2}] — the measured capacitance of the membrane referred to the unit area of the membrane;

C_1, C_2 [μF cm^{-2}] — the capacitance of the membrane built by components 1 and 2, respectively, referred to the unit area of the membrane;

R_m^{-1} [Ω^{-1} cm^{-2}] — the measured conductance of the membrane referred to the unit area of the membrane;

R_1^{-1}, R_2^{-1} [Ω^{-1} cm^{-2}] — the conductance of the membrane built by components 1 and 2, respectively, referred to the unit area of the membrane;

c_1^s, c_2^s [mol m^{-2}] — the surface concentration of components 1 and 2, respectively, in the membrane;
S_1, S_2 [m^2 mol^{-1}] — the surface area, occupied by one mole of components 1 and 2, respectively;
x_1, x_2 – the molar fractions of components 1 and 2, respectively.

Elimination of c_1^s and c_2^s yields the linear equations:

$$(C_m - C_1)x_1 = -(S_2/S_1)(C_m - C_2)x_2 \quad (4a)$$
$$(R_m^{-1} - R_1^{-1})x_1 = -(S_2/S_1)(R_m^{-1} - R_2^{-1})x_2 \quad (4b)$$

Let us assume that there is the equilibrium in the system, regardless whether it is a monolayer or a bilayer. As a result of this equilibrium a chemical complex (compound 3) with the stoichiometry of 1:1 is formed (Cadenhead, 1970; Demel & de Kruyff, 1976). Then, Eqns. (1) are modified because the electric capacity and electric conductance are the sum of the contributions of all compounds:

$$C_m = C_1 c_1^s S_1 + C_2 c_2^s S_2 + C_3 c_3^s S_3 \quad (5a)$$
$$R_m^{-1} = R_1^{-1} c_1^s S_1 + R_2^{-1} c_2^s S_2 + R_3^{-1} c_3^s S_3 \quad (5b)$$

here:

$$K_R = c_3^s/(c_1^s \times c_2^s) \quad (6)$$
$$x_1 = (c_1^s + c_3^s)/(c_1^s + c_2^s + c_3^s) \quad (7)$$
$$c_{t1}^s = c_1^s + c_3^s \quad (8)$$
$$c_{t2}^s = c_2^s + c_3^s \quad (9)$$
$$x_1 + x_2 = 1 \quad (10)$$

where:

C_3 [μF cm^{-2}] — the capacitance of the membrane built by compound 3 referred to the unit area of the membrane;
R_3^{-1} [Ω^{-1} cm^{-2}] — the conductance of the membrane built by compound 3 referred to the unit area of the membrane;
c_3^s [mol m^{-2}] — the surface concentration of compound 3 in the membrane;
c_{t1}^s, c_{t2}^s [mol m^{-2}] — the total surface concentration of components 1 and 2, respectively, in the membrane;
S_3 [m^2 mol^{-1}] — the surface area, occupied by one mole of compound 3;
K_R [m^2 mol^{-1}] — the stability constant of compound 3.

Two substances can form complexes at different stoichiometries. However, due to the fact that the first stability constant in complexes, as the essential one, is usually the biggest and should be taken into consideration (Inczedy, 1976), we assume that a 1:1 complex is formed between two kinds of lipids in a bilayer membrane.

After solution of the equation system (5)–(10) the following basic equations are derived:

$$[(C_m - C_1)B_2x_1 + (C_m - C_2)B_1x_2][(C_3 - C_1)B_2x_1 + (C_3 - C_2)B_1x_2 + (C_1 - C_2)(x_1 - x_2)] = K_R S_3^{-1} B_1 B_2 [(C_m - C_1)(x_2 - x_1) + (C_3 - C_m)B_1x_2] \times [(C_m - C_2)(x_1 - x_2) + (C_3 - C_m)B_2x_1] \quad (11a)$$

and

$$[(R_m^{-1} - R_1^{-1})B_2x_1 + (R_m^{-1} - R_2^{-1})B_1x_2][(R_3^{-1} - R_1^{-1})B_2x_1 + (R_3^{-1} - R_2^{-1})B_1x_2 + (R_1^{-1} - R_2^{-1})(x_1 - x_2)] = K_R S_3^{-1} B_1 B_2 [(R_m^{-1} - R_1^{-1})(x_2 - x_1) + (R_3^{-1} - R_m^{-1})B_1x_2][(R_m^{-1} - R_2^{-1})(x_1 - x_2) + (R_3^{-1} - R_m^{-1})B_2x_1] \quad (11b)$$

in which:

$B_1 = S_3/S_1$ and $B_2 = S_3/S_2$.

Eqns. (11) are second-degree equations with respect to C_m, the complex composition as well as with respect to the constants: C_1, C_2, C_3, R_1^{-1}, R_2^{-1}, R_3^{-1}, B_1, B_2. Opening of the parentheses results in a great complexity of the equations, and it is troublesome, when directly applied to the determination of constants. The constants mentioned above can be determined in individual cases using simplified forms of these equations.

Eqns. (11) may be simplified taking into account the sufficiently high value of the stability constant of the complex. Based on this assumption, the dependencies of linear type are derived for small x_2 values ($x_2 < x_1$):

$$(C_1 - C_m)(x_1 - x_2)/x_2 = -B_1C_3 + B_1C_m \quad (12a)$$
$$(R_1^{-1} - R_m^{-1})(x_1 - x_2)/x_2 = -B_1R_3^{-1} + B_1R_m^{-1} \quad (12b)$$

while for the high x_2 values ($x_2 > x_1$) Eqns. (11) can be described as other linear expressions:

$$(C_2 - C_m)(x_2 - x_1)/x_1 = -B_2C_3 + B_2C_m \quad (13a)$$
$$(R_2^{-1} - R_m^{-1})(x_2 - x_1)/x_1 = -B_2R_3^{-1} + B_2R_m^{-1} \quad (13b)$$

Eqns. (11) can be simplified in some other way. In the case where $x_1 = x_2$, the following forms are assumed:

$$K_R(S_1^{-1})^2(S_2^{-1})^2S_3(C_m - C_3)^2 = [C_2S_1^{-1} + C_1S_2^{-1} - C_m(S_1^{-1} + S_2^{-1})](C_2S_1^{-1} + C_1S_2^{-1}) - [C_2S_1^{-1} + C_1S_2^{-1} - C_m(S_1^{-1} + S_2^{-1})](S_1^{-1} + S_2^{-1})C_3 \quad (14a)$$

and

$$K_R(S_1^{-1})^2(S_2^{-1})^2S_3(R_m^{-1} - R_3^{-1})^2 = [R_2^{-1}S_1^{-1} + R_1^{-1}S_2^{-1} - R_m^{-1}(S_1^{-1} + S_2^{-1})](R_2^{-1}S_1^{-1} + R_1^{-1}S_2^{-1}) - [R_2^{-1}S_1^{-1} + R_1^{-1}S_2^{-1} - R_m^{-1}(S_1^{-1} + S_2^{-1})](S_1^{-1} + S_2^{-1})R_3^{-1} \quad (14b)$$

The parameters describing the complex determined from equations (11) and (14) could be applied to present the agreement of Eqn. (11) with the experimental data using Eqns. (15):

$$K_R S_1^{-1} S_2^{-1}(a_1 + a_2)(a_3 - a_1)C_m^2 + [K_R S_1^{-1} S_2^{-1}(C_1a_1 - C_3a_3)(a_1 + a_2) - K_R S_1^{-1} S_2^{-1}(C_2a_1 - C_3a_2)(a_3 - a_1) + a_4 S_3^{-1}(a_3 + a_2)]C_m + K_R S_1^{-1} S_2^{-1} a_3 C_3(C_3a_2 + C_1a_2)$$

$$- K_R S_1^{-1} S_2^{-1} a_1 C_1 (C_2 a_1 + C_3 a_2) - a_4 S_3^{-1} (C_2 a_3 + C_1 a_2) = 0 \quad (15a)$$

where:

$$a_1 = S_3^{-1}(x_2 - x_1)$$
$$a_2 = S_2^{-1} x_1$$
$$a_3 = S_1^{-1} x_2$$
$$a_4 = [S_3^{-1}(C_1 - C_2)(x_2 - x_1) + (C_1 - C_3) x_1 S_2^{-1} + (C_2 - C_3) x_2 S_1^{-1}$$

and

$$K_R S_1^{-1} S_2^{-1}(a_1 + a_2)(a_3 - a_1)(R_m^{-1})^2 + [K_R S_1^{-1} S_2^{-1}(R_1^{-1} a_1 - R_3^{-1} a_3)(a_1 + a_2) - K_R S_1^{-1} S_2^{-1}(R_2^{-1} a_1 - R_3^{-1} a_2)(a_3 - a_1) + a_4 S_3^{-1}(a_3 + a_2)] R_m^{-1} + K_R S_1^{-1} S_2^{-1} a_3 R_3^{-1}(R_3^{-1} a_2 + R_1^{-1} a_2) - K_R S_1^{-1} S_2^{-1} a_1 R_1^{-1}(R_2^{-1} a_1 + R_3^{-1} a_2) - a_4 S_3^{-1} (R_2^{-1} a_3 + R_1^{-1} a_2) = 0 \quad (15b)$$

in which:

$$a_4 = [S_3^{-1}(R_1^{-1} - R_2^{-1})(x_2 - x_1) + (R_1^{-1} C_1 - R_3^{-1}) x_1 S_2^{-1} + (R_2^{-1} - R_3^{-1}) x_2 S_1^{-1}]$$

MATERIALS AND METHODS

Reagents and preparation of the forming solutions. Ninety-nine percent egg phosphatidylcholine was purchased from Fluka and it had the following fatty acid composition: 16:0 ~ 33%, 18:0 ~ 4%, 18:1 ~ 30%, 18:2 ~ 14%, 20:4 ~ 4%. Ninety-eight percent sheep brain ceramide was also obtained from Fluka. The lipids were dissolved in chloroform to prevent oxidation and mixed in appropriate proportions to achieve the desired molar fractions. The solvent was evaporated under a stream of argon. Dried residues were dissolved in a hexadecane/butanol mixture (10:1, v/v). The resultant solution used to form the model membrane contained 20 mg ml^{-1} of lipids in solution. During membrane formation, the solvent mixture was removed and the membrane created has the same proportion as in the resultant solution. The samples were stored for at least five days at 4°C before examination.

The solvents were of chromatographic standard grade: chloroform and butanol were from Aldrich; hexadecane was from Fluka.

Potassium chloride solution of 0.1 mol dm^{-3} was used as the electrolyte for experiments. KCl was analytical grade and was heated prior to use at 400°C for 4 h to remove traces of organic material. Water purified by Milli-Qll was used to make the electrolyte and in all cleaning procedures.

Preparation of the bilayer membranes. Bilayer membranes were obtained as bubbles at the Teflon cap constituting a measuring vessel component. The use of hexadecane as the solvent makes it possible to obtain membranes of thickness and capacity values similar to those of membranes formed of monolayers (Benz *et al*., 1975; Karolins *et al*., 1998); there is almost no solvent retained in the bilayer. A small quantity of butanol added has a negligible effect on the impedance parameters of the bilayers created; however, it considerably accelerates the formation of the membranes. The formation of the bilayers was monitored visually and electrically by measuring the membrane capacitance at low frequency. Capacity of the membranes increased with time after bilayer formation until a steady-state value was reached some 10–20 min later. The measurements were begun only after the low frequency capacitance was stable, increasing by less than 1% per hour. When the capacitance had stabilized, it was assumed that diffusion of solvent out of the bilayer was complete, although some hexadecane molecules would remain dissolved in the membrane interior. The bilayer areas were determined with a microscope employing a micrometer scale as 4×10^{-2}–8×10^{-2} cm^2 (the values were given for the bilayer area with subtracted margin).

Impedance analysis. The capacity of a black bilayer membrane is well defined when it is in the black state. The resistance may vary by at least one order of magnitude, possibly because of impurities of the bilayer, border leakage at the membrane support, the appearance of lipid "crystals" at the periphery of the bilayer, way of introducing the lipid solution (if the forming solution is introduced with a micro-syringe, instead of with a brush, the irreproducibility of bilayer can be minimized). The resistance of a single membrane, however, is usually constant until a short time before the membrane ruptures. Therefore, any changes in resistance due to addition of ions, proteins, drugs, etc., can be determined with a relatively high degree of accuracy (Tien, 1974).

The impedance technique was used in our study to characterize the membrane features as this method has been shown to be able to measure the membrane capacitance and resistance on bilayer lipid membranes accurately. Electrochemical impedance spectroscopy was performed with an AC impedance system (Model 388, EG&G, Princeton Applied Research) that included a personal computer, a two-phase lock-in amplifier (Model 5208) and a potentiostat/galvanostat (Model 273), in which a four-electrode input was applied within the pre-amplifier. The electrochemical cell contained two identical reversible silver-silver chloride electrodes and two identical current platinum electrodes (described exactly in (Naumowicz & Figaszewski, 2003; Naumowicz *et al*., 2003; 2005). The use of the four-electrode system in the studies of electric phenomena occurring in membranes makes it possible to reduce, considerably, the errors caused by electrode and electrolyte impedance (Kalinowski & Figaszewski, 1995). A 4-mV amplitude sine-wave signal perturbation was

applied in the 0.1–10000 Hz frequency range. The PowerSuite 2.4 software package was used for acquisition of impedance data. These data were analyzed using complex nonlinear least squares (CNLS) fit to a model represented by an equivalent electrical circuit. The CNLS program used in this work was ZSimpWin 3.21. All experiments were carried out at room temperature 20 ± 1°C.

RESULTS AND DISCUSSION

The effect of ceramide on capacitance and resistance (reciprocal of conductance) of the phosphatidylcholine bilayer was examined in all the concentration range using electrochemical impedance spectroscopy. The mean values of the determined parameters were obtained from six independent measurements of the lipid bilayer. Based on numerous results given in the literature and our own experimental results, we assume that the membranes created by us do not contain solvent. If some of these quantities, which are not large in number, are contained in the membranes, then one should treat them as traces of impurities. As it is impossible to determine the quantity of these impurities, it is impossible to make a thorough qualitative determination of their nature and so one cannot take them into account in quantitative considerations (except as a possible qualitative indication). If quantitative analysis were possible, we would take into account the possibility of solvent's presence in the derived equations.

Figure 1 presents the results of impedance measurements conducted with the phosphatidylcholine, phosphatidylcholine-ceramide (1:1 molar ratio) and ceramide membranes. Very simple impedance diagrams were obtained for all examined membranes. They had the form of semicircles in the entire analyzed frequency range. Their centers lie on the real axis, provided that the lipid bilayers are considered as dielectric layers with leakage. The pure phosphatidylcholine bilayers have higher impedance than the phosphatidylcholine-ceramide membranes, confirming that ceramide has been successfully incorporated into the phosphatidylcholine bilayers and has an effect on the electrical properties of the membranes. It caused both C_m and R_m to decrease. The equivalent circuit used for data analysis (inset in Fig. 1) consists of a parallel arrangement of capacitance C_m and resistance R_m, attributed to the electrical properties of the bilayer, completed by a serial resistance R_0 for the conductivity of the bulk. The possibility of misinterpretation of the recorded data is reduced by simplicity of the circuit. This electric circuit is characteristic for an artificial lipid membrane only when ionophore systems, specific channels — pores and adsorption are absent (Krysiński, 1982). Based on this equivalent circuit, the complex nonlinear least squares analysis was used to simulate the impedance plots. Then the values of C_m and R_m were extracted from the fit. The CNLS fits are represented by the solid lines in Fig. 1. They are in good agreement with the data obtained.

The dependencies of the capacitance and the conductance of phosphatidylcholine (component 1)

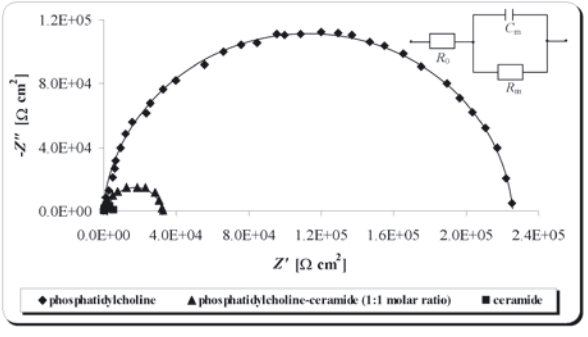
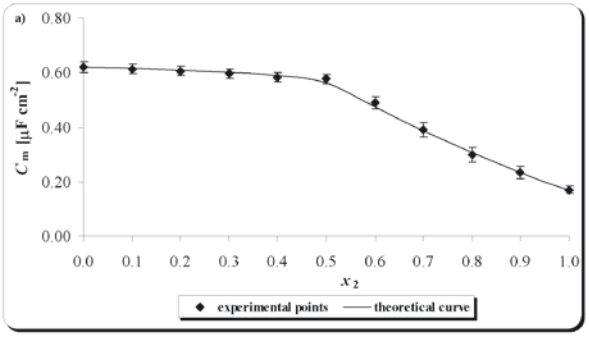


Figure 1. Complex plane impedance diagrams of phosphatidylcholine, phosphatidylcholine-ceramide and ceramide membranes and the applied equivalent circuit: R_0 – the resistance of the electrolyte, R_m – the resistance of the membrane, and C_m – the capacitance of the membrane. The solid lines represent the results of the fitting procedure.



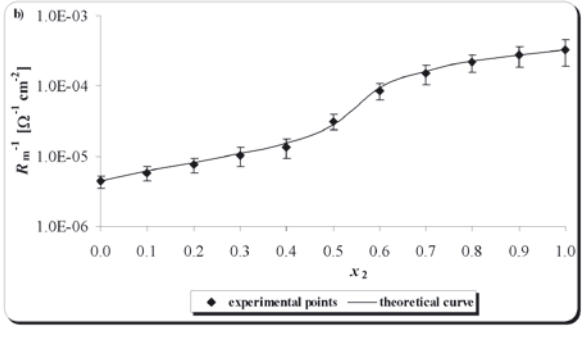


Figure 2. Dependencies of the capacitance C_m (a) and the conductance R_m^{-1} (b) of the phosphatidylcholine-ceramide membrane on the molar fraction of ceramide x_2. Error bars indicate the experimental scatter. The experimental values are denoted by points and the theoretical ones, calculated according to Eqns. (15), by curves.

– ceramide (component 2) membranes are illustrated in Fig. 2 as functions of a molar fraction of ceramide. The experimental values are marked by points and the theoretical ones obtained from Eqns. (15) by solid lines – the values of C_3, R_3^{-1}, S_3 and K_R whose determination will be described in further parts of this article were required for the calculation of these theoretical values. The resulting dependencies deviate from linearity, indicating that some bonds are formed in the membrane. The capacitance and the conductance values of a pure phosphatidylcholine bilayer are equal to 0.620 ± 0.019 μF cm^{-2} and (4.35 ± 0.913)×10^{-6} Ω^{-1} cm^{-2}, respectively, while for pure ceramide bilayer they are equal to 0.170 ± 0.014 μF cm^{-2} and (3.29 ± 1.35)×10^{-4} Ω^{-1} cm^{-2}, respectively.

Figure 3 presents the dependencies $(C_m - C_1)x_1$ *versus* $-(C_m - C_2)x_2$ and $(R_m^{-1} - R_1^{-1})x_1$ *versus* $-(R_m^{-1} - R_2^{-1})x_2$ described by Eqns. (4). According to Eqns. (4), as in the case when the membrane components do not form chemical compounds, the values of these functions should form straight lines. As one can see, this is not the case, which suggests that there is a complex or other chemical compound formation in the phosphatidylcholine-ceramide bilayer. Since Eqns. (4) do not describe the system under study sufficiently, the creation of a complex in such a system was assumed. Because the existence of a 1:1 complex is essential for others, as was written earlier, formation of a 1:1 phosphatidylcholine-ceramide complex was assumed.

Consequently, Eqns. (5) and the stability constant K_R, describing a third compound formed in this system, broaden the theoretical description. After simple modifications of Eqns. (5), one can obtain information of great interest from our point of view, presented by Eqns. (11). The capacitance and the conductance values of membranes formed from pure components were measured directly and their values have been given above. The other constants B_1, B_2, C_3, R_3^{-1}, were determined assuming that the value of the stability constant of the phosphatidylcholine-ceramide complex was sufficient with respect to the simplified Eqns. (11) to Eqns. (12) and (13).

The plots of functions (12a), (13a), (12b) and (13b) are presented in Figs. 4a, 4b, 5a and 5b, respectively. The presented dependencies are transformed into straight lines when the stability constant of the complex is high and the values x_2 are low (Figs. 4a and 5a) and when the stability constant of the complex is high and the values x_2 are high (Figs. 4b and 5b). If at least three following points are found on a straight line, one can accept, that the circumstances of the simplification of Eqns. (11) become realized and straight lines passing through these three points are described by Eqns. (12) and (13).

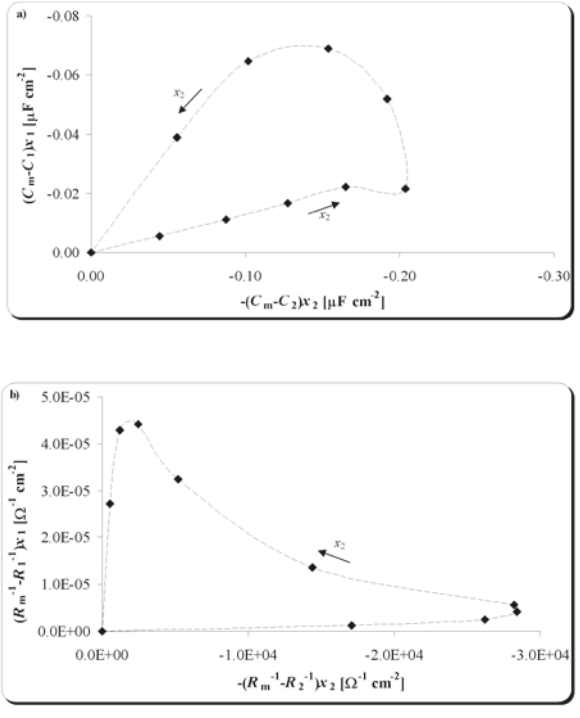


Figure 3. Dependencies of $(C_m - C_1)x_1$ *versus* $-(C_m - C_2)x_2$ (a) and $(R_m^{-1} - R_1^{-1})x_1$ *versus* $-(R_m^{-1} - R_2^{-1})x_2$ (b) described by Eqns. (4).

The arrows denote the direction of the increasing x_2 values and the dashed lines indicate the order of points.

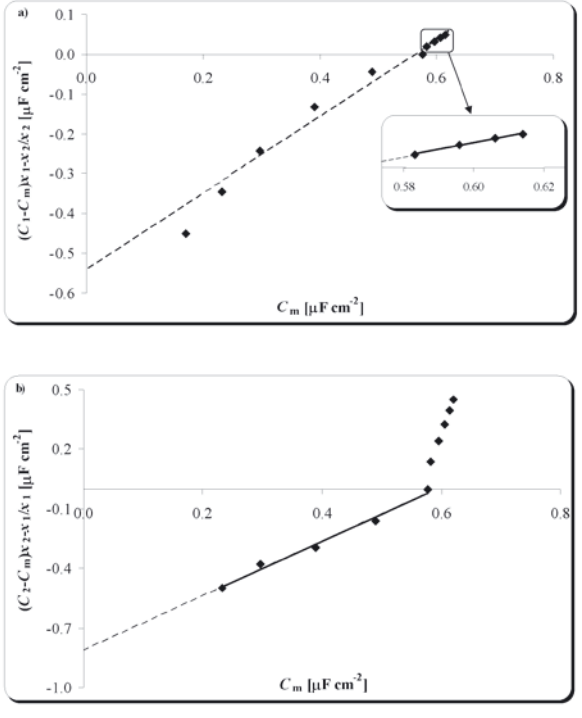


Figure 4. Plots illustrating the dependencies for phosphatidylcholine-ceramide complex described with Eqns. (12a) (a) and (13a) (b).

Straight lines join the points, on this basis B_1, B_2, S_3 and capacitance C_3 can be determined.

These points, which fulfill both the aforementioned limitations of x_2 values and form straight lines, are joined together in Figs. 4 and 5. The B_1 values, determined from the line slopes, are equal to 0.96 ± 0.02 (Fig. 4a) and 0.80 ± 0.03 (Fig. 5a). The B_2 values, obtained in the same way, are equal to 1.37 ± 0.02 (Fig. 4b) and 1.58 ± 0.03 (Fig. 5b). The intersections of the straight lines with an ordinate yield the $-B_1C_3$ and $-B_2C_3$ values as well as $-B_1R_3^{-1}$ and $-B_2R_3^{-1}$, respectively. The mean C_3 and R_3^{-1} values, obtained from these points, are equal to 0.58 ± 0.01 μF cm^{-2} and (1.30 ± 0.13)×10^{-5} Ω^{-1} cm^{-2}.

Knowing that the surface area of the phosphatidylcholine molecule amounts to 85 Å^2 per molecule (Petelska & Figaszewski, 2000) and the surface area occupied by the ceramide molecule equals to 50 Å^2 per molecule (Imura *et al*., 2000) Eqns. (12) and (13) could also be applied to calculate the surface area per a single phosphatidylcholine-ceramide molecule. The resulting S_3 value amounts to 75 Å^2 per molecule and is lower than the sum of areas occupied by the phosphatidylcholine and ceramide (135 Å^2) molecules.

The deviations of the additive behavior of mean molecular area indicate that the analyzed membranes are non-ideal, exhibiting strong condensation that reveals molecular interactions and "miscibility". The variation of mean molecular area and average surface potential/molecule, with respect to the ideal behavior, in a binary system can be due to changes of the molecular parameters of one, the other, or both lipid components (Maggio *et al*., 1997; Carrer & Maggio, 2001). Which of the components contributes more predominantly to the deviations from the ideal behavior can be inspected by analysis of the variation with composition of the partial mean molecular area and surface potential/molecule (Maggio *et al*., 1997; Carrer & Maggio, 2001). Mixed films of ceramide with ganglioside GM3 (the simplest ganglioside, and a key point for divertion of enzymatic routes for ganglioside biosynthesis (Maccioni *et al*., 2002; Yu *et al*., 2004) show molecular area condensation with ideal behavior of the surface potential/molecule (Maggio, 2004). This suggests a "molecular cavity" effect (Maggio *et al*., 1997; Carrer & Maggio, 2001; Diociaiuti *et al*., 2004), consistent with reduction of the mean molecular area and essentially unchanged dipolar properties of the lipids and of the film elasticity compared to an ideally mixed film. The interactions of ceramide with ganglioside GD3, located at a further divertion point for the biosynthesis of complex gangliosides (Maccioni *et al*., 2002; Yu *et al*., 2004), are of a similar type than those found for the mixtures with GM3. With more complex gangliosides, the mixed films with ceramide show condensation of the mean molecular area accompanied by interfacial depolarization, as indicated by the negative deviations from ideality of the average surface potential/molecule. The increase in polar head group complexity of the ganglioside (in the series GM2, GM1, GD1a and GT1b) brings about an increase of the magnitude of molecular condensation, depolarization and an increasingly reduced in-plane elasticity at similar surface pressures, compared to the ideally mixed films (Maggio, 2004).

Relatively recent data (Massey, 2001) indicate that the addition of ceramide to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) bilayers that were in the liquid-crystalline phase, resulted in a linear increase in acyl chain order and decrease in membrane polarity. The addition of ceramide to DPPC and sphingomyelin bilayers also resulted in a linear increase in the gel to liquid-crystalline phase transition temperature. The magnitude of the change was dependent upon ceramide lipid composition and was much higher in sphingomyelin bilayers than DPPC bilayers. The results are interpreted as the formation of DPPC/ceramide and sphingomyelin/ceramide lipid complexes. Goni and Alonso (2006) demonstrate that ceramides have two main effects, when mixed with phospholipid monolayers or bilayers: they increase the molecular order of phospholipids, and they give rise to lateral phase separation and domain formation. Moreover,

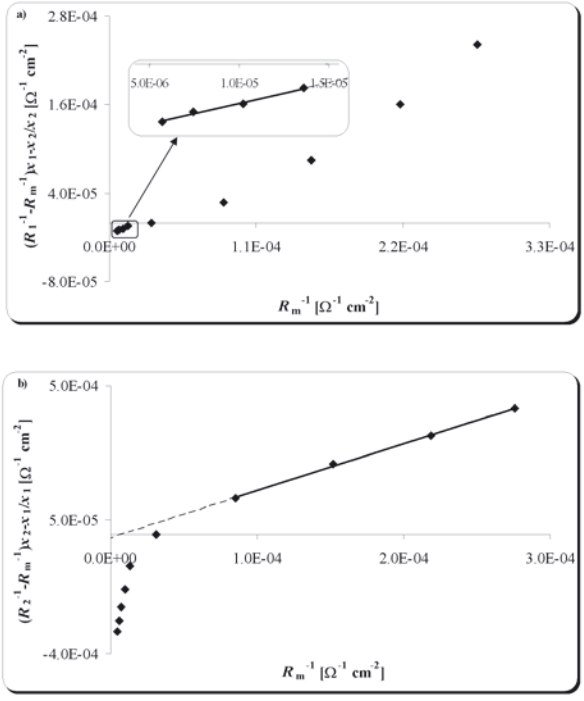


Figure 5. Plots illustrating the dependencies for phosphatidylcholine-ceramide complex described with Eqns. (12b) (a) and (13b) (b).
Straight lines join the points, on this basis B_1, B_2, S_3 and conductance R_3^{-1} can be determined.

ceramides in bilayers have the additional effects of inducing membrane permeabilization, transbilayer (flip-flop) lipid movements, and transition to non-lamellar phases. ^{2}H-NMR studies by Hsueh *et al*. (2002), of mixtures of C16:0 ceramide and d_{31}-POPC found that gel and liquid-crystalline (fluid) phases coexisted over a wide range of temperatures and compositions, with domains of different composition and physical state being present at physiological temperatures. However, no evidence of liquid-liquid phase separation in the fluid phase was found.

The presence of ceramide provokes changes in the phosphatidylcholine bilayer, for example the mobility of the alkyl chains is reduced, the membrane is more ordered and more stiff and its permeability is lower. Many other compounds impose such properties to the membrane, among them cholesteryl esters e.g. cholesteryl linoleate, cholesteryl oleate (Malcolmson *et al*., 1997), polar carotenoids e.g. zeaxanthin, violaxanthin, lutein (Wisniewska *et al*., 2006), and some phosphate derivatives of polyisoprenols (e.g. hexadecaprenyl diphosphate, dolichyl phosphate (Janas *et al*., 2000). These compounds are either membrane-spanning molecules able to reinforce the lipid bilayer by bracing together the two leaflets of the bilayers as rivets, or they make just one leaflet stiffer. Some of them are lipid bilayer membrane stabilizers in eukarya, bacteria and archaea (Ourisson & Nakatani, 1994). The above-cited compounds can be useful as natural, non-toxic stabilizers in many applications, for example in drug delivery systems.

Only one parameter remained to be determined – the stability constant of the phosphatidylcholine-ceramide complex. It could be determined by substituting C_m or R_m^{-1} equal to the arithmetic mean values of capacitance or conductance of the pure component into Eqns. (14). The resulting mean value of the stability constant is $(8.76 \pm 0.42)\times10^7$ m^2 mol^{-1}. This value is relatively high, giving additional evidence for the prevailing of the 1:1 complex in mixed phosphatidylcholine-ceramide bilayers. This value also confirmed that the assumptions used to simplify Eqns. (11) were correct.

The parameters describing the complex determined from Eqns. (11) and (14) were applied to present the agreement of the Eqns. (11) evaluated data (solid lines) with the experimental data (points) in Fig. 2a and 2b using Eqns. (15). Both of Eqns. (15) can yield two solutions, as they are quadratic equations. The values, ensuring better agreement of the experimental points with the predicted ones by the equations describing the complex formation between membrane lipid components, were chosen. It can be seen from this figure that the agreement between experimental and theoretical points is good, which verifies the assumption about the formation of a 1:1 phosphatidylcholine-ceramide complex in the lipid membrane.

CONCLUSIONS

The following conclusions can be drawn on the ground of the obtained parameter values describing the phosphatidylcholine-ceramide complex:

1. The stability constant of the complex amounts $(8.76 \pm 0.42)\times10^7$ m^2 mol^{-1}. The high value confirms the legitimacy of the basis to simplify Eqns. (11).

2. The experimental area occupied by one studied complex amounts to 75 Å^2 per molecule and is lower than the sum of areas occupied by the phosphatidylcholine and the ceramide (135 Å^2) molecules (strong condensation effect).

3. Good agreement of the experimental and theoretical points verifies the assumption of formation of a 1:1 complex in the lipid membrane. The lack of variances between points indicates that complexes at different stoichiometries or associates are possible in the phosphatidylcholine-ceramide membranes, but one can neglect their influence on the studied system.

In conclusion, we would like to emphasize that, to our knowledge, the value of the stability constant of the 1:1 phosphatidylcholine-ceramide complex has not been obtained so far.

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