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Impedance Spectroscopic Investigation of the Bilayer Lipid Membranes Formed from the Phosphatidylserine–Ceramide Mixture

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Abstract Electrochemical impedance spectroscopy was used for the study of two-component lipid membranes. Phosphatidylserine and ceramide were to be investigated because they play an important biochemical role in cell membranes. The research on biolipid interaction was focused on a 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 a two-component system forming bilayers was assumed, which could explain the deviation from the additivity rule. The molecular area and the equilibrium constant of the complex were determined.

Keywords Electrochemical impedance spectroscopy · Bilayer lipid membrane · Phosphatidylserine · Ceramide · Complex formation · Molecular area · Equilibrium constant

Introduction

The inspiration for lipid bilayer research, without question, comes from the biological world. Although the first report on self-assembled bilayer lipid membranes in vitro was reported in 1961, experimental scientists including surface, colloid, and bioscientists have been dealing with these interfacial phenomena since Robert Hooke's time (1672). Bilayer lipid membranes have been used in a number of applications ranging from basic membrane biophysics studies to the conversion of solar energy via water photolysis, and to biosensor development that uses supported bilayer lipid membranes (Tien and Ottova 2001).

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 nonionic lipids are used as the basic lipids for the preparation of bilayers. These lipids can be categorized into three principal types: phospholipids, sphingolipids, and cholesterol. They each play different roles in the membranes. Sphingolipids differ from phospholipids in being based on a lipophilic amino alcohol (sphingosine) rather than glycerol. Ceramides, whose construction is presented in Fig. 1a, are N-acylsphingosines. Free ceramides are only found in large amounts in the skin stratum corneum (Bouwstra and Ponec 2006). They exist in much smaller proportions in cell membranes, in which they occur primarily as intermediates in the metabolism of the more complex sphingolipids, and where they play an important role in cell signaling (Kolesnick et al. 2000; Futerman and Hannun 2004). Although phosphatidylserine, depicted in Fig. 1b, is distributed widely among animals, plants, and microorganisms, it is usually less than 10% of the total phospholipids, with the greatest concentration being in myelin from brain tissue. However, it may comprise 10 to 20% of the total phospholipid in the cell membrane bilayer (Freysz et al. 1982), where it exerts important functions.

The complexity of biological membranes makes it virtually impossible to draw detailed physical conclusions

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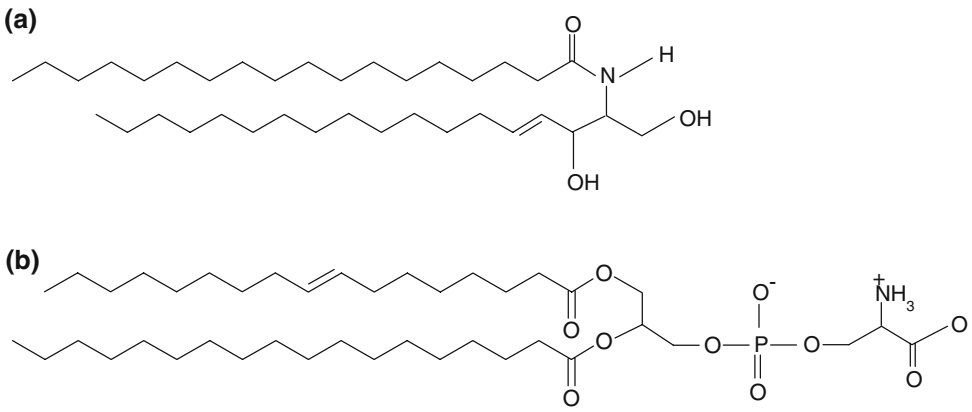

Fig. 1 Molecular structures of ceramide (**a**) and phosphatidylserine (**b**)

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.

Beginning in the 1970s, research electrochemists and materials scientists began to discover the power of electrochemical impedance spectroscopy as a tool for studying difficult and complicated systems. Even today, impedance often provides the only noninvasive method for detailed structural–functional studies of these systems. This is especially so of systems in which important processes occur at the molecular level, such as those processes associated with biological and synthetic membranes and interfaces that form between solutions and various solids, e.g., metals and colloid particles (Coster et al. 1996). In the present work, we used electrochemical impedance spectroscopy to investigate the effect of ceramide on the capacitance and resistance of the phosphatidylserine membranes. Our results show that the complex between membrane components is formed with a stoichiometry of 1:1. The determination of the area occupied by one phosphatidylserine–ceramide complex molecule and the stability constant of the complex is the final research result. The equations we present here can be successfully used for the quantitative determination of area and stability constant of 1:1 complexes formed in any two-component system.

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 (Naumowicz and Figaszewski 2005a, 2005b; Petelska et al. 2006), assumes that in cases where the membrane components do not form chemical compounds, any two-component system, regardless of whether it forms a monolayer or a bilayer, can be described by the equations expressing additivity of electric capacity and electric conductance:

$$C_m = C_1 c_1^s S_1 + C_2 c_2^s S_2 \tag{1a}$$

and

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

here:

$$x_1 = \frac{c_1^s}{c_1^s + c_2^s} \tag{2}$$

$$x_1 + x_2 = 1 \tag{3}$$

where

C_m [μF cm^{-2}] = the measured capacitance of the membrane
C_1, C_2 [μF cm^{-2}] = the capacitance of the membrane built by components 1 and 2, respectively
R_m^{-1} [Ω^{-1} cm^{-2}] = the measured conductance 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
c_1^s, c_2^s [mol m^{-2}] = the surface concentrations 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

After solution of the equations system (1)–(3), the following linear dependences are derived:

$$(C_m - C_1)x_1 = -\frac{S_2}{S_1}(C_m - C_2)x_2 \tag{4a}$$

$$(R_m^{-1} - R_1^{-1})x_1 = -\frac{S_2}{S_1}(R_m^{-1} - R_2^{-1})x_2 \tag{4b}$$

Because the first stability constant in complexes, as the most essential one, is usually the biggest and should be

taken into consideration (Inczedy 1976), the existence of 1:1 complex (compound 3) in phosphatidylserine–ceramide system was assumed. Then, the set of equations (1)–(3) is modified because the impedance parameters (electric capacity and electric conductance) are the sum of the contributions of all the compounds (Naumowicz et al. 2006; Naumowicz and Figaszewski 2006):

$$C_m = C_1 c_1^s S_1 + C_2 c_2^s S_2 + C_3 c_3^s S_3 \tag{5a}$$

$$R_m^{-1} = R_1^{-1} c_1^s S_1 + R_2^{-1} c_2^s S_2 + R_3^{-1} c_3^s S_3 \tag{5b}$$

here:

$$K_R = \frac{c_3^s}{c_1^s \cdot c_2^s} \tag{6}$$

$$x_1 = \frac{c_1^s + c_3^s}{c_1^s + c_2^s + 2c_3^s} \tag{7}$$

$$c_{t1}^s = c_1^s + c_3^s \tag{8}$$

$$c_{t2}^s = c_2^s + c_3^s \tag{9}$$

$$x_1 + x_2 = 1 \tag{10}$$

where

C_3 [μF cm^{-2}] = the capacitance of the membrane built by compound 3;
R_3^{-1} [Ω^{-1} cm^{-2}] = the conductance of the membrane built by compound 3;
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 concentrations 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.

After solving equations system (5)–(10), the following basic equations are derived:

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

and

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

in which

$$B_1 = \frac{S_3}{S_1} \quad \text{and} \quad B_2 = \frac{S_3}{S_2}.$$

Equation 11 are the second-degree equations with respect to C_m, to the complex composition as well as with respect to the constants: $C_1, C_2, C_3, R_1^{-1}, R_2^{-1}, R_3^{-1}, B_1, B_2$. The 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 by means of simplified forms of these equations.

Equation 11 may be simplified taking into account the sufficiently high value of the stability constant of the complex (in limit $\to \infty$). The criterion of rightness of the accepted assumption is the agreement between theoretical and experimental values.

With the above assumption, the dependences of linear type are derived for small x_2 values ($x_2 \ll x_1$):

$$(C_1 - C_m)\frac{x_1 - x_2}{x_2} = -B_1C_3 + B_1C_m \tag{12a}$$

$$\left(R_1^{-1} - R_m^{-1}\right)\frac{x_1 - x_2}{x_2} = -B_1R_3^{-1} + B_1R_m^{-1} \tag{12b}$$

while for the case of high x_2 values ($x_2 \gg x_1$) Eq. 11 can be described as other linear expressions:

$$(C_2 - C_m)\frac{x_2 - x_1}{x_1} = -B_2C_3 + B_2C_m \tag{13a}$$

$$\left(R_2^{-1} - R_m^{-1}\right)\frac{x_2 - x_1}{x_1} = -B_2R_3^{-1} + B_2R_m^{-1} \tag{13b}$$

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

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

and

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

The next Eq. 15 can be used for verification of the calculated values against experimental ones obtained on the basis of Eqs. 12–14. Good agreement between them will mean that the system is well described by the above equations. To verify this agreement, Eq. 11 should be presented in the following forms:

$$\begin{aligned}&K_RS_1^{-1}S_2^{-1}\left(a_1+a_2\right)\left(a_3-a_1\right)C_m^2\\&+\left[K_RS_1^{-1}S_2^{-1}\left(C_1a_1-C_3a_3\right)\left(a_1+a_2\right)\right.\\&-K_RS_1^{-1}S_2^{-1}\left(C_2a_1+C_3a_2\right)\left(a_3-a_1\right)+a_4S_3^{-1}\left(a_3+a_2\right)\left.\right]C_m\\&+K_RS_1^{-1}S_2^{-1}a_3C_3\left(C_3a_2+C_1a_2\right)-K_RS_1^{-1}S_2^{-1}a_1C_1\\&\times\left(a_1C_2+a_2C_3\right)-a_4S_3^{-1}\left(C_2a_3+C_1a_2\right)=0\end{aligned} \tag{15a}$$

where

$$a_1=S_3^{-1}(x_2-x_1)$$

$$a_2=S_2^{-1}x_1$$

$$a_3=S_1^{-1}x_2$$

$$a_4=\left[S_3^{-1}(C_1-C_2)(x_2-x_1)+(C_1-C_3)x_1S_2^{-1}+(C_2-C_3)x_2S_1^{-1}\right]$$

and

$$\begin{aligned}&K_RS_1^{-1}S_2^{-1}\left(a_1+a_2\right)\left(a_3-a_1\right)(R_m^{-1})^2\\&+\left[K_RS_1^{-1}S_2^{-1}\left(R_1^{-1}a_1-R_3^{-1}a_3\right)\left(a_1+a_2\right)\right.\\&-K_RS_1^{-1}S_2^{-1}\left(R_2^{-1}a_1+R_3^{-1}a_2\right)\left(a_3-a_1\right)\\&+a_4S_3^{-1}\left(a_3+a_2\right)\left.\right]R_m^{-1}+K_RS_1^{-1}S_2^{-1}a_3R_3^{-1}\\&\times\left(R_3^{-1}a_2+R_1^{-1}a_2\right)-K_RS_1^{-1}S_2^{-1}a_1R_1^{-1}\left(a_1R_2^{-1}+a_2R_3^{-1}\right)\\&-a_4S_3^{-1}\left(R_2^{-1}a_3+R_1^{-1}a_2\right)=0\end{aligned} \tag{15b}$$

in which

$$a_4=\left[S_3^{-1}\left(R_1^{-1}-R_2^{-1}\right)\left(x_2-x_1\right)+\left(R_1^{-1}-R_3^{-1}\right)x_1S_2^{-1}+\left(R_2^{-1}-R_3^{-1}\right)x_2S_1^{-1}\right]$$

Materials and Experimental Details

Reagents and Preparation of the Forming Solutions

The lipid bilayer was formed from the Fluka product of 98% sheep brain phosphatidylserine and from 98% sheep brain ceramide, also produced by Fluka. Both substances 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. The dried residues were dissolved in a hexadecane–butanol mixture (10:1 by volume). The resultant solution used to form the model membrane contained 20 mg ml^{-1} of substances in solution. This solution containing the membrane components was unsaturated; therefore, it contained any proportion of the components. During membrane formation, the solvent mixture was removed, and the created membrane had the same proportion as that in the resultant solution. The samples were stored for at least 5 days at 4°C before examination.

The solvents were of chromatographic standard purity grade; chloroform and butanol were purchased from Aldrich, hexadecane from Fluka.

Potassium chloride solution of 0.1 mol dm^{-3} was used as the electrolyte for experiments. KCl was analytical purity grade and was heated before use at 400°C for 4 h to remove traces of organic material. Water purified by Milli-Qll (18.2 M, Millipore, USA) was used to make the electrolyte and in all cleaning procedures.

Preparation of the Bilayer Membranes

Bilayer membranes were formed as bubbles. They were obtained at the Teflon cap constituting a measuring vessel component. The use of hexadecane as the solvent allows

obtaining membranes with thickness and capacity values similar to those of membranes formed of monolayers (Benz et al. 1975; Karolins et al. 1998). A small quantity of butanol has a negligible effect on the impedance parameters of the bilayers created, whereas it considerably accelerates the formation of the membranes. The thinning of the membranes was monitored by visual observation in transmitted light and by recording the membrane capacitance. The capacity of the membranes increased with time after the bilayers' formation until a steady-state value was reached some 10–20 min later. The measurements were started 20–30 min after the membranes turned completely black. The bilayers area was determined with a microscope that used a micrometer-level scale as 4×10^{-2} to 8×10^{-2} cm^2 (the values were given for the bilayers area without margin).

Impedance Analysis

Electrochemical impedance spectroscopy was performed with an AC impedance system (EG&G, Princeton Applied Research, Model 388) that included a personal computer, a two-phase lock-in amplifier (Model 5208), and a potentiostat/galvanostat (Model 273). The electrochemical cell was connected with a potentiostat via a self-constructed four-electrode preamplifier with high-impedance inputs; the measuring cell has been previously described (Naumowicz and Figaszewski 2003; Naumowicz et al. 2003, 2005). The four-electrode potentiostat assured passage of current between the two identical current platinum electrodes (CE_1 and CE_2) in such a manner as to hold constant amplitude of voltage between the two identical reversible silver–silver chloride electrodes (RE_1 and RE_2) and measured intensity and phase of current in the circuit CE_1–CE_2. The four-electrode system cannot "see" the impedances of the current electrodes and the resistances of solutions between the current and the reference silver–silver electrodes (Figaszewski 1982; Figaszewski et al. 1982).

An electrochemical impedance software, Power Sine 2.4, was used to carry out impedance measurements between 10 mHz and 10 kHz. The AC amplitude voltage used for the experiments was 4 mV. The impedance spectra were further analyzed by ZSimpWin 3.21 (Princeton Applied Research). The modeling process was iterative, with the χ^2 value used for the entire model and the percentage error values for each circuit component to determine the fit of a given model to the experimental data. The circuit elements were chosen on the basis of theories from electrochemical cell studies and used the Boukamp suggestion that each component addition should reduce the χ^2 value by one order of magnitude. The χ^2 value was minimized when the experimental data points correlated with the theoretical data points. This was performed by first calculating the difference between the experimental and calculated data points. The difference was squared to give larger variances a greater significance. The differences for all data points were summed and then divided by a weighing factor. According to the literature (Cui and Martin 2003), a χ^2 value of on the order of 1×10^{-3} or below was acceptable for a given model.

All experiments were carried out at room temperature (20 ± 1°C).

Results and Discussion

To obtain information on the interaction between ceramide and phosphatidylserine, the effect of ceramide on capacitance and resistance (reciprocal of conductance) of the phosphatidylserine membranes was examined in all the concentration range. The impedance technique was used in our study to characterize the membrane features because this method has been shown to accurately measure the capacitance and resistance of bilayer lipid membranes. The mean values of the determined parameters were obtained on the basis of six independent measurements of the lipid bilayer. In view of 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 solvents are contained in the membranes, then one should treat them as trace impurities. Because it is impossible to determine their quantity and their nature, one cannot take them into account in quantitative considerations (except as a possible qualitative indication). In the opposite case, we would take into account the possible presence of any solvent in the derived equations.

Figure 2 shows the results of impedance measurements conducted with the phosphatidylserine, phosphatidylserine–ceramide (1:1 molar ratio) and ceramide membranes (inset of Fig. 2). Very simple impedance diagrams were obtained for all the examined membranes; they have the form of semicircles in the entire analyzed frequency range.

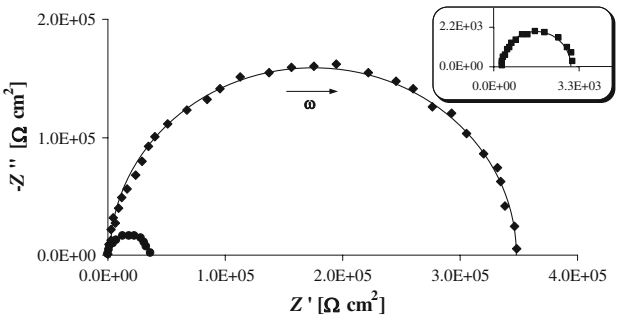

Fig. 2 Complex plane impedance diagrams of ceramide (*inset*) (■), phosphatidylserine–ceramide (1:1 molar ratio) (●), and phosphatidylserine (◆) membranes. *Solid lines* represent the results of the fitting procedure

The centers of the semicircles lie on the real axis, provided that the lipid bilayers are considered as dielectric layers with leakage. The pure phosphatidylserine bilayers have higher impedance than the phosphatidylserine–ceramide membranes, confirming that ceramide has been successfully incorporated into the lipid bilayers and has a contrast effect on the electrical properties of the membranes. It caused both capacitance of the membrane and resistance of the membrane to decrease. The validity of the results obtained by electrochemical impedance spectroscopy was verified by means of the electrical equivalent circuit model, which is presented in Fig. 3. This equivalent circuit 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 for misinterpretation of the recorded data is reduced by the simplicity of the circuit. This electric circuit is characteristic for an artificial lipid membrane only when ionophore systems, specific channel pores, and adsorption are absent (Krysiński 1982). The electrochemical parameters of the circuit were evaluated by ZsimpWin software. A very high correlation was observed between experimental results and the results calculated with the best-fitting electrical equivalent circuit model, where χ^2 was minimized below 10^{-3}. An examination of the data obtained for analyzed systems indicate that the proposed equivalent circuit can be used to describe the experimental results (Fig. 2).

Dependences of the capacitance and the conductance of phosphatidylserine (component 1)–ceramide (component 2) membranes are illustrated in Fig. 4a, b as functions of the molar fraction of ceramide. The resulting dependences deviate from linearity, indicating that specific interactions between membrane components are presented in the membrane. The capacitance and the conductance values obtained for a pure phosphatidylserine bilayer are equal to 0.624 ± 0.023 μF cm^{-2} and $(2.88 \pm 0.60) \times 10^{-6}$ Ω^{-1} cm^{-2}, respectively. The capacitance and the conductance values for a pure bilayer of ceramide are equal to 0.170 ± 0.014 μF cm^{-2} and $(3.29 \pm 1.35) \times 10^{-4}$

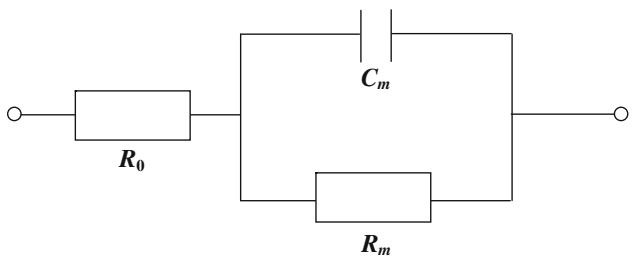

Fig. 3 An equivalent circuit representing electric properties of the phosphatidylserine membrane modified with ceramide. R_0, resistance of the electrolyte; C_m, capacitance of the membrane; R_m, resistance of the membrane

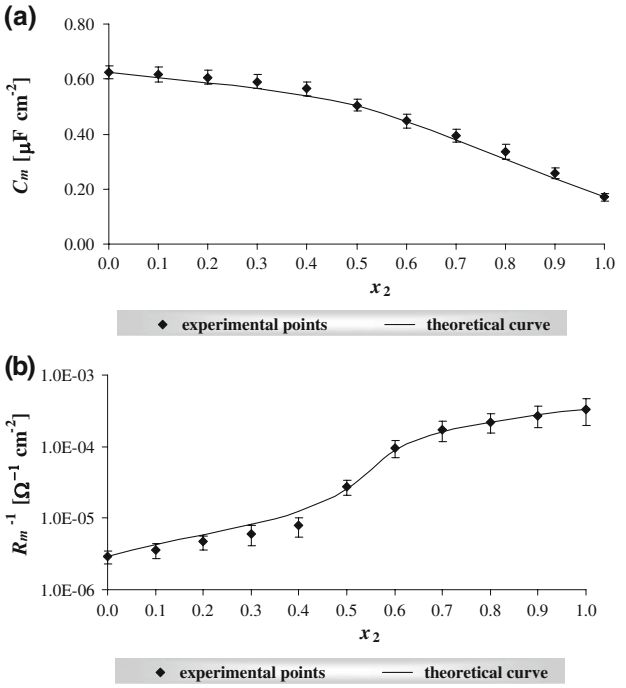

Fig. 4 Dependences of the capacitance C_m (**a**) and the conductance R_m^{-1} (**b**) of the phosphatidylserine–ceramide membrane on the molar fraction of ceramide x_2. Error bars indicate the experimental scatter. The experimental values are denoted by points and the theoretical ones, calculated according to Eq. 15, by curves

Ω^{-1} cm^{-2}, respectively. Values of resistance often definitely show greater scattering than values of capacity, and therefore effects of measurements of the conductance are most often treated as supplementary data. The conductance is burdened with random errors caused by the presence of the solvent and ions in a bilayer. The presence of the solvent and ions always carries a meaningful, easily noticeable error as well as scattering to results. This effect is not occurring in such a visually perceptible manner in the capacity. As can be seen in Fig. 4a, b, the inclusion of ceramide molecules into the membrane results in the increase of the membrane thickness. The increase in the membrane thickness results in decrease in its electrical capacity. Such an increase represents a main manifestation of the ceramide condensing effect on the membranes. Figure 4a, b also shows the theoretical values, presented with a curve, obtained from Eqs. 15a and 15b (describing the complex formation process). These theoretical values were calculated by values whose determination will be described below. That the theoretical values agree well with the experimental data in the whole analyzed concentration range suggests the existence of phosphatidylserine–ceramide complex in the examined membranes.

Figure 5a, b present the dependences $(C_m - C_1)x_1$ vs. $-(C_m - C_2)x_2$ and $(R_m^{-1} - R_1^{-1})x_1$ vs. $-(R_m^{-1} - R_2^{-1})x_2$ described by Eqs. 4a and 4b, respectively. The

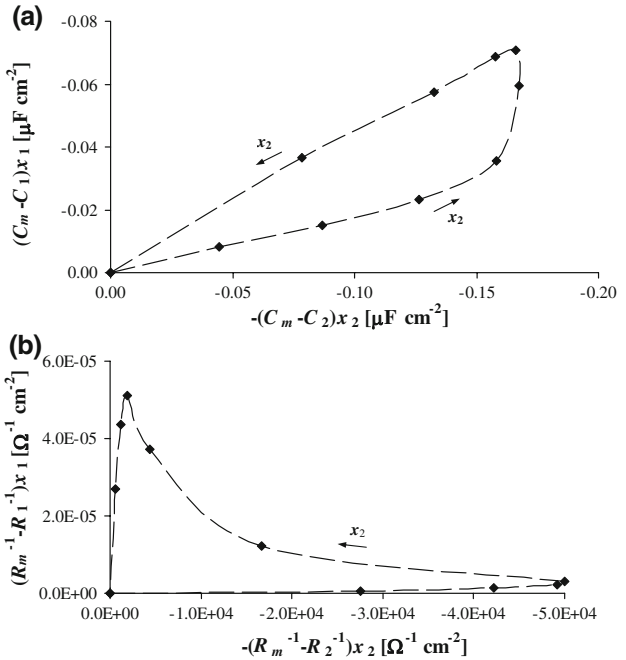

Fig. 5 Dependences of $(C_m - C_1)x_1$ vs. $-(C_m - C_2)x_2$ (**a**) and $(R_m^{-1} - R_1^{-1})x_1$ vs. $-(R_m^{-1} - R_2^{-1})x_2$ (**b**) described by Eq. 4. *Arrows* denote the direction of the increasing x_2 values; *dashed lines* indicate the order of points

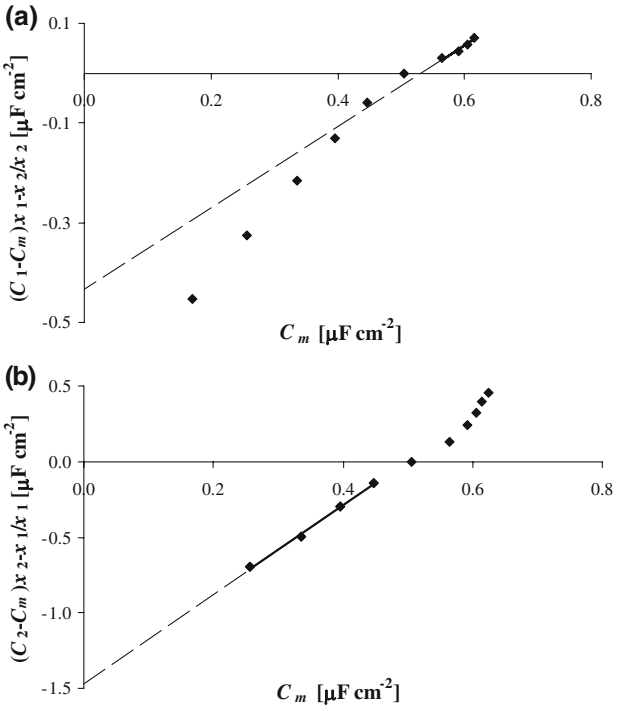

Fig. 6 Plots illustrating the dependences for phosphatidylserine–ceramide complex described with Eq. 12a (**a**) and Eq. 13a (**b**). Straight lines join the points, based on which B_1, B_2, S_3 and capacitance C_3 can be determined

dependences are expressed in the coordinate systems in which the plots should be straight lines in the case when they are lacking specific interactions between membrane components. Their actual shapes prove that they do not correspond to Eqs. 4a and 4b, suggesting that there are specific interactions in the phosphatidylserine–ceramide bilayer.

Therefore, the formation of a complex in this system was assumed. Because the existence of a 1:1 complex is a typical case (Inczedy 1976), the formation of a 1:1 phosphatidylserine–ceramide complex was accepted.

Consequently, Eqs. 5a and 5b, and the stability constant K_R, describing a complex formed in this system, complete the theoretical description. After simple modifications of Eqs. 5a and 5b, one can obtain information of great interest from our point of view, presented by Eqs. 11a and 11b. The capacitance and the conductance values of the 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 obtained assuming that the value of the stability constant of the phosphatidylserine–ceramide complex was sufficient with respect to the simplified Eq. 11 to Eqs. 12 and 13.

The plots of functions (12a), (13a), (12b), and (13b) are shown in Figs. 6a, b and 7a, b, respectively. The presented dependences are transformed into straight lines when K_R is high and the values x_2 are low (Figs. 6a and 7a) or the values x_2 are high (Figs. 6b and 7b). If at least three of the following points are found on a straight line, one can accept the circumstances of the simplification of Eq. 11 become realized and straight lines passing through these three points are described by Eqs. 12 and 13.

These points, which fulfill both the aforementioned limitations of x_2 values and form straight lines, are joined together in Figs. 6 and 7. From the B_1 and B_2 constants, which were determined on the basis of on these equations, it was possible to calculate the capacitance value of the complex C_3 and the conductance value of the complex R_3^{-1}. The mean values are equal to 0.515 μF cm^{-2} and 7.87 × 10^{-6} Ω^{-1} cm^{-2}, respectively.

Equations 12 and 13 could also be applied to calculate the surface area per a single phosphatidylserine–ceramide molecule S_3. The values of the surface area, occupied by one mole of components 1 and 2, are necessary for this calculation. In our case, we chose the S_1 value, determined in our laboratory as 68.5 Å^2 (Petelska and Figaszewski 2003). The surface area occupied by the ceramide molecule, reported in the literature, is equal to 50 Å^2 (Imura et al. 2000). The resulting S_3 value amounts to 88.5 Å^2 per molecule and is lower than the sum of areas occupied by each component of the complex (118.5 Å^2).

The deviations of the additive behavior of mean molecular area indicate that the analyzed membranes are

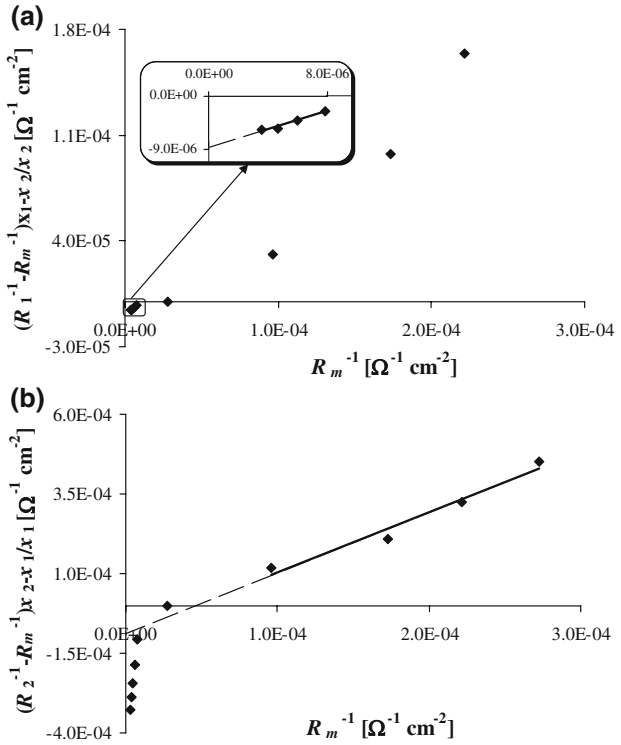

Fig. 7 Plots illustrating the dependences for phosphatidylserine–ceramide complex described with Eq. 12b (**a**) and Eq. 13b (**b**). Straight lines join the points, based on which B_1, B_2, S_3 and conductance R_3^{-1} can be determined

nonideal, 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 (Carrer and Maggio 2001; Maggio et al. 1997). Which of the components contributes more predominantly to the deviations from the ideal behavior can be inspected by the analysis of the variation with the composition of the partial mean molecular area and surface potential/molecule (Carrer and Maggio 2001; Maggio et al. 1997). Mixed films of ceramide with ganglioside GM3 (the simplest ganglioside, and a key point for changes of directions 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 (Carrer and Maggio 2001; Diociaiuti et al. 2004; Maggio et al. 1997), consistent with the reduction of the mean molecular area and essentially unchanged dipolar properties of the lipids and of the film elasticity compared with an ideally mixed film. The interactions of ceramide with ganglioside GD3, located at a further diversion 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 the ideal 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 increasingly reduced in-plane elasticity at similar surface pressures, compared with 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 on 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.

The only value to be determined was the stability constant of the phosphatidylserine–ceramide complex. It could be determined on the basis of Eqs. 14a and 14b, for $x_1 = x_2 = 0.5$, leading to $(4.18 \pm 1.35) \times 10^7$ m^2 mol^{-1}. This value is relatively high, giving additional evidence for the prevailing of the 1:1 complex in mixed phosphatidylserine–ceramide bilayers. This value also confirmed that the assumptions, used to simplify Eq. 11, were correct.

The parameters determined on the basis of Eqs. 11 and 14 were applied to represent the agreement of the data, evaluated from Eqs. 11 (solid lines) with the experimental data (points) in Fig. 4a, b by means of Eqs. 15. Both of Eqs. 15 can yield two solutions, as they are of a second order. The values that ensured 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 Fig. 3a, b that the agreement between experimental and theoretical points is good, which verifies the assumption about the formation of a 1:1 phosphatidylserine–ceramide complex in the lipid membrane.

We emphasize that to our knowledge, the value of the stability constant of the 1:1 phosphatidylserine–ceramide complex has not been obtained so far. Application of impedance spectroscopy to the study of the electrochemical behavior of lipid bilayers allows a quantitative description of equilibria in a two-component membrane. On the basis of derived mathematical equations, the existence of a 1:1

complex of high stability constant between phosphatidylserine and ceramide was proved. The complex formation is the main reason, for which deviation from rectilinearity of the parameters of the system described by the additivity rule is observed.

Adequate equations let us calculate and verify such parameters of the complex as capacitance, conductance, molecular area, and stability constant of the complex molecule. This data obtained from the mathematical derivation and confirmed experimentally are of primary interest for structural biology where the origins and functions of complexes at different stoichiometries are currently intensively discussed. In our opinion, this information can help us better understand the physicochemical properties of cell membranes and their associated physiology.

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