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Impedance Analysis of Complex Formation Equilibria in Phosphatidylcholine Bilayers Containing Decanoic Acid or Decylamine

Monika Naumowicz · Aneta Dorota Petelska · Zbigniew Artur Figaszewski

Abstract Bilayer lipid membranes composed of phosphatidylcholine and decanoic acid or phosphatidylcholine and decylamine were investigated using electrochemical impedance spectroscopy. Interaction between membrane components causes significant deviations from the additivity rule. Area, capacitance, and stability constant values for the complexes were calculated based on the model assuming 1:1 stoichiometry, and the model was validated by comparison of these values to experimental results. We established that phosphatidylcholine and decylamine form highly stable 1:1 complexes. In the case of decanoic acid-modified phosphatidylcholine membranes, complexes with stoichiometries other than 1:1 should be taken into consideration.

Keywords Electrochemical impedance spectroscopy · Complex formation · Phosphatidylcholine membrane · Decanoic acid · Decylamine

M. Naumowicz (✉) · A. D. Petelska · Z. A. Figaszewski
Institute of Chemistry, University of Bialystok, Al.
J. Pilsudskiego 11/4, 15-443 Bialystok, Poland
e-mail: monikan@uwb.edu.pl

A. D. Petelska
e-mail: aneta@uwb.edu.pl

Z. A. Figaszewski
e-mail: elchem@uwb.edu.pl

Z. A. Figaszewski
Laboratory of Electrochemical Power Sources, Faculty
of Chemistry, University of Warsaw, Pasteur Street 1,
02-093 Warsaw, Poland

Introduction

Models based on synthetic bilayer lipid membranes were introduced in the early 1960s and since then have contributed enormously to the current understanding of biological membranes. These systems have been used in applications ranging from basic membrane biophysics to solar energy conversion [1].

Phospholipids are the major fractions of lipids found in biological membranes. They have two fatty acids themselves, which are esterified to glycerol. In this context, fatty acids are significant components of membranes and affect properties such as flexibility, fluidity, and material transfer [2]. Although the level of free fatty acids is generally low in body tissue and fluids, they affect a variety of physiological functions of biomembranes. The effect of fatty acids on the physiological functions should more or less result from the modification of physical properties of biomembranes caused by the presence of fatty acids. Thus, a number of investigations have been reported for the effect of fatty acids on physical properties of model membranes, particularly on the phase behavior of hydrated phospholipid bilayers [3].

The phase behavior of the phospholipid-fatty acid mixtures most extensively studied so far is concerned with the mixture of diacylphosphatidylcholine and saturated fatty acids with C_{14}-C_{18} chain lengths in which the phase diagrams over the whole composition range have been reported for some mixture systems [4–6]. All the phase diagrams have exhibited the formation of a molecular compound in the gel phase with the stoichiometry of diacylphosphatidylcholine:fatty acid (1:2), which means that a strong attractive interaction acts between the two components in the gel phase bilayer.

Inoue et al. [3] used differential scanning calorimetry and Fourier transform infrared spectroscopy to investigate

the effect of fatty acids on the phase behavior of hydrated 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine bilayers determining that long-chain saturated and *trans*-monounsaturated fatty acids raise the main phase transition temperature of hydrated phosphatidylcholine bilayers and stiffen the lipid acyl chain in gel phase bilayers, whereas *cis*-monounsaturated fatty acids such as oleic acid exhibit minimal perturbing effects on the physical properties of phosphatidylcholine bilayers. Moreover, they demonstrated that in systems composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and *cis*- or *trans*-monounsaturated fatty acids, a 1:1 complex is formed instead of the 1:2 complex observed in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine/long-chain saturated fatty acid mixtures.

In this article, we describe model biomembranes prepared using two-component bilayer systems containing phosphatidylcholine (PC) from egg yolk and decanoic acid (DA) or decylamine (DE). PC was selected because it is the most abundant phospholipid in mammalian cell membranes, comprising 40–50% of the total phospholipid content. DA was chosen as a component to determine whether its effect on bilayer membranes was similar to long-chain saturated fatty acids or to monounsaturated fatty acids. Liposoluble compounds such as organic acids are commonly used as antimicrobial food additives, for example, DA is added as an adjuvant to sulfur dioxide for biological stabilization of sweet wines [7]. The mechanisms underlying DA toxicity and the development of tolerance in yeast have been the focus of various studies [8–10]. The undissociated toxic form of the weak acid enters the cell by passive diffusion across the plasma membrane and dissociates in the neutral cytoplasm causing the decrease of intracellular pH. The insertion of the liposoluble acid form inside the plasma membrane is thought to decrease the spatial organization of the membrane, affecting its function as a selective barrier [10] and as a matrix for enzymes [11]. DE was used in the experiment mainly because it has the same number of carbon atoms as DA. Amines and fatty acids are commonly used to study the mechanisms (i.e., modification of solubility or partition coefficient, barrier disruption, or increased solvent permeation) by which these compounds increase skin permeability [12]. Fatty acids and amines contain the same functional groups that are present in natural membranes and are suitable for introduction into model membranes. Their simple structures make them invaluable sources of information that may be extrapolated to more complex biological membranes.

This work continues our systematic study of the electrical properties of lipid bilayers using electrochemical impedance spectroscopy with the general goal of confirming the formation of complexes in lipid bilayers [e.g. 13–17]. Bilayer lipid membranes may be assembled

from two components capable of forming complexes of varying stoichiometry. In the initial stage of complexation, a 1:1 complex is formed, whereas in subsequent stages, complexes with other stoichiometries may appear. However, because the first-stability constant in these complexes is usually the largest [18], we have assumed that our model membranes would primarily contain 1:1 complexes.

In reference [16], the equations describing 1:1 PC–stearic acid complex equilibria were sufficient for the entire concentration range. To adequately characterize the molecular interactions between PC and DA in bilayers, complexes with stoichiometries other than 1:1 must be considered when formulating mathematical models. Next, suitable modifications should be applied in equations. We present evidence for the formation of a 1:1 complex in bilayers formed from PC and DE. Using equations from [16] and from earlier studies [14, 15], the stability constant and area occupied by a single complex were calculated. This is to our knowledge the first report of a stability constant value for any lipid–DE complex formed in bilayer lipid membranes.

Theory

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

The model, which has been presented in full detail previously [15, 17], assumes that in the cases where the membrane components do not form chemical compounds, their interaction in any two-component system, regardless if it is a monolayer or a bilayer, can be described by the equation expressing additivity of the capacitance:

$$C_{\mathrm{m}} = C_1 c_1^{\mathrm{s}} S_1 + C_2 c_2^{\mathrm{s}} S_2. \tag{1}$$

Here,

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

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

where C_{m} ($\mu\mathrm{F\ m}^{-2}$) is the measured capacitance of the membrane; C_1 and C_2 ($\mu\mathrm{F\ m}^{-2}$) are the capacitance of the membrane built by components 1 and 2, respectively; c_1^{s}, c_2^{s} ($\mathrm{mol\ m}^{-2}$) are the surface concentration of components 1 and 2, respectively, in the membrane; S_1 and S_2 ($\mathrm{m}^2\ \mathrm{mol}^{-1}$) are the surface area occupied by one mole of components 1 and 2, respectively; and x_1 and x_2 are the molar fraction of components 1 and 2 in the solution forming the membrane, respectively.

Elimination of c_1^{s} and c_2^{s} yields the linear equation:

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

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 [18], we assume that the complexes are primarily of 1:1 stoichiometry.

Each of the individual chemical compounds contained in the membrane (component 1, component 2, and the complex—compound 3) occupies a fraction of the surface. Thus, according to the additivity rule, the surface corresponding to each compound has a certain electric capacity. The capacities are electrically parallel, so the total capacity is the sum of the contributions from each compound:

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

and

$$K_R = \frac{c_3^{\mathbf{s}}}{c_1^{\mathbf{s}} \times c_2^{\mathbf{s}}}, \tag{6}$$

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

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

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

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

where C_3 ($\mu F\ cm^{-2}$) is the capacitance of the membrane built by compound 3; c_3^s ($mol\ m^{-2}$) is the surface concentration of compound 3 in the membrane; $c_{t1}^{\mathbf{s}}$ and $c_{t2}^{\mathbf{s}}$ ($mol\ m^{-2}$) are the total surface concentration of components 1 and 2, respectively, in the membrane; S_3 ($m^2\ mol^{-1}$) is the surface area occupied by one mole of compound 3; K_R [$m^2\ mol^{-1}$] is the stability constant of compound 3.

After solving equation systems 5–10, the following basic equation is obtained:

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

in which

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

The Eq. 11 is an equation of second-degree, with respect to C_m, to the complex composition as well as with respect to the constants: C_1, C_2, C_3, B_1, and B_2. Attempts to solve this equation result in too complicated expressions. The capacitance values of the membranes formed from pure components 1 and 2 can be measured directly (or C_2 can be

determined in such a way as presented in Results and Discussion). The constants C_3, B_1, and B_2 can be determined in individual cases using simplified forms of the Eq. 11. This equation was simplified by taking into account the sufficiently high stability constant of the complex and approximated x_2 to low or high values. The criterion of rightness of the accepted assumption is the agreement between theoretical and experimental values.

At $x_2 \to 0.0$ (a bilayer formed from pure component 1), the equation represents a straight line:

$$(C_1 - C_m)\frac{x_1 - x_2}{x_2} = -B_1 C_3 + B_1 C_m. \tag{12}$$

At $x_2 \to 1.0$ (a bilayer formed from pure component 2), the equation represents another straight line:

$$(C_2 - C_m)\frac{x_2 - x_1}{x_1} = -B_2 C_3 + B_2 C_m. \tag{13}$$

When calculating the stability constant for the complex, Eq. 11 can be simplified to $x_1 = x_2$ [15, 17]:

$$\left[C_2 S_1^{-1} + C_1 S_2^{-1} - C_m\left(S_1^{-1} + S_2^{-1}\right)\right]\left(C_2 S_1^{-1} + C_1 S_2^{-1}\right)$$
$$- \left[C_2 S_1^{-1} + C_1 S_2^{-1} - C_m\left(S_1^{-1} + S_2^{-1}\right)\right]\left(S_1^{-1} + S_2^{-1}\right)C_3$$
$$= K_R \left(S_1^{-1}\right)^2 \left(S_2^{-1}\right)^2 S_3 (C_m - C_3)^2. \tag{14}$$

The parameters describing the complex (K_R, C_3, and S_3), determined using Eqs. 12–14, 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.):

$$K_R S_1^{-1} S_2^{-1}(a_1 + a_2)(a_3 - a_1)C_m^2$$
$$+ \Big[K_R S_1^{-1} S_2^{-1}(C_1 a_1 - C_3 a_3)(a_1 + a_2)$$
$$- K_R S_1^{-1} S_2^{-1}(C_2 a_1 + C_3 a_2)(a_3 - a_1) + a_4 S_3^{-1}(a_3 + a_2)\Big]$$
$$\times C_m + K_R S_1^{-1} S_2^{-1} a_3 C_3 (C_3 a_2 + C_2 a_1)$$
$$- K_R S_1^{-1} S_2^{-1} a_1 C_1 (a_1 C_2 + a_2 C_3) - a_4 S_3^{-1}(C_2 a_3 + C_1 a_2)$$
$$= 0 , \tag{15}$$

where

$$a_1 = S_3^{-1}(x_2 - x_1); \quad a_2 = S_2^{-1} x_1; \quad a_3 = S_1^{-1} x_2;$$
$$a_4 = \Big[S_3^{-1}(C_1 - C_2)(x_2 - x_1) + (C_1 - C_3)x_1 S_2^{-1}$$
$$+ (C_2 - C_3)x_2 S_1^{-1}\Big].$$

For bilayer membranes assembled from two 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 electrical capacitance of molecules and complexes. The accuracy of

the models was verified by comparison to experimental results.

Materials and Experimental Details

Chemicals and Preparation of the Forming Solutions

Egg-yolk 3-sn-PC (99%), DA (≥98%), and DE (≥99.5%) were supplied by Fluka (Neu-Ulm, Germany). PC had the following fatty acids composition: 16:0~33%, 18:0~4%, 18:1~30%, 18:2~14%, and 20:4~4%. All substances were dissolved in chloroform 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 mixture of n-hexadecane and n-butanol (10:1 by volume) to produce a concentration of 20 mg ml^{-1}. This concentration was sufficiently low that it could contain any proportion of the components without saturation. During membrane formation, the solvent mixture was removed, resulting in a membrane with the same composition as the solution. Samples were stored at 4°C for less than a week. The preparation and storage methods provided reproducible electrochemical properties when samples prepared at different times were examined using impedance spectroscopy.

All solvents were chromatographic standard grade: chloroform and n-butanol were from Aldrich (Milwaukee, WI) and n-hexadecane was from Fluka (Neu-Ulm, Germany).

Potassium chloride solution of 0.1 mol dm^{-3} was used as the electrolyte for experiments. KCl produced by POCh (Gliwice, Poland) 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 Bilayer Membranes

Bilayer membranes were obtained as bubbles at the Teflon cap comprising a portion of the measuring vessel. The use of n-hexadecane as a solvent made it possible to obtain membranes with thickness and capacity values similar to those of monolayer membranes [19, 20]. The small quantity of n-butanol had a negligible effect on the impedance parameters of the bilayers, yet it considerably accelerated membrane formation.

Thinning of the membranes was monitored using reflected light microscopy with a high-brightness yellow LED source. The microscope and the LED were mounted on supports enabling placement of the illuminator,

measuring vessel, and microscope on the optical axis. The distance of the microscope from the measuring cell could also be adjusted to focus on the membrane located deep within the vessel. The bilayer areas were calculated taking into consideration the spherical nature of the surface and using the equations provided in [21]. The area of the bilayer membranes was between 4×10^{-2} and 8×10^{-2} cm^2 (the values were given for the bilayer area with subtracted margin).
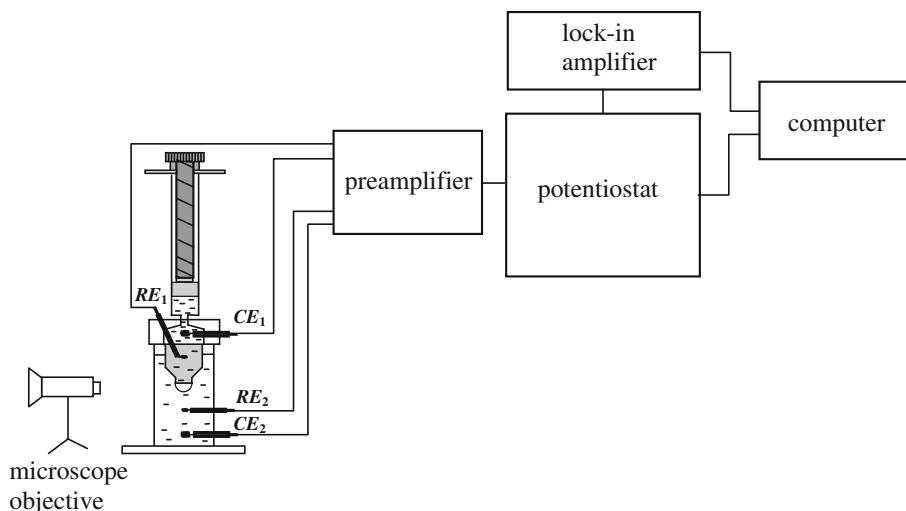
Bilayers formation was also monitored electrically by measuring the membrane capacitance at low frequency. The capacitance of the membranes increased with time after bilayer formation until a steady-state value was reached after approximately 10–20 min. Measurements were begun 20–30 min after the membranes turned completely black. All experiments were carried out at room temperature 293 ± 1 K.

Electrochemical Impedance Spectroscopy and Modeling

The general architecture of the system used for electrochemical impedance spectroscopy measurements is shown in Fig. 1. The setup included a personal computer, a two-phase lock-in amplifier (EG&G, Princeton Applied Research, model 5210), and a potentiostat/galvanostat (EG&G, Princeton Applied Research, model 273A). 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 [13, 22]. 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 the constant amplitude of voltage between the two identical reversible silver–silver chloride electrodes (RE_1 and RE_2) and measured the 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 [23]. A 4-mV amplitude sine-wave signal perturbation was applied in the 0.1–10,000 Hz frequency range.

ZSimpWin V 3.21 (Princeton Applied Research) was used to develop a circuit model from the spectroscopy impedance data. Data acquired in the PowerSuite 2.4 software package were inputted directly into ZSimpWin. The modeling process was iterative, using the chi-square (χ^2) value for the entire model and the percent error values for each circuit component to determine the fit of a given model to the experimental data. Components were chosen based on theories from electrochemical cell studies and using 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 correlate with the theoretical data points. This was done 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 [24], a χ^2 on the order of 1×10^{-3} or less was acceptable for a given model. The χ^2 value was calculated by means of the procedure described in [25].

Results and Discussion

The effects of DA and DE on the capacitance and resistance of the PC bilayers were examined using electrochemical impedance spectroscopy. The DA content was changed in the range from 0 to 0.52 molar fractions. Only in this range of concentration, the bilayer membrane formation was possible, because the forming solution with more than 0.52 molar fraction of fatty acid was granulated. The DE content was varied up to a 0.88 molar fraction; more than this limit, the amine induced disorder of the acyl chains of PC, and we were not able to form a bilayer stable enough to carry out measurements on it. The impedance technique was used in our study to characterize the membrane features as this method has been shown to measure the capacitance and resistance of bilayer lipid membranes accurately [26, 27]. The mean values of the determined parameters were obtained based on 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. As it is impossible to determine their quantity and nature, one cannot take them into account in quantitative considerations (except for a possible qualitative indication). In the opposite case, we would take into account the possible presence of any solvent in the derived equations.
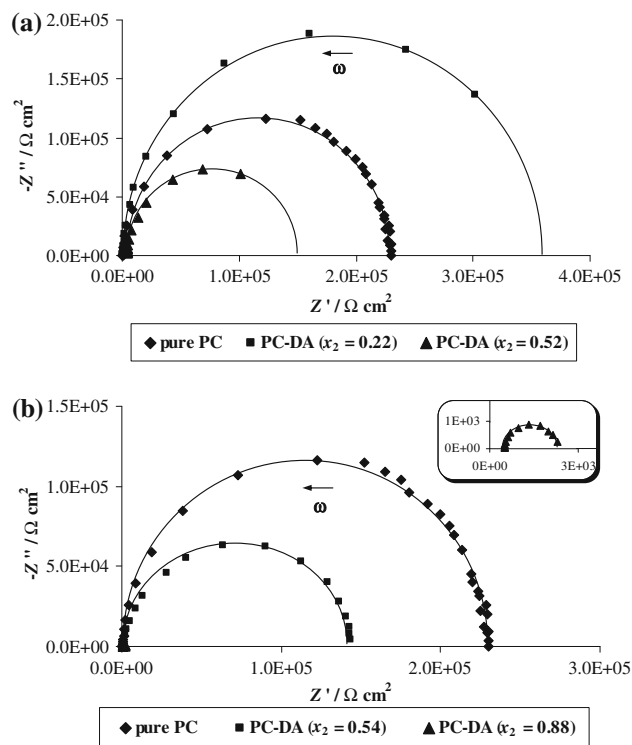

Fig. 2 Plots of the imaginary component of impedance-Z'' versus the real component of impedance Z' over a frequency range of 0.1 Hz–10 kHz for a phosphatidylcholine-decanoic acid (**a**) and phosphatidylcholine-decylamine (**b**) membranes. A different content of decanoic acid and decylamine (expressed as a molar fraction x_2) is illustrated by the different point's shapes of the impedance spectra. The *solid lines* represent the results of the fitting procedure
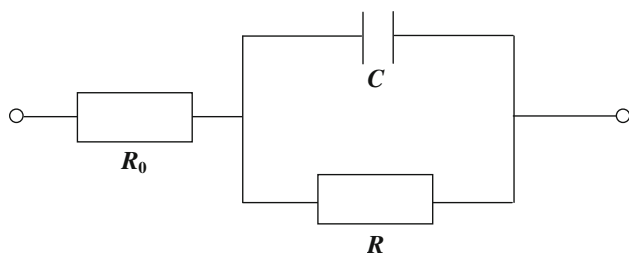
Fig. 3 An equivalent circuit used for impedance data analysis: R_0 resistance of the electrolyte, C capacitance of the membrane, and R resistance of the membrane

Figure 2 represents typical impedance spectra of the PC membranes, pure and containing different amounts of DA (Fig. 2a) or DE (Fig. 2b). Very simple impedance diagrams were obtained for all examined membranes; they had the form of semicircles in the entire analyzed frequency range (the point distribution is logarithmic). The centers of the semicircles lie on the real axis, provided that the lipid bilayers are considered as dielectric layers with leakage [22, 26]. These impedance spectra confirm that DA and DE have been successfully incorporated into the PC bilayers and have considerable effect on the electrical properties of the membrane. The validity of the results obtained by electrochemical impedance spectroscopy was verified using electrical equivalent circuit model that is presented in Fig. 3. This equivalent circuit consists of a parallel arrangement of capacitance C and resistance R, attributed to the electrical properties of the bilayer, completed by a serial resistance R_0 for the resistance of the electrolyte. This electric circuit is characteristic for an artificial lipid membrane only when ionophore systems, specific channels, pores, and adsorption are absent [28]. The values of membrane capacitance C_m and resistance R_m were calculated from the experimental complex impedance after subtraction of R_0 and were then recalculated as a set of parallel components and normalized for area. The electrochemical parameters of the circuit were evaluated by employing the 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 less than 10^{-3}. An examination of the data obtained for analyzed systems indicates that the proposed equivalent circuit can be used to describe the experimental results.

Figure 4 shows graphs of $(C_m - C_1)x_1$ versus $-(C_m - C_2)x_2$ for the two systems: PC–DA (Fig. 4a) and PC–DE (Fig. 4b). According to Eq. 4, these functions should yield straight lines when the membrane components do not interact. This is clearly not the case, which suggests that a complex or other structure exists in PC–DA and PC–DE bilayers. Because the use of Eq. 11 presupposes the existence of 1:1 complexes, our initial assumption was that the
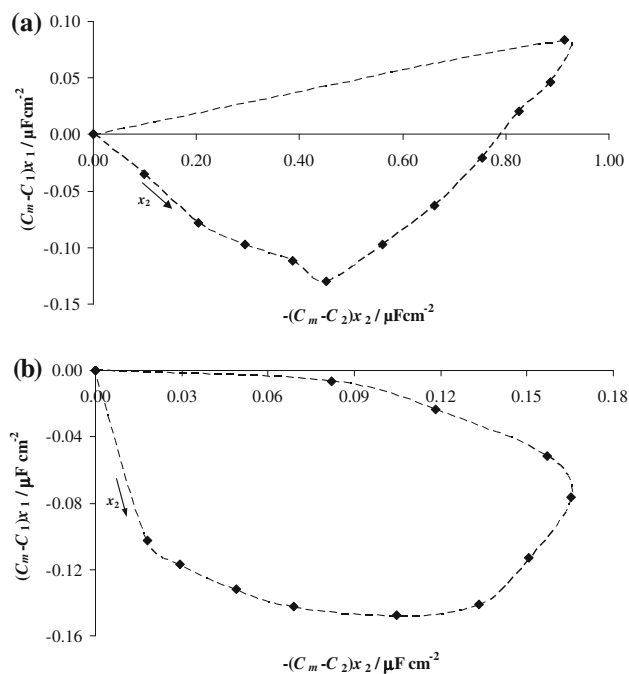

Fig. 4 Graph of Eq. 4 for phosphatidylcholine-decanoic acid (**a**) and phosphatidylcholine-decylamine (**b**), where x_2 is the molar fraction of component 2 (decanoic acid or decylamine)

complexes formed were 1:1. The capacitance of the lipid membrane was studied over a wide range of lipid compositions.

Figure 5 is a graph of the relationship between membrane capacitance and fatty acid or amine mole fraction. The capacitance value of pure PC bilayer (component 1) C_1 was measured directly and presented earlier [14], which is equal to 0.62 ± 0.02 μF cm^{-2}. There is no accurate literature data on capacitance values for the pure component 2 (DA or DE), because these components do not form the bilayer membrane. However, to characterize the course of the experimental curves, the C_2 value for the pure components is necessary, which will be used in the calculations. In this case, the capacitance hypothetical values for membranes built from DA and DE were determined adjusting the experimental curve with the polynomial of the other mark extrapolating the $x_2 = 1$ value, which is presented in the insets in Fig. 5. The capacitance values obtained in this way for pure DA and DE are equal to 2.552 ± 0.046 and 0.652 ± 0.047 μF cm^{-2}.

The resistance value of pure PC bilayer is equal to $2.30 \pm 0.25 \times 10^5$ Ω cm^2. The highest concentration of DA tested ($x_2 = 0.52$) modified this value by $1.43 \pm 0.50 \times 10^5$ Ω cm^2. However, it is well known [3] that PC bilayers became more rigid in the presence of long-chain saturated fatty acids (tetradecanoic acid, hexadecanoic acid, or octadecanoic acid) and unsaturated fatty acids containing *trans* double bonds (e.g. *trans*-9-decenoic acid).
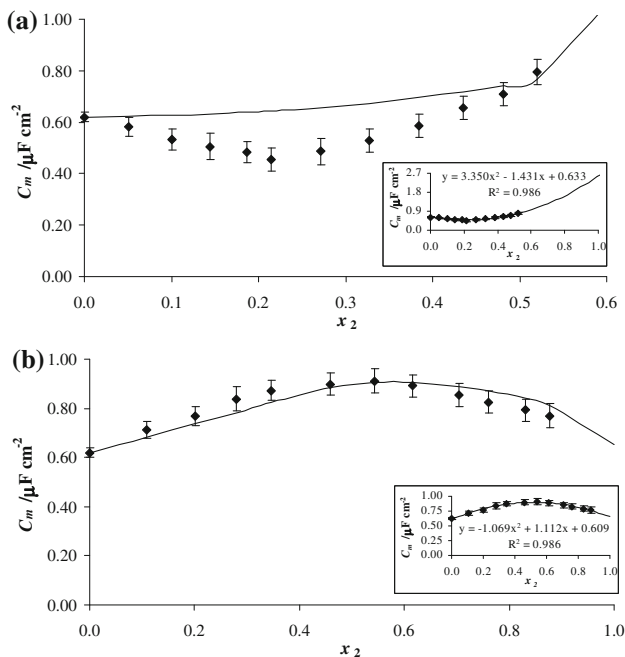
Fig. 5 The capacitance C_m of the phosphatidylcholine bilayers modified with decanoic acid (**a**) and decylamine (**b**) as a molar fraction of decanoic acid or decylamine x_2. *Error bars* indicate the experimental scatter. The *solid lines* represent the "theoretical" values calculated from Eq. 15. Plots illustrating a method for evaluating C_2 for analyzed systems are shown in the insets

In contrast, *cis*-9-octadecenoic acid, the unsaturated fatty acids with *cis* double bond in the acyl chain, exerts a minimal perturbing effect on the physical properties of the bilayer. The resistance value of PC bilayer modified with the largest content of DE ($x_2 = 0.88$) amounts to $2.28 \pm 0.74 \times 10^3 \, \Omega \, cm^2$. A quantitative description of membrane equilibrium based on conductance is not provided because of the spread in the R_m measurements and the uncertainty of the results. Resistance measurements often exhibit greater scattering than capacitance measurements, and therefore conductance values are most often treated as supplementary data. Conductance measurements are burdened with random errors caused by the presence of solvent molecules and ions in the bilayer, resulting in a noticeable error and introducing scatter in the results. This effect is smaller in capacitance measurements.

Equation 4 predicts that in the absence of interactions, the plots in Fig. 4 should yield straight lines. The nonlinear nature of the plots indicates some form of interaction between PC and DA as well as between PC and DE. The curves presented in Fig. 5 also deviate from linearity, indicating that specific interactions between membrane components are presented in the membrane. Such interaction in analyzed systems can be explained in terms of complexes. The behavior of mixtures of PC with long-chain fatty alcohols or amines is very similar to that of fatty

acid–phospholipids mixture, with evidence for 1:1 complex formation [29]. It was argued that the phase behavior of fatty acid–phospholipid mixtures is strongly influenced by hydrogen bonding between the fatty acids and the PC headgroups, even in the presence of excess water [30].

The 1:1 complex is formed in the initial stage of complexation followed by other compositions in subsequent stages. Based on the literature, an assumption was made that a 1:1 complex forms the most prevalent structure and is characterized by a maximal stability constant [18]. Given this assumption, the dependence of capacitance on membrane composition is described by Eq. 11. The values of capacitance C_1 and C_2 employed in this equation have been provided above. The constants B_1, B_2, and C_3 were obtained assuming that the values of the stability constants of the PC–fatty acid and the PC–amine complexes were sufficient with respect to the simplified Eqs. 11–13.

Figure 6a and b presents the experimental data for the entire range of DE concentrations examined in these experiments. In accordance with Eqs. 12 and 13, the curves become straight lines when the stability constant of the complex is high and x_2 values are low (Fig. 6a) and when the stability constant of the complex is high and x_2 values are high (Fig. 6b). If at least three successive points fall on the same straight line, it is reasonable to assume that the conditions for simplification of Eq. 11 have been met and
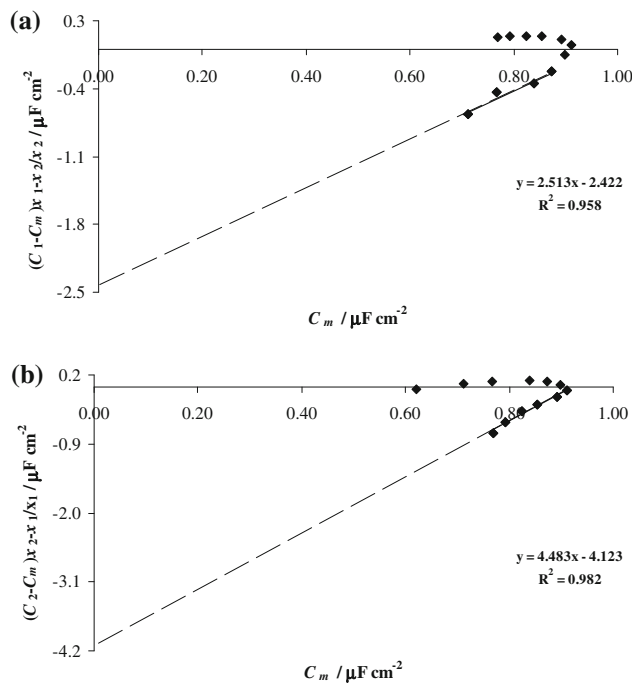
Fig. 6 Plots representing the dependences for phosphatidylcholine-decylamine complex described with Eqs. 12 and 13. *Straight lines* join the points, based on which parameters B_1, B_2, and capacitance C_3 can be determined

the lines passing through these points may be described by Eqs. 12 and 13.

Points fulfilling the limitations on the x_2 value and forming straight lines are joined in Fig. 6a and b. The values of B_1 (2.513) and B_2 (4.483) were determined from the slopes of the lines. The intersections of the straight lines with the ordinate provide $-B_1C_3$ and $-B_2C_3$, which can be used to determine C_3. The mean value obtained from both points is 0.942 μF cm^{-2}. The B_1 and B_2 values, obtained in the same way for PC–DA system, are equal to 2.816 and -6.052 (the last negative value was rejected). The resulting C_3 value amounts to 0.326 μF cm^{-2}.

Equations 12 and 13 may also be used to calculate the surface area S_3 of a single PC–DA or PC–DE complex. The values of the surface area, occupied by one mole of components 1 and 2, are necessary for this calculation. The surface area occupied by the PC molecule depends on the way the phospholipid is prepared, because this affects the length, conformation, and degree of unsaturation of the fatty acids chains. Therefore, the values in the literature range between 0.54 and 0.99 nm^2 [31, 32]. We chose the S_1 value amounting 0.85 nm^2 [33]. In the early work of Pockels [34] and Langmuir [35], fatty acids films with different chain lengths were found to occupy the same molecular area (approximately 0.20 nm^2). This same value has also been reported in more contemporary works [36, 37]. In our case, we estimated the surface areas occupied by DA and DE molecules to be 0.22 and 0.20 nm^2, respectively [38]. The resulting S_3 values for the PC–DA and PC–DE complexes were 2.39 and 1.52 nm^2, respectively. These are much larger than the amount of the surface area occupied by each component of the complexes. It is probably connected with the arrangement of PC molecules in the complex and also connected with the structural construction of such complexes. In the study [39], we suggested the arrangement of the PC molecules in a bilayer membrane at pH >5. In these media, one particle from the PC molecules in the bilayer (orientated in this way) has two straightened chains; however, the next molecule of PC has one straightened and another chain fastened to the membrane surface. An association of ions occurs in such conditions with OH$^-$ from the electrolyte solution. How these ions were characterized was previously reported [39]: These ions are strongly solvated and they produce a separation of PC particles in the bilayer which is an influence on the increasing surface occupied by the single molecule of PC.

The stability constants of PC–DA and PC–DE complexes were determined from Eq. 14 by setting $x_1 = x_2 = 0.5$. The stability constants of PC–DA and PC–DE complexes are 2.82×10^5 and 2.06×10^7 m^2 mol^{-1}, respectively. The K_R value obtained for PC–DE complex is relatively high, giving additional evidence for the

prevailing of the 1:1 complex in mixed PC–amine bilayers. This value also confirms that the assumption used to simplify Eq. 11 was correct. The verification of the assumption about the formation of 1:1 complexes in the PC–DA and PC–DE membranes is shown in Fig. 5. The experimental values are marked by points and compared with the values calculated from Eq. 15. These calculated ("theoretical") results are presented as the *solid lines*. It can be seen that, in the case of PC–DE membranes, the agreement between experimental and calculated points is good. This agreement means that our theoretical model (presented in Sect. Theory earlier) is sufficient to describe the interaction in PC–DE system. Our results indicate that a 1:1 complex with high stability constant forms in mixed membranes of PC and DE, similar to the complex between PC and unsaturated fatty acids containing a *trans* or *cis* double bond in the acyl chain [3]. The results also verify our choice of C_2 value (for component of the membrane). The agreement between the experimental results and the model predictions justifies the statement that other complexes do not represent a significant component of this system. Still, the fact remains that we cannot detect other complexes based on our experimental data.

On the contrary, the lack of agreement between the experimental results and values calculated from Eq. 15 for PC–DA bilayers indicates that complexes of stoichiometry other than 1:1 are present in these membranes. Amphiphiles such as fatty acids or amines are known to strongly modify the structures of biological membranes and their functions such as transport [40] or enzyme adsorption [41]. Such effects may be investigated by examining the molecular interaction of fatty acids or amines with phospholipids in the bulk [42–44] or in monolayers [38]. Calorimetric studies [43, 44] of phospholipid–fatty acid mixtures in the bulk phase have shown that saturated fatty acids partition preferentially into the solid-like domains of the lipids; a 1:1 or 1:2 complex formation was also suggested. It can be seen from the Fig. 5a that the agreement between experimental values and theoretical curve is very poor, which does not verify the assumption of formation of a 1:1 PC–DA complex in the bilayer lipid membrane. We suppose that the existence of a 1:2 complex (PC–DA) is the most probable explanation, as in the case of the mixtures of PC with longer-chain (C$_{12}$–C$_{20}$) fatty acids [29, 43]. Numerous carboxylic acids are known to form dimeric hydrogen-bonded units in the gas phase as well as solid phase [45–48]. In addition, through the near-infrared spectroscopy and vapor-pressure osmosis measurements, it has been revealed that *cis*-9-octadecenoic acid and *n*-fatty acids (octanoic, nonanoic, decanoic, and undecanoic) in their liquid state exist as dimers even at 363 K [48, 49]. The degree of dissociation value for nonanoic acid is ∼1% at 290 K and increases with the rise in temperature but

even at 363 K, only $\sim$3.2% of the acid dimers dissociate into the monomeric species in the liquid state. Most molecules of nanoic acid in the liquid state still remain as dimer even at such relatively high temperatures. The other fatty acids also gave similar results to the nonanoic acid. Namely, the dimers are the units in intramolecular or intermolecular movements in the liquid and solid states. In addition, the distribution function curves obtained from the X-ray diffraction data suggest that, in the pure liquid state, rod-like fatty acid dimers highly aggregate in parallel and probably make clusters that would be randomly aligned [49]. Through the electron spin resonance, nuclear magnetic resonance, density, viscosity, differential scanning calorimetry, fluorescence polarization, and X-ray diffraction measurements of the fatty acids such as *cis*-6-octadecenoic, *cis*-9-octadecenoic, *cis*-11-octadecenoic, *trans*-9-octadecenoic, and octadecanoic acids in their pure liquids, it has also been revealed that the dimers of all of these fatty acids aggregate to form clusters possessing the structure of a quasi-smectic liquid crystal: The dimerized acid molecules arrange longitudinally and alternately to make an interdigitated structure in the clusters [50, 51].

The interactions between PC and fatty acids of different chain length (DA, palmitic acid, and stearic acid) as well as PC and DE were examined in monolayers at the air/water interface [38]. The existence of 1:2 complexes was proved as a result of strong hydrogen bonding between acids chain and polar group of PC. A 1:1 PC–DE complex formation was also demonstrated. The complex formation energy (Gibbs free energy) and stability constants of complexes were calculated. It was shown that there is a minimum of Gibbs' energy at such composition of complexes. The stability constant of the PC–DA complex is given as 4.47×10^5 m^2 mol^{-1}, whereas the stability constant of the PC–DE complex was 1.01×10^6 m^2 mol^{-1}. It should be emphasized that the stability constant value is higher for complexes in bilayers. The larger value of stability constant for bilayer probably results from the roughness between the hydrophobic layers in the bilayer. Additional complexes between two leaflets of bilayer can be formed because of the roughness of this interface [52]. A monolayer is a two-dimensional system forming a plane at the air/water interface, whereas a bilayer possesses a third dimension and is additionally stabilized by hydrophobic chains. Thus, the value of the K_R for PC–DE complex (2.06×10^7 m^2 mol^{-1}) proposed in this study validates the assumption of 1:1 complex formation in the PC bilayer containing DE. The K_R value obtained for PC–DA complex (2.82×10^5 m^2 mol^{-1}) demonstrated that complexes with stoichiometry other than 1:1 are created in PC bilayer modified by DA.

During the course of our investigations, we assumed the formation of PC–DA and PC–DE complexes with 1:1 stoichiometry. These complexes arise by producing a connection between the $-N^{(+)}(CH_3)_3$ group from the molecule of PC and $-COO^{(-)}$ groups from DA, in the case of the complex PC–DA, and between the $-PO^{(-)}$ group from PC and $-N^{(+)}H_3$ group from DE. The dissociation constants of the $-N^{(+)}(CH_3)_3$ group from PC and $-COO^{(-)}$ groups from the DA are equal $10^{-5.7}$ [33] and about 10^{-5} [53], respectively. It should be noted that the dissociation constants of $-PO^{(-)}$ group from PC and $-N^{(+)}H_3$ group from DE are equal $10^{-2.6}$ [33] and about 10^{-10} [53], respectively. Therefore, the connection between PC and DA is stronger, and it is possible to expect that the stability constant of the PC–DA should be higher than the stability constant of the PC–DE complex. Thus, to correctly characterize the interactions between PC and DA in the bilayers, complexes with stoichiometry other than 1:1 should be taken into consideration during derivation of mathematical formulas. Next, suitable modifications should be applied in equations.

Conclusions

Noninvasive electrochemical impedance spectroscopy method has been used to characterize the capacitive properties of lipid bilayers and provide a quantitative description of equilibria in a two-component membrane. The conductance values were treated as supplementary data and were not used for equilibrium calculations. Based on mathematical-modeling results, the existence of a highly stable 1:1 complex between PC and DE is highly likely. Complex formation is the main reason for the deviations from linearity observed in the system parameters. Accurate modeling enables us to calculate and verify parameters such as molecular area and stability constant. The presence of complexes with stoichiometries other than 1:1 was postulated for the PC–DA bilayers.

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