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Impedance characteristics of the lipid membranes formed from a phospholipid-fatty acid mixture

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Electrochemical impedance spectroscopy was used for the study of two-component lipid membranes. Phosphatidylcholine and stearic acid were to be investigated, since they play 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 this two-component system forming bilayers was assumed, that could explain the deviation from additivity rule. The molecular area and the equilibrium constant of the complex were determined.

Key words: Phosphatidylcholine, stearic acid, complex formation, equilibrium constant, bilayer lipid membrane, electrochemical impedance spectroscopy.

INTRODUCTION

The inspiration for lipid bilayer research work, without question, comes from the biological world. Although the first report on self-assembled bilayer lipid membranes in vitro appeared 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 using supported bilayer lipid membranes [1].

Bilayer lipid membranes are made predominantly of amphiphiles, a special class of surface-active molecules, which are characterized by having a hydrophilic and a hydrophobic group in the same molecule [2]. Usually, a zwitterionic or non-ionic lipid is used as the basic lipid for the preparation of bilayers. Phosphatidylcholines, whose construction is represented in Fig. 1a, are the most widely used bilayer-forming molecules because of their relevance to the behaviour of these components in cell membranes. They contain two fatty acids themselves, which are esterified to glycerol. The interaction between different acyl chains within a phospholipid molecule or among the different phospholipid molecules in the bilayer should determine the physical properties of biomembranes. The bilayer membranes mostly consist of either natural or synthetic phospholipids, but other double-tail surfactants such as dialkyl quaternary ammonium compounds in pharmaceutical applications are also used. In addition, minor amounts of single-tail surfactants, such as fatty acids (stearic acid depicted in Fig. 1b belongs to the class of fatty acids), may be added to affect specific characteristics such as the membrane permeability or electric charge density [3].

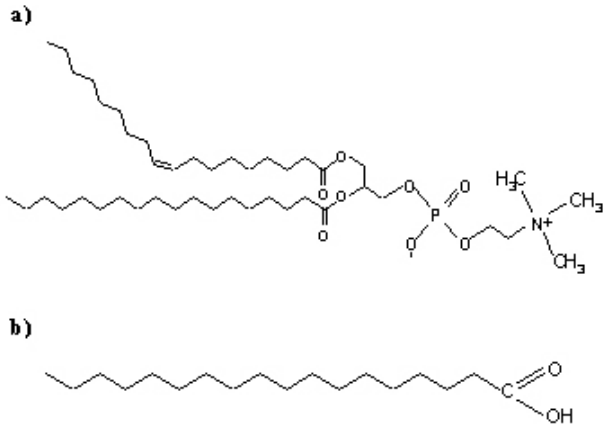

Fig. 1. Comparison of phosphatidylcholine and stearic acid molecular structures.

The physico-chemical studies of the phospholipid-fatty acid mixture may have an additional significance other than the interest to the alteration of membrane function caused by fatty acid. The study of the phase behaviour of the hydrated bilayer,

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composed of a phospholipid-fatty acid mixture would be useful to understand the acyl-acyl interactions playing such an important role in phospholipid bilayers [4].

In this work, the electric capacity and the electric conductance of the phosphatidylcholine-stearic acid membranes were determined within the composition range, where the bilayer formation was possible. A 1:1 complex has been proposed to exist in the examined bilayers. The aims of these investigations were to study the mixed phosphatidylcholine-stearic acid bilayers, to characterize the molecular interaction between the phospholipid and fatty acid and to determine the values of parameters of the complex: the equilibrium constant and the molecular area.

The data presented in this work, mathematically obtained result and confirmed experimentally, are of great importance for the interpretation of the phenomena occurring in lipid membranes. The knowledge of equilibrium represented by the complexing reaction lets us understand the processes that take place on 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 for 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 represented in full detail previously [5–7] assumes that in the cases, where the membrane components do not form chemical compounds, any two-component system, regardless if it forms a monolayer or a bilayer, can be described by the equation expressing additivity of the capacitance:

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

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·m^{-2}] – the measured capacitance of the membrane; C_1, C_2 [μF·m^{-2}] – the capacitance of the mem-brane 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 mol 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 equation:

$$\left(C_{\mathrm{m}} - C_1\right) x_1 = -\frac{S_2}{S_1}\left(C_{\mathrm{m}} - C_2\right) x_2 \tag{4}$$

Since the first stability constant in complexes, as the most essential one, is usually the biggest and should be taken into consideration [8], the existence of 1:1 complex (compound 3) in lipid-fatty acid system was assumed. Then, the set of Eqs (1)–(3) is modified because the electric capacity is the sum of the contributions of all the compounds [9, 10]:

$$C_{\mathrm{m}} = C_1 c_1^s S_1 + C_2 c_2^s S_2 + C_3 c_3^s S_3 \tag{5}$$

here:

$$K_{\mathrm{R}} = \frac{c_3^s}{c_1^s \cdot c_2^s} \tag{6}$$

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

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

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

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

where: C_3 [μF·m^{-2}] – the capacitance 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 mol of compound 3; K_{R} [m^2·mol^{-1}] – the stability constant of compound 3.

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

$$\left[\left(C_{\mathrm{m}} - C_1\right) B_2 x_1 + \left(C_{\mathrm{m}} - C_2\right) B_1 x_2\right]_{\times}$$

$$\left[\left(C_3 - C_1\right) B_2 x_1 + \left(C_3 - C_2\right) B_1 x_2 + \left(C_1 - C_2\right)\left(x_1 - x_2\right)\right] =$$

$$= K_{\mathrm{R}} S_3^{-1} B_1 B_2 \left[\left(C_{\mathrm{m}} - C_1\right)\left(x_2 - x_1\right) + \left(C_3 - C_{\mathrm{m}}\right) B_1 x_2\right]_{\times}$$

$$\left[\left(C_{\mathrm{m}} - C_2\right)\left(x_1 - x_2\right) + \left(C_3 - C_{\mathrm{m}}\right) B_2 x_1\right] \tag{11}$$

in which: $B_1 = \frac{S_3}{S_1}$ and $B_2 = \frac{S_3}{S_2}$.

The Eqn. (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. The opening of the parentheses results in a great complexity of the equation, 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 this equation.

The Eqn. (11) may be simplified taking into account the sufficiently high value of the stability constant of the complex. With this assumption it represents a straight line for small x_2 values ($x_2 < x_1$):

$$\left(C_1 - C_m\right)\frac{x_1 - x_2}{x_2} = -B_1C_3 + B_1C_m \qquad (12)$$

while for the case of high x_2 values ($x_2 > x_1$) Eqn. (11) can be expressed by another straight line:

$$\left(C_2 - C_m\right)\frac{x_2 - x_1}{x_1} = -B_2C_3 + B_2C_m \qquad (13)$$

The Eqn. (11) can be simplified in some other way. In the case where $x_2 = x_1$, the following form is assumed:

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

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

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

where:

$$a_1 = S_3^{-1}\left(x_2 - x_1\right);\ a_2 = S_2^{-1}x_1;\ a_3 = S_1^{-1}x_2;$$
$$a_4 = \left[S_3^{-1}\left(C_1 - C_2\right)\left(x_2 - x_1\right) +\right.$$
$$\left. + \left(C_1 - C_3\right)x_1S_2^{-1} + \left(C_2 - C_3\right)x_2S_1^{-1}\right].$$

MATERIALS AND EXPERIMENTAL DETAILS

Reagents and preparation of the forming solutions

The lipid bilayer was formed from the Fluka product of 99% egg lecithin (3-sn-phosphatidylcholine) and from stearic acid (97%) 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 was used to form the model membrane containing 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 solvents were of chromatographic standard purity 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 purity grade and was heated prior to 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 obtained as bubbles at the Teflon cap constituting a measuring vessel component. The use of hexadecane as a solvent allows one to obtain membranes of thickness and capacity values similar to those of membranes formed of monolayers. A small quantity of butanol has negligible effect on the impedance parameters of the bilayers created, whereas it accelerates considerably the formation of membranes. The process of membrane formation was monitored by visual observation in transmitted light and by measuring the membrane capacitance. The bilayer area was determined with a microscope employing a micrometer scale as $4\times10^{-2} - 8\times10^{-2}$ cm^2 (the values were given for the bilayer area without margin).

Impedance analysis

Electrochemical impedance spectroscopy was performed with an a.c. 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), in which a four-electrode input

was fed to the pre-amplifier. The electrochemical cell contained two identical reversible silver-silver chloride electrodes and two identical platinum electrodes (described exactly in [11−13]). 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 were analyzed by using the complex nonlinear least squares (CNLS) fitting to a model represented by an equivalent electrical circuit. The CNLS program used in this work was ZSimpWin 3.21. All the experiments were carried out at room temperature of 20 ± 1°C.

RESULTS AND DISCUSSION

The effect of stearic acid on capacitance and resistance of the phosphatidylcholine bilayer was examined in the presence of different amounts of the fatty acid by using electrochemical impedance spectroscopy. The stearic acid content was varied up to a 0.80 molar fraction, above this limit the acid induced disorder of the acyl chains of phosphatidylcholine and we were not able to form a bilayer stable enough to carry out measurements on it. The capacity of a 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 in the bilayer, border leakage at the membrane support, the appearance of lipid "crystals" at the periphery of the bilayer, or way of introducing the lipid solution (if the forming solution is introduced with a micro-syringe, instead of a brush, the irreproducibility of the 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 the resistance due to addition of ions, proteins, drugs, etc., can be determined with a relatively high degree of accuracy [3]. 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. 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 their 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.

Figs. 2a,b represent typical impedance spectra of the phosphatidylcholine membranes, pure and containing stearic acid. Very simple diagrams were obtained for all the examined membranes; they have the form of semicircles in the entire analyzed frequency range. The centres of the semicircles lie on the real axis, provided that the lipid bilayers are considered as dielectric layers with leakage and the apexes of the semicircles satisfy the equation $C_m R_m \omega = 1$. The CNLS fits (according to the equivalent circuit illustrated in Fig. 3) are represented by solid lines and are in good agreement with the data obtained.

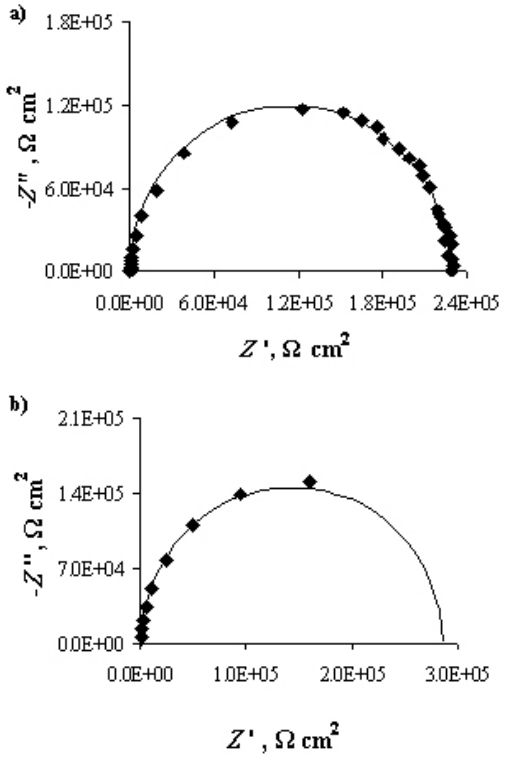

Fig. 2. Plots of the imaginary component of impedance – Z" versus the real component of impedance Z' over a frequency range of 0.1 Hz – 10 kHz for a phosphatidylcholine bilayer modified with stearic acid (x_2 = 0.80). (-) CNLS fit to the equivalent circuit shown in Fig. 3.

An equivalent circuit depicted in Fig. 3 describes the electric properties of the analyzed lipid membrane. The impedance of the phosphatidylcholine bilayers modified with stearic acid is represented by the electrolyte solution resistance R_0, which is in series with a paralleled circuit, composed of the resistance of the membrane R_m and of the membrane capacitance C_m. The possibility of misinterpretation of the recorded data is reduced by the simplicity of the circuit. This electric circuit is characteristic of an artificial lipid membrane only when ionophore

systems, specific channels, pores and adsorption are absent [14].

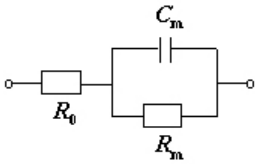

Fig. 3. An equivalent circuit representing electric properties of the phosphatidylcholine membrane modified with stearic acid: R_0 – resistance of the electrolyte, C_m – capacitance of the membrane, R_m – resistance of the membrane.

The dependence of the capacitance of phosphatidylcholine membranes containing stearic acid is illustrated in Fig. 4 as a function of the molar fraction of fatty acid. The resulting dependence deviates from linearity, indicating that some bonds are formed in the membrane. The capacitance value of pure phosphatidylcholine bilayer (component 1) C_1 was measured directly and represented earlier [7, 10, 12] as 0.62 ± 0.02 μF·cm^{-2}. There are no reliable literature data on capacitance values for the pure component 2 (stearic acid), because it does not form a bilayer membrane.

However, in order to characterize the course of the experimental curve, the C_2 value for the pure component is necessary, which will be used in the calculations. In this case, the hypothetical capacitance value for membrane built from stearic acid was estimated by fitting the experimental curve with a straight line for four different molar fractions of fatty acid and extrapolating $x_2 = 1$ value. The capacitance value obtained in this way for pure stearic acid is equal to 0.48 μF·cm^{-2}.

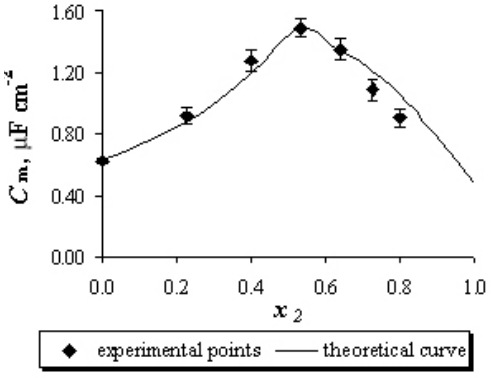

Fig. 4. Dependence of the capacitance C_m of the phosphatidylcholine-stearic acid membrane on the molar fraction of stearic acid x_2. Error bars indicate the experimental scatter. The solid line represents the theoretical values calculated from Eqn. (15).

The resistance value of pure phosphatidylcholine bilayer is equal to (2.30 ± 0.25)·10^5 Ω cm^2. The resistance value of phosphatidylcholine bilayer modified with the highest content of stearic acid (x_2 = 0.88) amounts to (2.99 ± 1.05)·10^5 Ω cm^2. The quantitative description of equilibrium in the phosphatidylcholine-stearic acid membrane, based on values of conductance, is not given here because of the very close values of resistances obtained for the analyzed bilayers and the irreproducibility of results.

Fig. 5 represents the dependence of $(C_m–C_1)x_1$ versus $–(C_m–C_2)x_2$ described by Eqn. (4). According to Eqn. (4), as it is in the case where the membrane components do not form chemical compounds, the values of this function should lie on a straight line. As one can see, this is not the case, which suggests that there is a complex or other chemical compound formation in the phosphatidylcholine bilayers containing stearic acid.

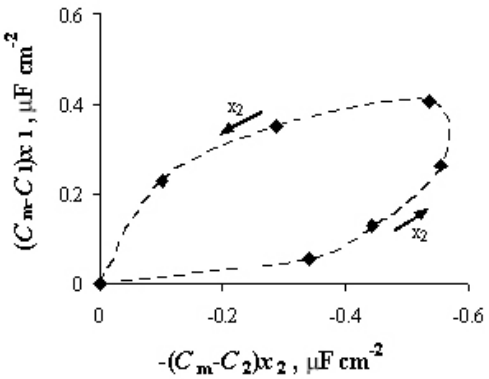

Fig. 5. Plot of $(C_m–C_1)x_1$ vs $(C_m–C_2)x_2$; C_m − capacitance of the membrane, C_1 − capacitance of the phosphatidylcholine membrane, C_2 − capacitance of the stearic acid membrane, x_1 – molar fraction of the phosphatidylcholine and the x_2 − molar fraction of stearic acid. The arrows denote the direction of the increasing x_2 values and the dashed line indicates the order of points.

Therefore, the formation of a complex in this system was assumed. Because the existence of a 1:1 complex is a typical case [8], the formation of a 1:1 phosphatidylcholine-stearic acid complex was accepted.

Thus, Eqn. (5) and the stability constant K_R, describing a third compound formed in this system, broaden the theoretical description. After a simple modification of Eqn. (5), one can obtain information represented by Eqn. (11). The capacitance values of C_1 and C_2 have been given above. The other constants B_1, B_2 and C_3 were obtained assuming that the value of the stability constant of the phosphatidylcholine-stearic acid complex was sufficient with respect to the simplified Eqns. (12) and (13). From the B_1 and B_2 constants, which were determined based on these equations it was possible to calculate the capacitance value of the complex C_3. The mean value is equal to 1.52 μF·cm^{-2}.

Eqns. (12) and (13) could also be applied to calculate the surface area per a single phosphatidylcholine-stearic acid molecule S_3. The values of the surface area, occupied by one mol of components 1 and 2, are necessary for this calculation. The surface area occupied by the phosphatidylcholine 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 54 and 99 Å^2 [15, 16]. In our case, we chose the S_1 value, determined in our laboratory as 85 Å^2 [17]. The surface area occupied by the stearic acid molecule, reported in the literature, is equal to 19 Å^2 [18]. The resulting S_3 value amounts to 122 Å^2 and it is bigger than the sum of areas occupied by each component of the complex (104 Å^2). It is probably connected with the arrangement of lecithin molecules in the complex and with its structure.

The only value to be determined was the stability constant of the phosphatidylcholine-stearic acid complex. It could be determined based on Eqn. (14) for $x_1 = x_2 = 0.5$ leading to 2.87×10^7 m^2·mol^{-1}. This value is relatively high, giving additional evidence for the prevailing of the 1:1 complex in mixed phosphatidylcholine-stearic acid bilayers. This value also confirmed that the assumptions, used to simplify Eqn. (11), were correct.

The parameters determined based on Eqns. (11) and (14) were applied to represent the agreement of the data, evaluated from Eqn. (11) (solid lines) with the experimental data (points) in Fig. 4 by using Eqn. (15). As Eqn. (15) is of second order, it can yield two solutions. The values yielding a better agreement of the experimental points with equation describing the complex formation between membrane components were chosen. Good agreement between experimental and theoretical points verifies the assumption about the formation of a 1:1 phosphatidylcholine-stearic acid complex in the lipid membrane as well as the assumption of the correct choice of the C_2 value for component of the membrane. The small variances between the experimental and the theoretical capacitance values indicate that complexes of different stoichiometries or associates are also possible in the phosphatidylcholine-stearic acid bilayer.

The phase behaviour 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 phase diagrams over the whole composition range have been reported for some mixture systems [19−23]. All the phase diagrams have exhibited the formation of a molecular compound (or phase 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. Although an agreement is documented for complex formation, there seems to be a discrepancy in the phase diagrams reported for the composition ranges of both low and high fatty acid concentrations [19, 20, 22].

In our previous paper [24], we represented the dependence of interfacial tension on the composition for the phosphatidylcholine-stearic acid membrane. A 1:1 complex has been assumed to exist in the bilayer, but the existence a fatty acid in the form a dimer was taken also into account. The value of stability constant of such complex is 2.18×10^9 m^2·mol^{-1}, whereas the experimental by determined area occupied by one phosphatidylcholine-stearic acid complex molecule is 187 Å^2 [24] (the only values available in the literature).

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ИМПЕДАНСНИ ХАРАКТЕРИСТИКИ НА ЛИПИДНИ МЕМБРАНИ ОБРАЗУВАНИ ОТ СМЕС НА ФОСФОЛИПИД И МАСТНА КИСЕЛИНА

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(Резюме)

Чрез електрохимична импедансна спектроскопия са изучени двукомпонентни липидни мембрани. Изследвани са фосфатидилхолин и стеаринова киселина, тъй като те играят важна биохимична роля в клетъчните мембрани. Изследването на биолипидните взаимодействия е фокусирано върху количественото описание на процесите, които протичат в двойния слой. Приетите модели на взаимодействие между амфифилните молекули и равновесието което се установява в изследваните системи са описани с математически уравнения. Допуска се възможността за образуване на комплекс в тази двукомпонентна система, която образува двойни слоеве, с което може да се обясни отклонението от правилото за адитивност. Определени са молекулната площ и равновесната константа на комплекса.