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Chronoamperometry Insight into the pH Effect on the Electrical Properties of Bilayer Lipid Membrane Formed from Phosphatidylcholine

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This paper discusses the effect of adsorption of hydrogen and hydroxide ions which are present in the solution upon electrical capacitance and electrical resistance of the bilayer membrane formed by phosphatidylcholine. Both electrical parameters of the membrane were determined as a function of pH from chronoamperometric measurements. Two models were tested to explain the phenomena occurring on the membrane surface. The models display changes in the capacitance and the resistance values, particularly in the ranges distant from the isoelectric point. In Model I, the surface is continuous with uniformly distributed functional groups constituting the centers of the hydrogen and hydroxide ions adsorption. In Model II, the surface is built of lipid molecules, free or with adsorbed H^+ and/or OH^-. In both models, the contributions of the individual lipid molecule forms to the electrical parameters of the membrane were assumed to be additive. Theoretical equations were derived to describe the electrical parameters as a function of pH for proposed models.

Keywords: chronoamperometry; electrical capacitance; electrical conductance; pH; bilayer lipid membrane; phosphatidylcholine; adsorption equilibria

1. INTRODUCTION

Biological membranes are extremely interesting, nonetheless complicated, research systems, containing many elements which influence their electric properties to a considerable extent. Studies of such complex structure is difficult, because of various kinds of interactions occurring between its components. For this reason, models of the membrane are used, e.g. bilayer lipid membranes (BLMs) which are simplified structures reflecting properties of natural membranes. BLMs are made

predominantly from amphiphiles, a special class of surfo-active molecules which are characterized by having a hydrophilic and a hydrophobic group on the same molecule [1]. The properties of BLMs and their subsequent applicability depend on the physical and physicochemical characteristics of the bilayer membrane. Usually, a zwitterionic or non-ionic lipid is used as the basic lipid for the preparation of bilayers [2].

The most widely used BLM-forming molecules are phosphatidylcholines (PCs) because of their relevance to the behavior of these components in cell membranes. They are zwitterionic at physiological values of pH because the quaternary ammonium group is neither basic nor acidic in these pH ranges [1]. The bilayer membranes mostly consist of either natural or synthetic phospholipids, but the application of other double-tail surfactants such as dialkyl quaternary ammonium compounds in pharmaceutical applications is also increasing used [3].

Components of membranes possess groups which contain an electric charge e.g. $-PO_4^-$, $-NH_3^+$, $-COO^-$. These groups are most often acidic-alkaline properties groups of which charge is dependent upon pH of the electrolyte solution [4]. The equilibria existing at the membrane surface occur between functional groups of the membranes and outer medium components. The electric properties of the membrane are affected by this kind of equilibria and by their progress. The equilibria can be affected by e.g. adsorption leading to a membrane capacitance variation [5].

The studies on acid-base equilibria between the BLMs and solutions around membranes are of great importance in understanding the phenomena which take place in living organisms. The pH inside the human body varies from about 1 to 8. The membranes of the cells from gastrointestinal tract continuously force different pH values. Gastric walls produce secretion whose pH oscillates between 1 and 2.5 [6], the pH of the duodenal content differs over range 4.8-8.2 [7], the pH of the small intestine is 5.1-7.8 [6,8], whilst the pH of the caecum is 6.4-6.7 [6,8] and finally the pH in the colon changes within range 6.5-7.0 [6,8]. Moreover, there are many more examples of pH which deviates from physiological value (7.4), for instance: blood pH fluctuates between 6.8 to 7.6 [9], the pH of bile is 6.8-7.0 [7], the pH of human breast milk range from 6.6 to 7.6 [7] and finally pH of the human waste products, urine and feces is 4.8-8.4 [7]. Otherwise, the pH solution impacts many membrane parameters, such as dipole and zeta potential [10], bending stiffness [10] or surface tension [11]. It is well-known that many membrane-mediated processes are affected by pH changes, e.g. phase transition between gel and liquid-crystal [12,13], acid induced membrane fusion [14] or drug-membrane interaction [15]. Although literature is full of examples on how pH affects model and biological membranes there are still only few publications which describe the effect of pH on electrical properties of lipid bilayers [16,17].

This paper describes the application of chronoamperometry to the research of the pH effect on the electrical capacitance and the electrical resistance of BLMs as a continuation of the physicochemical investigations undertaken by Naumowicz and co-workers [5]. The BLMs were formed from PC, which was selected mainly because it is present in biological membranes and it fulfill essential functions in lively organisms. We have shown that the registration of chronoamperometric curves allows a simple estimation of the electric parameters of the membrane. Proposed method of determination of electrical properties of membrane is simple and precise. It is also

quick what has especially meaning during examination of bilayers which properties are changing in time.

It has been proved in previous studies [5], using electrochemical impedance spectroscopy, that electrical capacitance of the PC is affected by pH. A model supposing the existence of four different forms of phosphatidylcholine as a result of adsorption of H^+ and OH^- ions on the bilayer surface was presented and experimentally confirmed. In this paper, the model presented earlier and an alternative model displaying changes in values of electrical parameters (particularly in the ranges distant from the isoelectric point) were checked utilizing chronoamperometry and validated by comparison of theoretical values to experimental results.

2. THEORY

2.1. The effect of pH on the phosphatidylcholine bilayer

PC molecule is a neutral, zwitterionic phospholipid with an amphiphilic character so it can participate in equilibrium reactions with both hydrogen ions and hydroxide anions. The phosphatidylcholine layer observed from the aqueous solution side has uniformly distributed $–PO^{(-)}$ and $–N^{(+)}(CH_3)_3$ groups because it is built of molecules each having one $–PO^{(-)}$ group and one $–N^{(+)}(CH_3)_3$ group. Therefore, the PC membrane surface can be modeled in two ways. In model I, the membrane surface is continuous with uniformly distributed functional groups being the active centers of adsorption of H^+ and OH^-. In model II, the bilayer surface is composed of nonbonded PC molecules and of molecules with bonded H^+ and OH^- ions.

2.1.1. Model I

The electrical capacitance of the lipid membrane results from acid-base equilibria existing between uniformly distributed active centers at the membrane surface and solution ions. Assuming that H^+ and OH^- are adsorbed at the PC surface, the adsorption equilibria can be characterized by the equations:

$$A^- + H^+ \rightleftarrows AH \quad (1)$$

$$B^+ + OH^- \rightleftarrows BOH \quad (2)$$

in which:

A^- is $–PO^{(-)}$ group of PC and B^+ is $–N^{(+)}(CH_3)_3$ group of PC.

Consequently, the four groups: A^-, AH, B^+ and BOH are present at the layer surface.

The lipid is present in the membrane only. Therefore, the surface concentration of the lipid is equal to its amount related to the membrane surface area. These concentrations, and the concentrations of H^+ and OH^-, determine the acid-base constants according to the relationships [18]:

$$K_A = \frac{a_{AH}}{a_{A^-} a_{H^+}} \quad (3)$$

$$K_B = \frac{a_{BOH}}{a_{B^+} a_{OH^-}} \tag{4}$$

Taking into account the acid-base equilibria – Eqs. (1) and (2), the surface concentration of the phosphatidylcholine *s* can be expressed as [18]:

$$a_{A^-} + a_{AH} = s \tag{5}$$

$$a_{B^+} + a_{BOH} = s \tag{6}$$

here:

$a_{A^-}, a_{AH}, a_{B^+}, a_{BOH}$ [mol m^{-2}] – the concentrations on the membrane surface of the membrane components, respectively.

Assuming additivity of the contributions of the individual forms of the PC molecule to the electrical capacitance of membrane C_m, the following expression can be obtained:

$$C_m = C_{A^-} + C_{AH} + C_{B^+} + C_{BOH} \tag{7}$$

where:

$$C_{A^-} = C_{oA^-} \frac{a_{A^-}}{s} \tag{8}$$

$$C_{AH} = C_{oAH} \frac{a_{AH}}{s} \tag{9}$$

$$C_{B^+} = C_{oB^+} \frac{a_{B^+}}{s} \tag{10}$$

$$C_{BOH} = C_{oBOH} \frac{a_{BOH}}{s} \tag{11}$$

in which:

$C_{oA^-}, C_{oAH}, C_{oB^+}, C_{oBOH}$ [μF cm^{-2}] – the specific capacitances of the membrane components, respectively.

The Eqs. (1)-(11) form an equation system from which the $a_{A^-}, a_{AH}, a_{B^+}, a_{BOH}$ values could be eliminated, yielding the equation:

$$C_m = C_{oA^-}\left(\frac{1}{1+K_A a_{H^+}}\right) + C_{oAH}\left(\frac{K_A a_{H^+}}{1+K_A a_{H^+}}\right) + C_{oB^+}\left(\frac{1}{1+K_B a_{OH^-}}\right) + C_{oBOH}\left(\frac{K_B a_{OH^-}}{1+K_B a_{OH^-}}\right) \tag{12}$$

Eq. (12) describes the dependence of the electrical capacitance of the PC bilayer on the pH of the electrolyte solution.

The equations above ((7)-(12)) can be derived in an analogous manner, taking into account the membrane conductance R_m^{-1} (instead membrane capacitance C_m). As a result, the R_m^{-1} dependence on the pH value based on Model I is obtained:

$$R_m^{-1} = R_{oA^-}^{-1}\left(\frac{1}{1+K_A a_{H^+}}\right) + R_{oAH}^{-1}\left(\frac{K_A a_{H^+}}{1+K_A a_{H^+}}\right) + R_{oB^+}^{-1}\left(\frac{1}{1+K_B a_{OH^-}}\right) + R_{oBOH}^{-1}\left(\frac{K_B a_{OH^-}}{1+K_B a_{OH^-}}\right) \tag{13}$$

where:

$R_{oA^-}^{-1}, R_{oH}^{-1}, R_{oB^+}^{-1}, R_{oBOH}^{-1}$ [Ω^{-1} cm^{-2}] – the specific conductances of the membrane components, respectively.

2.1.2. Model II

This model, which has been presented in full detail previously [5], assumes that PC molecule possessing zwitterionic character can participate in acid-base equilibria with both H^+ and OH^-:

$$PC + H^+ \rightleftarrows PCH^+ \quad (14)$$

$$PC + OH^- \rightleftarrows PCOH^- \quad (15)$$

$$PC + HOH \rightleftarrows PCHOH \quad (16)$$

Thus, Eqs. (14)-(16) can be considered as the description of an adsorption process. As a result of adsorption of H^+ and OH^- on the surface of phosphatidylcholine layer, the PC molecule can exist in four different forms: PCH^+ with H^+ adsorbed, $PCOH^-$ with OH^- adsorbed, PCHOH with both H^+ and OH^- ions adsorbed on the surface and a free phosphatidylcholine molecule PC i.e. with no ions adsorbed. A phosphatidylcholine bilayer is assumed to consist of these four forms. The relative contributions of above forms are dependent on pH, according to Eqs. (14)-(16).

Assuming that the individual forms constituting the phosphatidylcholine bilayer contribute to the electrical capacitance of membrane additively, the following equation can be presented:

$$C_m = C_{PCHOH} + C_{PCH^+} + C_{PCOH^-} + C_{PC} \quad (17)$$

The surface concentrations of the groups postulated in Model I can be written depending on the forms of the PC molecule postulated in Model II [5,18]:

$$a_{A^-} = a_{PC} + a_{PCOH^-} \quad (18)$$

$$a_{AH} = a_{PCH^+} + a_{PCHOH} \quad (19)$$

$$a_{B^+} = a_{PC} + a_{PCH^+} \quad (20)$$

$$a_{BOH} = a_{PCOH^-} + a_{PCHOH} \quad (21)$$

here:

$a_{PC}, a_{PCOH^-}, a_{PCH^+}, a_{PCHOH}$ [mol m^{-2}] – the concentrations on the membrane surface of the membrane components, respectively.

Then, the association acid constant of the groups PC and $PCOH^-$ with the H^+ ions (Eq. 3) may be expressed as [5,18]:

$$K_A = \frac{a_{PCH^+} + a_{PCHOH}}{a_{H^+}\left(a_{PC} + a_{PCOH^-}\right)} \quad (22)$$

and the association base constant of the groups PC and PCH^+ with the OH^- ions (Eq. 4) may be presented as [5,18]:

$$K_B = \frac{a_{PCOH^-} + a_{PCHOH}}{a_{OH^-}\left(a_{PC} + a_{PCH^+}\right)} \quad (23)$$

On the basis of Eqs. (18)-(21), the surface concentration of the lipid can be written as:

$$a_{PCHOH} + a_{PCH^+} + a_{PCOH^-} + a_{PC} = s \quad (24)$$

The relationship between the surface concentrations of the membrane components, the surface concentration of the lipid and the electrical capacitance values results in:

$$C_m = C_{0PCHOH}\frac{a_{PCHOH}}{s} + C_{0PCH^+}\frac{a_{PCH^+}}{s} + C_{0PCOH^-}\frac{a_{PCOH^-}}{s} + C_{0PC}\frac{a_{PC}}{s} \quad (25)$$

where:

$C_{0PCHOH}, C_{0PCH^+}, C_{0PCOH^-}, C_{0PC}$ [μF cm^{-2}] – the specific capacitances of the membrane components, respectively.

The equations connecting surface concentrations of the lipid forms $a_{PC}, a_{PCOH^-}, a_{PCH^+}, a_{PCHOH}$ with surface concentrations of the groups $a_{A^-}, a_{AH}, a_{B^+}, a_{BOH}$ can be written as [5,18]:

$$\frac{a_{PCHOH}}{s} = \frac{a_{AH}}{s}\frac{a_{BOH}}{s} \quad (26)$$

$$\frac{a_{PCH^+}}{s} = \frac{a_{AH}}{s}\left(1 - \frac{a_{BOH}}{s}\right) \quad (27)$$

$$\frac{a_{PCOH^-}}{s} = \frac{a_{BOH}}{s}\left(1 - \frac{a_{AH}}{s}\right) \quad (28)$$

$$\frac{a_{PC}}{s} = \left(1 - \frac{a_{AH}}{s}\right)\left(1 - \frac{a_{BOH}}{s}\right) \quad (29)$$

Based on Eqs. (22)-(24), the following expressions can be obtained [5,18]:

$$a_{AH} = a_{PCH^+} + a_{PCHOH} = \frac{K_A a_{H^+} s}{1 + K_A a_{H^+}} \quad (30)$$

$$a_{BOH} = a_{PCOH^-} + a_{PCHOH} = \frac{K_B a_{OH^-} s}{1 + K_B a_{OH^-}} \quad (31)$$

Substituting Eqs. (30)-(31) to Eqs. (26)-(29) yields the dependence:

$$C_m = C_{oPCHOH}\left(\frac{K_A a_{H^+}}{1 + K_A a_{H^+}}\right)\left(\frac{K_B a_{OH^-}}{1 + K_B a_{OH^-}}\right) + C_{oPCH^+}\left(\frac{K_A a_{H^+}}{1 + K_A a_{H^+}}\right)\left(\frac{1}{1 + K_B a_{OH^-}}\right) +$$
$$C_{oPCOH^-}\left(\frac{1}{1 + K_A a_{H^+}}\right)\left(\frac{K_B a_{OH^-}}{1 + K_B a_{OH^-}}\right) + C_{oPC}\left(\frac{1}{1 + K_A a_{H^+}}\right)\left(\frac{1}{1 + K_B a_{OH^-}}\right) \quad (32)$$

Eq. (32) represents the dependence of the electrical capacitance of the lipid bilayer on the pH of the electrolyte solution.

The expression describing the R_m^{-1} dependence on the pH value can be obtained in an analogous manner, taking into account the membrane conductance instead the membrane capacitance:

$$R_m^{-1} = R_{oPCHOH}^{-1}\left(\frac{K_A a_{H^+}}{1 + K_A a_{H^+}}\right)\left(\frac{K_B a_{OH^-}}{1 + K_B a_{OH^-}}\right) + R_{oPCH^+}^{-1}\left(\frac{K_A a_{H^+}}{1 + K_A a_{H^+}}\right)\left(\frac{1}{1 + K_B a_{OH^-}}\right) +$$
$$R_{oPCOH^-}^{-1}\left(\frac{1}{1 + K_A a_{H^+}}\right)\left(\frac{K_B a_{OH^-}}{1 + K_B a_{OH^-}}\right) + R_{oPC}^{-1}\left(\frac{1}{1 + K_A a_{H^+}}\right)\left(\frac{1}{1 + K_B a_{OH^-}}\right) \quad (33)$$

where:

$R_{oPCHOH}^{-1}, R_{oPCH^+}^{-1}, R_{oPCOH^-}^{-1}, R_{oPC}^{-1}$ [Ω^{-1} cm^{-2}] – the specific conductances of the membrane components, respectively.

2.2. Determination of the electrical properties of the bilayer lipid membranes from chronoamperometric measurements

Electrically, the conventional BLM system is represented by a parallel arrangement of capacitance C_m and resistance R_m, completed by a serial resistance R_0 for the electrolyte solution resistance (Fig. 1). This system can be characterized by several dependences resulting from Kirchhoff's laws and Ohm's law:

$$U = R_m I_{Rm} + IR_0 \tag{34}$$

$$I = I_{Rm} + C_m \frac{dU_{Cm}}{dt} \tag{35}$$

$$\frac{dU_{Cm}}{dt} = \frac{dU}{dt} - R_0 \frac{dI}{dt} \tag{36}$$

in which:

U - voltage applied to the measuring electrodes;

U_{Cm} - voltage across the membrane;

I - total current flowing through the electrodes and the membrane;

I_{Rm} - resistance current of the membrane.

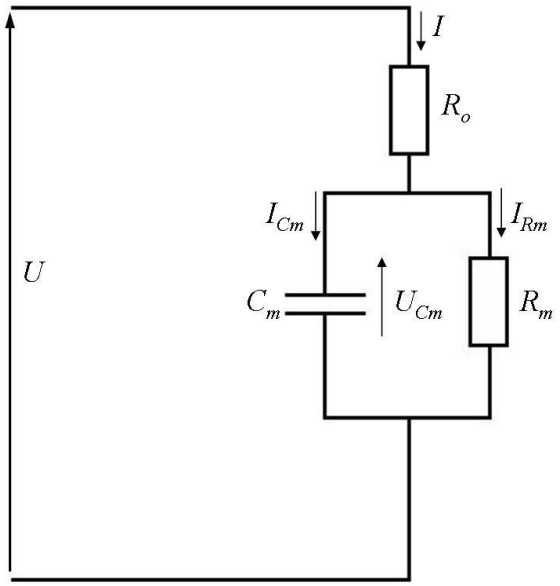


Figure 1. An equivalent circuit describing the phosphatidylcholine membrane: C_m - capacitance of the membrane, R_m - resistance of the membrane; R_0 - resistance of the electrolyte; U - voltage applied to the measuring electrodes; Uc_m – voltage across of the membrane; I - total current flowing through the electrodes and the membrane; I_{Rm} - resistance current of the membrane; I_{Cm} - capacitance current of the membrane.

From the above system of equations, the following expression is obtained:

$$\frac{U}{R_m} + C_m \frac{dU}{dt} = I\left(1 + \frac{R_0}{R_m}\right) + R_0 C_m \frac{dI}{dt} \tag{37}$$

Taking into account that in the chronoamperometric method:

$$\frac{dU}{dt} = 0 \tag{38}$$

where: $U = const$,

after inserting Eq. (38) into Eq. (37) and rearranged, inhomogeneous equation is received:

$$\frac{dI}{dt} = -\frac{I}{C_m}\left(\frac{1}{R_0} + \frac{1}{R_m}\right) + \frac{U}{R_0 R_m C_m} \tag{39}$$

which is then converted to the homogeneous equation:

$$\frac{dI}{dt} = -\frac{I}{C_m}\left(\frac{1}{R_0} + \frac{1}{R_m}\right) \tag{40}$$

Integral from Eq. (40) has the form:

$$I = C_1 \exp\left(-\frac{t}{C_m}\left(\frac{1}{R_0} + \frac{1}{R_m}\right)\right) \tag{41}$$

Assumption that the integral constant C_1 is a variable dependent on the time t and differentiation Eq. (41) leads to the relationship:

$$\frac{dI}{dt} = \frac{dC_1}{dt}\exp\left(-\frac{t}{C_m}\left(\frac{1}{R_0} + \frac{1}{R_m}\right)\right) - \frac{C_1}{C_m}\left(\frac{1}{R_0} + \frac{1}{R_m}\right)\exp\left(-\frac{t}{C_m}\left(\frac{1}{R_0} + \frac{1}{R_m}\right)\right) \tag{42}$$

Subsequently, after inserting Eqs. (41) and (42) into Eq. (39) and grouping similar terms, the following expression is obtained:

$$\frac{dC_1}{dt} = \frac{U}{R_0 R_m C_m}\exp\left(\frac{t}{C_m}\left(\frac{1}{R_0} + \frac{1}{R_m}\right)\right) \tag{43}$$

Integrating Eq. (43) yields:

$$C_1 = \frac{U}{R_0 + R_m}\exp\left(-\frac{t}{C_m}\left(\frac{1}{R_0} + \frac{1}{R_m}\right)\right) + C_2 \tag{44}$$

where:

C_2 - the integral constant.

Then, it is necessary to insert Eq. (44) into Eq. (41):

$$I = \frac{U}{R_0 + R_m} + C_2 \exp\left(-\frac{t}{C_m}\left(\frac{1}{R_0} + \frac{1}{R_m}\right)\right) \tag{45}$$

Denoting the current value registered at the time $t = 0$ as I_{max}, the following initial condition can be written:

$$I_{max} = \frac{U}{R_0} \text{ for } t = 0 \tag{46}$$

Then, insertion above assumption into Eq. (45) allows to determine the value of C_2:

$$C_2 = U\left(\frac{1}{R_0} - \frac{1}{R_0 + R_m}\right) \tag{47}$$

Inserting Eq. (47) into (45) permits to obtain the dependence characterizing the chronoamperometric curve:

$$I = \frac{U}{R_0 + R_m} + U\left(\frac{1}{R_0} - \frac{1}{R_0 + R_m}\right)\exp\left(-\frac{t}{C_m}\left(\frac{1}{R_0} + \frac{1}{R_m}\right)\right) \tag{48}$$

Next it is necessary to consider the different parts of the function described by Eq. (48). At the infinitely long time of the measurement ($t \to \infty$), Eq. (48) simplifies to the form:

$$I_\infty = \frac{U}{R_0 + R_m} \tag{49}$$

in which:

I_∞ – the current value registered at the $t \to \infty$.

It is thus seen that at the sufficiently long time of the measurement chronoamperometric curve reaches a plateau with a value described by the Eq. (49). This allows for calculation of the membrane resistance if the value of the electrolyte resistance used in the study is also known. However, in most of the analyzed cases R_0 can be omitted as much smaller than R_m and Eq. (49) takes the form which enables to calculate the resistance of the bilayer:

$$R_m = \frac{U}{I_\infty} \tag{50}$$

Eq. (48) allows also on the determination of the membrane capacitance. Appropriate its transformations using of Maclaurin series expansion and keeping only the linear term lead to the following relationship:

$$I = \frac{U}{R_0 + R_m} + U\left(\frac{1}{R_0} - \frac{1}{R_0 + R_m}\right)\left[1 - \left(\frac{1}{R_0} + \frac{1}{R_m}\right)\frac{t}{C_m}\right] \tag{51}$$

The equation for the straight line representing the initial part of the chronoamperometric curve is obtained after arranging the above expression:

$$I = \frac{U}{R_0} - \frac{tU}{R_0^2 C_m} \tag{52}$$

Thus, at the point of intersection of the straight line described by the Eq. (52) with the abscissa axis (when $I = 0$) this equation allows to obtain the following relationship:

$$t_{(I=0)} = R_0 C_m \tag{53}$$

Using Eq. (46) and of Fig. 2 it is possible to write the expression on the electrolyte resistance:

$$R_0 = \frac{U}{I_{\max}} \tag{54}$$

The combination of Eqs. (53) and (54) leads to form:

$$C_m = \frac{t_{(I=0)} I_{\max}}{U} \tag{55}$$

Based on above-mentioned expressions, straight line characterized by Eq. (52) can be presented as:

$$I = I_{\max} - \frac{dI}{dt}t \tag{56}$$

here:

$\frac{dI}{dt}$ - slope of the line representing the initial part of the chronoamperometric curve (described by Eq. (52)).

Using Eq. (56), the value of the time in place where straight line intersects the abscissa (when $I = 0$) can be calculated:

$$t_{(I=0)} = \frac{I_{\max}}{dI/dt} \tag{57}$$

Substituting Eq. (57) to Eq. (55) yields:

$$C_m = \frac{\left(\frac{I_{\max}}{dI/dt}\right) I_{\max}}{U} = \frac{I_{\max}^2}{\frac{dI}{dt} U} \tag{58}$$

Thus, it is evident that reading of the slope of the initial part of the curve and the maximum value of the electric current (at time $t = 0$) and knowing the value of the voltage applied to the measuring electrodes, which is set at the beginning of the experiment, it is possible to determine the electrical capacitance of the analyzed lipid bilayer.

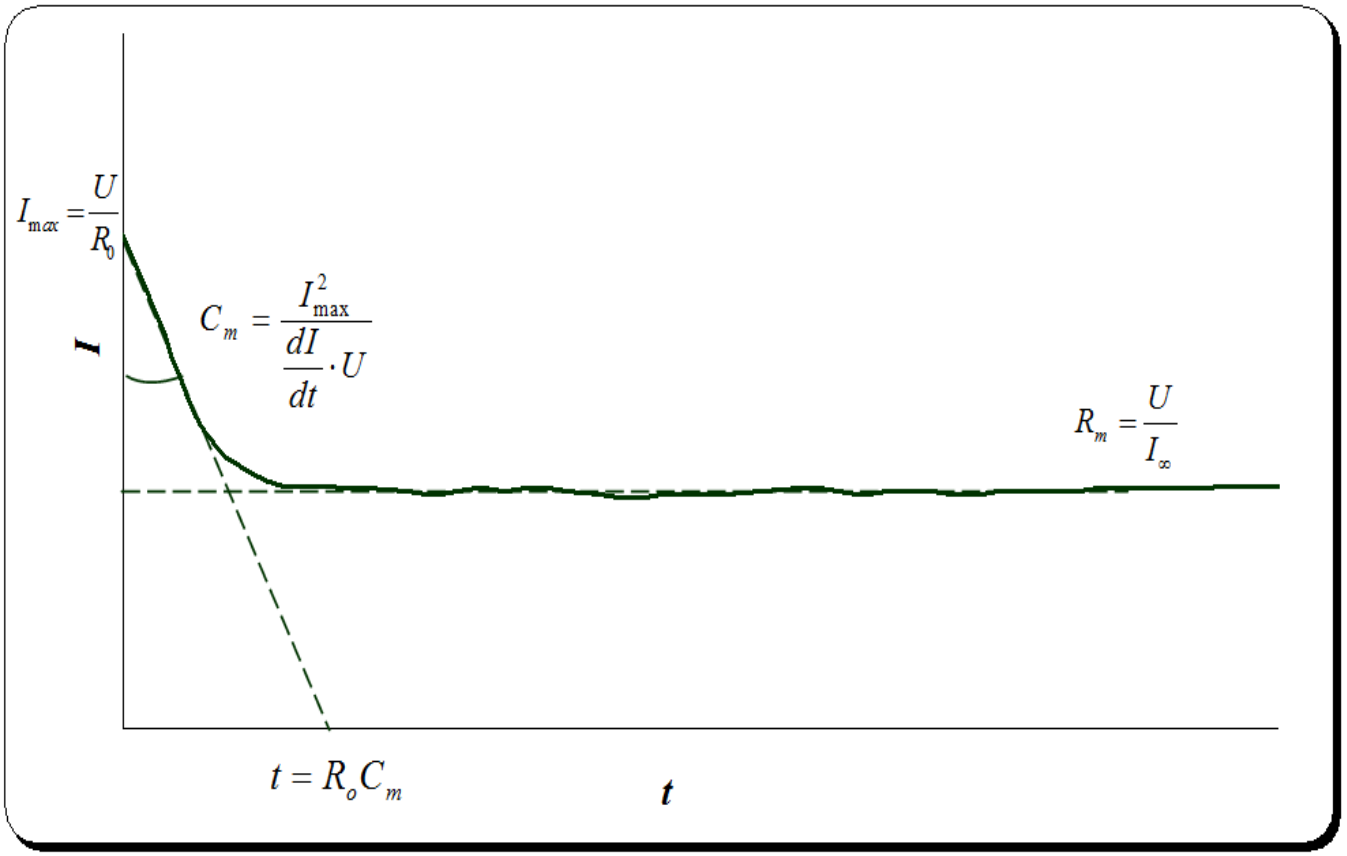


Figure 2. Chronoamperometric curve for the circuit equivalent to that shown in Fig. 1.

3. MATERIALS AND EXPERIMENTAL DETAILS

3.1. Chemicals and preparation of the forming solutions

Egg yolk 3-sn-phosphatidylcholine was purchased from Sigma (No. 61755). The lipid was dissolved in chloroform to prevent oxidizing. Next, the solvent was evaporated under a stream of argon. Dried residues were dissolved in a n-hexadecane - n-butanol mixture (10:1 by volume). The resultant solution used to form the model membrane contained 20 mg ml^{-1} of lipids in solution. During membrane formation, the solvent mixture was removed and the membrane created has the same proportion as in the resultant solution. The samples were stored for at least four days at 4°C before examination. The preparation and storage methods provided reproducible electrochemical properties when samples prepared at different times were examined using chronoamperometric method.

Potassium chloride solution of 0.1 mol dm^{-3} was used as the electrolyte for experiments and was prepared using triple-distilled water (second distillation was made with $KMnO_4$ and KOH to remove organic impurities) and KCl produced by POCh (Poland). The KCl was calcined to remove traces of organic material.

The solvents were of chromatographic standard grade: hexadecane was purchased from Fluka (Neu-Ulm, Germany) and chloroform and butanol were obtained from Aldrich (Milwaukee, WI, USA).

3.2. Preparation of the bilayer membranes

BLMs were formed as bubbles. They were obtained at the Teflon cap constituting a measuring vessel component. The use of n-hexadecane as a solvent made it possible to obtain membranes with thickness and capacity values similar to values determined from studies of bilayer membranes formed from lipid monolayers [19]. The small quantity of n-butanol had a negligible effect on the electrical 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 in order to focus on the membrane located deep within the vessel.

BLM formation was also monitored electrically by measuring the membrane capacitance at a low frequency. The capacitance of the membranes increased with time after bilayer formation until a steady-state value was reached after approximately 5-10 min. Measurements were initiated 10-15 min after the membranes turned completely black. When the capacitance stabilized, it was assumed that diffusion of solvent out of the bilayer was complete, although some hexadecane molecules might remain ‘‘dissolved’’ in the membrane interior.

Membrane images were captured with a color CCD camera using the WinFast PVR program (http://winfastpvr.software.informer.com). The bilayer areas were calculated from the photographs, taking into consideration the spherical nature of the surface and using the Makroaufmassprogram

program (http://ruedig.de/tmp/messprogramm.htm). The area of the bilayer membranes was about $6 \cdot 10^{-2}$ cm^2.

3.3. Chronoamperometric measurements

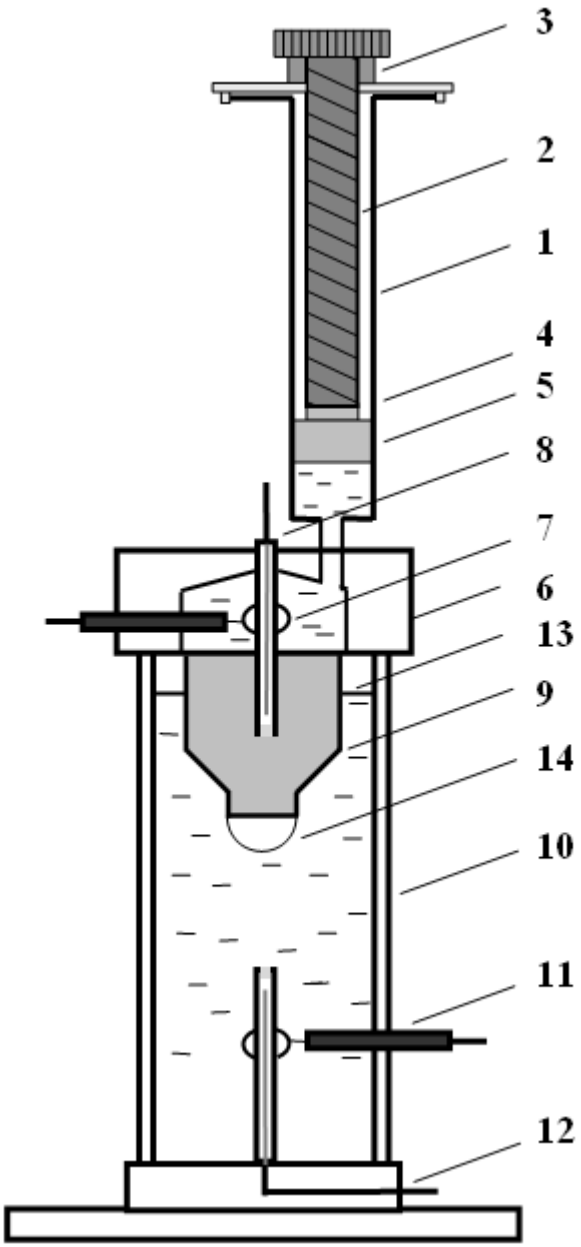


Figure 3. The measuring vessel.

The general architecture of the system used to chronoamperometric measurements was essentially the same as that proposed by us to other electrochemical measurements [e.g. 20,21]. 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), in which a four-electrode input was applied within the self-constructed electrometer. The use of

the four-electrode system in the studies of electric phenomena occurring in membranes makes it possible to considerably reduce the errors caused by electrode and electrolyte impedance [22].

The self-constructed measuring vessel depicted in Fig. 3 was placed in a Faraday cage during the measurement in order to decrease the background noise. A syringe (1) with an external thread screw (2) and with a handwheel (3) was located in its upper part. An acid-resistant steel tube (4) with a tight Teflon piston (5) was at the other end of the screw. A connector (6) made of glass with a platinum current electrode (7) and a reversible silver-silver chloride electrode with a salt bridge (8) was fixed to the syringe cone. The connector ended with a tight Teflon attachment (9). The lower part of the vessel (10) made of glass contained a second platinum current electrode (11) and a second reversible silver-silver chloride electrode with a salt bridge (12). The lateral side of the vessel was a flat glass plate allowing to observe formed membranes. The syringe, the connector, and the Teflon attachment formed a tight setup, which could be filled with electrolyte solution. The forming solution was placed at the Teflon cap tip and the setup was placed in the lower part of the measuring vessel, also filled with electrolyte solution. As a result, the Teflon attachment was immersed in the electrolyte solution, the approximate level of which (13) is marked in Fig. 3. A drop of electrolyte could be squeezed out from the cap by rotating the handwheel (3) and forming sphere (14) could be simultaneously observed under microscope. N-butanol contained in the forming solution dissolved in aqueous solution, and n-hexadecane able to wet Teflon shifted into the cap. As a result, a bilayer in the form of a bubble built of lipids was produced.

Chronoamperometry measurements were performed using PowerSTEP software (part of the PowerSuite package, EG&G Princeton Applied Research). The data were analyzed with the computer program Excel (Microsoft, Redmond, WA).
All experiments were carried out at room temperature 293 ± 1 K.

4. RESULTS AND DISCUSSION

The effect of pH on the capacitance and the resistance of lipid bilayer formed by PC was examined using chronoamperometry, with acetate buffer as the electrolyte. The minimum electrical capacitance value was 0.397 ± 0.049 μF cm^{-2} at pH equal to 4.1. The minimum electrical resistance value was (1.70 ± 0.59)·10^6 Ω cm^2 at the same pH. The pH of the electrolyte solution was carefully controlled during the measurements in the range from pH 3.5 to 6.0. Although the bilayer formation was only sufficiently stable for measurements within this pH range, this range was of greatest interest, given that it covers the pH of the isoelectric point of the analyzed membrane. Presumably, the large electrostatic forces due to the dissociation of polar groups at the interface may prevent the formation of a stable membrane. The arithmetic mean and standard deviation of the determined parameters (electrical capacitance and electrical resistance) were obtained from measurements conducted on eight lipid bilayers.

The chronoamperometric characteristics ($I = f(t)$, current flowing through the bilayer as a function of time) of analyzed membranes were recorded under constant voltage conditions. Voltage applied to the membrane caused membrane charging, with the speed of this charging dependent on the voltage value. As a result, the current flowing through the bilayer decreased exponentially to a constant value.

Fig. 4 depicts typical chronoamperometric curves registered sequentially for one PC membrane at several various voltage values (from 20 to 120 mV) in pH 4.1. The following sequence of measurements was applied for registration of these curves: after recording of the first curve, the measurements were terminated for 120 s, and a second curve was registered at higher voltage value. After the same time of termination, the third chronoamperometric curve was registered. It is thus seen that chronoamperometry is relatively safe for membranes and enables recording of several chronoamperometric curves for a single bilayer. When combining chronoamperometric curves obtained for the same membrane at different voltage values such as in Fig. 4, it was apparent that the speed at which changes in current flowing through the bilayer occurred was dependent on the voltage – higher voltage value resulted in slower decrease in membrane current.

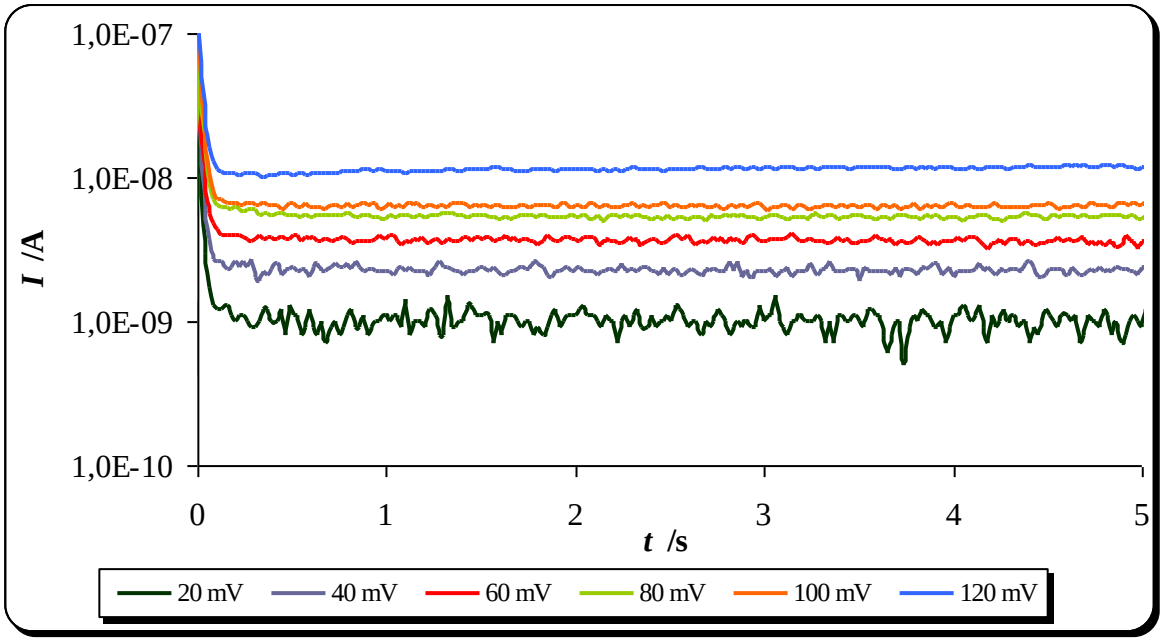


Figure 4. Chronoamperometic characteristics of bilayer lipid membrane registered for different voltage value (voltage value are shown inside the figure).

From the $I = f(t)$ curves, the values of the capacitance and the resistance of the BLMs were estimated using the method described in Subsection 2.2. - the C_m was calculated from the slope of the curves according to Eq. (58), the R_m was calculated considering Eq. (50) and these steady state values appearing as the linear, final part of the $I = f(t)$ curves. All membrane measurements performed for the same pH at different voltage values yielded similar results, indicating good reproducibility of the electrical behavior. Although it was not possible to completely control the membrane formation process and every membrane was slightly different, the R_m and C_m values were consistent.

Fig. 5 shows the plot of the capacitance and the conductance of the PC membrane against the pH of the electrolyte solution. Experimental values are marked as points, the total values calculated from Eqs. (12) and (13) are presented by solid lines and the C_m and the R_m^{-1} of the individual bilayer components, i.e. A^-, B^+, AH and BOH are marked with broken lines. This figure refers to the above-

described Model I of the PC membrane surface, in which the functional groups were assumed to be uniformly distributed on the surface from the aqueous solution side.

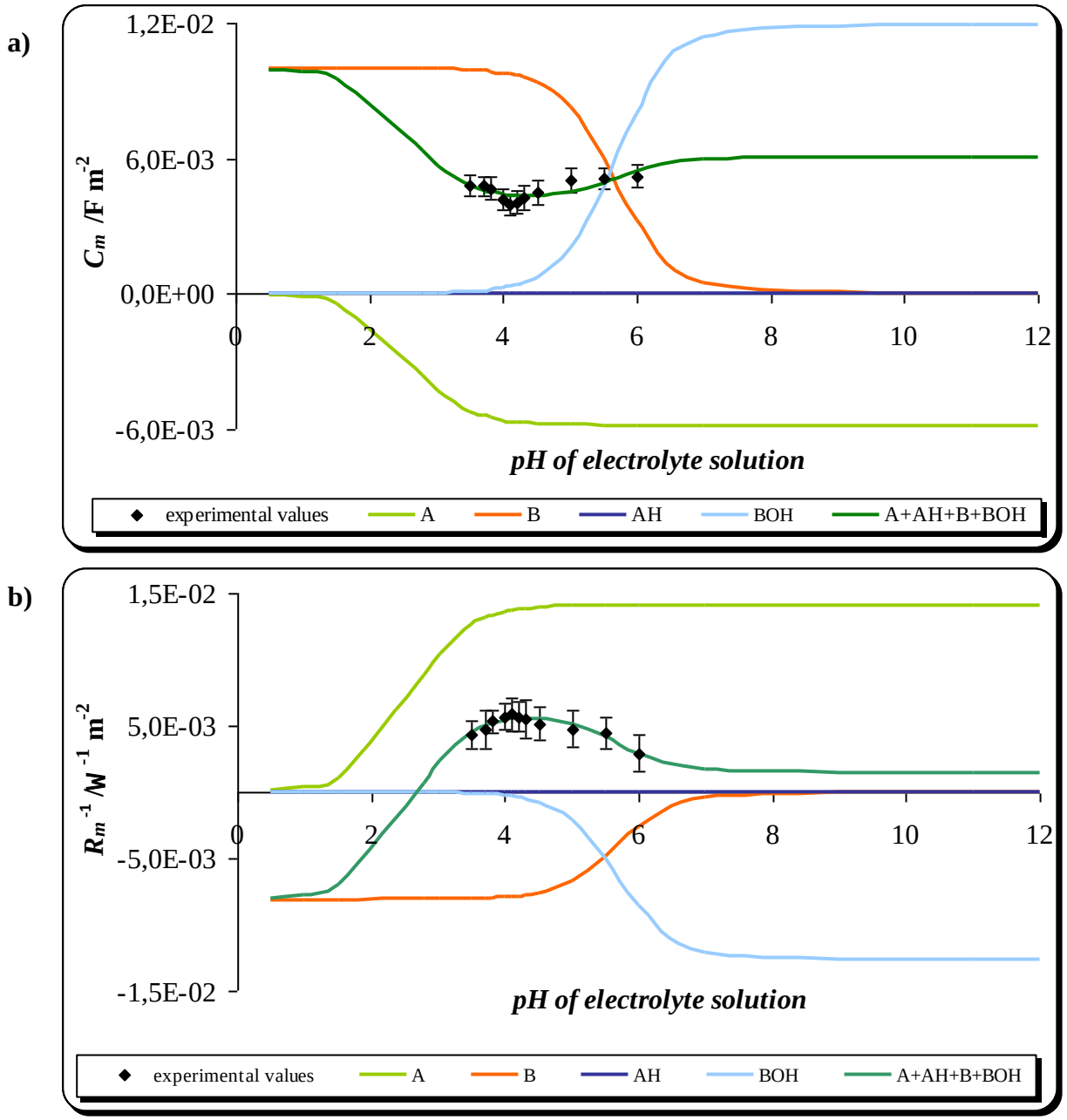


Figure 5. The participation of the A and B groups, calculated from the Model I, in associated and dissociated forms in the membrane capacitance (a) and the membrane conductance (b), as a function of pH of the electrolyte solution.

Fig. 5 shows that the capacitance had a minimum value around pH 4.1 and the conductance had a maximum value around the same pH. The values of both electrical parameters changed almost symmetrically towards low or high pH – capacitance increased and conductance decreased (similar variation in C_m with pH was reported in [23]). Disturbances of the symmetry may be caused by difference in the sizes of the positive and negative ionized groups. The minimum electrical capacitance

and the maximum electrical conductance of the analyzed membranes appeared at the isoelectric point of the PC, which corresponds to the pH at which the surface formed by PC has no net electrical charge, or where the negative and positive charges are equal [24].

Based on Eqs. (12)-(13), the total capacitance and the total conductance values of the PC bilayer are the sum of the capacitances and the conductances of its components. To calculate the values of the specific capacitance or the specific conductance of these components, the equilibrium constants of adsorption processes of H^+ and OH^- on PC must be known. Acid-base equilibrium constants for PC membrane equal $K_A = 10^{2.581}$ and $K_B = 10^{5.687}$ were determined in the work [25] and were assigned to the $–PO^{(-)}$ and $–N^{(+)}(CH_3)_3$ groups. From comparison of the association constants it appears that OH^- is more strongly adsorbed than H^+. The K_A and K_B values were substituted into Eq. (12) and Eq. (13) to calculate the specific capacitance and specific conductance values of the individual forms of the PC bilayer. The values of specific parameters calculated using the linear regression method were -0.583 ± 0.351 μF cm^{-2}, 0.000 ± 0.001 μF cm^{-2}, 0.999 ± 0.335 μF cm^{-2}, 1.190 ± 0.366 μF cm^{-2} for $C_{oA^-}, C_{oAH}, C_{oB^+}, C_{oBOH}$, respectively, and $(-1.26 \pm 0.38)\cdot 10^{-7}$ Ω^{-1} cm^{-2}, $(-0.81 \pm 0.34)\cdot 10^{-7}$ Ω^{-1} cm^{-2}, 0.0 Ω^{-1} cm^{-2}, $(1.41 \pm 0.36)\cdot 10^{-7}$ Ω^{-1} cm^{-2} for $R^{-1}_{oA^-}, R^{-1}_{oAH}, R^{-1}_{oB^+}, R^{-1}_{oBOH}$, respectively. When the specific capacitances or specific conductances of the membrane components have zero or negative values it is possible to suppose that the bilayer formed from these forms does not exist.

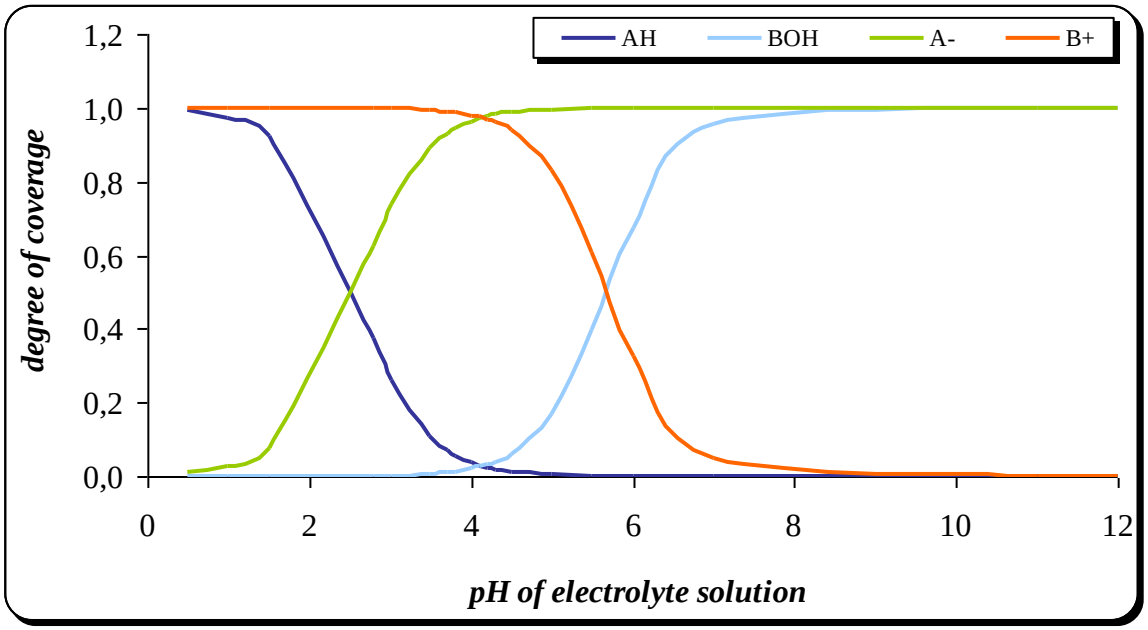
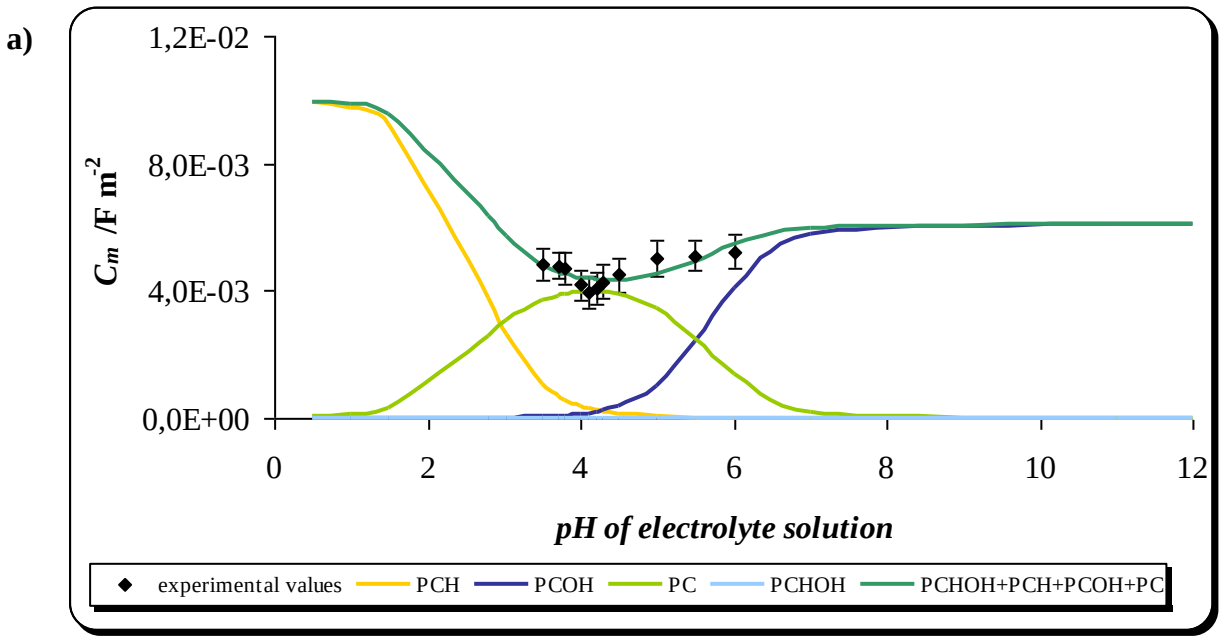


Figure 6. The degree of coverage of the phosphatidylcholine bilayer surface, calculated from the Model I, with associated and dissociated forms of the A and B groups as a function of pH of the electrolyte solution.

The degree of coverage of the PC bilayer surface by H^+ and OH^- as a function of the pH of the electrolyte solution is presented in Fig. 6. As can be seen, the degree of coverage of the membrane by the H^+ ions was over 0.9 at extreme acidic pH's, e.g. in this pH range the bilayer was covered by H^+. As the pH changed to less acidic values, the concentration of the adsorbed protons decreased e.g. AH

groups began to lose partially their proton ($AH \rightarrow A^-$). Around the isoelectric point of PC (i.e. pH 4.1), the surface was almost not covered by H^+. In the proximity of isoelectric point of PC, there was also almost no coverage of the membrane by OH^-. As the pH of the electrolyte solution increased up to basic values, the concentration of hydroxide ions in solution grew and therefore such ions began to bind with B^+ groups ($B^+ \rightarrow BOH$). The coverage with the OH^- was favored at basic pH's, it was almost one at pH > 7.

Fig. 7 also demonstrates the electrical capacitance and the electrical conductance of the PC bilayer plotted against the pH of the electrolyte solution. Points present the experimental values, the total values of C_m and R_m^{-1} calculated from Eqs. (32)-(33), derived according to Model II, are presented by a continuous lines and the capacitance and the conductance values of PC membrane components are marked with broken lines. Based on Eqs. (32)-(33), the total membrane capacitance and the total membrane conductance are the sum of the capacitances and the conductances of its individual components, i.e. PC, PCH^+, $PCOH^-$ and PCHOH. Specific capacitance and specific conductance values of individual components of the PC bilayer were determined by the linear regression method. The $C_{oPC}, C_{oPCH^+}, C_{oPCOH^-}$ and C_{oPCHOH} values obtained in this way were 0.416 ± 0.189 μF cm^{-2}, 0.999 ± 0.336 μF cm^{-2}, 0.607 ± 0.435 μF cm^{-2} and 0.000 ± 0.002 μF cm^{-2}, respectively. The resulting values of $R_{oPC}^{-1}, R_{oPCH^+}^{-1}, R_{oPCOH^-}^{-1}$ and R_{oPCHOH}^{-1} were (0.60 ± 0.02)·10^{-7} Ω^{-1} cm^{-2}, (-0.81 ± 0.35)·10^{-7} Ω^{-1} cm^{-2}, (0.15 ± 0.05)·10^{-7} Ω^{-1} cm^{-2} and 0.0 Ω^{-1} cm^{-2}, respectively. When the capacitance and the conductance of bilayer lipid membranes formed from individuals only have negative or zero values, it is possible to suppose that the bilayer membrane formed from these forms does not exist.



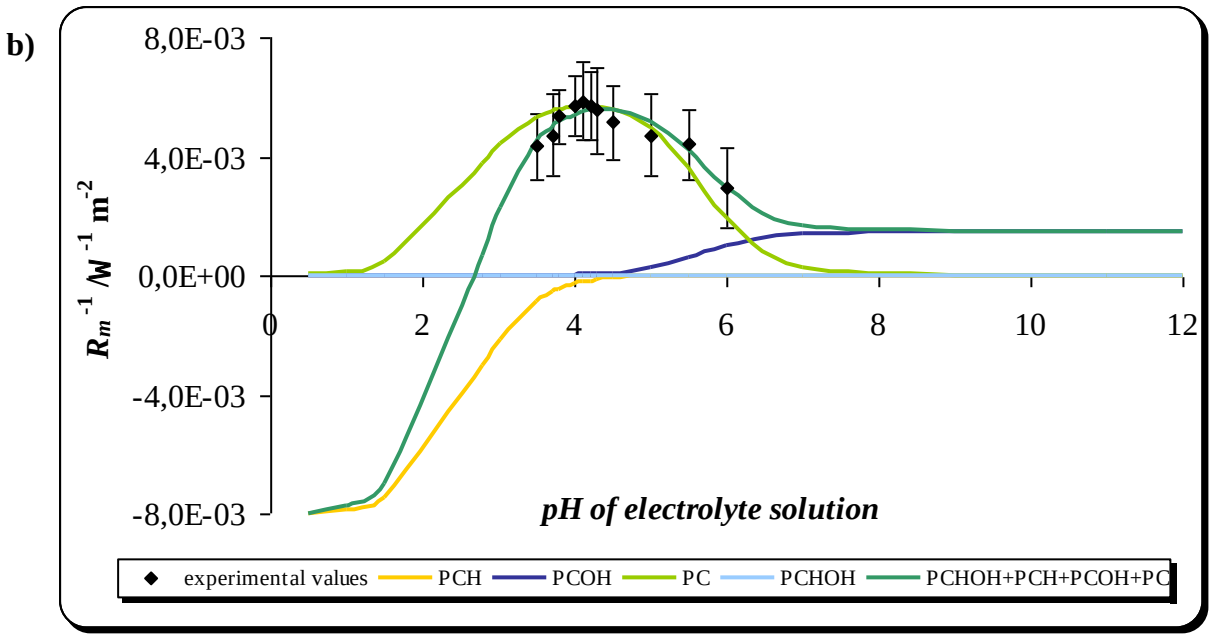


Figure 7. The participation of the individual forms of the phosphatidylcholine molecules, calculated from the Model II, in the membrane capacitance (a) and the membrane conductance (b), as a function of pH of the electrolyte solution.

The degree of coverage of the PC membrane surface by associated and dissociated forms of the groups present at the membrane surface as function of pH of the electrolyte solution is plotted in Fig. 8.

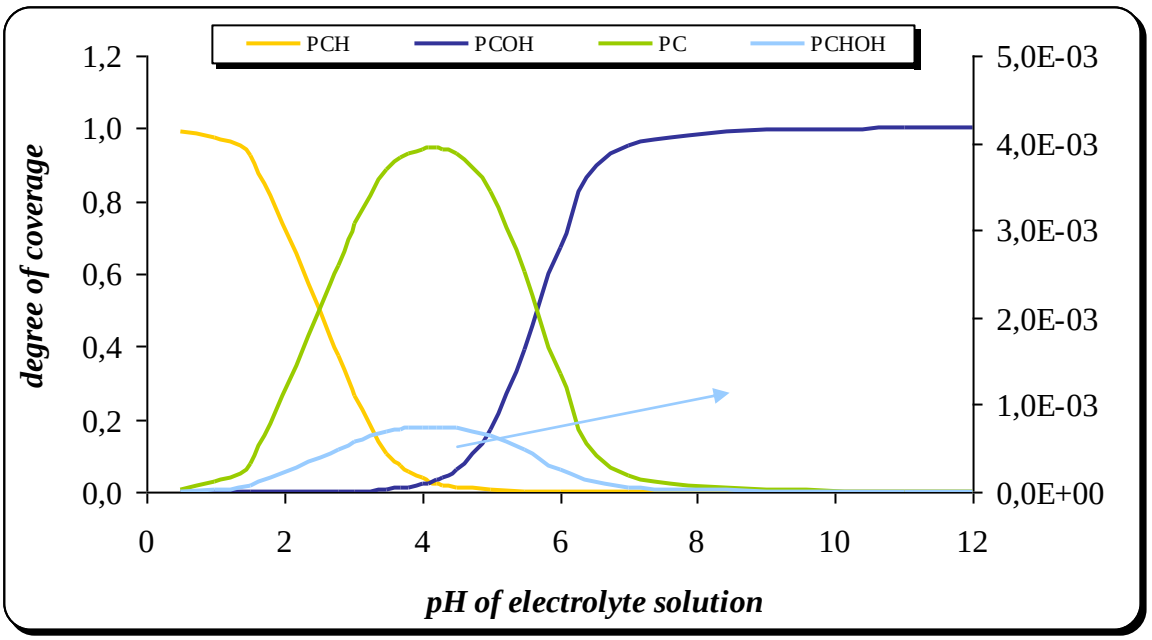


Figure 8. The degree of coverage of the phosphatidylcholine bilayer surface, calculated from the Model II, with the individual forms of the molecules as a function of pH of the electrolyte solution.

The PC form predominated in the membrane surface in the proximity of its isoelectric point, i.e. at pH equal to about 4 (like in Fig. 6); the membrane surface was almost not covered by the hydrogen and hydroxide ions there. In both descriptions, the degree of coverage of the PC membrane surface by the H^+ and OH^- remained unchanged in the ranges below 1.5 and above 7.5. A head group of amphoteric PC contains two separated oppositely charged moieties. Then, there is a possibility of strong electrostatic attraction between hydrophobic parts and appropriate local charge. The negative charge of the phosphate group is distributed among four oxygen atoms, while the positive charge of ammonium group is concentrated on a single nitrogen atom, which is favorable for electrostatic interaction with anions [26].

Various techniques, such as EPR and NMR spectroscopy [27], Langmuir film balance [28], and fluorescence spectroscopy [29], have documented changes in many membrane properties, including liposome stability, lateral phase separation, and the interdigitated gel-to-bilayer gel phase transition [27-29], in response to changes in pH. In particular, calorimetric studies have established that low pH environments ($pH \leq 2$) can increase phosphatidylcholine membrane phase-transition temperatures [30]. From the mechanical standpoint, the effect of pH on membrane interfacial tension has been studied by measuring the curvature change of a phospholipid drop exposed to different pH environments [18,25,30]. The interfacial tension of phosphatidylcholine [18,25], phosphatidylserine [18] and phosphatidylethanolamine [31] was observed to increase near pH 4.

Zhou and Raphael [10] demonstrated that the solution pH affects both the membrane mechanical and interfacial electrical properties, and that alterations in membrane surface charge density and the Debye length can account for the experimentally measured changes in the membrane bending stiffness. Those authors measured also changes in the intramembrane (dipole) potential as a function of solution pH and the results obtained by them suggests that either protons or hydroxide ions play a dominant role in affecting intramembrane potential. Clarke and Lüpfert [32] established that the effectiveness of an ion in reducing the membrane dipole potential depends on the free energies of hydration of the ion as well as whether the ion is positively or negatively charged. It was found that hydrophobic anions and hydrophilic cations tend to be more effective at altering the membrane dipole potential [32]. Theoretical calculations estimate that the free energies of hydration for protons and hydroxide ions is about - 260 kJ/mol [33]. This value indicates that these ions are relatively hydrophobic compared to other ions, such as Mg^{2+} with about - 2000 kJ/mol [32]. Thus, it is likely that the relatively hydrophobic OH^- is more effective at interacting with lipids than an equally hydrophobic H^+. This also agrees with the studies of Ninham and colleagues [34,35], who suggest that the affinity of a particular ion for membranes depends upon the polarizability of the ion, or the ionic dispersion coefficient. A much more polarizable hydroxide ion should interact with membranes more favorably than a proton, which is not polarizable [34,35]. In addition, both inorganic anions and cations decrease the dipole potential [32].

In the present paper, two models describing the surface of the BLM build from phosphatidylcholine were presented. Model I assumed the equilibria between $-PO^{(-)}$ and $-N^{(+)}(CH_3)_3$ groups uniformly distributed on membrane surface and hydrogen and hydroxide ions. Model II supposed the existence of four different forms of PC as a result of adsorption of H^+ and OH^- on the bilayer surface. Figs. 5-8 show that Model II gave more reliable results than Model I, in which the

negative value of the specific capacitance of $-PO^{(-)}$ form showed that this model was not likely from the physicochemical point of view.

5. CONCLUSIONS

In conclusion, the chronoamperometric technique was used to characterize the electrical properties of the PC bilayers and to provide a quantitative description of the acid-base equilibria at the interface separating the electrolyte solution and bilayer. It was found that the electrical capacitance of the bilayer had a minimum value around pH 4.1 and the electrical conductance had a maximum value around the same pH. The value of both parameters changed as the pH of the solution decreased or increased. As a result of adsorption of hydrogen and hydroxide ions on the surface of phosphatidylcholine bilayer, the phosphatidylcholine molecule could exist in four different forms: PCH^+ with H^+ adsorbed, $PCOH^-$ with OH^- adsorbed and PCHOH with both H^+ and OH^- ions adsorbed on the surface and a free phosphatidylcholine molecule PC i.e. with no ions adsorbed. The relative contributions of the above forms were dependent on the pH.

The analysis of data obtained by chronoamperometry method provided clues to better understanding of the physico-chemical properties of lipid membranes and their associated physiology. Chronoamperometry allowed on simple and precise estimation of the membrane electrical parameters. The proposed method was also quick, which is particularly important during the examination of time-dependent phenomena in bilayers.

References

1. D. D. Lasic, *Liposomes from Physics to Applications*, Elsevier, Amsterdam (1993).
2. F. Frézard, *Braz. J. Med. Biol. Res*., 32 (1999) 181.
3. A. Hubbard, *Electrophoresis of Liposomes in Encyclopedia of Surface and Colloid Science*, Marcel Dekker, New York (2002).
4. H. T. Tien, *Bilayer lipid Membrane (BLM). Theory and Practice*, Marcel Dekker, New York (1974).
5. M. Naumowicz and Z. A. Figaszewski, L. Półtorak, *Electrochim. Acta*, 91 (2013) 367.
6. D. F. Evans, G. Pye, R. Bramley, A. G. Clark, T. J. Dyson and J. D. Hardcastle, *Gut*, 29 (1988) 1035.
7. D. R. Lide (Ed.), *CRC Handbook of Chemistry and Physics, Internet Version 2005*, http://www.hbcpnetbase.com, CRC Press, Boca Raton, FL (2005).
8. A. McDowell and B. J. McLeod, *Adv. Drug Del. Rev*., 59 (2007) 1121.
9. N. A. Rosen, W. E. Charash and E. F. Hirsch, *J. Surg. Res*., 106 (2002) 282.
10. Y. Zhou, R.M. Raphael, *Biophys. J*., 92 (2007) 2451.
11. A. D. Petelska, M. Naumowicz and Z. A. Figaszewski, *Langmuir*, 28 (2012) 13331.
12. S. Furuike, V. G. Levadny, S. J. Li and M. Yamazaki, *Biophys. J*., 77 (1999) 2015.
13. R. N. A. H. Lewis and R. N. McElhaney, *Biophys. J*., 79 (2000) 2043.
14. N. Düzgüneş and S. Nir, *Adv. Drug Del. Rev*., 40 (1999) 3.
15. J. M. Carrozzino and M. G. Khaledi, *J. Chromatogr. A*, 1079 (2005) 307.
16. T. J. Mclntosh, A. D. Magid and S. A. Simon, *Biophys. J*., 57 (1990) 1187.

17. A. K. Berkovich, E. P. Lukashev and N. S. Melik-Nubarov, *Biochim. Biophys. Acta*, 1818 (2012) 375.
18. A. D. Petelska and Z. A. Figaszewski, *Biochim. Biophys. Acta*, 1561 (2002) 135.
19. M. Montal and P. Mueller, *Proc. Natl. Acad. Sci. USA*, 69 (1972) 3561.
20. M. Naumowicz, A. D. Petelska and Z. A. Figaszewski, *Cell Biochem. Biophys*., 61 (2011) 145.
21. M. Naumowicz and Z. A. Figaszewski, *J. Electrochem. Soc*., 160 (2013) H166.
22. Z. Figaszewski, *J. Electroanal. Chem*., 139 (1982) 309.
23. S. Ohki, *Biophys. J*., 9 (1969) 1195.
24. A. W. Adamson, *Physical Chemistry of Surfaces, Wiley and Sons*, New York (1976).
25. A. D. Petelska and Z. A. Figaszewski, *Biophys. J.*, 78 (2000) 812.
26. V. L. Shapovalov, *Thin Solid Films*, 327/329 (1998) 599.
27. W. W. Sulkowski, D. Pentak, K. Nowak and A. Sulkowska, *J. Mol. Struct*., 744 (2005) 737.
28. F. Petriat and S. Giasson, *Langmuir*, 21 (2005) 7326.
29. S. Furuike, V. G. Levadny, S. J. Li and M. Yamazaki, *Biophys. J*., 77 (1999) 2015.
30. J. Stumpel, K. Harlos and H. Eibl, *Biochim. Biophys. Acta*, 599 (1980) 464.
31. A. D. Petelska and Z. A. Figaszewski, *Biochim. Biophys. Acta*, 1567 (2002) 79.
32. R. J. Clarke and C. Lüpfert. 1999. *Biophys. J*., 76 (1999) 2614.
33. G. J. Tawa, I. A. Topol, S. K. Burt, R. A. Calwell and A. A. Rashin, *J. Chem. Phys*. 109 (1998) 4852.
34. M. Bostrom, D. R. Williams and B. W. Ninham, *Phys. Rev. Lett*. 87 (2001) 168103.
35. M. Bostrom, D. R. Williams, P. R. Stewart and B. W. Ninham, *Phys. Rev. E Stat. Nonlin. Soft Matter Phys*., 68 (2003) 041902.