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Chronopotentiometric Technique as a Method for Electrical Characterization of Bilayer Lipid Membranes

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Abstract The basic electrical parameters of bilayer lipid membranes are capacitance and resistance. This article describes the application of chronopotentiometry to the research of lipid bilayers. Membranes were made from egg yolk phosphatidylcholine. The chronopotentiometric characteristic of the membranes depends on the current value. For low current values, no electroporation takes place and the voltage rises exponentially to a constant value. Based on these kinds of chronopotentiometric curves, a method of the membrane capacitance and the membrane resistance calculations are presented.

Keywords Chronopotentiometry · Bilayer lipid membrane · Phosphatidylcholine · Capacitance · Resistance

Introduction

All biomembranes are composed of a lipid bilayer intercalated with other constituents such as proteins, carbohydrates and their complexes of lipids. Insofar as can be determined, biomembranes are liquid-like and in a dynamic state. Thus, it is not surprising that today bilayer lipid membranes (BLMs), since the first report in 1961, are the most used model systems for biomembrane studies, as evidenced by the vast literature that now exists (e.g., Disalvo and Simon 1995; Hianik and Passechnik 1995; Iglic 2010; Jain 1972; Katsaras and Gutberlet 2001; Tien 1974; Walz et al. 2004).

The main reason for the sustained interest in experimental lipid bilayers is twofold: (1) basic scientific studies and (2) potential practical applications. The former is because cellular functions are membrane-bound, and the lipid bilayer is the central structural component of all biomembranes (Jain 1972; Tien 1974). Further, the vast majority of physiological/biochemical reactions involve a high degree of molecular recognition. These reactions are collectively known as "ligand–receptor interactions," for which the BLM system has often been used (Castellana and Cremer 2006; Helm et al. 1991; Nakashima et al. 1989).

Further, the BLM system allows the exquisite investigation of electrical properties (membrane potentials, resistance, current–voltage curves and membrane capacitance). Unlike most other model membranes, BLMs are dynamic, ultrathin and liquid-crystalline. Indeed, studies of the BLMs facilitate the initial testing of working hypotheses, which have generated guidelines for a better choice of reconstituted membrane experiments and have led to potential applications (Tien and Ottova-Leitmannova 2003a, b).

The advantages of the BLM are its high electrical resistance and high capacitance. Capacitance is a characteristic and well-reproducible parameter of BLMs. Membrane capacitance measurements are made for various purposes including, e.g., membrane thickness measurements (Hianik and Passechnik 1995), studies of membrane stability and of its formation process (Koronkiewicz et al. 1999), studies of surface potentials (Schoch et al. 1979), toxicological studies (Stern et al. 1992), investigation of photo effects (Stelzle et al. 1993) and studies of transport

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phenomena through the membrane (Alonso-Romanowski et al. 1995). Membrane capacitance can be measured by various methods, differing in the way of capacitance determination and the type of measurement signal applied to the membrane: measurements with a sinusoidal signal (Naumowicz et al. 2003), measurements with a triangular signal (Cherny et al. 1980), bridge methods (White 1970), pulse methods (Feldberg and Kissel 1975), compensation methods (Alvarez et al. 1983), conversion of capacitance to frequency (Kalinowski and Figaszewski 1992) and measurements of the minimum capacitance potential (Sargent and Hianik 1994).

Resistance (reciprocal of conductance) as an electrical property of nonpermeabilized planar lipid bilayer can be measured only during application of voltage or current signal (Pavlin et al. 2008). Galluci et al. (1996) developed the system for measuring conductance and capacitance simultaneously and continuously as a function of time. This method allows measurement of electrical properties of nonpermeabilized planar BLM as well as during the process of defect formation and electroporation (*electroporation* is a significant increase in the electrical conductivity and permeability of the membrane caused by an externally applied electrical field).

Chronopotentiometry is used mainly for investigations of the electroporation phenomenon in BLMs. Correctly selected current intensity allows generation of a single, stable and long-lived pore in the membrane, without its destruction (Koronkiewicz et al. 2001, 2002). A more advanced technique is programmable chronopotentiometry, where software controls the intensity and direction of current. This technique is useful for the investigation of electroporation and pore resealing and allows analysis of the recovery of membrane continuous structure (Koronkiewicz et al. 2002).

The present article continues our systematic study of the electrical properties of lipid bilayers. In the series of reports (e.g., Naumowicz and Figaszewski 2003, 2005, 2009; Naumowicz et al. 2003; Petelska et al. 2006), our general goal was to measure membrane capacitance and membrane resistance using electrochemical impedance spectroscopy. The application of chronopotentiometry in studying the process of electroporation has been described elsewhere (Genco et al. 1993; Kalinowski et al. 1998; Koronkiewicz and Bryl 1999; Koronkiewicz and Kalinowski 2004; Koronkiewicz et al. 2001, 2002; Robello and Gliozzi 1989). In this report, we show that the registration of chronopotentiometric curves at low-intensity current conditions allows a simple estimation of the electric capacity of the membrane and of the electric resistance of the membrane. The proposed method of determining membrane electrical properties is simple and precise. It is also quick, which is especially important during the examination of bilayers with properties that change over time. Both intervention of electrical signal (negligible,small) and disturbances of electrical properties are visible in the response curve. This method allows observation during the longer disturbances in electrical properties of BLMs in conditions of constant current flowing through the BLMs.

Theory

From an electrical point of view, a BLM can be regarded as a leaky capacitor model, as shown in Fig. 1, which is composed of a membrane capacitance, C_m, in parallel with a membrane resistance, R_m. The current flowing through electrodes creates a membrane voltage, U_m, which reaches a constant value in a few seconds (Fig. 2).

There are two components of the current flowing through the membrane, I, namely, the resistance current, I_R, and the charging current, I_C:

$$I_R = \frac{U_m}{R_m} \tag{1}$$

$$I_C = C_m \cdot \frac{dU_m}{dt} \tag{2}$$

where t denotes time.

Thus, according to Kirchhoff's law, the net current passing through the membrane can be expressed as follows:

$$I = I_R + I_C = \frac{U_m}{R_m} + C_m \frac{dU_m}{dt} \tag{3}$$

On the basis of the above equations, the following differential equation is obtained:

$$C_m \cdot R_m \frac{dU_m}{I \cdot R_m - U_m} = dt \tag{4}$$

which, after integration with initial conditions $t = t_0$ and $U_m = U_0$, can be converted into the following form:

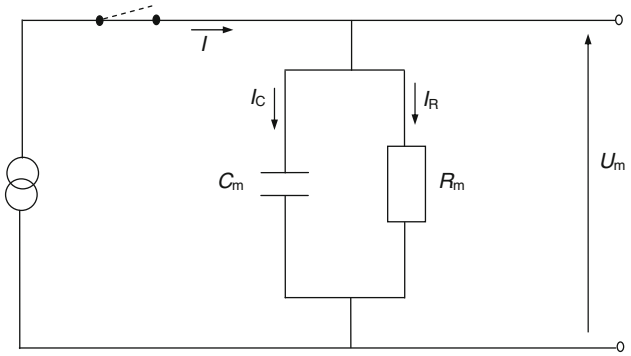

Fig. 1 An equivalent circuit describing the phosphatidylcholine membrane: R_m, resistance of the membrane; C_m, capacitance of the membrane; U_m, potential of the membrane; I_R, resistance current of the membrane; I_C, capacitance current of the membrane; I, total current flowing through the electrodes and the membrane

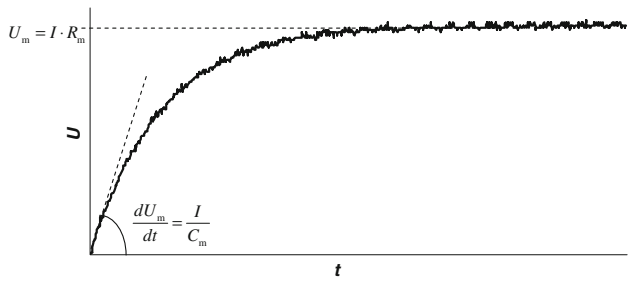

Fig. 2 Chronopotentiometric curve for the circuit equivalent to that shown in Fig. 1

$$U_m = I \cdot R_m - (I \cdot R_m - U_0) \cdot e^{\frac{t_0 - t}{C_m \cdot R_m}} \quad (5)$$

Assuming that $t_0 = 0$ and $U_0 = 0$, Eq. 5 can be simplified as follows:

$$U_m = I \cdot R_m - I \cdot R_m \cdot e^{-\frac{t}{C_m \cdot R_m}} \quad (6)$$

Considering the course of Eq. 6, one can state the following:

$$\frac{dU_m}{dt} = \frac{I}{C_m} \quad \text{when } t = 0 \quad (7)$$

$$U_m = I \cdot R_m \quad \text{when } t \to \infty \quad (8)$$

Figure 2 is a graph of the relationship between membrane voltage, U_m, and time, t. It is a typical chronopotentiometric curve for the equivalent circuit as presented in Fig. 1. In accordance with Eq. 7, when I is known, the value of membrane capacitance may be calculated from the slope of the curve. The smooth, linear part of the chronopotentiometric curve can be used for the calculation of membrane resistance according to Eq. 8.

Materials and Experimental Details

Chemicals and Preparation of the Forming Solutions

Egg yolk 3-*sn*-phosphatidylcholine was obtained from Sima (61755; St. Louis, MO). The lipid was dissolved in a mixture of *n*-hexadecane and *n*-butanol (10:1 by volume) to produce a concentration of 20 mg ml^{-1}. 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 1 week. The preparation and storage methods provided reproducible electrochemical properties when samples prepared at different times were examined using chronopotentiometric methods.

The electrolyte solution contained 0.1 mol dm^{-3} potassium chloride 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 (Gliwice, Poland). The KCl was calcined to remove any organic impurities.

All solvents were chromatographic standard grade. Hexadecane was purchased from Fluka (Neu-Ulm, Germany), and chloroform and butanol were obtained from Aldrich (Milwaukee, WI).

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 (Benz et al. 1975; Karolins et al. 1998). 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.

Bilayer 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. 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://winfast-pvr.software.informer.com). The bilayer areas were calculated from the photographs, taking into consideration the spherical nature of the surface and using the equations provided in Bronsztejn and Siemiendiajew (1996). The area of the bilayer membranes was about 6×10^{-2} cm^2.

Chronopotentiometric Measurements

The general architecture of the system used for chronopotentiometric measurements is shown in Fig. 3. The setup included a personal computer, a two-phase lock-in amplifier (EG&G Princeton Applied Research, Oak Ridge, TN; model 5210) and a potentiostat/galvanostat (EG&G Princeton Applied Research, model 273A), in which a

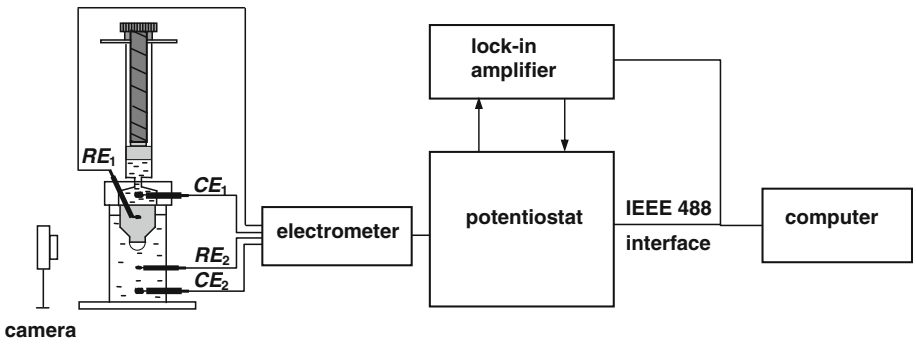

Fig. 3 General architecture of the measurement setup

four-electrode input was applied within the self-constructed electrometer.

The electrochemical cell used for chronopotentiometric measurement with a BLM system was essentially the same as that proposed by us for impedance measurements (Naumowicz et al. 2003; Naumowicz and Figaszewski 2003) and was placed in a Faraday cage during the measurement in order to decrease the background noise. The electrochemical cell contained two identical reversible silver–silver chloride electrodes (RE_1 and RE_2) and two identical current platinum electrodes (CE_1 and CE_2). The four-electrode potentiostat assured passage of current between the two platinum electrodes in such a manner as to hold constant the amplitude of voltage between the two reversible electrodes and measured the intensity and phase of current in the circuit CE_1–CE_2. The use of the four-electrode system in the studies of electrical phenomena occurring in membranes makes it possible to considerably reduce the errors caused by electrode and electrolyte impedance (Figaszewski 1982; Figaszewski et al. 1982).

Constant-current chronopotentiometric 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.

Results and Discussion

The chronopotentiometric characteristics $U = f(t)$, membrane potential as a function of time, of phosphatidylcholine membranes were recorded under constant-current conditions. The flow of constant current through bilayers causes charging of the membranes, with the speed of this charging depending on the value of the capacitance current. As a result, the membrane potential increases.

Three types of chronopotentiometric curves can be registered with different constant currents flowing through the lipid bilayer (Kalinowski et al. 1998; Koronkiewicz and Bryl 1999; Koronkiewicz and Kalinowski 2004). Figure 4 shows typical chronopotentiometric curves registered for analyzed phosphatidylcholine bilayers with different constant currents flowing through membranes: I, 5 nA; II, 17 nA; and III, 23 nA. The shape of these curves strongly depends on the current intensity. At low current intensity (Fig. 4, curve I), the membrane voltage increases within a few seconds, reaching a constant value described by Ohm's law. Higher current intensity (Fig. 4, curve II) causes a fast voltage increase until a certain, characteristic critical value and then a decrease to a characteristic value and oscillations around this value. However, the membrane is not destroyed. These oscillations were interpreted as generation (by constant current) of a pore changeable in size (Koronkiewicz et al. 2001, 2002). The third region of current intensities (Fig. 4, curve III) represents the currents causing membrane destruction—a fast voltage increase is followed by its sudden drop to zero.

The chronopotentiometric curves, similar to that presented by curve II in Fig. 4, are very useful in examining the process of pore formation, the changes in size over time and pore closing leading to complete membrane recovery. Moreover, this kind of chronopotentiometric curve may be the basis for calculating pore diameter (Kalinowski et al. 1998).

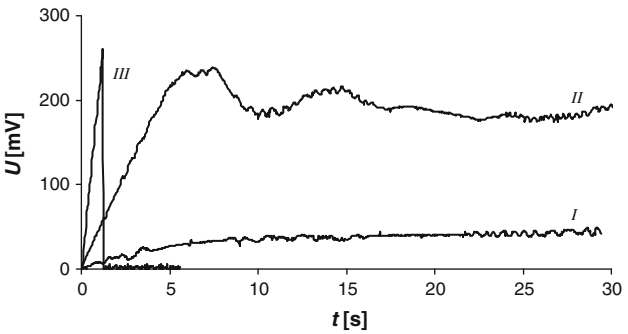

Fig. 4 Chronopotentiometric characteristics of BLMs at different constant-intensity currents: I, low current intensity (5 nA); II, intermediate current intensity (17 nA); III, highest current intensity (23 nA)

It should be emphasized that certain phenomena (in this case pore generation) can be more easily observed by the proper choice of current intensity and, more importantly, by proper programming of the current application—a constant current intensity throughout the experiments.

The curve shape depends on the current value. For low current values no electroporation takes place and the voltage rises exponentially to a constant value. Figure 5 shows examples of chronopotentiometric curves registered sequentially for one membrane at several different constant current intensities—the applied current values ranged 1–15 nA. The shape of these curves is very similar to curve I in Fig. 4: The voltage rises exponentially to a constant value. The curves presented in Fig. 5 demonstrate the sequence of measurements: After registration of the first curve, at 1 nA, measurements were terminated for 30 s. Then, the next chronopotentiometric curves were registered at higher constant-intensity currents. Registration was stopped at the moment the curve pointed at the beginning of the electroporation process in the analyzed membrane. It was possible to register several chronopotentiometric curves for one membrane. The same tendency was observed for all measurements.

The values of electric capacity and electric resistance of the membrane were derived from the $U = f(t)$ curves by least squares fitting. Capacitance was calculated from the slope of the curves according to Eq. 7. Considering Eq. 8 and the fact that R_m without pores obeys Ohm's law, resistance was calculated from the linear part of the chronopotentiometric curves. Next, both parameters were normalized for area of the bilayer. The dependence of the capacitance and resistance of phosphatidylcholine membranes is illustrated in Fig. 6 as a function of current intensity. The mean values of the determined parameters were obtained from six independent measurements of the lipid bilayer. Measurements of all analyzed membranes gave similar results, indicating a good reproducibility of the electrical behavior. As it is not possible to completely

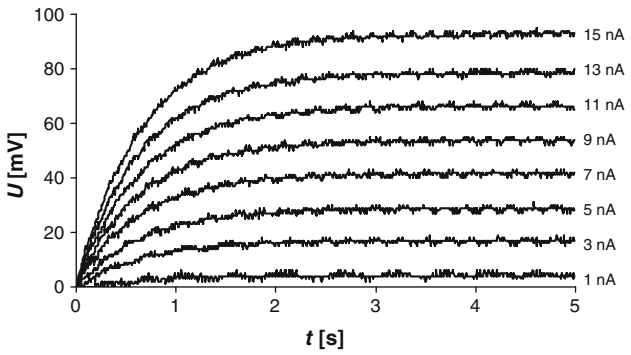

Fig. 5 Chronopotentiometric curves registered for a phosphatidylcholine bilayer. Current intensities are shown inside the figure

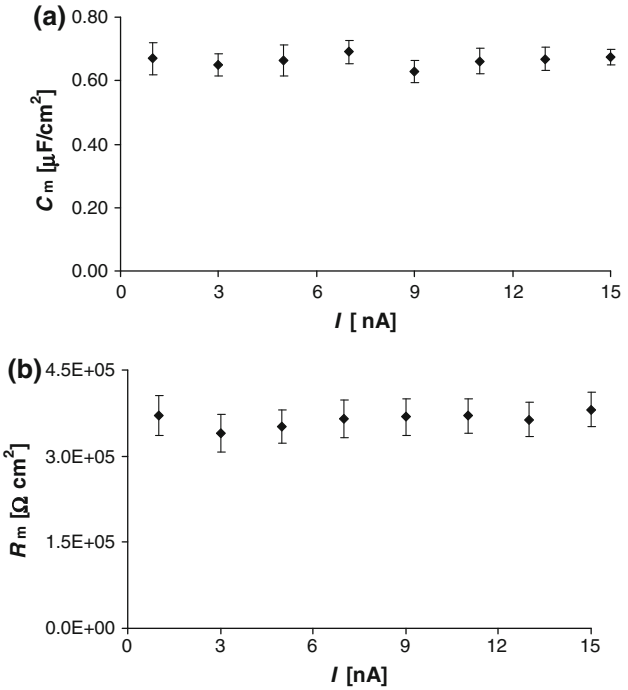

Fig. 6 Dependence of the capacitance, C_m, **a** and the resistance, R_m, **b** of the phosphatidylcholine membrane on the intensity current, I. Each point represents the mean value (± SD) obtained from six different BLMs

control the membrane formation process, every membrane is different; however, the C_m and R_m values are preserved. The mean capacitance value of a pure phosphatidylcholine bilayer is equal to 0.66 ± 0.04 μF cm^{-2}. This value has been reported in the literature (Coster 2003; Genco et al. 1993; Pavlin et al. 2008; Robello and Gliozzi 1989). The value of membrane resistance obtained for phosphatidylcholine membrane equals (3.64 ± 0.32) × 10^5 Ω cm^2. It should be noted that results similar to those obtained for membranes at very low current values (no electroporation) were obtained for passive *RC* circuit, with electrical parameters corresponding to the typical bilayer membranes formed in our laboratory (Naumowicz and Figaszewski 2005, 2009; Petelska et al. 2006).

Capacitance is a parameter that is considered to be the best tool for probing the stability and formal goodness of the lipid bilayer (Pavlin et al. 2008). Resistance may vary by at least one order of magnitude, possibly because of impurities in the BLM, border leakage at the membrane support, the appearance of lipid "crystals" at the periphery of the BLM or the method of introducing the lipid solution (if the forming solution is introduced with a microsyringe, instead of with a brush, the irreproducibility of bilayer can be minimized). The resistance of a single membrane, however, is usually constant until a short time before the membrane ruptures. Therefore, any changes in resistance due to addition of ions, proteins, drugs, etc., can be

determined with a relatively high degree of accuracy (Tien 1974).

The newly formed bilayer by our technique reaches stable resistance and capacitance values within 30–40 min. The reason that it takes 30–40 min until the newly formed bilayer displays stable R_m and C_m values can be attributed to the fact that the hydrophobic interior has not yet reached its completely ordered configuration. Residues of organic solvents (mostly hexadecane) must redistribute between the membrane core and the lipid deposit on the Teflon rim (pretreatment procedure), forming the Gibbs-Plateau border. Drainage of solvent residues to that border is accompanied by an increasing order of the hydrocarbon chains forming the hydrophobic interior of the bilayer.

The high capacitance values obtained for phosphatidylcholine membranes (Fig. 6) leave no doubt that the membrane is really a bilayer. In our earlier article (Naumowicz and Figaszewski 2003) we inferred that gramicidin D causes the formation of transmembrane ion channels in the membranes we created, which is an ultimate proof for the bilayer status of the lipid membrane (the gramicidin dimmer is too short to penetrate membranes thicker than bimolecular ones [Andersen 1984; Urry et al. 1971]). Based on our experimental results and numerous literature data (Benz et al. 1975; Smondyrev and Berkowitz 2001; Ziegler et al. 1998), we assume that our membranes do not stay solvent. If some of these quantities, which are not large in number, are contained in the membranes, then one should treat them as traces of impurities. Since it is impossible to determine the quantity of these impurities, it is impossible to make a thorough qualitative determination of their nature, so one cannot take them into account in quantitative considerations (except as a possible qualitative indication). If quantitative analysis were possible, we would take into account the possibility of the solvent's presence in the derived equations.

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