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Programmable chronopotentiometry as a tool for the study of electroporation and resealing of pores in bilayer lipid membranes

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Abstract

This paper presents the application of chronopotentiometry in the study of membrane electroporation. Chronopotentiometry with a programmable current intensity was used. The experiments were performed on planar bilayer phosphatidylcholine and cholesterol membranes formed by the Mueller–Rudin method. It was demonstrated that a constant-intensity current flow through the bilayer membranes generated voltage fluctuations during electroporation. These fluctuations (following an increase and decrease in membrane conductance) were interpreted as a result of the opening and closing of pores in membrane structures. The decrease in membrane potential to zero did not cause the pore to close immediately. The pore was maintained for about 200 s. The closing of the pore and recovery of the continuous structure of the membrane proceeded not only when the membrane potential equalled zero, but also at membrane potentials up to several tens of millivolts. The fluctuations of the pore were possible at values of membrane potential in the order of at least 100 mV. The size of the pore changed slightly and it closed after some time below this potential value.

Keywords: Electroporation; Resealing of pores; Lifetime of pores; Bilayer lipid membrane; Chronopotentiometry

1. Introduction

The application of electric fields of sufficient strength can cause biomembranes to enter a transient permeability state. In this high permeability state, the membrane can allow the flow of ions and macromolecules. This is consistent with the formation of pores and is commonly termed 'electroporation' [1].

Electroporation has numerous applications in molecular biology, biotechnology, and medicine. The most frequent applications are: (a) the transfection of cells [2,3], (b) introduction (incorporation) of fluorescent probes, enzymes and antibodies for research purposes [4–6], (c) loading cells with molecules for drug delivery purposes [7], (d) transporting molecules into and out of tissue for therapeutic purposes [8]. In spite of the widespread use of the electroporation technique in fundamental research, the detailed mechanism of pore formation and regeneration of the membrane after electroporation is not clear. Particularly, the kinetics and extent of pore expansion and its subsequent contraction are still poorly understood. For practical purposes, it is also important to know the pore size, the number of pores, how long they remain open and how they close.

Abbreviations: PC, phosphatidylcholine; BLM, bilayer lipid membrane

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On approaching the problems of electroporation, there are two opposing requirements. In basic research it is necessary to devise an experimental system which allows for the generation of pores that will live long enough. However, if pores persist for a long time, chemical and osmotic imbalances can develop in the cells. These imbalances can result in cell death [4,9]. After electroporation, the cells should maintain their natural integrity in subsequent suspension or culture. Therefore, from the three main systems applied in electroporation experiments, (1) cell suspensions, (2) individual cells, and (3) planar bilayer lipid membranes (BLM), the latter is very effective for basic research. Hence, BLM has been used as a model system to investigate the electroporation process. However, applying a steady potential difference across a BLM will cause the membrane to break. Therefore, with this method, only the action of very short electric pulses can be investigated [10]. However, the voltage applied as high-voltage pulses for a short duration may easily cause membrane rupture.

Hence, there is a need to find other techniques to help in solving the above-mentioned problems. There are several hints in the literature indicating the potential application of chronopotentiometry in studying the process of electroporation [11–15], while chronopotentiometry has been mainly used as a method for qualitative and quantitative analysis in analytical chemistry and for studying the mechanisms of electrode reactions [16]. We have also found several advantages in applying it in the study of the process of electroporation [17–19]. The method allows for the generation of voltage fluctuations in bilayer lipid membranes during electroporation. These fluctuations (following an increase and decrease in membrane conductance) were interpreted as a result of the opening and closing of pores in membrane structures [17]. Under the assumption that the high conductance state is due to one single cylindrical pore, the chronopotentiometric curve may be the basis for calculating the pore diameter [17,18]. Chronopotentiometry may also help to examine the mechanism of pore resealing (membrane recovery) after an electrical breakdown [19]. The last point seems to be very important in view of the fact that there have been several theoretical and experimental studies on pore formation, but the process of membrane recovery has not been examined sufficiently.

This inspired us to elaborate a method for studying the electroporation and, particularly, the process of pore resealing. In this work, programmable chronopotentiometry as a tool for studying electroporation and resealing of pores in bilayer lipid membranes is presented.

2. Materials and methods

Egg yolk phosphatidylcholine (PC) was purchased from Fluka (Buchs, Switzerland). Cholesterol was obtained from Sigma (St. Louis, MO, USA). *n*-Decane was from Aldrich (Gillinghem-Dorset, UK). Analytical grade KCl was obtained from POCh (Gliwice, Poland). The forming solution for bilayer membrane preparation contained lipids (20 mg/ml) dissolved in *n*-decane. The electrolytes (0.1 M KCl) were buffered with HEPES (Aldrich) to pH 7.0. Ul-

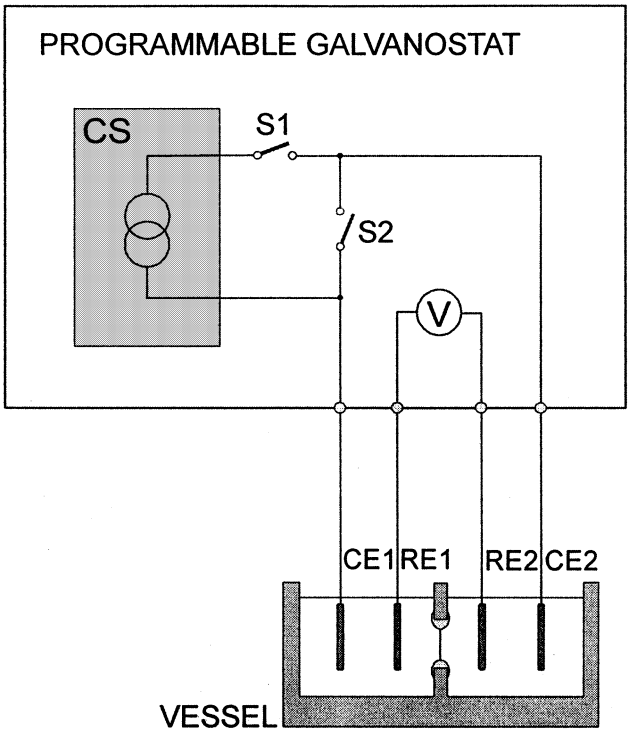

Fig. 1. Simplified diagram of the experimental setup. CS, programmable current source; V, potential meter; S1 and S2, switches; CE1 and CE2, current electrodes; RE1 and RE2, measuring electrodes.

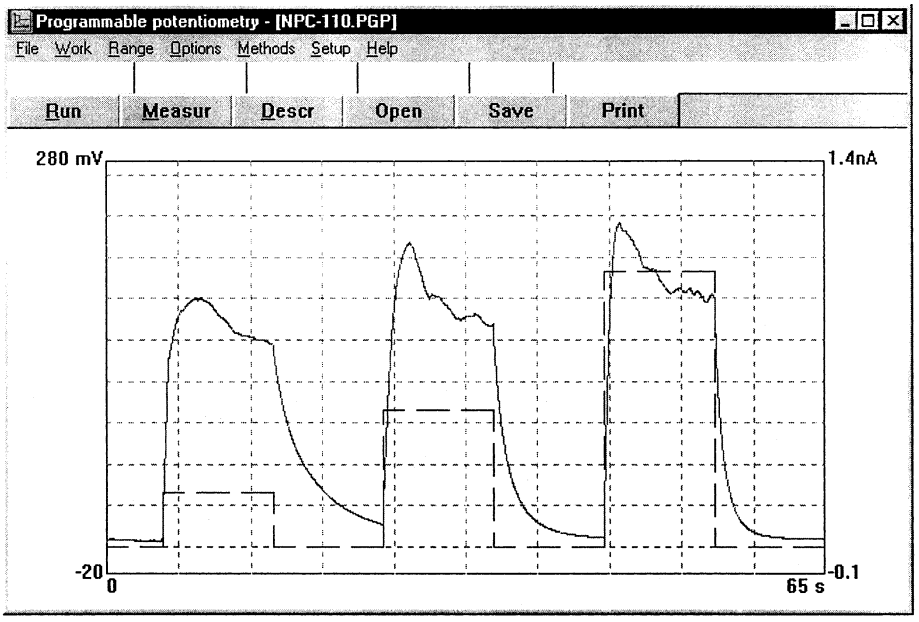

Fig. 2. The main window of the program for programmable chronopotentiometry. Solid line, left axis, potential changes; dashed line, right axis, programmed current changes.

trapure water was prepared with a Milli-Q system (Millipore).

The experiments were performed using planar bilayer membranes formed in an aperture in a septum separating two water solutions. The membranes were formed by the Mueller–Rudin method [20] in a vessel made from one piece of Teflon, consisting of two chambers with a volume of 10 cm^3 each. The septum between the chambers had a thickness of 0.3 mm and the diameter of the aperture was 1 mm. The process of spontaneous formation of the membrane was monitored by recording the membrane capacitance and by visual observation in transmitted light. The experiments were performed at a temperature of 23–25°C.

2.1. Measuring system and software

Programmable chronopotentiometry measurements were performed using the four-electrode potentiostat-galvanostat described earlier [21]. The measuring system was fully controlled by a PC computer and software working in a Windows (Microsoft) environment. A simplified diagram of the experimental setup is shown in Fig. 1. The system uses two pairs of (Ag-AgCl) electrodes. One of these pairs (CE1 and CE2) applies the current. They are connected to the current supply (CS) controlled by software using a 12-bit digital-to-analogue converter. The S1 switch causes switching of the on/off current flowing through electrodes. The S2 switch causes a shortening of the current electrodes CE1 and CE2 and as a result the membrane potential is forced equal to zero.

Two other electrodes (RE1 and RE2) are connected to the high input resistance amplifier and serve to measure the voltage across the bilayer (V). An analogue signal from the amplifier is converted

Table 1
Commands which can be used in a computer program for chronopotentiometric measurements

Command	Description
Level (i,t)	constant current of intensity i and duration t
Linear (i_1,i_2,s)	linear change of current from intensity i_1 to intensity i_2 with speed s
Open (t)	switch S1 (Fig. 1) is OFF and current equals zero for time t
Close (t)	switch S2 (Fig. 1) is ON and transmembrane potential is forced to equal zero for time t

by a 12-bit analogue-to-digital converter. The input resistance of the amplifier is higher than 10^{12} Ω and the input of the offset current is lower than 0.5 pA.

The main window of the program used for chronopotentiometric measurements is shown in Fig. 2. The current intensity during the experiment is drawn on the graphic area of the window. This current value may be set using any combination of four types of commands described in Table 1. The units in which the numbers existing in the commands are expressed and the units currently seen in the graphic window of the program are the same.

3. Results and discussion

The constructed setup and prepared software allowed chronopotentiometric measurements to be made in different programmable modes, which are named 'programmable chronopotentiometry'.

3.1. Constant-current conditions

The chronopotentiometric characteristics of bilayer lipid membranes were recorded under constant-current conditions. The flow of constant current through membrane causes the charging of the membrane with the speed of this charging depending on the value of the capacitance current. As a result, the membrane potential increases.

Depending on current intensity and membrane parameters, one out of three characteristic chronopotentiometric curves can be registered (Fig. 3). At low current, the membrane potential rises within seconds, reaching a constant value (Fig. 3, curve a). Higher current intensity causes a more rapid increase in membrane potential, which decreases after reaching a certain critical value. The membrane potential, however, does not reach the 0 mV value, indicating that the membrane is not destroyed. Instead, the membrane potential fluctuates at the preset level (Fig. 3, curve b). These fluctuations were interpreted as a generation (by constant current) of pore changeable in size [13,17]. The third region of current intensities represents the destruction of membranes induced by current (irreversible breakdown): the rapid increase in membrane potential is followed by its sudden disappearance (Fig. 3, curve c).

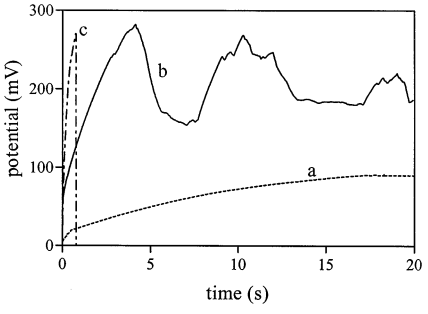

Fig. 3. Typical chronopotentiometric curves for bilayer lipid membranes registered under different constant currents: (a) 0.005 nA, (b) 0.2 nA, and (c) 2.0 nA. Forming solution: phosphatidylcholine/cholesterol (7:3 w/w). Electrolyte: 0.1 M KCl, HEPES pH 7.0.

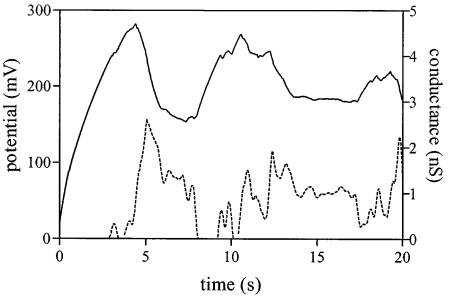

Fig. 4. Chronopotentiometric curve for bilayer lipid membrane registered under a constant current of 0.2 nA (solid line, left axis). Dashed line, right axis, calculated pore conductance. Mean conductance was about 1 nS, calculated pore diameter was about 2 nm. Forming solution: phosphatidylcholine/cholesterol (7:3 w/w). Electrolyte: 0.1 M KCl, HEPES pH 7.0.

The chronopotentiometric curves, similar to that presented in Fig. 3 (curve b), 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 the pore diameter. The procedure of calculation is described in detail elsewhere [17]. Under the assumption that the high conductance state is due to one single cylindrical pore, the calculated mean pore diameter described by the curves in Fig. 4 is $d=2$ nm.

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

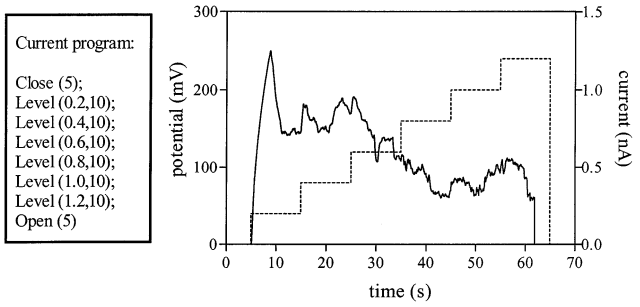

Fig. 5. Chronopotentiometric curve for bilayer lipid membrane registered under programmable current with stepwise changed intensity. Solid line, left axis, potential changes; dashed line, right axis, current changes. The intensity increased every 10 s and 0.2 nA. The frame on the left side of the figure contains the parameters of the current program. Forming solution: phosphatidylcholine/cholesterol (7:3 w/w). Electrolyte: 0.1 M KCl, HEPES pH 7.0.

3.2. Current with stepwise changed intensity

The application of the current can be changed stepwise with a programmed length of the step (time of current application) and height of the step (current intensity). Fig. 5 presents an example of a chronopotentiometric curve for membrane through which a current flows with a stepwise changed intensity: the intensity increased every 10 s and 0.2 nA up to 1.2 nA. The current with a starting intensity of 0.2 nA caused potential changes typical for electroporation of the membrane: a rapid increase in the potential up to about 250 mV, and its further decrease to a value of about 150 mV (compare Fig. 3, curve b). A stepwise increase of the current intensity provides interesting information on the current induced 'pore evolution'. The pore diameters calculated from the chronopotentiometric curve presented in Fig. 5 are shown in Fig. 6. It is easily observed that a higher intensity current generates a larger pore diameter. The question arises as to whether the observed changes reveal the consecutive accumulation of new pores or the expansion of a single pore. According to our previous work ([17] and discussion therein), the calculated pore diameter of approx. 5 nm (for 0.2 nA) was interpreted as the diameter of one pore. By changing the current intensity in separate experiments we could form pores with larger diameters [17]. Here, by changing the current intensity stepwise we could form a pore with a progressively enlarged diameter in one experiment. Hence, the 'current with stepwise changed intensity' mode of programmable chronopotentiometry may be applied for examining the process of pore evolution in time and pore expansion in space.

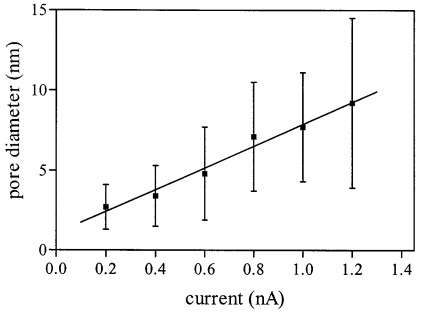

Fig. 6. Dependence of pore diameter on current intensity. The diameters were calculated from the data in Fig. 5. The standard deviations represent the changes of pore diameter (the amplitude of pore size fluctuations).

3.3. Current with regular interruptions for short circuit

Fig. 7 presents the chronopotentiometric characteristic when a constant current was supplied with interruptions to short-circuit the current electrodes. The following sequence of measurements was applied for this registration: 0.2 nA current flowing within 10 s, then 2 s interruption for the short circuit of current

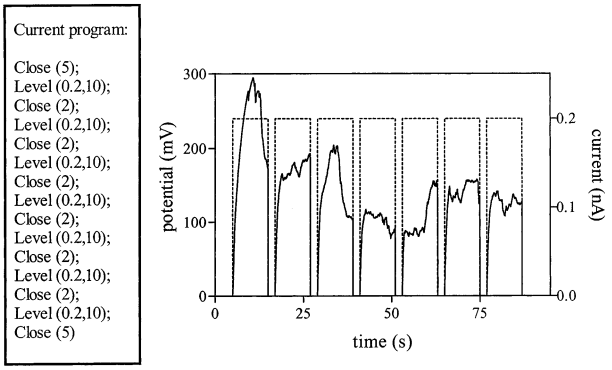

Fig. 7. Chronopotentiometric curves for bilayer lipid membrane registered under programmable current with regular interruptions for a short circuit of the electrodes. Solid line, left axis, potential changes; dashed line, right axis, current changes. Sequence of registration: 0.2 nA current flowing within 10 s, 2 s interruption for a short circuit of the current electrodes. The frame on the left side of the figure contains the parameters of the current program. Forming solution: phosphatidylcholine/cholesterol (7:3 w/w). Electrolyte: 0.1 M KCl, HEPES pH 7.0.

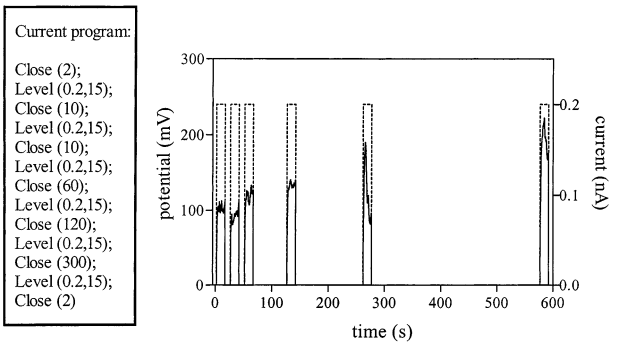

Fig. 8. Chronopotentiometric curves for bilayer lipid membrane registered under programmable current with changeable interruptions for a short circuit of the electrodes for an increasing period of time. Solid line, left axis, potential changes; dashed line, right axis, current changes. Sequence of registration: 0.2 nA current flowing within 15 s, interruption for a short circuit of the current electrodes. The time of interruption increases in consecutive repetitions. The frame on the left side of the figure contains the parameters of the current program. Forming solution: phosphatidylcholine/cholesterol (7:3 w/w). Electrolyte: 0.1 M KCl, HEPES pH 7.0.

electrodes. This sequence can be repeated and the number of repetitions can be regulated. The short-circuiting of the electrodes forced the membrane potential to zero. The chronopotentiometric characteristics indicate that the re-flow of current (after short-circuiting) induced membrane potential, whose value reached the level of potential just before the short-circuiting of the electrodes. Subsequently, further potential oscillations appeared. These results lead to the conclusion that the short-circuiting of current electrodes and forcing the membrane potential to zero caused the disappearance of the changes in pore size and the maintenance of its diameter. The membrane recovers its initial (before short-circuiting of current electrodes) conductance state.

A current with regular interruptions for short-circuiting is a novelty in the programmable chronopotentiometry approach to study membrane phenomena. It permits the examination of membrane phenomena under electrical potential and/or with a zero membrane potential.

3.4. Current with changeable interruptions for short circuit

Programmable chronopotentiometry also allows for an extension of the above method. The interruptions for short-circuiting can be consecutively applied and the timing of these interruptions can be changed. Fig. 8 demonstrates the following sequence of measurements: 0.2 nA current flowing within 15 s, followed by interruption for the short-circuiting of current electrodes. This sequence can be repeated and the number of repetitions can be programmed. The timing of the short-circuiting was changed in consecutive sequences. The chronopotentiometric characteristics indicate that the re-flow of current after a time not longer than 100 s induced the membrane potential, whose value reached the level of potential just before the short-circuiting of the electrodes. Based on the data obtained in this mode of measurements, it may be stated that the stable size of the pore (at membrane potential equal to zero) is maintained for at least 100 s, and by a time of 300 s the continuous membrane structure is fully recovered (the pores are fully closed).

The mode 'current with changeable interruptions for short circuit' of programmable chronopotentiometry provides information on the time required for pore resealing. The application of this technique to obtain basic data from artificial membrane systems is obvious. Moreover, it may be helpful in diagnosing

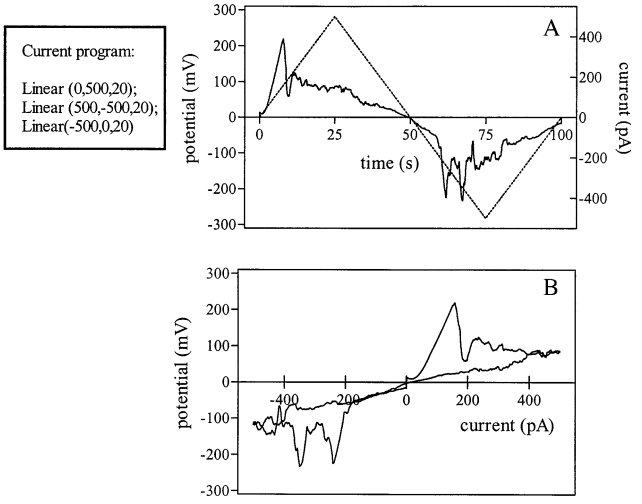

Fig. 9. (A) Chronopotentiometric curve for bilayer lipid membrane registered at programmable linearly changed current. Solid line, left axis, potential changes; dashed line, right axis, current changes. (B) Potential–current characteristics. The frame on the left side of the figure contains the parameters of the current program. Forming solution: phosphatidylcholine/cholesterol (7:3 w/w). Electrolyte: 0.1 M KCl, HEPES pH 7.0.

whether the membrane of a cell, for example, is 'recovered' or is still 'injured' after electroporation. It can also help in estimating the time required for full recovery.

3.5. Current with linearly changed intensity

The current intensity can be linearly changed: an increase within a certain period of time, followed by a decrease within the same period of time. This can be repeated with the current flowing in the opposite direction. The linear course of the current induces changes in the potential. Fig. 9A shows the changes in current and potential. The changes in current and potential as a function of time can be combined in the form of potential–current characteristics (Fig. 9B). The initial part of the curve is very similar to the potential–time characteristics registered under a constant current. An increase of membrane potential causes pore formation. Then, due to the increase in membrane conductivity, the membrane potential decreases. Decreasing current causes a decrease in potential and the disappearance of oscillations. A similar disappearance of oscillations (however, in current–voltage characteristics) was also observed by others [11,12]. For low current intensity, the dependence of the potential on current is almost linear. This indicates the constant resistance of the system. These observations lead to the conclusion that pores formed in membrane under small current intensities and small potentials have an invariable size in the period of time under study. A further increase in the intensity of current flowing in the opposite direction causes a sudden re-increase of the potential – indicating a decrease in membrane conductivity (a decrease in pore size). The shape of the curve leads to the conclusion that the pore was fully closed and was subsequently opened.

An analysis of potential–current characteristics suggests that: (a) a pore maintains almost a constant size for some time after the decrease in membrane potential, and (b) closing of the pore may take place at potentials of dozens of times higher mV and is a function mainly of time, but not potential.

The mode 'current with linearly changed intensity' supplements the above modes of programmable chronopotentiometry with a very useful function.

3.6. Summary

This paper presents programmable chronopotentiometry as a tool for the study of electroporation and resealing of pores in bilayer lipid membranes. The application of several modes of programmable chronopotentiometry is demonstrated.

The main conclusions can be summarised as follow: (a) the size of the pore changes to a slight degree after reducing the membrane potential to zero for at least several seconds; (b) the closing time of the pore and the time of regenerating the continuous structure of the membrane is up to hundreds of seconds; (c) pores close under membrane potentials of up to dozens of times of mV.

Programmable chronopotentiometry has also more general aspects. It enables the study of the effects of factors facilitating electroporation and regeneration of continuous membrane structures in model systems. The single registration of potential under a programmed current provides information not only on the process of pore formation, but also enables the analysis of the kinetics of pore closure.

Moreover, the obtained results should be important in the practical application of electroporation – defining the optimal conditions for the electroporation of cells, helping in designing systems for electroporation and protection against cell damage during this process.

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