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System for Measuring Planar Lipid Bilayer Properties

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Abstract We present a system for measuring planar lipid bilayer properties. The system is composed of a control unit, an output stage, an LCR meter, pumps for filling reservoirs, a bath with temperature regulation and a measurement chamber with four electrodes. The planar lipid bilayer is automatically formed using a folding method on apertures of different sizes. The automatization is assured by two syringes, which are clamped in actuators. Actuators are driven and controlled by a control unit via RS-232 communication. The temperature of the planar lipid bilayer can be regulated between 15 and 55 °C. The regulation is assured by insertion of the measurement chamber into the temperature-regulated bath. Different shapes of voltage- or current-clamp signals can be applied to the planar lipid bilayer. By measuring the response of the planar lipid bilayer to the applied signal, the capacitance and breakdown voltage of the planar lipid bilayer can be determined. The cutoff frequencies of the system output stage for voltage- and current-clamp methods are 11 and 17 kHz, respectively.

Keywords Electroporation · Capacitance · Breakdown voltage · Temperature regulation

Introduction

Electroporation is a phenomenon that describes the occurrence of structural changes in biological membranes as a consequence of applied electric pulses (Chen et al. 2006; Kotnik et al. 1997; Weaver and Chizmadzhev 1996). These structural changes are most often named "pores" and present an increase in cell membrane permeability. Electroporation is nowadays used in different fields like biology, medicine and biotechnology. Electroporation is divided into two different fields: irreversible electroporation and reversible electroporation. In irreversible electroporation, the cell membrane does not reseal pores after applied voltage and the cell dies. Irreversible electroporation is used in food production and preservation (Golberg et al. 2010), water cleaning (Vernhes et al. 2002) and tissue ablation (Davalos et al. 2005; Maor et al. 2009). In reversible electroporation, the cell membrane pores are resealed after application of electric pulses. It can be used to introduce substances into the cell. The best-known applications of reversible electroporation are electrochemotherapy (Sersa et al. 2008), transdermal drug delivery (Denet et al. 2004; Prausnitz 1999), gene therapy (Daud et al. 2008), cell fusion (Mekid and Mir 2000; Ogura et al. 1994) and insertion of proteins into membranes (Ouagari et al. 1995; Teissié 1998). The principles of pore formation are not yet fully elucidated. Recently, studies based on molecular dynamics proved that pores are formed in a lipid bilayer (Tieleman et al. 2003). When a lipid bilayer is exposed to an electric field, water wires are formed across the membrane. Then, the water wires expand into the water-filled pores, which are stabilized by reorganization of lipid molecules in the lipid bilayer (Levine and Vernier 2010). It is believed that the general picture of electroporation is the same for the planar lipid bilayer and biological cell membrane (Tarek 2005). Therefore, the lipid bilayer is considered the most important part of the cell membrane for studying pore formation.

Synthetic liposomes and vesicles are the simplest model of the biological cell membrane. They mimic the geometry of the biological cell membrane, but they do not have inner

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structures (Tekle et al. 2001). In comparison to synthetic liposomes and vesicles, planar lipid bilayers can have trapped solvent between the two bilayer leaflets. These can lead to differences in measured electrical properties and influences on pore formation in the lipid bilayer (White 1974, 1978). The planar lipid bilayer formed between two liquid solutions mimics a small fraction of the cell membrane, and it is accessible from both sides; therefore, the experiments are simpler than experiments on synthetic liposomes and vesicles (Benz et al. 1975; Huang et al. 1964; Mueller et al. 1963; Ottova and Tien 2002). Moreover, due to similar geometry usually modeled in molecular dynamic simulations, the results of both research methods can be combined and compared. Electroporation on small vesicles can also be performed using the molecular dynamic simulation.

Through the years, many planar lipid bilayer formation techniques between two liquid solutions have been developed: the tip-dip method (Coronado and Latorre 1983), the double-well chip method (Funakoshi et al. 2006), the cross-channel chip method (Funakoshi et al. 2006), the painting method (Mueller et al. 1963) and the folding method (Montal and Mueller 1972). The folding method is faster than other methods and can be easily automated. In the folding method, the lipid solution is spread on the liquid solution surface at each reservoir. In a few minutes, monolayers on the liquid solution surfaces are formed. After monolayer formation, the liquid solution levels in both reservoirs are raised. When the liquid solution surfaces cross the aperture between the reservoirs, the planar lipid bilayer is formed. This method is simple and quick and formation can be automated by computer-controlled syringe pumps.

From the electrical point of view, the planar lipid bilayer is considered a capacitor and resistor in parallel configuration. The capacitance and resistance are the most frequently measured electrical properties of a planar lipid bilayer. An additional electrical property of a planar lipid bilayer is breakdown voltage. It is one of the most important properties of a lipid bilayer when electroporation is under consideration. The capacitance is also a reference that the planar lipid bilayer is formed. If the capacitance of the planar lipid bilayer is lower than the expected value, then either multiple layers are formed or the planar lipid bilayer is not formed at all. Electrical properties of planar lipid bilayers are usually measured by two types of methods: voltage clamp and current clamp (Kramar et al. 2010). In the voltage-clamp method, a voltage signal is applied to the planar lipid bilayer and current, which flows through planar lipid bilayer, is measured. In the current-clamp method, a current signal is applied to the planar lipid bilayer and the voltage across the planar lipid bilayer is measured. The two methods use different-shaped signals like pulses, linear rising signals, sinusoids or triangular signals. Planar lipid bilayer capacitance, for example, is mostly measured using a discharge method (Kramar et al. 2010), a capacitance to period conversion method (Kalinowski and Figaszewski 1995) or an LCR meter (Punnamaraju and Steckl 2010).

Lipid bilayers can exist in a gel or liquid phase. The phase is defined by the mobility of the lipid molecules, which changes with temperature. The mobility of lipid molecules is higher in the liquid phase than in the gel phase; therefore, a lipid bilayer is in liquid phase at higher temperatures and in gel phase at lower temperatures. At a given temperature, a lipid bilayer can exist in either a liquid or a gel phase. With the phase transition also the thickness of the lipid bilayer is changed (Katsaras and Gutberlet 2010; Luckey 2008; Tokumasu et al. 2002). Because the capacitance of the planar lipid bilayer is inversely proportional to its thickness, also changes of the planar lipid bilayer capacitance have been observed (Antonov et al. 2003; Boheim et al. 1980). Moreover, Basu et al. (2001) showed that the conductance of the planar lipid bilayer is temperature-dependent.

To study the phenomenon of electroporation at various temperatures and provoked by various electrical signals, we developed a new system for measuring the properties of planar lipid bilayers. In the system, the folding method for forming planar lipid bilayers is implemented. The folding method is automated by two syringes, which raise and lower liquid solution levels in the measurement chamber. The temperature in the measurement chamber can be maintained at a constant value, which can be changed during the experiment. The system can be used to determine the planar lipid bilayer capacitance and breakdown voltage. The breakdown voltage can be measured by the voltage- or current-clamp method using a broad spectrum of signal shapes.

System Architecture

The system is composed of a control unit, an output stage, an LCR meter, pumps for filling reservoirs, a bath with temperature regulation and a measurement chamber with four electrodes. The control unit consists of an embedded PC, a control circuit, a digital to analog converter and an analog to digital converter (Figs. 1, 2).

Control Unit

The control unit consists of an embedded PC, a control circuit, an analog to digital converter and a digital to analog converter. This part of the system controls all switches, actuators and generators and acquires signals from sensors.

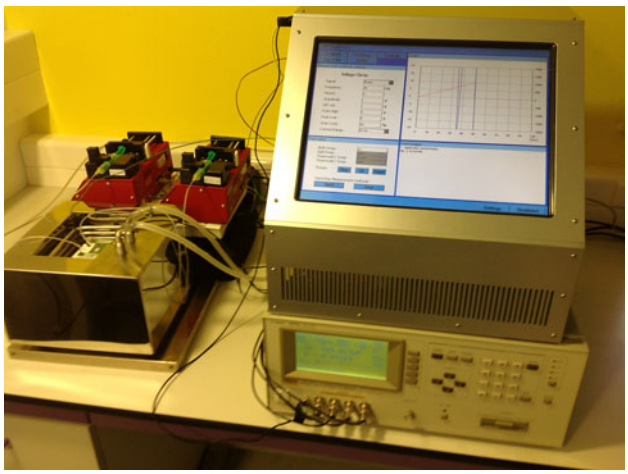

Fig. 1 The photography of the system for measuring the properties of planar lipid bilayers. On the left are two pumps for filling reservoirs and the bath with temperature regulation. The measurement chamber with four electrodes is inserted into the bath. The cables from the electrodes lead to the control unit, which is on the right. Below the control unit is the LCR meter

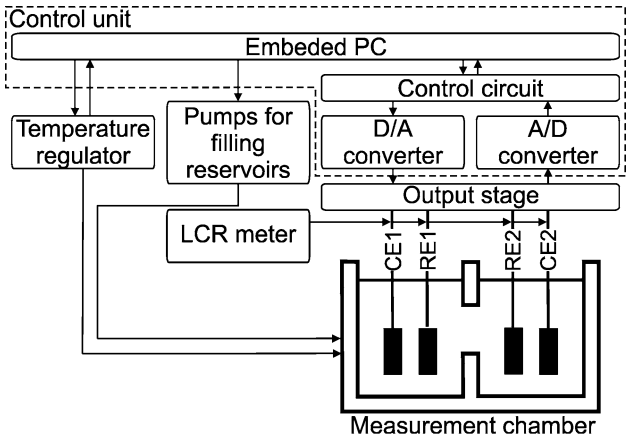

Fig. 2 The system for measuring the properties of planar lipid bilayer consists of the control unit, the LCR meter, the output stage, the pumps for filling reservoirs, the measurement chamber with four electrodes and the bath with temperature regulation. The control unit consists of the embedded PC, the control circuit, the digital to analog converter and the analog to digital converter

An embedded PC (Windows CE) is used as an interface between the human and the device. It has a graphical user interface, which allows setting of measurement method parameters and temperature. It also displays measured data and temperatures in both reservoirs and the bath. All acquired data are saved on a disc and can be accessible through Ethernet. By the press of a button, we can form planar lipid bilayers and start the measurement with the selected method.

The converters are a bridge between the analog and digital parts of the system. The digital to analog converter converts a digital signal from the control circuit to an analog signal, which is used by the output stage. The conversion is made at a frequency of 48 MHz with 14-bit resolution. It generates a bipolar signal between -2 and 2 V. These properties show that the generated signal is smooth and can contain high frequencies. The analog to digital converter converts an analog signal from the output stage to a digital signal, which is acquired by the control circuit. The conversion is made at a frequency of 150 kHz with 12-bit resolution. The analog voltage input can vary between -2 and 2 V. Both analog signals range between -2 and 2 V because the breakdown voltages of already measured planar lipid bilayers from the literature are in this range.

LCR Meter

The capacitance of a planar lipid bilayer is measured by an Agilent (Santa Clara, CA, USA) LCR meter 4284A. The LCR meter is connected directly to electrodes in the measurement chamber. The Agilent LCR meter 4284A can measure capacitance and resistance in different formations (parallel and serial). The LCR meter applies the sinus signal to the load and measures the response. We can set the parameters of the agitating sinus signal like frequency from 20 Hz to 1 MHz and the effective value from 0.005 to 2 V.

Output Stage

The output stage is a circuit, which combines the voltage- and current-clamp measuring circuits. The output stage has an input and an output that are connected to converters and four connectors for electrodes. Two of them are current electrodes (CE1 and CE2) and other two are reference electrodes (RE1 and RE2).

The voltage- and current-clamp methods that are implemented in our system are designed similarly. Both circuits have a current source and differential amplifier. The voltage-clamp method has closed-loop regulation, and the current-clamp method has open-loop regulation. The idea for the circuits was found in the literature (Kalinowski and Figaszewski 1995); our system has an additional resistor connected to the current electrodes for current source stabilization. The current source in our system generates current, which flows through an added resistor and planar lipid bilayer.

In the voltage-clamp method (Fig. 3a), the voltage is applied to the planar lipid bilayer and the current through the planar lipid bilayer and parallel resistor is measured. The differential amplifier measures the transmembrane voltage. The single-ended output of the differential amplifier is compared to the input voltage. The difference between the signals drives the current source, which forces

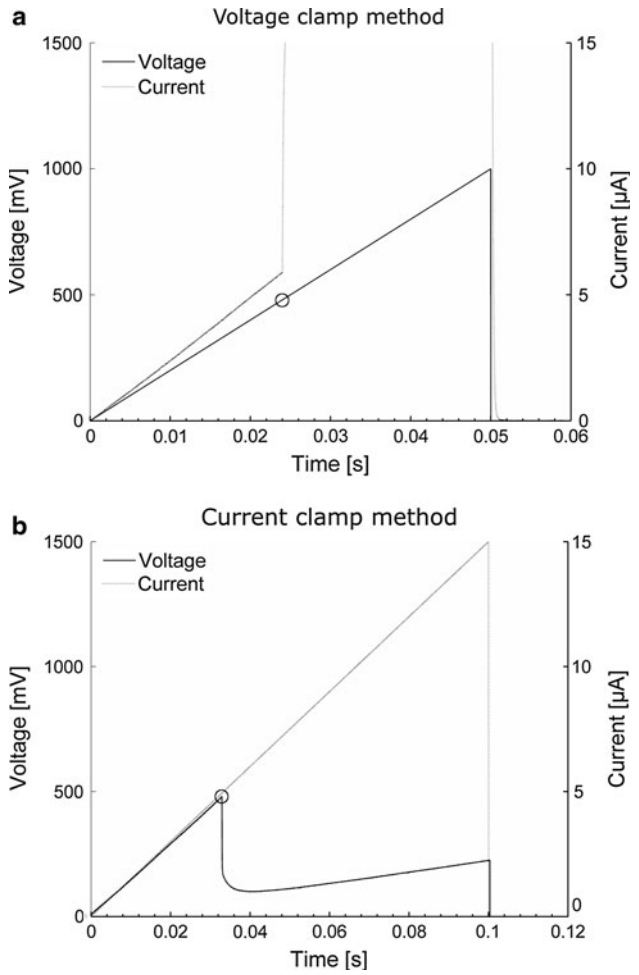

Fig. 3 The scheme of the circuit for the voltage-clamp method (**a**) and the scheme of the circuit for the current-clamp method (**b**). Both circuits consist of a differential amplifier and current source. The difference is only in the realization of the feedback loop. In the voltage-clamp method, the voltage is applied to the planar lipid bilayer and the current, which flows through the planar lipid bilayer and the resistor, is measured. In the current-clamp method, the current through the planar lipid bilayer and the resistor R_{I2} is forced and the voltage difference on the planar lipid bilayer is measured

current through the planar lipid membrane and resistor R_{I2}. The voltage at the output of the operational amplifier, which drives the current source, is proportional to the current which is forced through the planar lipid bilayer and resistor R_{I2}. The generated current is equal to the quotient between the driving voltage and resistor R_{I1}. The resistors R_{I1} and R_{I2} have a value of 100 kΩ. Capacitors C_{I1} and C_{I2} and resistor R_{I2} are added to the circuit to stabilize the current source and prevent oscillations. Capacitors C_{I1} and C_{I2} have a capacity of 33 and 100 pF, respectively.

The current-clamp method (Fig. 3b) is used to observe the transmembrane voltage response caused by forced current. The input voltage drives a current source, which forces current through the current electrodes and resistor R_{I2}. The voltage response on the planar lipid bilayer is measured by reference electrodes.

In both methods, different shapes of signals can be used, e.g., pulse, step change, linear rising signal or arbitrarily shaped signal. In the voltage-clamp method the output voltage range is between −1.5 and 1.5 V, with accuracy of 1 mV; the measured current ranges from −15 to 15 μA, with accuracy of 0.05 μA. In the current-clamp method, the output current range is between −15 and 15 μA, with accuracy 0.02 μA; the measured voltage ranges from −1.5 to 1.5 V, with accuracy 4 mV.

Pumps for Filling Reservoirs

The measurement chamber has two channels for filling reservoirs. Into each channel, a pipeline is inserted. Pipelines connect the reservoirs with the syringe filled with liquid solution. The syringes are clamped into actuators (Aladin-1000; World Precision Instruments, Sarasota, FL, USA). The Aladin-1000 is a syringe pump that can be driven via RS-232 communication. The syringe pumps are driven by an embedded PC, where we can set the volume which will be pumped into each reservoir. Each pump can be driven separately; therefore, we can avoid errors of liquid solution levels caused by asymmetry of the reservoirs. On the other hand, if asymmetric filing of the reservoirs is needed, the system allows setting this condition. Our system enables us to form planar lipid bilayers by the folding method by one press of a button.

Measurement Chamber

The measurement chamber is made of Teflon because it is highly resistant to chemicals and has a hydrophobic surface. The hydrophobic surface favors contact with lipid hydrophobic tails (Montal and Mueller 1972); therefore, the boundaries of the planar lipid bilayer can be linked to the edge of the aperture on the measurement chamber. The measurement chamber has two cubed reservoirs, which are connected with a round aperture. Each reservoir is made of a separate piece of Teflon. The round hole with a diameter of 3 mm connects the two reservoirs. Between the two reservoirs is a 25.40 μm-thin Teflon sheet with a round aperture of different sizes. The aperture is placed in the center of the connecting hole between the two reservoirs. The measurement chamber has two channels to each reservoir (Fig. 4a). In one channel the temperature probe is inserted, and in the other channel the pipe for filling the reservoir is inserted. The pipes are connected to the pumps, which fill or empty reservoirs.

Four electrodes made from Ag–AgCl (E255; IVM, Healdsburg, CA, USA) are inserted into the measurement chamber as shown in Fig. 4b. Two are current electrodes

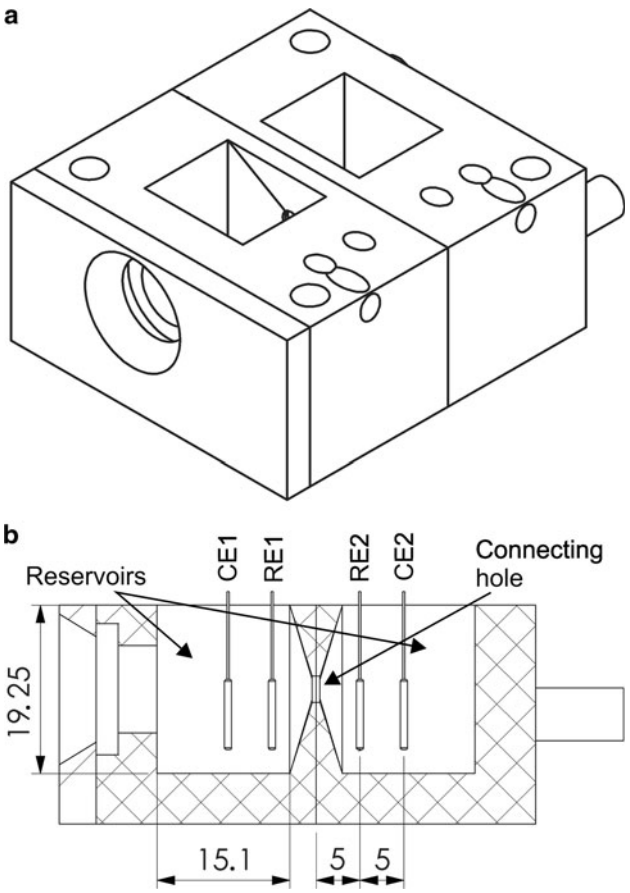

Fig. 4 The measurement chamber for formation of the planar lipid bilayer by the folding method. Perspective view reveals the construction of the measurement chamber (**a**). The Teflon sheet with aperture is inserted between the reservoirs. Lateral cut of the measurement chamber (**b**) reveals details and dimensions of the measurement chamber. *CE1*, *RE1*, *RE2* and *CE2* are Ag–AgCl electrodes. Chamber dimensions and electrode positions are in millimeters. The dimension of the reservoir perpendicular to the sketch is 17.8 mm. The hole, which connects the reservoirs, has a diameter of 3 mm

(CE1 and CE2), and the other two are reference electrodes (RE1 and RE2).

Temperature Regulation

The temperature of a planar lipid bilayer is ensured by a temperature-regulated bath. The bath is constructed of stainless steel. The inside dimensions of the bath are 150 × 150 × 100 mm. The bath is surrounded with 40-mm-thick insulation, and the bath cover has 20 mm of insulation. The coil, which is inserted into the bath, is used to heat or cool the medium and air in the bath. Through the coil flows medium with precisely regulated temperature. Regulation of the medium temperature is made by a Solid State (Wappingers Falls, NY, USA) ThermoCube 300. This

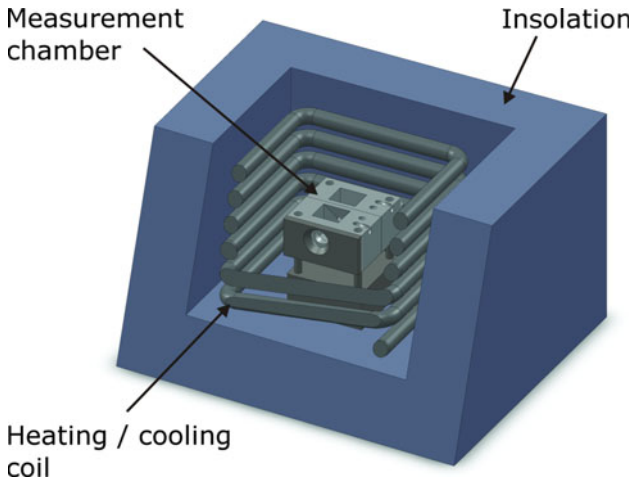

Fig. 5 The temperature-regulated bath with the measurement chamber. The bath is constructed of stainless steel and insolation. The heating coil and the measurement chamber are inserted into the bath

device can regulate medium temperature between 5 and 65 °C. The liquid solution that is in contact with the planar lipid bilayer can, however, achieve a temperature of 15–55 °C, which can be measured with an accuracy of 0.5 °C.

The measurement chamber is inserted in the temperature-regulated bath (Fig. 5). The temperature of the planar lipid bilayer is measured by two K-type thermocouple probes. They are inserted as close as possible to the planar lipid bilayer; therefore, the probes are inserted into reservoirs through the channels. One additional thermocouple probe is inserted into the bath. On the user interface, we set the temperature of the coil and measure the temperatures in the bath and both reservoirs.

System Evaluation

The system for measuring the properties of a planar lipid bilayer was evaluated using the frequency characteristic of the output stage, comparison of the measurement chambers with two sizes of the aperture and comparison of the voltage- and current-clamp measurement methods.

Chemicals

Lipids were prepared from 60 % lecithin (Fluka Analytical, Seelze, Germany), which was dissolved in a solution of hexane and ethanol at a ratio of 9:1. The mixture of hexadecane and pentane at a ratio of 3:7 was used for torus formation. The liquid solution consisted of 0.1 M KCl and 0.01 M HEPES in the same proportion. NaOH was added to obtain pH 7.4.

Methods

To evaluate the output stage, the electrode outputs, which lead into the same reservoir, were connected together (Fig. 4); the CE1 and RE1 outputs were connected together and the CE2 and RE2 outputs were connected together. On the input of the output stage, we applied the sinus signal with amplitude 1 V and frequencies from 1 Hz to 18 kHz generated by the Function/Arbitrary Waveform Generator 33250A. The input and output signals of the output stage were measured by a Tektronix (Beaverton, OR, USA) MSO4104 oscilloscope. We calculated gain and phase between output and input signals. Acquired data were analyzed using MATLAB software (Mathworks, Natick, MA, USA).

The parallel capacitance of the measurement chamber with and without formed lecithin planar lipid bilayers on two apertures with diameters of 126 and 197 μm was measured by the Agilent LCR meter 4284A. Measurements were done at 50 mV effective voltage, 1 kHz frequency and 25 °C temperature. The lecithin planar lipid bilayer is in liquid phase due to its mixture of unsaturated lipids, which have phase transition at low temperatures. At each aperture diameter, we performed 100 measurements. The difference between capacitances when the lecithin planar lipid bilayer was formed and when it was not present is the capacitance of the planar lipid bilayer. This value of the capacitance was divided by the area of the aperture. The result is specific capacitance of the lecithin planar lipid bilayer. At this point, it is not considered that the planar lipid bilayer has the Plateau-Gibbs border; therefore, the specific capacitance can be loaded with an error.

Voltage- and current-clamp methods to measure planar lipid bilayer breakdown voltage were tested on lecithin planar lipid bilayers formed on an aperture with a diameter of 126 μm at temperature 25 °C. During this test, we measured also the capacitance of each planar lipid bilayer to prove its correct formation. In the voltage-clamp method, we used linear rising voltage with slope 20 V/s. In the current-clamp method, we used a linear rising current with slope 150 μA/s. In each method, we performed three measurements. The mean values of breakdown voltage were calculated. Finally, the breakdown voltages obtained by the two methods were compared.

Results

Evaluation of the output stage has shown that, in the voltage-clamp and current-clamp methods, gain and phase between the output and input signals are close to 0 dB and 0° for frequencies from 1 Hz to 1 kHz. At higher frequencies, the gain and phase start to increase in the voltage-clamp method and decrease in the current-clamp method. This is expected because these two methods have inverted inputs and outputs. The voltage-clamp circuit reaches 3 dB gain at 11.0 kHz. The current-clamp circuit has −3 dB gain at 17 kHz. The phase of the circuits never reaches values of −45° or 45°. The frequency when the gain reaches −3 dB is called a "cutoff frequency." In our system, the voltage-clamp method has a lower cutoff frequency. The frequency characteristics for voltage and current clamp are shown in Fig. 6.

The lecithin planar lipid bilayer-specific capacitances were measured at 0.386 ± 0.027 and 0.381 ± 0.021 μF/cm^2 for apertures with diameters of 126 and 197 μm, respectively. The values are similar to the data reported in the literature (Naumowicz et al. 2003). The results also show that aperture size does not affect the specific capacitance.

The breakdown voltage was measured by applying linearly rising current or linearly rising voltage on the planar lipid bilayer. In the voltage-clamp method (Fig. 7a), planar lipid bilayer breakdown is detected by a dramatic increase of the current, while in the current-clamp method (Fig. 7b), planar lipid bilayer breakdown is detected by a sudden voltage drop. The breakdown voltage detected using the voltage-clamp method was 480.0 ± 5.0 mV. The breakdown voltage measured using the current-clamp method was 480.50 ± 6.5 mV. The specific capacitances of all formed planar lipid bilayers were 0.38 ± 0.01 μF/cm^2. The predominant species in lecithin is 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). It was determined that the breakdown voltage of the POPC planar lipid membrane is 400 ± 6 mV in 100 mM KCl surrounding medium and use of voltage pulses (Meier et al. 2000). Using a linear rising signal to determine breakdown voltage can avoid multiple exposures to an applied signal. Kramar et al.

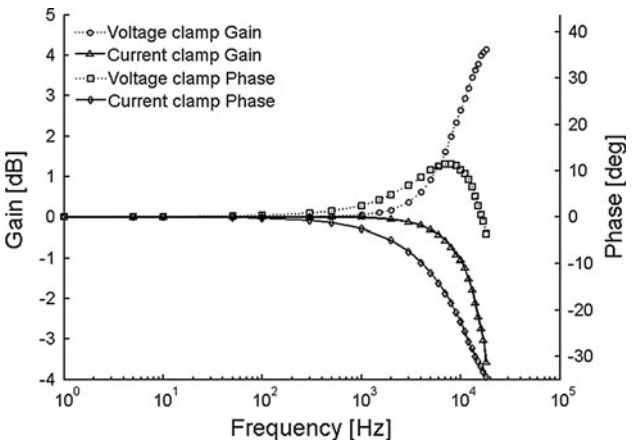

Fig. 6 Frequency characteristics for the voltage- and current-clamp circuits. The gain and phase for both circuits are shown for frequencies from 1 Hz to 18 kHz

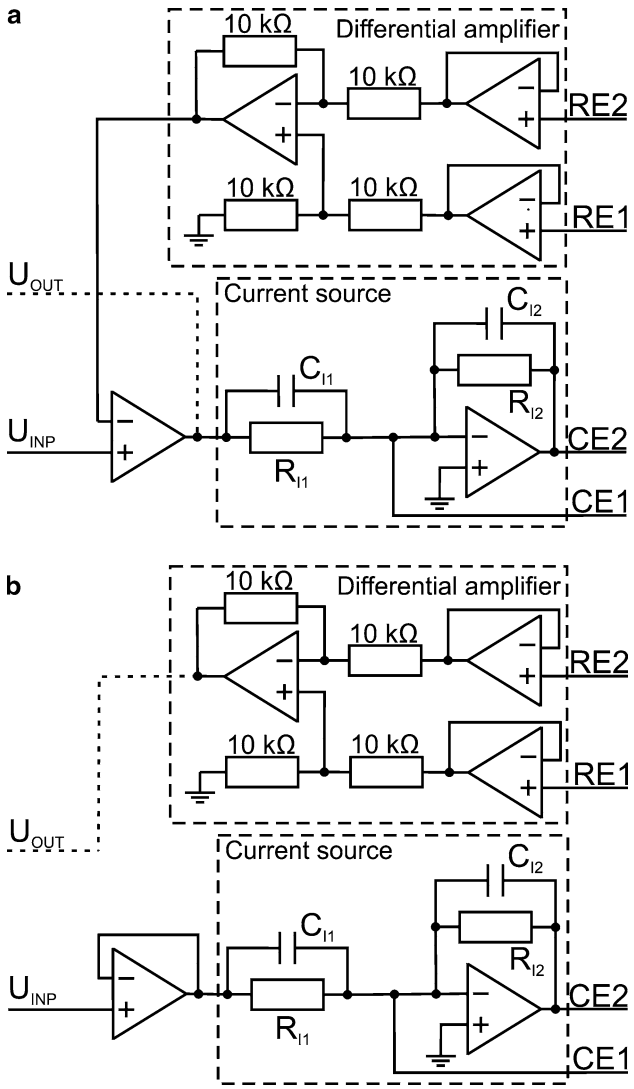

Fig. 7 The voltage and current signals acquired from the voltage- (**a**) and current- (**b**) clamp methods. In the voltage-clamp method, the rising voltage signal is applied. The current response is measured. At the beginning, the current rises proportionally to the applied voltage. When the current starts to increase more than before and goes out of the measured range, the planar lipid bilayer is broken. The value of voltage when this happens is voltage breakdown. In the current-clamp method the rising current signal is applied. The voltage response is measured, and it is proportional to the applied current. The planar lipid bilayer is broken when the measured voltage drops. The voltage value before the planar lipid bilayer is broken is breakdown voltage

(2007) found that breakdown voltage increases with increasing slope of the linear rising voltage signal. These measurements were performed using linear rising voltage signals with slopes from 4.8 to 48 kV/s. The minimum breakdown voltage of POPC in this study was 490 mV. In our experiment, we used a linear rising signal with slope of 20 V/s, due to expected lower values of the breakdown voltages. The measured breakdown voltages of the lecithin planar lipid bilayer using our new system are in the same range as the breakdown voltages of planar lipid bilayers using the other systems.

Discussion

We developed a system for formation of planar lipid bilayers and measuring planar lipid bilayer properties. The system allows the formation of planar lipid bilayers by the folding method. The electrical properties of a planar lipid bilayer which can be measured by this system are capacitance and breakdown voltage. Breakdown voltage can be determined by the voltage- and current-clamp methods. Using the voltage-clamp method we can generate voltage signals with 1 mV accuracy, while in the current-clamp method the system is able to measure voltage with 4 mV accuracy. The voltage and current signals can be generated as pulse, step change, linear rising signal or arbitrarily shaped signals. The cutoff frequencies of the system output stage are 11 and 17 kHz for the voltage-clamp and current-clamp methods, respectively. These two values show the dynamics of our system with open connectors. The voltage- and current-clamp methods were compared by measuring the breakdown voltage of lecithin planar lipid bilayers. In both cases, a linear raising signal was used to determine the breakdown voltage (Kramar et al. 2007). The results show that the two methods give similar breakdown voltages in similar conditions.

Planar lipid bilayers are automatically formed by the folding method. The automation is implemented by precise regulation of the liquid level in each reservoir. In this way, we are able to have the same hydrostatic pressure on both sides of the planar lipid bilayer, and each planar lipid bilayer is exposed to the same pressure conditions. This automation allows reproducible planar lipid bilayer formation and measurements at constant conditions. The measurement of planar lipid bilayer capacitance was tested on lecithin planar lipid bilayers at 25 °C. They were formed on apertures with diameters of 126 and 197 μm. The specific capacitances were 0.386 ± 0.027 and 0.381 ± 0.021 μF/cm^2, respectively. These values are similar to the literature data (Naumowicz et al. 2003). The results show that aperture size has no effect on measured specific capacitance.

The measurement chamber in our system is designed to form planar lipid bilayers by the folding method. The size of the aperture between the reservoirs, where the planar lipid bilayer is formed, is defined by the size of the aperture in the thin Teflon sheet which is inserted between the two parts of the chamber before experiments. Therefore, the size of the aperture can be easily changed by changing the Teflon sheet. The planar lipid bilayer can be formed on the aperture automatically. Moreover, we are able to have

the same pressure condition on a planar lipid bilayer at each formation by precisely regulating the liquid level in each reservoir.

Preliminary results confirm that the measuring system allows a broad spectrum of measurements. In particular, the temperature regulation can give new insights into planar lipid bilayer electroporation studies.

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