Bioelectrochemistry 144 (2022) 108004

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Effect of the cholesterol on electroporation of planar lipid bilayer

Peter Kramar *, Damijan Miklavčič

University of Ljubljana, Faculty of Electrical Engineering, Slovenia

ARTICLE INFO

Article history:
Received 7 June 2021
Received in revised form 18 November 2021
Accepted 21 November 2021
Available online 23 November 2021

Keywords:
Planar lipid bilayer
Cholesterol
Capacitance
Breakdown voltage

ABSTRACT

Electroporation threshold depends on the membrane composition, with cholesterol being one of its key components already studied in the past, but the results were inconclusive. The aim of our study was to determine behaviour of planar lipid bilayers with varying cholesterol concentrations under electric field. This would give us a better insight into cholesterol effect on membrane properties during electroporation process, since cholesterol is one of the major components of biological membranes and plays a crucial role in membrane organisation, dynamics, and function. Planar lipid bilayers were prepared from phosphatidylcholine lipids with 0, 20, 30, 50 and 80 mol% cholesterol. Capacitance was measured using the discharge method. Results show no statistical difference of c_{BLM} between the cholesterol concentrations. Breakdown voltage U_{br} of planar lipid bilayers was measured by means of linear rising voltage with seven different slopes. Obtained results were fitted to a strength-duration curve, where parameter U_{brmin} represents minimal breakdown voltage, and parameter τ_{RC} represents the inclination of the strength-duration curve. Adding cholesterol to planar lipid bilayer gradually increased its U_{brmin} until 50 mol% cholesterol concentration. Afterwards at 80 mol% U_{brmin} does not further increase, in fact it reduces by 20% of the U_{brmin} at 50 mol% cholesterol concentration.

1. Introduction

The interaction of the electric field with biological membranes and pure phospholipid bilayers has been extensively studied in the past decades [1-3]. A strong electric field can destabilize membranes and induce changes in their structure. The key parameter is the transmembrane voltage imposed by an external electric field due to the difference in the electric properties of the membrane and the external medium, known as Maxwell–Wagner polarization. According to the most widely accepted theory, the lipids in the membrane are then rearranged and aqueous pores are formed. This increases the conductivity of the membrane and its permeability to water-soluble molecules, which are otherwise deprived or have limited transmembrane transport mechanisms. This phenomenon is known as electroporation, sometimes referred to also as dielectric breakdown or electropermeabilization of the membrane [4-8]. The application of electroporation is emerging as a powerful technique for manipulation of cell membrane permeability, with wide-ranging use in biomedical and biotechnological applications [9,10], as well as food processing [11,12]. However, the fundamental biophysical processes and molecular-scale mechanisms involved in electroporation — the dynamics of electroporation-mediated molecular transport and the associated membrane structural rearrangements — are still not completely understood.

To improve understanding of the fundamental electroporation processes the experiments on artificial cell membrane models were used to mimic its behaviour. Phospholipid vesicles, small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and giant unilamellar (GUV) vesicles can be used to model the cell membrane geometry. Experimentally formed planar lipid bilayers can be considered as a non-curved part of a cell membrane assembled in controlled laboratory conditions. Planar lipid bilayers are often used to study electroporation phenomena since they are electrically accessible from both sides. They can be electrically represented as a resistor and a capacitor wired in parallel. Usually, two main characteristics of a planar lipid bilayer are measured: its capacitance and the voltage, which causes planar lipid bilayer rupture, *i.e.*, breakdown voltage. Measurement of capacitance provides information on the quality of the planar lipid bilayers after the self-assembly process while breakdown voltage determines its stability in the electric field [13]. In most studies' breakdown voltage of the planar lipid bilayer is determined by applying a rectangular voltage pulse, where amplitude of the voltage pulse is increased in small steps until the breakdown of the bilayer is obtained [14-17]. Therefore, the number of applied voltage pulses is not known in advance

* Corresponding author at: University of Ljubljana, Faculty of Electrical Engineering, Tržaška 25, 1000 Ljubljana, Slovenia.
E-mail address: peter.kramar@fe.uni-lj.si (P. Kramar).

https://doi.org/10.1016/j.bioelechem.2021.108004
1567-5394/

and each planar lipid bilayer is exposed to a voltage stress many times. Such a pre-treatment of the planar lipid bilayer affects its stability and consequently the determined breakdown voltage. In our study, a single voltage exposure to linear rising signal was used to obtain breakdown voltage [18]. On a molecular level, molecular dynamics (MD) simulations have been used modelling small patches (100 nm^2) of planar lipid bilayers containing hundreds of lipid molecules and associated interfacial and bulk water. MD simulation study provides visual information of molecule ordering in lipid systems, which cannot be seen in experiments. Basic Newton equations are solved based on provided energy relations between basic particles for example atoms when an all-atom simulation procedure is used, or coarse grain, where atoms are joined in bigger clusters. In MD simulation voltage across the lipid bilayer [19-21] induces the formation of an aqueous pore, which can be large and hydrophilic [22,23] or small and hydrophobic [24-26], and increases membrane permeability due to lipid peroxidation [27,28].

Cholesterol is one of the major components of biological membranes and plays a crucial role in its organisation, dynamics, and function. Therefore, its role in cell membrane electroporation is of great interest. Its effect has been studied on various levels of biological complexity as well as using MD simulations to understand the underlying mechanisms and the interactions between cholesterol and various types of lipids as primary components of cell membranes. Extensive reviews are available on cholesterol interactions with phospholipids in membranes [29,30]. Cholesterol has been studied on different model membranes such as vesicles, planar lipid bilayers, tethered lipid membranes, lattices, monolayers and computer MD studies as well as theoretical models. The foremost outcomes of performed studies are suggesting that cholesterol has the ability to modulate the physicochemical properties of phospholipids [31,32], embeds in lipid chains [33,34], forms micro and nanodomains [35,36], and changes the phase transition of the main lipid component [37-43], thickness of the membrane [33,35,39,44-48], permeability of the membrane [29,47-53], lipid dipole potential [54,55], dielectric constant [32,55], stiffness of the membrane [39,44,56], as well as breakdown voltage [50,57-60].

To emplace our experimental work, the most interesting studies of cholesterol interaction with membrane lipids are those, where an electric field is applied and its effect on cholesterol presence is studied. Raffy and Teissé analysed the stability of phospholipid vesicles under applied electric field and concluded that lipid membrane permeability is increased by increasing cholesterol content [52]. Needham and Hochmuth used the micropipette technique to determine the critical electric field strength for GUV breakdown as a function of the applied membrane tension. Their results show that the critical membrane voltage, required for breakdown increased with increasing cholesterol concentration [56]. Kakorin *et al.* studied membrane deformation and electroporation of SUV consisting of different phospholipid molecules with cholesterol. Both membrane bending rigidity and membrane stretching modulus increased with increasing cholesterol concentration. Furthermore, cholesterol also induced denser packing of the lipids and increased the lipid packing density [57]. Karolis *et al.* used low frequency impedance spectroscopy to determine the position of the cholesterol in the planar lipid bilayer [61]. Alobeedallah *et al.* studied capacitance and conductance of tethered bilayer lipid membranes at various cholesterol concentrations using electrical impedance spectroscopy [47]. In a recent study the presence of pore has been evaluated using voltage-current characteristics [48]. Koronkiewicz *et al.* observed the pore formation and their dynamics due to insertion of the cholesterol exposing planar lipid bilayer to constant current [62]. Naumowicz *et al.* measured change in the membrane capacitance and resistance at various cholesterol concentrations [49]. Corlovan *et al.* measured fluctuation of the lipids in planar lipid bilayers at various cholesterol concentrations [46]. Breakdown voltage dependence on the cholesterol concentration was also studied on planar lipid bilayers using voltage clamp pulses [17,63]. The effects of cholesterol lipid bilayers were also studied using MD simulation in different fields and interests, on which Róg and Vattulainen wrote an extensive review [30]. What is more, effects of the electric field on planar lipid bilayers during the process of electroporation were simulated [58,60]. Fernandez *et al.* observed an increase in thickness, reduction of area per lipid and dynamics of pore formation in applied electric field, in lipid bilayers with various cholesterol concentration [58]. Casciola *et al.* studied how low intensity millisecond pulses and high intensity nanosecond electric pulses effect the area per lipid, capacitance, breakdown voltage and pore formation dynamics for planar lipid bilayers with different cholesterol concentrations [60].

The aim of our study is to determine the value of specific capacitance and breakdown voltage of planar lipid bilayers using POPC lipids with various cholesterol concentrations. The stability of planar lipid bilayer under electric field is determined by the breakdown voltage, which is of ultimate interest for lipid bilayer electroporation studies. According to our measuring protocol, each lipid bilayer is exposed to the voltage signal only twice. First, the bilayer capacitance is determined by a discharge pulse method. Capacitance measurement reveals an intact lipid bilayer. In the next step, lipid bilayer breakdown is induced by a linear rising voltage signal. The obtained experimental results are compared to MD studies and other experimental studies that examine cholesterol influence on membrane properties such as capacitance, and stability in electric field applications.

2. Materials and methods

For planar lipid bilayer formation POPC lipids (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and Cholesterol (ovine wool, >98%) were used, both in powder (Avanti Polar-Lipids Inc. USA). Planar lipid bilayers were constructed using the Montal-Muller method [64]. In short, lipids were dissolved in a mixture of hexane and ethanol in 9:1 volumetric ratio. Mixture of hexadecane and pentane in volumetric ratio 3:7 was used for torus formation. When preparing two-component bilayers POPC and cholesterol were mixed together in appropriate ratio in small plastic tube before application. Planar lipid bilayer was made of POPC lipids with addition of 0, 20, 30, 50 and 80 mol% of cholesterol. KCl (0.1 M) and HEPES (0.01 M) were mixed together in a 1:1 volumetric ratio and applied as an aqueous electrolyte in the measurement chamber for planar lipid bilayer measurements. Its pH was adjusted to 7.4 by adding 1 M NaOH. Chamber, where planar lipid bilayers are built, is made of Teflon. It consists of two cubed reservoirs with volume of 5.3 cm^3 each. In the hole between two reservoirs, a thin 25 μm Teflon sheet with circular hole (100 μm in diameter) was inserted.

Measurement setup for following up electroporation of planar lipid bilayers was described previously [18]. Briefly, it consists of a signal generator, a Teflon chamber and a voltage and current amplifiers, which are used for measurements of transmembrane current and voltage using oscilloscope. Two Ag-AgCl electrodes, one on each side of the planar lipid bilayer, were immersed into the aqueous electrolyte. All the signals were processed offline using Matlab.

Measurement protocol consisted of two parts: i.) capacitance measurement and ii.) planar lipid bilayer breakdown voltage measurement. Capacitance and the breakdown voltage were determined for each formed planar lipid bilayer. The capacitance of

planar lipid bilayer was measured using discharge method as previously described [18]. The planar lipid bilayer capacitance (C_{BLM}) was normalised to the surface area of the orifice to obtain specific capacitance (c_{BLM}) for comparison with other studies. The breakdown voltage (U_{br}) of the planar lipid bilayer was determined by applying the linear rising signal. The slope of the linear rising signal (k) and the peak voltage of the signal has to be selected in advance. Seven different slopes were selected with slope of 4.8, 5.5, 7.8, 11.5, 16.7, 21.6, and 48.1 kV/s. Breakdown voltage was defined as the voltage at the moment t_{br} when sudden increase of transmembrane current was observed. Time of breakdown t_{br} was defined as a lifetime of the lipid bilayer at chosen slope of the linear rising signal [18].

To compare specific capacitances and breakdown voltages of the planar lipid bilayers exposed to voltage signals of different slopes (k) one-way ANOVA test was used. All pairwise multiple comparisons were made by Tukey's test. Pairwise multiple comparisons were performed by Dunn's method. When comparing the mean breakdown voltages at the same slopes (k) between one component lipid bilayers unpaired t-test was used. As the variances of mean breakdown voltages at slopes k = 7.8 kV/s and k = 11.5 kV/s were statistically different, the comparisons were made with Mann-Whitney's test. We rejected the null hypothesis of analyses if the p-value of the test was less than 0.05 ($p < 0.05$) regardless of the test used. Using nonlinear regression, a two-parameter strength-duration curve was fitted to the data [65]:

$$U = \frac{a}{1 - e^{-\frac{t}{b}}} = \frac{U_{brmin}}{1 - e^{-\frac{t}{\tau_{RC}}}} \quad (1)$$

where U was U_{br} measured at different slopes, t was corresponding t_{br}, and a and b are curve parameters [18]. The parameter a is an asymptote of the strength-duration curve, which corresponds to minimal breakdown voltage U_{brmin} for specific planar lipid bilayer. Parameter b governs the inclination of the strength-duration curve and, can be considered as a time constant of the system τ_{RC}. Since planar lipid bilayer can be simplified as capacitor and resistor in parallel, the time constant can be expressed as $\tau_{RC} = RC$. The upper and lower border of the 95% confidence interval of the fitted strength-duration curve was estimated using Matlab function *nlparci*. The pairwise comparison were performed for all obtained values for both parameters U_{brmin} and τ_{RC}. The null hypothesis of analyses was rejected if the p-value of the test was less then 0.05 ($p < 0.05$). For values that were not compliant with the hypothesis additional p-value of the test less then 0.2 was used ($p < 0.2$).

3. Results

In our study, specific capacitance c_{BLM} and breakdown voltage U_{br} of planar lipid bilayers consisting of POPC lipids and 0, 20, 30, 50 and 80 mol% of cholesterol.

Specific capacitance was measured before exposure to linear rising signal and breakdown voltage measurement by means of a discharge method. Values of the specific capacitance c_{BLM} were grouped according to cholesterol concentation. For each group mean value and standard deviation were obtained (Table 1). The pairwise comparison of the measured planar lipid bilayer specific capacitance c_{BLM} shows no significant difference between various cholesterol concentrations.

Breakdown voltage U_{br} was measured by means of linear rising voltage with seven different slopes: 4.8, 5.5, 7.8, 11.5, 16.7, 21.6, and 48.1 kV/s. Breakdown voltage U_{br} increases with increasing steepness k of the voltage applied as seen in Fig. 1A for POPC planar lipid bilayers. Pairwise statistical comparisons of the U_{br} were performed. In general, measured planar lipid bilayer breakdown voltage U_{br} was significantly higher when stepper slopes of linear rising voltage were applied. At slope k = 21.6 kV/s breakdown voltage U_{br} was statistically significantly higher than U_{br} measured at slopes k = 11.5 kV/s or less. At slope k = 16.7 kV/s U_{br} was statistically significantly higher from U_{br} measured at slopes k = 7.8 kV/s or less. Mean U_{br} values measured at slopes k = 4.8 kV/s, 5.5 kV/s and 7.4 kV/s showed no statistical difference. Similar statistical analysis was performed for mean breakdown voltages for all membrane compositions and similar statistical results, with respect to the linear rising signal voltage slopes were obtained irrespective of the membrane composition (Fig. 1B-E).

The measured data of breakdown voltage U_{br} and a lifetime t_{br} were fitted to strength-duration curve (Eq. (1)) to obtain parameters U_{brmin} and τ_{RC}. For all regression curves in Fig. 1A-E the 95% confidence interval of the fit upper and lower border has been plotted by thin solid lines. Obtained values of parameters U_{brmin} and τ_{RC} with their 95% confidence interval for different cholesterol concentrations are presented in Table 1.

Result shows that minimal breakdown voltage U_{brmin} depends on the composition of the planar lipid bilayer (Fig. 1F). Adding cholesterol to POPC planar lipid bilayer gradually increases its minimal breakdown voltage U_{brmin} until cholesterol concentration of 50 mol% is reached. Minimal breakdown voltage U_{brmin} of the planar lipid bilayer then decreases at 80 mol% cholesterol concentration (Fig. 2A). Statistical comparison between obtained values of U_{brmin} are significantly different, and the p-value of the test was less then0.05 ($p < 0.05$), except for the comparison between the POPC mixtures with 20 and 30 mol% of cholesterol concentrations where the p-value of the test was less then 0.2 ($p < 0.2$) and denoted with * on Fig. 2A.

The values of parameter τ_{RC} obtained by fitting data of strength-duration curve (Eq. (1)) at different cholesterol concentrations are presented on Fig. 2B. Statistical pairwise comparison between the values show no significant difference between various cholesterol concentrations except for 0 and 30 mol% cholesterol concentrations where the p-value of the test was less then 0.05 ($p < 0.05$), denoted with # on Fig. 2B and for 0 and 20 mol% cholesterol concentrations where the p-value of the test was less then 0.2 ($p < 0.2$), denoted with * on Fig. 2B.

4. Discussion

The aim of our study was to determine specific capacitance and breakdown voltage of planar lipid bilayers consisting of POPC lipids and 0, 20, 30, 50 and 80 mol% of cholesterol. POPC lipids were cho-

Table 1
Mean values of the measured planar lipid bilayer specific capacitance (c_{BLM}) with their standard deviation and fitted parameters (U_{brmin}) and (τ_{RC}) with 95% confidence interval obtained by means of strength-duration curve (eq. (1)). Number of measurements (N) for each planar lipid bilayer composed of POPC lipid molecules and five different cholesterol concentrations are noted as well.

POPC + Cholesterol mol%	c_{BLM} /μF/cm^2	U_{brmin}/V	τ_{RC}/μs	N
0	0.54 ± 0.13	0.55 ± 0.04	14.3 ± 3.8	48
20	0.53 ± 0.14	0.80 ± 0.09	25.2 ± 8.6	35
30	0.49 ± 0.13	0.97 ± 0.11	28.6 ± 10.2	21
50	0.55 ± 0.16	1.32 ± 0.09	19.1 ± 8.3	40
80	0.52 ± 0.12	1.17 ± 0.07	21.2 ± 6.2	25

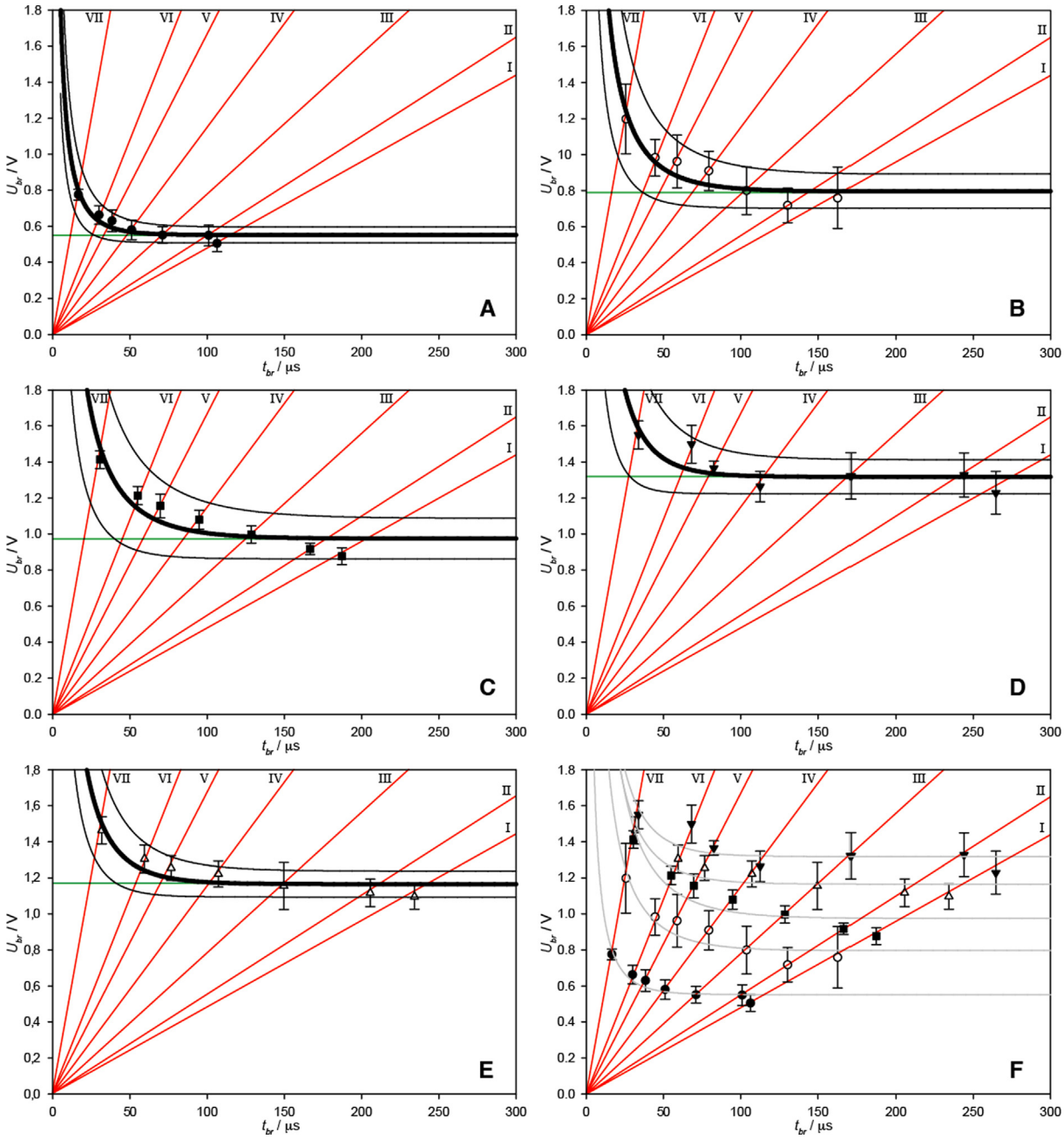

Fig. 1. Dependence of the planar lipid bilayer breakdown voltage U_{br} and lifetime t_{br} on steepness k of the applied linear rising voltage. All seven slopes of applied voltage are denoted with roman numbers I-4.8 kV/s, II-5.5 kV/s, III-7.8 kV/s, IV-11.5 kV/s, V-16.7 kV/s, VI-21.6 kV/s, VII-48.1 kV/s and plotted in red line. The breakdown voltage U_{br} of each planar lipid bilayer composition of POPC and cholesterol is represented: A) for pure POPC (●), B) POPC with cholesterol 20 mol% (○), C) POP with cholesterol 30 mol% (■), D) POPC with cholesterol 50 mol% (▼), E) POPC with cholesterol 80 mol% (△). The black thick solid line represents the fitted two-parameter curve (Eq. (1)) on the data measured. Corresponding thin solid lines represent the 95% confidence interval of the fit (upper border and lower border). The green line represents the value of parameter U_{brmin} which corresponds to minimal breakdown voltage. The error bars in U_{br} measurements represent standard deviation of the data. F) All measured data of the planar lipid bilayer breakdown voltage with their standard deviation. The grey solid lines represent the fitted two-parameter curve (Eq. (1)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sen because they are considered as model lipids for biophysical experiments, what is more their phase transition temperature is low enough to not have an effect on our experiments. Each lipid bilayer was exposed to the voltage signal only twice. First, to measure the capacitance of the planar lipid bilayer employing a discharge pulse method, and second to determine the breakdown voltage value by means of linear rising voltage method using seven different slopes: 4.8, 5.5, 7.8, 11.5, 16.7, 21.6, and 48.1 kV/s. Measured data of pure POPC planar lipid bilayers was fitted to a strength-duration curve (Eq. (1)), and is consistent with our previously published data [18,66].

Measurement of capacitance provides the information on the quality of the planar lipid bilayers after self-assembly process is finished. Value of capacitance indicates possible multilayer formation. The measurements of planar lipid bilayer capacitance were grouped according to the cholesterol molarity and normalised to the surface area of the orifice to obtain specific capacitance. The average and standard deviation values of specific capacitance

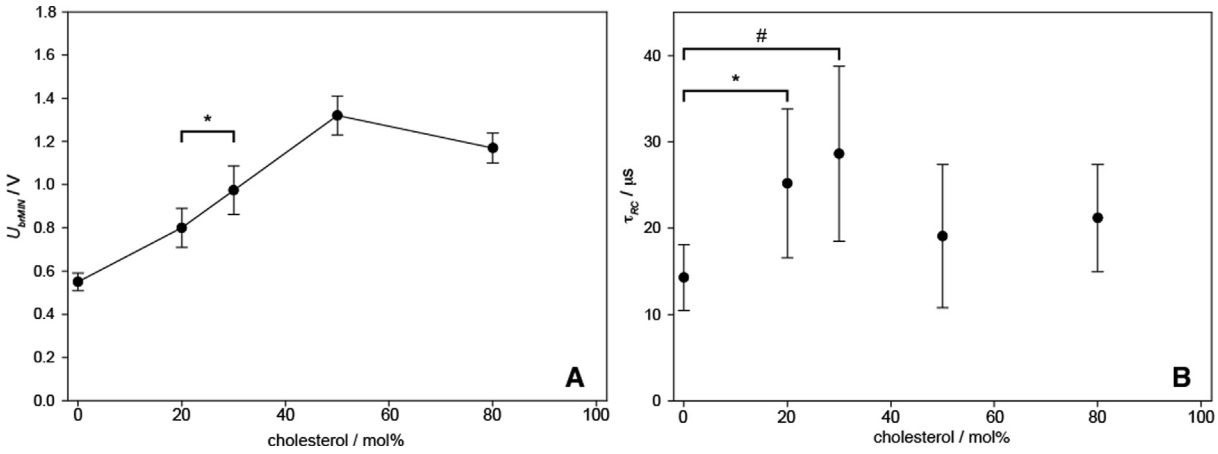

Fig. 2. (A) The parameter U_{brmin} of the fitted strength-duration curve (Eq. (1)) defined as minimal breakdown voltage at various cholesterol concentrations (0, 20, 30, 50 and 80 mol%). The error bars represent the 95% confidence interval of the fitted values. Pairwise comparison between obtained values of U_{brmin} are significantly different, and the p-value of the test was less than 0.05 ($p < 0.05$). For values denoted with * the p-value of the test was less then 0.2 ($p < 0.2$). (B) The parameter τ_{RC} represents the inclination of the fitted strength-duration curve and it is considered as a time constant of the system (Eq. (1)) at various cholesterol concentrations (0, 20, 30, 50 and 80 mol%). The error bars represent the 95% confidence interval of the fitted values. Pairwise comparison between for obtained value of τ_{RC} shows significant difference between 0 and 30 mol% of cholesterol concentration, denoted with #, where the p-value of the test was less then 0.05 ($p < 0.05$) and for 0 and 20 mol%, denoted with * where the p-value of the test was less than 0.2 ($p < 0.2$). All other comparisons with between obtained values of the τ_{RC} show no significant difference between various cholesterol concentrations.

(c_{BLM}) were calculated. Results do not show significant changes due to altered cholesterol concentrations. Our results are in the range of several other comparable published studies. We were able to compare our experimental measurements of planar lipid bilayer capacitance with three different studies found in the literature. Van Uitert *et al.* measured planar lipid bilayer capacitance using cholesterol concentrations up to 45 mol%. The measurement method consisted of an applied triangular voltage of amplitude 150 mV and frequency 50 Hz and measuring current across planar lipid bilayer. Measured capacitance was given as an average of all analysed planar lipid bilayers as 40 ± 11 pF. Planar lipid bilayers were built on the aperture of diameter 150 µm. Therefore, specific capacitance can be calculated as 0.23 µF/cm^2 (Fig. 3) which is considerably smaller than ours [17]. Corvalán *et al.* studied the capacitance of planar lipid bilayers in the presence of 0, 30, 40 or 50 mol % cholesterol concentration. Specific capacitance of analysed planar lipid bilayers was measured using voltage clamp pulses in discrete 20 mV steps from −200 to 200 mV. The applied voltage amplitude affected planar lipid bilayer thickness. Measured capacitance was found in quadratic relationship to applied voltage. The measured values of planar lipid bilayer capacitances were voltage dependent and in range from 0.37 µF/cm^2 for planar lipid bilayer with no cholesterol at 0 V to maximum value of capacitance 0.54 µF/cm^2 for planar lipid bilayer with 50 mol% of cholesterol and 200 mV. Capacitance of planar lipid bilayer increased due to increasing cholesterol concentration [46]. However, capacitance values at 0 mV were not statistically different between the neighbouring cholesterol concentrations. At values higher than 100 mV the statistical difference was observed between pure lipid and whichever mixture of the lipids with cholesterol. Statistical comparison between the lipid mixtures with cholesterol did not show statistical difference between measured capacitance value. Hence we were able to read out the values of measured planar lipid bilayer capacitances from Corvalán *et al.* paper for 0 mV and 100 mV with their standard deviation and normalise them to the aperture surface of 150 µm where planar lipid bilayers were formed [46] and present them in Fig. 3. Naumowicz and Figaszewski performed experiments on planar lipid bilayers composed of egg lecithin phosphatidylcholine and 0, 0.33, 0.46 and, 0.73 mol% cholesterol concentrations. In their study, planar lipid bilayer capacitance was determined using chronopotentiometry method. The planar lipid bilayers were exposed to low amplitude of DC current. At initial step of the current, slope of the voltage across the planar lipid bilayers were measured [49,67]. Results show that both, capacitance and resistance of the planar lipid bilayer rose with higher cholesterol concentration (Fig. 3). Using MD simulation Casciola *et al.* determined specific capacitance of planar lipid bilayer using POPC bilayer charge imbalance with 0, 20, 30 and 50 mol% cholesterol content [60] (Fig. 3).

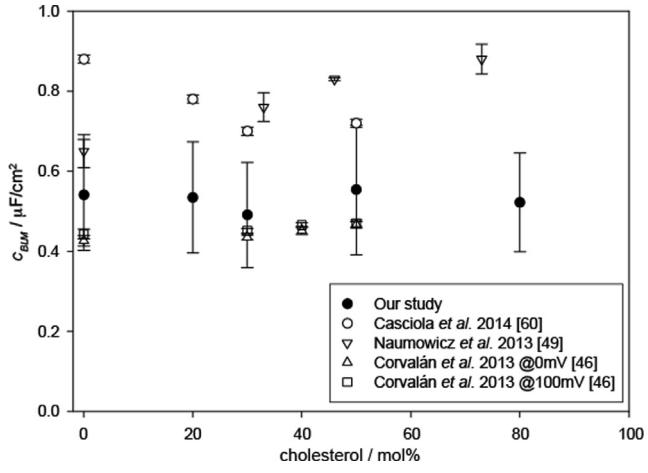

Fig. 3. Planar lipid bilayer specific capacitance (c_{BLM}) with its standard deviation in dependence of cholesterol concentration from our study (●) and compared to experimental studies of Naumowicz *et al.* [49] (▽), Corvalán *et al.* [46] (△,□) and MD simulation study of Casciola *et al.* [60] (○).

The planar lipid bilayer stability under applied electric field can be determined by measurement of its breakdown voltage. Breakdown voltage is one of the most important properties of planar lipid bilayers when studying electroporation. Result shows that breakdown voltage depends on the composition of the planar lipid bilayer [17,63]. In our current study, linear rising voltage method with seven different slopes: 4.8, 5.5, 7.8, 11.5, 16.7, 21.6, and 48.1 kV/s was used to measure planar lipid bilayer breakdown voltage. Breakdown voltage was defined as the voltage at the moment t_{br} when sudden increase of transmembrane current was observed. Time of breakdown t_{br} was defined as a lifetime of

the lipid bilayer at a chosen slope of the linear rising voltage signal. Measured data of different linear rising slopes were fitted to a strength-duration curve (Eq. (1)). The outcome, parameters U_{brmin} and τ_{RC}, where U_{brmin} governs minimal breakdown voltage and τ_{RC} governs inclination of the strength-duration curve were analysed according to planar lipid bilayer cholesterol content. Adding cholesterol to POPC planar lipid bilayer gradually increased its minimal breakdown voltage U_{brmin} until 50 mol% of cholesterol was reached. Afterwards at 80 mol% U_{brmin} does not further increase, in fact it reduces by 20% of the U_{brmin} at 50 mol% cholesterol concentration. Our experimental data of planar lipid bilayer breakdown voltage can be compared to other studies performed experimentally or using MD simulation, in which breakdown voltage was determined as a function of cholesterol concentration. Experimentally van Uitert *et al.* measured breakdown voltage using 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) planar lipid bilayers with various cholesterol concentrations ranging from 0 to 63 mol%. Contrary to our results they observed higher breakdown voltage of planar lipid bilayer at low cholesterol amounts and lower breakdown voltage for cholesterol amounts higher than 20 mol% [17]. The values of the planar lipid bilayer breakdown voltages varied between 250 mV and 150 mV. Similar effects were also observed by Watala *et al.* using increasing sweeping potential to analyse stability of planar bilayer lipid composed of L-α-phosphatidylcholine (from egg yolk) and 3–2-*sn*-phosphatidylethanolamine (from bovine brain) with cholesterol content from 0 to 30 mol%. It was demonstrated that fluctuating pores in structure of planar lipid bilayer caused higher current flow. Similar to our results presence of cholesterol in the membrane caused an increase in the value of the breakdown voltage at values of 10 and 20 mol% cholesterol concentration and a decrease of breakdown voltage at their highest 30 mol% cholesterol concentration [63]. Using MD simulations, planar lipid bilayer composed from POPC lipids only and with addition of the cholesterol was studied by Casciola *et al.* [60]. The voltage needed to form the pore in planar lipid bilayer in MD simulation was compared to experimentally obtained breakdown voltage where planar lipid bilayer is completely destroyed due to the voltage application. Direct comparison of those results agrees with our previous studies comparing MD simulations [23,24]. The values of breakdown voltages in MD simulations are much higher than in our current experimental study. However, the trend of increasing breakdown voltage with increasing cholesterol concentration is the same. Fernandez *et al.* also observed that addition of cholesterol increases the minimal voltage needed to form pores in planar lipid bilayers composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) lipid molecules [58,59] Fig. 4.

The parameter τ_{RC}, obtained from fitted strength-duration curve (Eq. (1)), can be considered as a time constant of the system, that represents capacitance and resistance of planar lipid bilayer, the resistance of the used aqueous solution and capacitance of the chamber where the planar lipid bilayer is formed. Since planar lipid bilayer can be simplified as capacitor and resistor in parallel, the parameter τ_{RC} is considered as a time constant, which is expressed as $\tau_{RC} = RC$. Therefore, resistance R of planar lipid bilayer is calculated from measured capacitance and measured breakdown voltage (Fig. 5). The changes of the parameter τ_{RC} are the consequences of the planar lipid bilayer composition e.g. cholesterol concentration. Here we have to also consider that the parameter τ_{RC} is obtained at a breakdown voltage, where planar lipid bilayer is no longer intact. On the other hand, specific capacitance is measured in intact state for each planar lipid bilayer before the application of linear rising voltage. Due to applied voltage water pores can be formed in the planar lipid bilayer[68], further changing capacitance and resistance of planar lipid bilayer. Therefore, comparison between studies of planar lipid bilayer for values of resistance is not warranted. Due to the high resistance of planar lipid bilayer the specific measurement system used unfortunately significantly contributes to the obtained values. Two studies have been compared to our calculated values for bilayer resistance with varying cholesterol concentration. Watala *et al.* analysed the value of L-α-phosphatidylcholine (from egg yolk) and 3-2sn-phosphatidy-lethanolamine (from bovine brain) planar lipid bilayer resistance with cholesterol content from 0, 10, 20, and 30 mol% as 305, 675, 659, and 723 MΩ respectively. The values were obtained from low voltage application and current measurements where planar lipid bilayer was in intact state [63]. The results show a rise of the resistance with increasing cholesterol concentration. However, absolute values of this study had greater values probably due to different measurement system used. Naumowicz and Figaszewski used chronopotentiometric measurements at low current amplitudes, where planar lipid bilayer structure also remains intact, to study the capacitance and resistance of the planar lipid bilayer [49]. Their measured values are closer to our results. However, with adding cholesterol above 30 mol% Naumowicz and Figaszewski observed an increase in resistance values, while in our study no significant change is observed in resistance (Fig. 5). One of the possible explanations

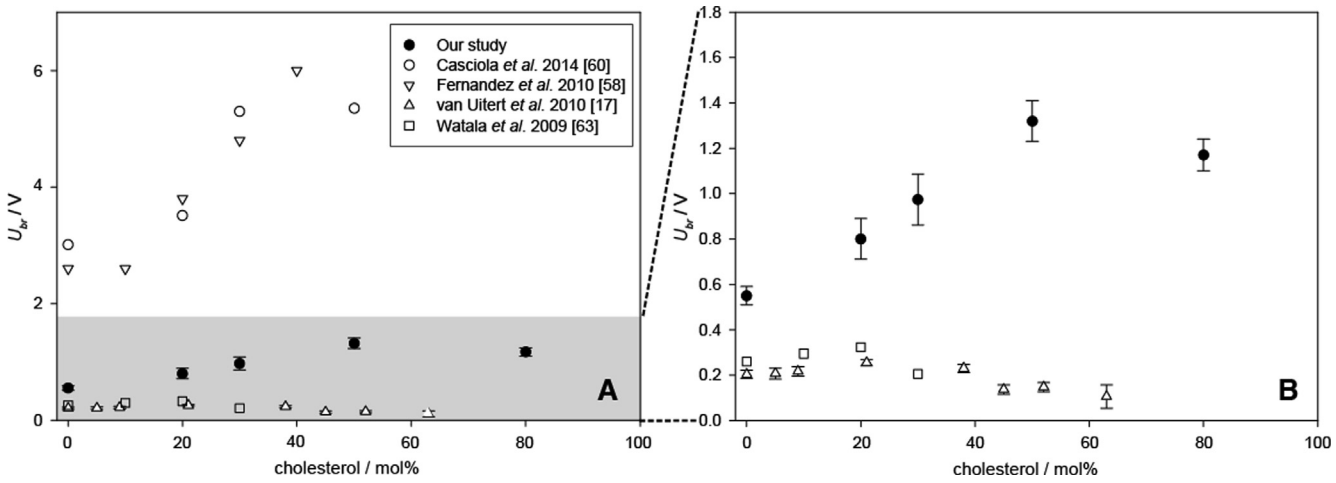

Fig. 4. A) Breakdown voltage (U_{br}) of planar lipid bilayers in dependence of cholesterol concentration from our study (●) and compared to MD study of Casciola *et al.* [60] (○) and Fernandez *et al.* [58] (▽), and experimental studies of van Uitert *et al.* [17] (△), and Watala *et al.* [63] (□). B) Close-up plot of experimental studies.

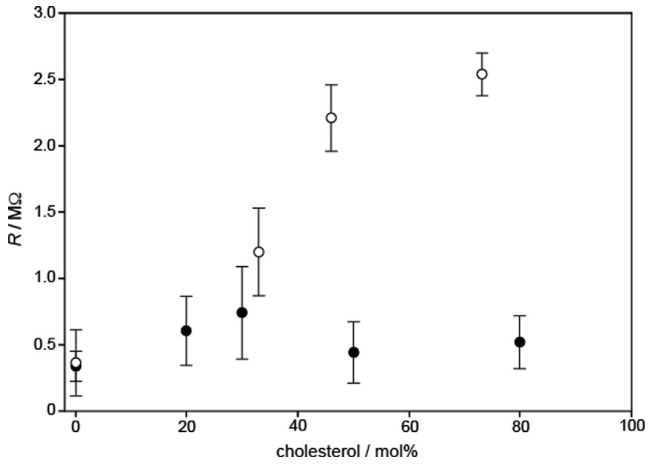

Fig. 5. Planar lipid bilayer resistance in dependence of cholesterol concentration. Values from our study (●) were obtained from parameter τ_{RC}, from fitted strength-duration curve (Eq. (1)), considered as time constant and measured capacitance of planar lipid bilayer. The error bars represent 95% confidence interval. Our study were compared with experimental study of Naumowicz *et al.* (○), where error bars represents standard deviation [49].

can be in obtained parameter τ_{RC}. Due to the higher stability of the planar lipid bilayer, the inclination of the curve is shifted (Fig. 1F). Therefore, steeper linear rising signal is necessary to obtain appropriate values of parameter τ_{RC} and consequently resistance R of planar lipid bilayer.

5. Conclusion

Biological membranes are a complex mixture of various phospholipid molecules, with different melting points and lengths of hydrophobic chains, including cholesterol and proteins. They exhibit phase separation and domain separation phenomena, as well as complex breakdown voltage behaviour. The presence of cholesterol in the planar lipid bilayer affects its properties and was mainly studied as a factor affecting membrane melting temperature and appearance of lipid rafts. Available experimental studies clearly show that addition of cholesterol into a lipid membrane increases its breakdown voltage, and this effect generally translates into the rupture of the planar lipid bilayer. Membrane properties also depend on the composition of the membrane, consequently, results at different lipid compositions are difficult to compare. In our study we focused on measurement of planar lipid bilayer capacitance and breakdown voltage using linear rising signal at various cholesterol concentrations. The results were then compared to the other similar experimental and MD studies, where planar lipid bilayers were exposed to voltage to determine its electrical properties.

The measurements of planar lipid bilayer capacitance were grouped according to cholesterol molarity in planar lipid bilayer and normalised to the surface area of the orifice to obtain specific capacitance. The average and standard deviation values were determined. Results do not show significant changes throughout the whole range of cholesterol concentrations used. Comparison of specific capacitance results in different studies gives us contradictory results. Interestingly, study of Naumowicz *et al.* [49] shows increasing capacitance, MD simulation studies of Casciola *et al.* [60] predict a decrease of specific capacitance at rising cholesterol concentration, and study of Corvalán *et al.* [46] shows no difference. The observed discrepancy of results may likely originate from difference in planar lipid bilayer formation method. Furthermore, the cause for the variance in capacitance measurements can be found in two processes. First, the level of the filled aqueous electrolyte solution in the chamber, which might vary. These variations were described by Benz *et al.* [69]. However, this does not affect breakdown voltage measurements since the level of the aqueous solution does not play a role in the breakdown voltage of the planar lipid bilayer. The second might be the measurement of capacitance with a discharge pulse. In the future, more accurate process for planar lipid bilayer formation needs to be determined in order to eliminate variability of planar lipid bilayer specific capacitance. A possible solution is to use a controlled system for rising of the aqueous solution level in the chamber as proposed by Karolis *et al.* [61]. Furthermore, experiments using longer chain length solvent should be carried out to study the effect of the solvent on the membrane thickness and consequently capacitance measurements. The planar lipid bilayer thickness may be altered by the loss of solvent due to electrostriction during applied transmembrane voltage. What is more the cholesterol itself can change the amount of the retained solvent and therefore also influence the planar lipid bilayer thickness.

The planar lipid bilayer stability under electric field was determined by measuring the breakdown voltage U_{br} and planar lipid bilayer lifetime t_{br} using linear rising voltage with seven different slopes. Measured data for different linear rising slopes were fitted to a strength-duration curve (Eq. (1)), where parameters U_{brmin} governs minimal breakdown voltage and τ_{RC} governs inclination of the strength-duration curve and were analysed according to cholesterol concentration.

Minimal breakdown voltage results of planar lipid bilayers were compared to previous studies. All available results show that planar lipid bilayer breakdown voltage increases with increased cholesterol concentration. The changed breakdown voltage due to cholesterol concentration in the planar lipid bilayer is mostly dependent on main lipid molecules. Various studies show that increasing the cholesterol concentration governs increasing stability of planar lipid bilayer and results in higher breakdown voltage. However, the chemical properties of the main lipid molecule determine incorporation of the cholesterol in the planar lipid bilayer. For more consistent results various lipid molecules should be tested under the equal experimental conditions.

The parameter τ_{RC}, can be considered as a time constant of the system, which can be expressed as a product of planar lipid bilayer resistance and capacitance. We have to consider that the parameter τ_{RC} is obtained at breakdown voltage, when planar lipid bilayer is no longer intact. Therefore, comparison between studies of planar lipid bilayer for values of resistance is not warranted. On the other hand, capacitance is measured in intact state for each planar lipid bilayer before the application of linear rising voltage. Nevertheless, the resistance obtained from parameter τ_{RC} and capacitance does not show significant difference at various cholesterol concentration. Measured resistance in other studies, where resistance of intact planar lipid bilayer was measured, however shows that values increase with increasing cholesterol concentration. One of the possible explanations of the deviation of our results might be in obtained parameter τ_{RC}. Due to the higher stability of the planar lipid bilayer, the inclination of the curve is shifted. Therefore, in the future experiments with steeper linear rising signal are necessary to obtain appropriate values of parameter τ_{RC} and consequently resistance R of planar lipid bilayer.

We were able to show that composition of planar lipid bilayers does have an effect on minimal breakdown voltage U_{brmin} and τ_{RC} and therefore voltages needed for electroporation. What is more the study of the broad compositions of planar lipid bilayer is essential to better understand the behaviour of the cell membrane in electric field and requires further studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by Slovenian Research Agency (ARRS) [research core funding No. P2-0249]. Experiments were performed within Infrastructure Program: Network of research infrastructure centres at University of Ljubljana (MRIC UL IP-0510).

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