Measurement protocol for detection of lipid peroxidation induced changes in planar lipid bilayers

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Merilni protokol za detekcijo sprememb ravninskih lipidnih dvoslojev zaradi lipidne oksidacije

***Abstract**: Namen študije je preučevanje vpliva lipidne oksidacije na povečano prevodnost celične membrane pri elektroporaciji. Elektroporacija je pojav, pri katerem z visokonapetostnimi pulzi kratkotrajno povečamo prevodnost celične membrane za molekule, ki drugače membrane ne prehajajo. Visokonapetostni pulzi lahko povzročijo številne stranske procese, ki so v določenih primerih neželeni, kot je na primer oksidacija lipidnih molekul. S preprostim modelom celične membrane – ravninskim lipidnim dvoslojem – želimo preučevati vpliv oksidacije na električne lastnosti ravninskega lipidnega dvosloja. Preučevali bomo upornost in prevodnost ter kapacitivnost ravninskih lipidnih dvoslojev zgrajenih iz oksidiranih ali neoksidiranih lipidnih molekul.*

1 Introduction

Exposure of biological cells to the electric field is a useful tool for manipulating the cell membrane permeability and has a widespread use in several medical and biotechnological applications as well as in food industry [1-3]. Even short-term exposure to electric field is stipulated to cause structural changes in biological membranes due to the formation of hydrophilic pores [4]. As a result, the membrane becomes temporarily more permeable for molecules, which lack the mechanism to cross the membrane [5]. This phenomenon is known as electroporation. The chemical and physical processes taking place at the molecular level during electroporation are relatively well studied; however, various side processes still remain unknown.

The main side processes of electroporation are electrochemical reactions taking place at the electrode-electrolyte interface, electrolysis and lipid peroxidation [8-9]. Lipid peroxidation is a chain reaction that causes degradation of lipids. Exposure of cells to electrical pulses is known to cause the formation of reactive oxygen species and oxidative damage to unsaturated lipid molecules which leads to chemical changes and results in long-lasting permeability of the cell membrane [8]. Numerous studies show that cell membrane permeability lasts considerably longer than the application of electrical pulses, which means that in addition to physical changes in the form of hydrophilic pores there must also be different chemical changes such as lipid peroxidation, to explain long-lasting membrane permeability [4-5].

In vitro cell membrane models such as planar lipid bilayers can be used to study lipid peroxidation processes at the molecular level. The artificial lipid bilayer is a simple, but still in certain specific aspects, a satisfactory model of the cell membrane [9]. Since we can assume that the electric field affects only a very small patch of the entire cell membrane, electroporation processes can well be studied using planar lipid bilayers. Planar lipid bilayers represent an electrophysiological technique for measuring the properties of phospholipid molecules, in a controlled environment. The advantage of planar lipid bilayers is their chemical and electrical accessibility from both sides [10]. The composition of the lipid bilayer can be arbitrarily changed to mimic the composition of a true cell membrane.

Lipid peroxidation is involved in the pathogenesis of various diseases [11]. What is more, oxidized phospholipids are known to destabilize the structure of biological membranes, therefore the study of lipid oxidation effects on cell membrane are of more general interest. Unfortunately, oxidized phospholipids are rarely commercially available, and we must synthesize them ourselves. Using chemical oxidants we were able to oxidize lipid molecules using protocol by Zschornig and his colleague [12]. Oxidation products can be generated by the Fenton reaction (H_2O_2 + Fe^{2+}) or via the $KMnO_4$ induced oxidation. The obtained oxidation products can be identified by MALDI–TOF MS, which is a simple and convenient method to characterize lipids and their oxidation products [13]. Lipid oxidation can also very well be carried out by leaving phospholipids to dry completely on atmospheric air. The degree of oxidation is determined by the incubation time, longer the incubation time more oxidation products will be obtained, furthermore, different oxidation products are produced.

Due to lipid peroxidation, cell membrane alters its structure [14]. Oxidized lipid tails can be shorter and more polar, due to the presence of oxidation products such as aldehydes and lipid peroxides [15]. Damage and destruction of lipid membrane due to lipid peroxidation leads to changes in fluidity and permeability of lipid bilayers [16]. Lipid bilayers become less stable, area per lipid increases, and leakage of molecules can occur [17]. In the presence

of lipid peroxides the membranes become less densely packed, less ordered, thinner and more permeable to water [15].

The aim of the study is to develop a measurement protocol to determine changes in planar lipid bilayer's electrical properties such as resistance and capacitance due to lipid peroxidation, using different peroxidation techniques.

2 Materials and methods

2.1 Chemicals

N-decane (ReagentPlus, ≥ 99.00 %) and hexane (ACS reagent) were purchased from Sigma Aldrich (Steinheim, Germany). Chloroform (Spectronorm quality) was obtained from VWR BDH Chemicals (Roncello, Italy), potassium chloride (KCl) and 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES) from Merck (Darmstadt, Germany). Nitrogen gas was from Messer (Gumpoldskirchen, Austria). The lipids 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) were from Avanti Polar Lipids (Alabaster, AL, USA).

2.2 Lipid oxidation

POPC lipids were chemically oxidized prior to bilayer formation using the protocol described in the literature [12]. Briefly, oxidation was carried out with atmospheric air, where lipids in chloroform were dried under a N_2 stream to obtain a dry thin lipid film at the bottom of the flask. Lipids were then left to oxidized under atmospheric air for 1 day. Chloroform was then added, and lipids were used for further experiments. Subsequently, chemical oxidants such as $KMnO_4$ were used for lipid oxidation. A 2M $KMnO_4$ solution was added to the dry thin lipid film at the bottom of the flask and incubated for 10 min at 37°C. Afterwards a 1 ml of chloroform: methanol [1:1 volume ratio] was added to stop the oxidation. Vortexing and centrifuging was used to separate the polar and non-polar phase and to extract the oxidized lipids, which were dissolved in chloroform and used for further experiments.

2.3 Planar lipid bilayer formation

Planar lipid bilayers were formed using the painting method [18]. The corresponding amount of lipids dissolved in chloroform were dried under a N_2 stream and subsequently dissolved in n-decan to obtain a final 20 mM lipid concentration. A Delrin measurement chamber from Warner Instruments (Hamden, CT, USA) with 150 µm diameter aperture separating the two cuvettes was used for planar lipid bilayer formation. The aperture was pretreated with 20 mM lipids dissolved in hexane. After evaporation of hexane, each compartment was filled with 4 ml of 100 mM KCl and 10 mM HEPES in 1:1 volume ratio electrolyte solution. For planar lipid bilayer formation, a small drop of lipids dissolved in n-decane was applied to the aperture using a glass rod. A stable planar lipid bilayer was formed after approximately 30 min when capacitance was stable.

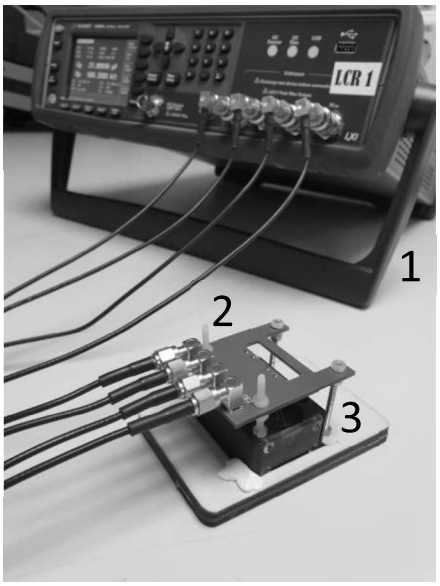

Figure 1: *Instrumentation setup for measurement of planar lipid bilayer's electrical properties. The setup consists of an LCR meter (1), electrodes (2) and a measurement chamber (3).*

2.4 Planar lipid bilayer analysis

The planar lipid bilayer is electrically represented as an electrical circuit of a capacitor and a resistor wired in parallel. Electrical measurements on planar lipid bilayers were performed using a LCR meter E4980A from Keysight (Santa Rosa, CA, USA) connected to four Ag/AgCl electrodes (In vivo metric, Healdsburg, CA, USA) immersed in the electrolyte solution in the Delrin measurement chamber (Warner Instruments, USA) as shown on Figure 1. The LCR meter inner model was set up to measure resistance R_p (Ω) and capacitance C_p (F) in parallel. The AC voltage was set to 20 mV and the frequency to 2 kHz. Data points were acquired each quarter of a second to obtain measurements of the resistance and capacitance over time. The LCR meter was connected through an Ethernet connection. The measured capacitance and resistance of each planar lipid bilayer were processed with MATLAB R2019a (MathWorks, Natick, MA, USA) using its Instrument toolbox. Measured data were accessed using a SCPI protocol. To compare capacitance measurements, specific capacitance c was calculated as measured capacitance C_p normalised to the area of the aperture in the chamber. Conductance σ (S) is the inverse of measured resistance R_p.

To study the effect of lipid peroxidation on the conductivity of planar lipid bilayers, either oxidized or non-oxidized lipids were used. Four measurements using POPC lipids were done for each oxidation protocol. Controlled oxidation of

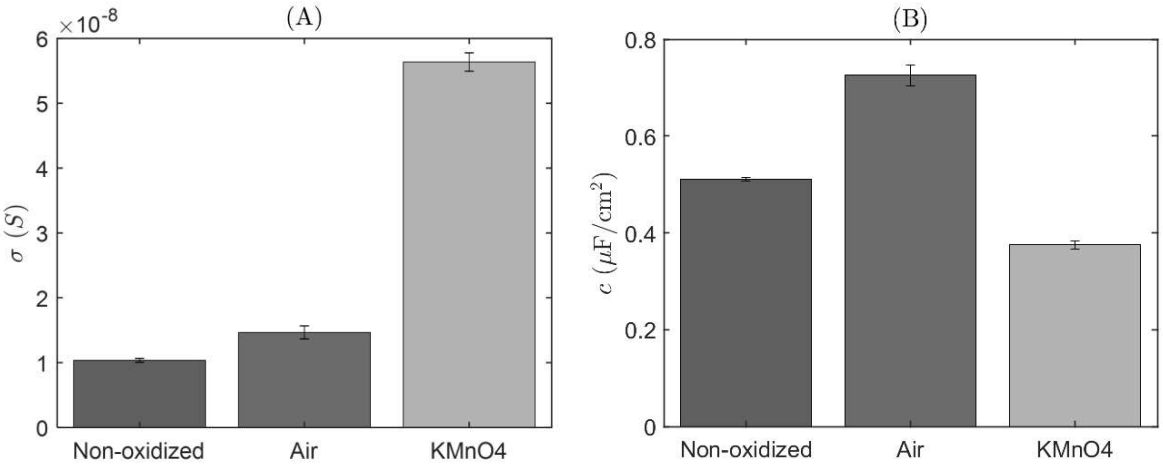

Figure 2: *Preliminary results of electrical measurements of oxidized and non-oxidized planar lipid bilayers. (A) Bilayer conductance increases in the lipid bilayer made of oxidized lipid molecules. The conductance increased more for $KMnO_4$ oxidation, since $KMnO_4$ is a stronger oxidant than air. The bar plots present the mean conductance of four measurements and its standard deviation. (B) Capacitance increased when lipids were oxidized on air and decreased when lipid were oxidized using $KMnO_4$. The bar plots present the mean capacitance of four measurements and its standard deviation.*

lipid molecules was carried out using chemical oxidants such as air and $KMnO_4$. Changes in conductance σ (S) and specific capacitance c ($\mu F/cm^2$) of the planar lipid bilayer formed of non-oxidized and oxidized planar lipid bilayers were studied.

3 Preliminary results

Preliminary results show that we are able to measure changes in electrical properties of POPC planar lipid bilayers, built from non-oxidized or from oxidized lipid molecules. A much higher conductance (Figure 2A) of the oxidized planar lipid bilayer was measured thus indicating that lipid oxidation can be the cause for increased membrane permeability. $KMnO_4$ is a much stronger oxidant than air; therefore, it was expected to observe greater changes in conductance of the $KMnO_4$ oxidized bilayers. Capacitance increased (Figure 2B) when lipids were oxidized on air, which has been reported previously and our results are in accordance with these findings [19]. Oxidation changes the hydrophobic tail region of the planar lipid bilayers, which should influence the dielectric constant of the bilayer and consequently results in a measureable increase of the lipid bilayer's capacitance. However, a decrease in capacitance was observed when lipids were oxidized using $KMnO_4$.

4 Discussion

Using electrical measurements of planar lipid bilayers, we observed an increase in conductance of the oxidized planar lipid bilayer, when compared to the non-oxidized bilayer. The conductance of the bilayer measures the ability for electrical current and ions to pass the bilayer. With increasing lipid oxidation, the core of the bilayer will become more polar, due to chemical changes and consequently more permeable [20]; therefore, an increase in conductivity is as expected. However, changes in capacitance measurements of oxidized planar lipid bilayer are not as expected. Oxidation affects the tails of the lipid molecules; therefore, the hydrophobic core of the planar lipid bilayer will be under attack. The oxidation of lipid tails will change the bilayer's dielectric constant due to formation of polar regions in the non-polar bilayer interior, therefore the capacitance of oxidized bilayers should increase with increasing dielectric constant. When lipids were dried and left to oxidize on air, an increase capacitance was measured compared to non-oxidized bilayers. However, oxidation with $KMnO_4$ did not give expected results since capacitance decreased compared to non-oxidized bilayers. This could be due to lipid extraction after incubation with $KMnO_4$, where some of the $KMnO_4$ molecules could still be dissolved in non-polar phase.

5 Conclusion

Lipid membranes are under constant attack of free radicals that may lead to lipid peroxidation in conditions of oxidative stress. The oxidative attack on fatty acid chains by reactive oxygen species may lead to structural and chemical changes in the lipid bilayer, which can alter the membrane properties. Lipid oxidation affects the hydrophobic interior of the bilayer, thus increasing the permeability and the dielectric constant due to formation of polar regions. Cell membranes have a natural response to the oxidative damage of their lipid constituents and try

to prevent it using antioxidants. Antioxidant defence consist of scavenging, quenching and removal of active oxidants, repair of damage sections and excretion of toxic oxidation products [21]. In our study, we were able to demonstrate that oxidation leads to an overall increase in the planar lipid bilayer conductance, thus showing the possibility of increased membrane permeability due to lipid oxidation. Capacitance measurements were not as straightforward, since capacitance increased as expected for lipid oxidation using air, while capacitance decreased with $KMnO_4$ oxidation.

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