

# Initial steps of pore formation in planar lipid bilayers made of phospholipids and archaeal lipids

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## Vzpostavitev vodnih por v ravninskih lipidnih dvoslojih zgrajenih iz fosfolipidnih in arheolipidnih molekul

**Povzetek.** Plazemska membrana je biološka membrana, ki ločuje notranjo celično vsebino od zunanjega okolja. Zato je zgradba plazemske membrane odvisna od okolja, v katerem se celica običajno nahaja. Arheje so se tudi z zgradbo plazemske membrane prilagodile na ekstremne pogoje v katerih živijo. V študiji smo preučevali proces nastajanja vodnih por med elektroporacijo ravninskih lipidnih dvoslojev zgrajenih iz lipidnih molekul pridobljenih iz arheje *Aeropyrum pernix K1* in ravninskih lipidnih dvoslojev zgrajenih iz fosfolipidnih molekul 1-pamitoyl 2-oleoyl fosphatidylcholone (POPC), 1-pamitoyl 2-oleoyl fosphatidylserine (POPS) in mešanice POPC:POPS v razmerju 1:1. Izmerili smo specifično kapacitivnost ravninskih lipidnih dvoslojev ter porušitveno napetost, i.e. napetost, ki povzroči porušitev ravninskega lipidnega dvosloja ob prisotni transmembranski napetosti. Porušitveno napetost smo določili z izpostavitvijo ravninskih lipidnih dvoslojev linearno naraščajoči napetosti. Specifična kapacitivnost ravninskih lipidnih dvoslojev je v veliki meri odvisna od dolžine verige ogljikovodikov, saj najmanjšo specifično kapacitivnost izkazujejo ravninski lipidni dvosloji narejeni iz lipidov arheje *Aeropyrum pernix K1*, največjo pa ravninski lipidni dvosloji narejeni iz POPC. Porušitvena napetost ravninskih lipidnih dvoslojev zgrajenih iz lipidov arheje *Aeropyrum pernix K1* je bila pri vseh uporabljenih naklonih linearno naraščajoče napetosti večja, kot porušitvena napetost ravninskih lipidnih dvoslojev narejenih iz POPC, POPS ali POPC:POPS (1:1). Pri vseh obravnavanih ravninskih lipidnih dvoslojih zavzamejo vodne pore manj kot 1% površine ravninskega lipidnega dvosloja. Na podlagi zgradbe obravnavanih ravninskih dvoslojev sklepamo, da je proces nastajanja vodnih por odvisen od urejenosti lipidnih molekul v ravninskem lipidnem dvosloju.

## 1 Introduction

The composition of plasma membrane is not the same in all cells. Phospholipids, glycolipids and sterols are the most common lipid molecules in plasma membranes of eukaryotic cells and bacteria. But archaeal membranes, for example, contain glycerol ether lipids with saturated

chains containing methyl branches [1]. Unique characteristic of archaeal membranes are the reason for diversity of studies suggesting their use in various biomedical applications [2]. Among others, archaeosomes are proposed for using as a drug carrier. In this case drug release can be enhanced by electroporation [3]. Considering such application, the behaviour of the archaeal lipid membrane in electric field is important in addition to membrane's mechanical and chemical properties.

In this study we focused on the initial processes of pore formation during electroporation in planar lipid bilayers that constitute the membrane of the aerobic hyperthermophilic archaeon *Aeropyrum pernix K1*. The detailed structure of constituents, 2,3-di-O-sesterterpanyl-sn-glycerol-1-phospho-1'-(2'-O- $\alpha$ -D-glucosyl)-myo-inositol (AGI) and 2,3-di-O-sesterterpanyl-sn-glycerol-1-phospho-myo-inositol (AI), was elucidated by Morii et al. in 1999 [4]. These two lipids usually compose archaeal membrane in the mol% ratio 91:9. The important feature of both lipids is C25-isoprenoid as a hydrophobic part, while in the head of the lipid molecule inositol is linked on the phosphate group in AI and glucosylinositol in AGI. Hydroxyl groups are present on all available C-atoms in the sugar rings.

Archaeosomes prepared from lipids isolated from *A. pernix K1* exhibit large negative surface charge (-50 to -110 mV, increasing with diameter) in broad pH range (2.5 to 12) [5]. Typical gel to liquid phase transition in the temperature range from 0°C to 100°C has not been detected [5]. Electron paramagnetic resonance spectra have shown that the archaeosome membranes are heterogeneous, and are composed of several regions with three types of fluidity characteristics. Presence of each fluidity type depends on pH and temperature. In general, increase in membrane fluidity with temperature has been also noticed [5].

To explore the possible differences in initial steps of pore formation in planar lipid bilayers of different compositions, we formed planar lipid bilayers using lipids extracted from archaea *A. pernix K1*, 1-pamitoyl 2-oleoyl phosphatidylcholine (POPC) - lipid molecules with a zwitterionic head group and almost zero spontaneous curvature, negatively charged 1-pamitoyl 2-oleoyl phosphatidylserine (POPS) molecules with negative spontaneous curvature and a mixture of POPC and POPS in 1:1 ratio. For each planar lipid bilayer composition we determined the capacitance  $C$  of planar lipid bilayer. Then, planar lipid bilayers were exposed to

linearly rising voltage signals of different slopes  $k_u$  and the voltage  $U_{br}$  and time  $t_{br}$  at which planar lipid bilayer rupture occurs were measured. From these measurements we evaluated the reduction of  $C$  that corresponds to presence of water pores in planar lipid bilayer and is a consequence of significant difference in dielectric constant values of lipid bilayer and water. We also estimated the fraction of planar lipid bilayer area that is occupied by water pores, the fraction of planar lipid bilayer area that is occupied by water pores.

## 2 Materials and Methods

### 2.1 Chemicals

The extraction of archaeal lipids was done at University of Ljubljana, Biotechnical Faculty, Slovenia. The lipids POPC and POPS were purchased in powder form (Avanti Polar-Lipids Inc. USA). A solution of 10 mg/ml of each lipid was prepared in a 9:1 mixture of hexane and ethanol. POPS:POPS (1:1) mixture was prepared by pipetting the same amount of both lipid solutions in a small plastic tube and mixing by repeated pipetting immediately before the experiments. The mixture of hexadecane and pentane in the ratio of 3:7 was used for torus formation. The electrolyte was prepared from 0.1 M KCl and 0.01 M Hepes in the volume ratio of 1:1. A few drops of 1 M NaOH were added to obtain a pH of 7.4.

### 2.2 Experimental setup and measurements

Planar lipid bilayers were formed by the Montal-Muller method across a circular hole 117  $\mu\text{m}$  in diameter ( $d$ ) as described previously [6]. All experiments were performed at room temperature (24°C).

The measurement protocol consisted of planar lipid bilayer formation and  $C$ ,  $U_{br}$ , and  $t_{br}$  determination.  $C$  was measured by the planar lipid bilayer discharge method and normalized to the area of the hole ( $A = 1.075 \cdot 10^{-8} \text{ m}^2$ ) to calculate the specific capacitance of the planar lipid bilayer  $c_{BLM}$ . Transmembrane voltage on planar lipid bilayer was build-up using a linearly rising voltage signal. Seven different slopes of the linearly rising voltage  $k_u$  (4.8 kV/s, 5.5 kV/s, 7.8 kV/s, 11.5 kV/s, 16.7 kV/s, 21.6 kV/s and 48.1 kV/s) were used. At the time  $t_{br}$ , when a sudden increase in transmembrane current was detected,  $U_{br}$  was measured.

### 2.3 Experimental data analysis

The specific capacitance  $c_{BLM}$  was determined for at least 18 planar lipid bilayers of each lipid composition. Mean value and standard deviation were calculated for each experimental group.

Using nonlinear regression, a two-parameter strength-duration curve

$$U = \sqrt[4]{a + \frac{b}{t}} \quad (1)$$

based on the viscoelastic model of Dimitrov [7] and proposed by Sabotin et al. [8] was fitted to experimentally obtained points ( $t_{br}$ ,  $U_{br}$ ) measured at different slopes of the linearly rising voltage. The parameter  $\sqrt[4]{a}$  is an asymptote of the strength-duration curve corresponding to minimal breakdown voltage  $U_{brmin}$  for planar lipid bilayers of a given lipid composition. The parameter  $b$  determines the inclination of the strength-duration curve. As in the case of strength-duration curve for excitable cell membranes of nerves and muscles, we can define for each lipid composition a kind of a chronaxie time constant  $t_c = b/(15 \cdot a)$  as the minimum time required for a stimulus of twice  $U_{brmin}$  to cause rupture of the planar lipid bilayer.

In the short interval before  $t_{br}$  we can assume that non-conductive water pores are already present in the planar lipid bilayer [9]. The capacitance of planar lipid bilayer  $C_{br}$  can be represented as

$$C_{br} = \sum_{i=1}^n C_{pi} + \sum_{j=1}^m C_{lj} \quad (2)$$

where  $C_{pi} = \varepsilon_p \varepsilon_0 A_{pi}/D$  is the capacitance of the  $i$ -th water pore and  $C_{lj} = \varepsilon_l \varepsilon_0 A_{lj}/D$  is the capacitance of  $j$ -th patch of the still intact planar lipid bilayer.  $\varepsilon_p$  is a dielectric constant of water ( $\varepsilon_p = 80$ ),  $\varepsilon_l$  is a dielectric constant of planar lipid bilayer ( $\varepsilon_l = 2$ ) and  $\varepsilon_0$  is vacuum permittivity ( $\varepsilon_0 = 8.854 \cdot 10^{-12} \text{ Fm}^{-1}$ ). The thickness  $D$  of the planar lipid bilayer was considered 6.00, 3.75, 4.32 and 4.00 nm for *A. pernix* K1, POPC, POPS, and POPC:POPS 1:1 respectively. The values were obtained from molecular dynamic (MD) simulation as the head to head distance between two electron density profiles [10, 11].

Change in planar lipid bilayer capacitance  $\Delta C$  due to the water pore formation is  $C_{br} - C$ , if we assume that capacitance of intact planar lipid bilayer at the beginning of the experiment is  $C = \varepsilon_l \varepsilon_0 A/D$ . To obtain the fraction of the planar lipid bilayer that is occupied by pores ( $A_{wat}/A$ ),  $\sum_i A_{pi}$  is calculated from  $\Delta C$  and normalized on the whole area of planar lipid bilayer  $A$ .  $\Delta C$  was evaluated from experiments as described previously [6].

## 3 Results and discussion

In Table 1  $c_{BLM}$  and  $U_{brmin}$  determined for planar lipid bilayers made of different lipid molecules are given. The specific capacitance of planar lipid bilayer is related to its geometrical dimensions; i.e. planar lipid bilayer area and thickness. All planar lipid bilayers were formed on the hole of the same size, therefore the thickness is the main geometrical parameter that affects  $c_{BLM}$ . Planar lipid bilayers made of *A. pernix* K1 have considerably smaller  $c_{BLM}$  than planar lipid bilayers made of POPC, POPS or POPC:POPS (1:1) mixture. It seems that the reason is in longer hydrocarbon chains in both archaeal lipids ( $C_{25,25}$ ) than in POPC and POPS lipids ( $C_{16,18}$ ) that leads to thicker planar lipid bilayer made of *A. pernix* K1.

Table 1. Specific capacitance ( $c_{BLM}$ ) and minimal breakdown voltage ( $U_{brmin}$ ) for planar lipid bilayers made of different lipid molecules. The values are given as mean  $\pm$  standard deviation (number of measurements).

Lipid composition	$c_{BLM}$ [ $\mu\text{F}/\text{cm}^2$ ]	$U_{brmin}$ [V]
<i>A. pernix</i> K1	$0.16 \pm 0.06$ (18)	$0.61 \pm 0.36$
POPC	$0.51 \pm 0.17$ (80)	$0.42 \pm 0.23$
POPS	$0.41 \pm 0.14$ (76)	$0.53 \pm 0.24$
POPC:POPS 1:1	$0.31 \pm 0.07$ (60)	$0.42 \pm 0.23$

Application of linearly rising voltage of different slopes  $k_u$  resulted in typical “strength-duration” behavior of  $U_{br}$ . The steeper the slope of the linearly rising stimulus, the higher transmembrane voltage is needed for planar lipid bilayer rupture. We fitted the strength–duration curves (1) to ( $t_{br}$ ,  $U_{br}$ ) points measured for each and every lipid compositions (Figure 1). Archeal planar lipid bilayers exhibit the highest  $U_{br}$  at all slopes  $k_u$  of linearly rising voltage. The lowest values of  $U_{br}$  were obtained for POPC planar lipid bilayers, where, according to MD simulations, hydrophilic pores occur before final membrane rupture, and planar lipid bilayers formed from a POPC:POPS (1:1) mixture.

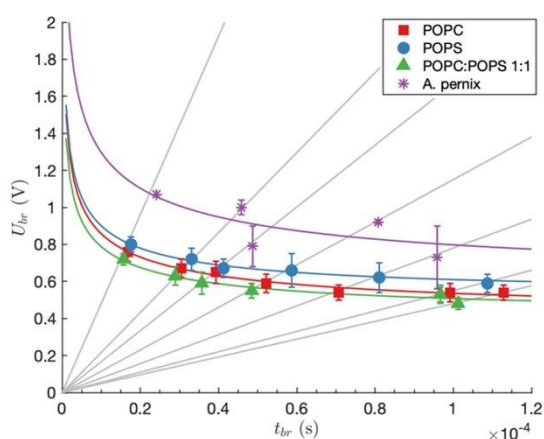


Figure 1. Breakdown voltages of specific lipid compositions measured during voltage-controlled experiments. The gray lines show different slopes  $k_u$  of linearly rising signal. Colored curves represent two-parameter strength-duration curves (1) fitted to the data obtained by voltage-controlled measurements on POPS, POPC, POPC:POPS mixture 1:1 and *A. pernix* K1, respectively.

In Figure 2, the fraction of the planar lipid bilayer area that is occupied by water pores at the moment of planar lipid bilayer rupture is presented as a function of  $U_{br}$ . Aqueous fractional area in the planar lipid bilayer due to an exponential pulse, bipolar square pulse, or a square pulse was theoretically evaluated by Freeman et al. in 1994 [12]. According to their calculations, only 0.1% of the membrane is occupied by pores, which is in good agreement with our experimental results obtained when the transmembrane voltage was built up slowly. MD simulations have shown that in the case of planar lipid

bilayers made of archaeal lipids and in the case of POPS planar lipid bilayers only hydrophobic pores are created [11, 13]. Wodzinska et al. claim that formation of the pore must keep the overall area of the membrane constant [14], which means that hydrophobic pores cannot expand and consequently lead to final planar lipid bilayer breakdown.

The properties of lipids which contribute to the stability of lipid bilayers when subject to transmembrane voltage are numerous and diverse, and include at least the structure of the lipid tails, the chemical nature and charge of the head group and the nature of the head to tail linkage (ester or ether) [11]. Measurements based on 2H nuclear magnetic resonance [15] have shown that the lipid hydrocarbon chains in pure PC and PS membranes have different order parameters at room temperatures. Namely, although both were in a lamellar liquid crystalline phase, PS was more ordered than PC. On the other hand, it was shown that the archaeosome membranes are heterogeneous, and are composed of nanodomains with different fluidity characteristics at room temperatures [5]. Archaeal lipids also have special head groups carrying inositol and glucose functions. In comparison to simple phosphatidylcholine (PC) head groups these carbohydrates are larger moieties that move much more slowly and are also involved in hydrogen bonds that contribute to stability and order of these bilayers. It can be assumed that planar lipid bilayers made of *A. pernix* K1 and POPS are more ordered than POPC planar lipid bilayers which leads to harder formation of water pores and lipid bilayer rupture.

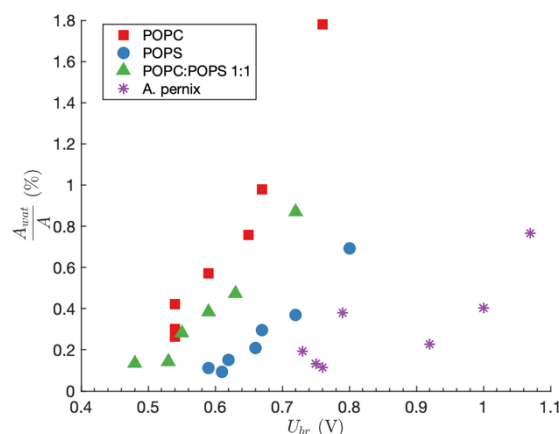


Figure 2. The fraction of the planar lipid bilayer area that is occupied by water pores as a function of  $U_{br}$ .

## 4 Conclusions

In the study planar lipid bilayers were formed using lipids extracted from archaea *A. pernix* K1, zwitterionic POPC lipids, negatively charged POPS lipids and POPC:POPS mixture in ratio 1:1. For each planar lipid bilayer capacitance was measured to monitor quality of the membrane model at forming. Planar lipid bilayers were exposed to linearly rising voltage and  $U_{br}$  and  $t_{br}$  were

determined. Using an analytical method of planar lipid bilayer substitute circuit we estimated the change of the capacitance at the breakdown and the fraction of planar lipid bilayer area occupied by water. Our results confirm the results of pore formation theories and MD models. The fraction of area occupied by water pores at the moment of planar lipid bilayer rupture and  $U_{br}$  is smaller when the transmembrane voltage is built up slower. In more disordered planar lipid bilayers, water pores are easily formed.

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