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IMPEDANCE ANALYSIS OF AGAR-SUPPORTED BILAYER LIPID MEMBRANES MODIFIED WITH ALAMENTHICIN

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Abstract

The aim of the work was to study an agar-supported lipid bilayer membrane (s-BLM) by electrochemical impedance spectroscopy (EIS). S-BLMs with high electrical resistance satisfy the criteria of ease and reproducibility of preparation, long-term stability, and low sensitivity to the electrical and mechanical disturbances, which enables their application in microchip technology. The insertion of polypeptide alamethicin (Ala) after the formation of s-BLM was used to corroborate the formation of a bilayer and show the applicability of agar s-BLM for measurement of ion channel activity. Ala is able to form spontaneously stable voltage gated ionic channels. An equivalent electrical circuit was used for analysis and extraction of main membrane parameters from impedance data.

Key words

Agar-supported bilayer lipid membrane, Alamethicin, Electrochemical impedance spectroscopy

INTRODUCTION

Supported bilayer lipid membranes, self-assembled mainly on the Pt, Au, and stainless steal support, overcame the essential shortcoming of the classical BLM – the fragility and sensitivity to electrical and mechanical disturbances (*1,2*). Although s-BLM represents a stable and long-lasting model of biological membrane, the membrane deposited in particular on the freshly created metallic surface does not represent an ideal continuous bilayer. S-BLM resistance is smaller and its specific capacitance 20–50 times higher than that of a conventional, freestanding BLM (*3*). It seems reasonable to expect that the novel type of membrane support based on agar gel (*4*) can improve the membrane properties and makes s-BLM an interesting element of nanodevices.

Alamethicin is a 20-residue-long natural polypeptide produced by *Trichoderma viridae*, which forms voltage-gated ion channels in lipid bilayers (*5*). Ala is predominantly α-helical; the sequence of polypeptide contains a number of helicogenic α-amino isobutyric acid residues. The slightly amphipathic nature of Ala molecules should allow them to self-associate in lipid bilayers forming helical

parallel bundles with hydrophobic exterior and hydrophilic water-filled interior. The multi-conductance behaviour of Ala channels is generally explained in terms of the "barrel stave" model.

The aim of the study was to obtain a membrane system that is on the one hand attached to the solid support so that it can be applied in chip technology. On the other hand, the system should exhibit membrane resistance high enough for the study of interactions of the ion channel forming polypeptides with the membrane. The insertion of polypeptide Ala after the formation of s-BLM also corroborates the formation of a bilayer and shows the applicability of this membrane type for measurement of ion channel activity.

S-BLM can be accessed by a variety of sensitive surface analysis tools such as EIS, provided that the support is electrically conductive. The EIS data analysis, by fitting to a model represented by an equivalent electrical circuit, allows assessment of the basic parameters of agar s-BLM and their applicability to the study of membrane – Ala interactions.

MATERIALS AND METHODS

1,2-Diacyl-sn-glycero-3-phosphocholine (PC), n-dodecane, and Ala were purchased from Sigma-Aldrich. The membrane forming solution was prepared by mixing ethanol solution of PC with n-dodecane to attain a final 5 % lipid concentration. Ala was added to the electrolyte from a 10^{-5} M stock solution in ethanol after bilayer formation, resulting in a nominal peptide concentration of $\sim 10^{-7}$ M. Other chemicals were of the highest quality possible, and were used without further purification. The water used was prepared with Milli-Q (R>18 MΩ.cm^{-1}, pH 5.5). As the membrane support served a tip of agar-filled (3 % agar in 1 M KCl) Teflon tube (0.5 mm inner diameter).

S-BLM was investigated by means of EIS. AC (alternating current) analysis was carried out with a Zahner IM6e electrochemical analyser (Kronach) providing fully computer-controlled impedance spectroscopy. The absolute values of the impedance $|Z|(f)$ and the phase shift angle $\phi(f)$ were recorded within the frequency range from 10^{-2} to 10^{6} Hz. EIS data were obtained at +70 mV DC (direct current) polarisation potential superimposed to a small sinusoidal AC voltage of 10 mV to avoid non-linear responses. EIS data were analysed by means of a complex non-linear regression least square (CNRLS) fit to a model represented by an equivalent electrical circuit. A two-electrode cell containing an agar-filled Teflon tube with Ag/AgCl wire and a platinum wire was applied for the electrochemical experiments. The electrolyte used was 100 mM KCl for all experiments. A measuring cell with a water jacket for maintaining stable temperature, placed into a Faraday's cage, was used. All experiments were carried out at room temperature (21±0.2) °C.

RESULTS AND DISCUSSION

The self-assembly of s-BLM on agar was followed by means of monitoring the electrical impedance $|Z|$ and the phase shift angle ϕ in time. The thinning process of the droplet adsorbed on the agar surface took $\sim$30 min. After this period impedance was reduced and both $|Z|$ and ϕ reached stable values. The impedance achieved was stable for 24 hours in most experiments. It is well known that voltage pulses of sufficient amplitude and duration tend to cause dielectric breakdown of the lipid bilayer. To test the membrane stability, transmembrane voltage pulses were

applied. After pulses of up to 1 V, which cause breakdown of the classical BLM, an abrupt change of membrane impedance was observed, but s-BLM was rarely disrupted completely. Instead, a slow relaxation of the membrane was detected. The phenomenon is probably due to the occurrence of metastable single pores observed also in the unmodified planar lipid bilayer (*6*).

In *Fig. 1* typical impedance spectra of s-BLM on agar are shown. Both impedance curves recorded for the same membrane in one-hour interval indicate that the insulating lipid layer is stable. To extract main membrane parameters from EIS data, an equivalent circuit model to the electrical behaviour of a lipid bilayer was used (*Fig. 1*). For all the data treated with this model the mean relative error of $|Z|$ did not exceed 2.9 %, the mean error of ϕ did not overstep 0.9 deg. For eight membrane preparations, membrane resistances extracted from the impedance data with a mean value of R_2=203.5±22.3 M□ were obtained. The membrane capacitance led to a value of C_2=537.4±162.9 pF. The mean value of s-BLM resistance related to the area S=1.96x10^{-3} cm^2 reached 0.4 M□.cm^2. For the specific membrane capacitance a value of 0.3 μF.cm^{-2} was obtained. The values of the specific capacitance are consistent with those reported for classical BLM. The

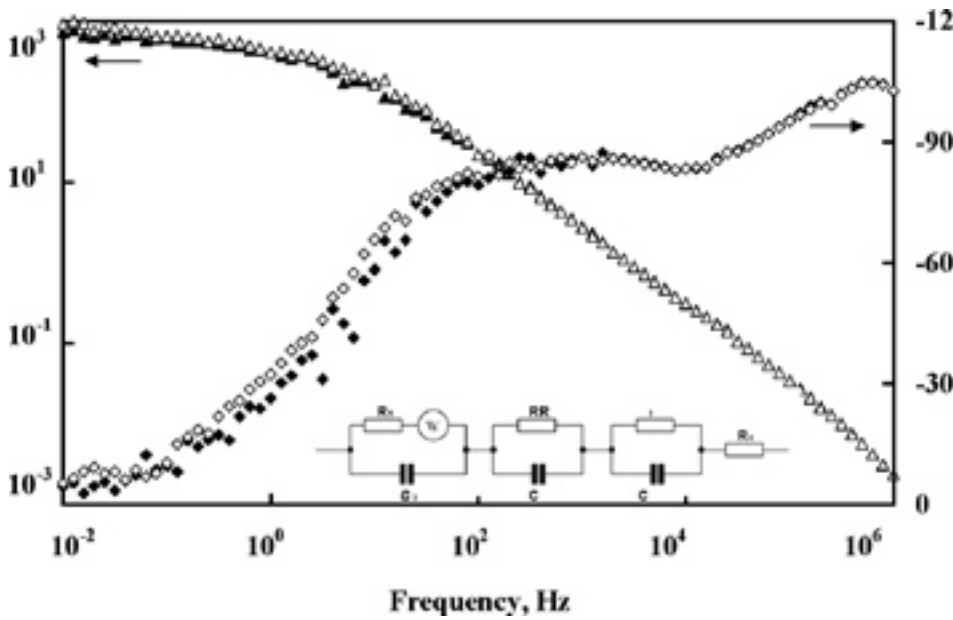

Fig. 1.

EIS spectra of agar s-BLM (amplitude of AC signal U=10 mV, U_{DC}=70 mV). The symbols represent $|Z|$ (▲,△) and φ (◆,◇) of spectra recorded repeatedly in one-hour intervals. Embedded picture: Equivalent electrical circuit – resistance R_1, Warburg impedance W, and capacitance C_1 correspond to the agar/lipid interface; - R_2, C_2 represent the resistance and double layer capacitance of the lipid bilayer; - R_3, C_3 correspond to the membrane/electrolyte interface; - R_4 is the resistance of the electrolyte

highest value of the specific capacitance of BLM reported is 1.5 μF.cm^{-2}, although most researchers use an upper limit of 1.0 μF.cm^{-2}. The lower limit of 0.3 μF.cm^{-2} corresponds to bilayers that are highly expanded, probably containing organic solvent between the phospholipid tails. The values of the specific capacitance for agar s-BLM do not differ from those reported for lipid bilayers spread across apertures (*7*). In contrast to the capacitance, the area-related membrane resistance for supported membranes in comparison with planar BLM is generally different. The defect-free unsupported membranes exhibit a membrane resistance of the order of 10^7–10^8 Ω.cm^2. The analysis of impedance data led to an area-related membrane resistance comparable with that for s-BLMs immobilised on gold electrodes (10^{-3}-1 MΩ.cm^2) (*8*). However, for the incorporation of channel-forming polypeptides into the lipid bilayer, the membrane resistance seems to play a part more important than the specific resistance. While the specific resistance may only reach values of ~5–400 Ω.cm^2, for membranes suspended across the apertures with diameters of 0.6–7 μm the corresponding resistance attains 10^9 Ω (*9*). The membrane resistance extracted from EIS data is comparable to that reported for metal supported bilayers (*8*). Assuming a dielectric constant of the non-polar hydrocarbon core of about ε=2, an estimate of a lipid film thickness d from $C_2=\varepsilon\varepsilon_0 S/d$, where ε_0 is the dielectric coefficient of free space and S is the surface area, yields an approximate value of 6.5 nm.

The suitability of agar s-BLM for biosensor development can be demonstrated by incorporation of channel-active Ala into the lipid bilayer. Since Ala is mainly surface orientated in the absence of membrane potential, and the application of the potential enhances the likelihood of transmembrane orientation, a DC potential of +70 mV was applied to the membrane during experiments (*10*). *Fig. 2* shows the time-dependent change of |Z| and ϕ of s-BLM after addition of the peptide. Induced by the applied positive potential, Ala molecules were inserted into the bilayer. As a result, a rapid change of the system impedance |Z| at the 1 Hz applied AC signal was observed. In a short time interval (~5 min) the system reached the new stable state. Thereafter impedance spectra of s-BLM modified with Ala were recorded. *Fig. 3* representatively illustrates the theoretical fit of EIS spectra on an unmodified s-BLM (curves a) and s-BLM in the presence of 6.10^{-7} M Ala (curves b). For comparison, *Fig. 4* shows the Nyquist plot of both agar s-BLM and Ala treated agar s-BLM. It can be seen from the figures that the interaction of Ala with s-BLM eventuates in a rapid increase of membrane conductance. Approximately 10 min after the addition of polypeptide into the KCl solution, the properties of s-BLM depicted in *Fig. 3* extracted by fitting were changed as follows from *Table 1.* The membrane resistance rapidly decreased due to the Ala insertion into the membrane. On the other hand, only a slight increase of membrane capacitance and a negligible thinning of the lipid bilayer were observed.

Ala has two binding states in a lipid bilayer, a surface state and a pore-forming state (*11*). Depending on peptide concentration, lipid composition, and applied

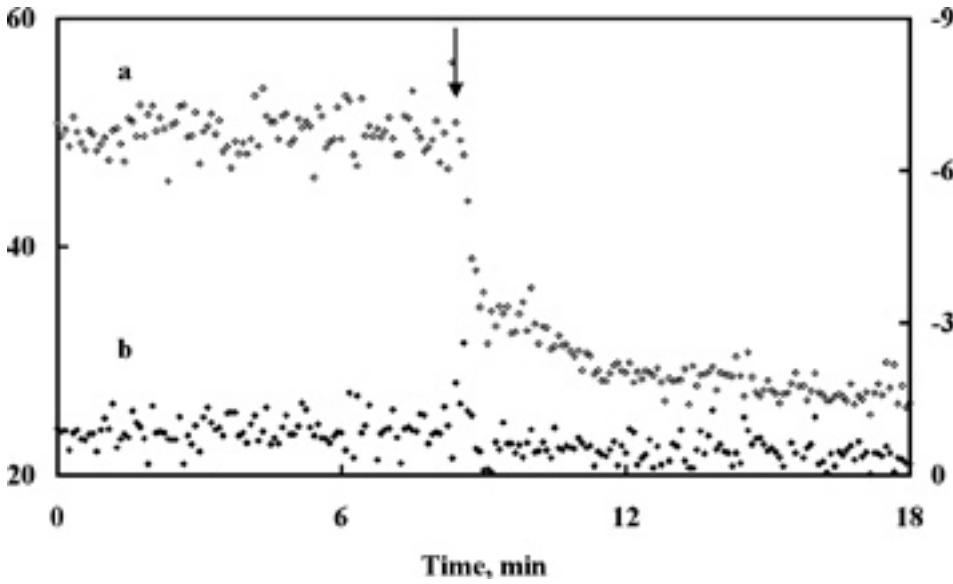

Fig. 2.
Time-dependent change of impedance |Z| (a) and phase shift angle φ (b) of agar s-BLM, amplitude of AC signal U=10 mV, f=1 Hz, U_{DC}=70 mV after addition of 2.10^{-7} M alamethicin (arrow).

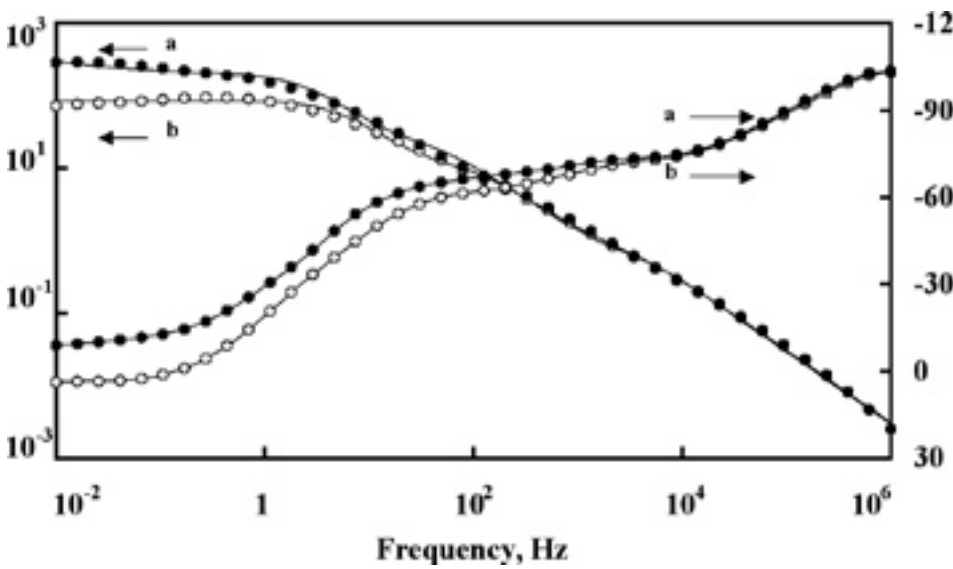

Fig. 3.
Impedance spectra of (a) agar s-BLM, (b) agar s-BLM in the presence of 6.10^{-7} M Ala, amplitude of AC signal U=10 mV, U_{DC}=70 mV, symbols – smoothed data, solid lines – fit to the equivalent electrical circuit *(Fig. 1).*

voltage, the peptide molecules may remain in the surface state or subsequently change into the state wherein the peptide molecules form transmembrane channels, apparently a mechanism to kill cells. The parameters of agar s-BLM allowed us to incorporate Ala into the lipid bilayer. Molecules of polypeptide probably formed channels across the bilayer, as the resistance of Ala-modified membrane extracted from the impedance spectra was significantly decreased, while only a slight increase of membrane capacitance was observed. The negligible thinning of the lipid bilayer is probably due to a local deformation of the lipid bilayer, as Ala α-helix is 34 long, and to an expansion of the membrane area depending on the values of bilayer elastic constants (*11*).

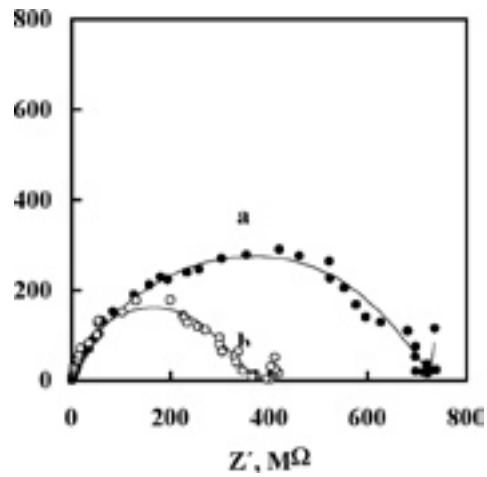

Fig. 4.
Impedance spectra (Nyquist plot) of a) agar s-BLM, b) agar s-BLM treated with Ala, amplitude of AC signal U=10 mV, U_{DC}=70 mV. The frequency range was 10^{-2}-10^{6} Hz, symbols – measured data, solid lines – smoothed data.

Table 1
Representative illustration of the parameters of unmodified and Ala treated (*Fig. 3*) s-BLM extracted from the fit to the equivalent electrical circuit

	R_2 [M]	C_2 [pF]	d [nm]
s-BLM	173.9	442.5	7.8
s-BLM+6.10^{-7}M alamethicin	77.1	454.1	7.6

A c k n o w l e d g e m e n t s

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ANALÝZA IMPEDANCE DVOJVRSTVÉ LIPIDOVÉ MEMBRÁNY S PODPOROU AGARU MODIFIKOVANÉ ALAMENTHICINEM

S o u h r n

Cílem práce bylo studium dvojvrstvé lipidové membrány s podporou agaru (s-BLM) elektrochemickou impedanční spektroskopií. Lipidové membrány (s-BML) o vysoké elektrické rezistenci splňují kritéria snadnosti a reprodukovatelnosti přípravy, dlouhodobé stability a nízké citlivosti na elektrické a mechanické poruchy, což umožňuje jejich nasazení v mikročipové technologii. Zavedení polypeptidu alamethicinu (Ala) po vytvoření s-BLM bylo použito k posílení tvorby dvojvrstvy a k průkazu použitelnosti agarové s-BLM pro měření aktivity iontových kanálů. Alamethicin je schopen vytvářet spontánně stabilní iontové kanály s napěťovým hrazením. K analýze a získání hlavních parametrů membrány z impedančních dat bylo použito ekvivalentního elektrického obvodu.

REFERENCES

1. *Tien HT, Salamon Z*. Formation of self-assembled lipid bilayers on solid substrates. Bioelectrochem Bioenerg 1989; 22: 211–218.
2. *Sabo J, Ottova A, Laputkova G, et al.* A combined AC-DC method for investigation supported lipid membranes. Thin Solid Films 1997; 306: 112–118.
3. *Passechnik VI, Hianik T, Ivanov SA, Sivak B*. Specific capacitance of metal supported lipid membranes. Electroanalysis 1998; 10: 295–302.
4. *Lu XD, Ottova AL, Tien HT*. Biophysical aspects of agar-gel supported bilayer lipid membranes: A new method for forming and studying planar bilayer lipid membranes. Bioelectrochem Bioenerg 1996; 39: 285–289.
5. *Cafiso D.* Alamethicin: A peptide model for voltage gating and protein-membrane interactions. Annu Rev Biophys Biomol Struct 1994; 23: 141–165.
6. *Melikov KC, Frolov VA, Shcherbakov A, et al.* Voltage-induced nonconductive pre-pores and metastable single pores in unmodified planar lipid bilayer. Biophys J 2001; 80: 1829–1836.
7. *Peterman MC, Ziebarth JM, Braha O, et al*. Ion channels and lipid bilayer membranes under high potentials using microfabricated apertures. Biomed Microdevices 2002; 4: 231–236.
8. *Steinem C, Janshoff A, Galla HJ, Sieber M.* Impedance analysis of ion transport through gramicidin channels incorporated in solid supported lipid bilayers. Bioelectrochem Bioenerg 1997; 42: 213–220.
9. *Schmidt C, Mayer M, Vogel H.* A chip-based biosensor for the functional analysis of single ion channels. Angew Chem Int Ed 2000; 39: 3137–3140.
10. *Römer W, Steinem C*. Impedance analysis and single-channel recordings on nano-black lipid membranes based on porous alumina. Biophys J 2004; 86: 955–965.
11. *Chen FY, Lee MT, Huangy HW*. Evidence for membrane thinning effect as the mechanism for peptide-induced pore formation. Biophys J 2003; 84: 3751–3758.