

Impedance Spectroscopy of Bilayer Lipid Membranes Self-assembled on Agar Support – Interaction with HDL

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Summary

The electrical properties of the supported lipid bilayer membrane (s-BLM) of egg phosphatidylcholine (PC) self-assembled on agar surface were examined. To characterize the insulating properties of s-BLMs, electrochemical impedance spectroscopy (EIS) was used. The analysis of impedance spectra in terms of an equivalent circuit of agar/electrolyte and agar/s-BLM/electrolyte in the frequency range of 0.1 Hz–10 kHz was performed. The high-density lipoproteins (HDL)/s-BLM interaction in the concentration range from 20 µg/ml to 80 µg/ml of HDL was investigated. It is evident that treatment of s-BLM with HDL resulted in an increase of the lipid film resistance and a decrease of membrane capacitance.

Key words

Agar-supported bilayer membrane • High-density lipoprotein • Electrochemical impedance spectroscopy

Introduction

An electrode-agar gel system as a support for the fabrication of s-BLM was used here. Agar surfaces offer a wide range of attractive physical properties that are important for the design of technical applications (Matsuno *et al.* 2004). Agar support allows overcoming one of the main drawbacks of the membrane systems on solid substrates - asymmetry of the two layers of the membrane. Other properties, such as fluidity and self-sealing, make them similar to the conventional BLMs.

Agar s-BLM can be accessed by a variety of surface analysis tools. In a number of reports it has been shown that EIS is useful as a nondestructive and sensitive method for investigation of lipid films on solid surfaces

(Wiegand 2000). The theoretical analysis of EIS data by fitting to a model represented by an equivalent electrical circuit allows the understanding of processes associated with the biomaterial-functionalized electrode surfaces.

The motivation of the present work was the implementation of EIS as an investigative tool for agar s-BLM/HDL interaction. HDL are highly heterogeneous lipoprotein particles consisting of an approximately equal amount of protein and lipid, mainly phospholipids and unesterified cholesterol. As a result of qualitative and quantitative differences in lipid, protein and enzyme content, HDL particles are multi-shaped complexes with varying density, fluidity, and charge. They consist of a hydrophobic core surrounded by a surface monolayer of phospholipids, unesterified cholesterol and apolipo-

proteins. HDL apolipoproteins have amphipathic properties, conferred by regions containing both polar and nonpolar amino acid residues. Hydrophobic forces whereby the fatty acyl chains and the nonpolar amino acids side chains are excluded from the aqueous environment drive the association of polar lipids and apolipoproteins in HDL. The apolipoproteins and the unesterified cholesterol have appreciable water solubility and can exchange easily between lipoproteins or with other lipid surfaces (Calabresi *et al.* 2006). The height of the HDL complex is consistent with a phospholipid bilayer structure. Furthermore, it has been demonstrated (Carlson *et al.* 1997) that HDL fuse when adsorbed to mica. Even if HDL binding proteins identified in the plasma membrane (Graham and Oram 1987) have been considered essential for HDL-membrane interaction, some studies indicate that HDL can interact with a protein-free lipid bilayer (Johnson *et al.* 1988, Tricerri *et al.* 1998). It is important to emphasize that most of HDL functions are exerted not by most apoproteins, but rather by lipids or enzymes associated with HDL (Nofer *et al.* 2002).

Methods

Materials

L- α -Phosphatidylcholine from egg yolk and n-dodecane were purchased from Sigma. The membrane forming solution was prepared by mixing an ethanol solution of PC with n-dodecane to attain a final 5 % lipid concentration. Lyophilised HDL from human plasma (Fluka) were rehydrated by lipoprotein refolding buffer (Fluka). Other chemicals (NaCl, Tris, EDTA) were of p.a. quality, and were used without further purification. The water used was Millipore-filtered ($R > 18 \text{ M}\Omega\cdot\text{cm}$, pH 5.5). As a standard buffer for electrochemical measurements, an aqueous solution of 150 mmol/l NaCl containing 10 mmol/l Tris and 1 mmol/l EDTA was used.

Method for supported lipid layer formation

The formation of s-BLM was in accordance with the procedure developed and further optimized by Lu *et al.* (1996). An agar-filled Teflon tube was used as a solid substrate. The tube was cut with a scalpel and dipped in a membrane-forming solution for about 1 s. Subsequently the tip was immersed in an aqueous solution. The exposure to the electrolyte promoted an alignment of the amphiphilic molecules leading to the formation of a bilayer.

Electrochemical measurements

AC analysis was carried out with a Zahner IM6e (Kronach) electrochemical analyzer providing fully computer-controlled EIS. The absolute values of the impedance $|Z(f)|$ and the phase shift angle $\varphi(f)$ were recorded in a frequency range from 10^{-1} to 10^4 Hz. A small sinusoidal voltage of 20 mV was applied and the current response of the system was measured. EIS data were analyzed by means of complex non-linear regression least-square (CNRLS) fit to a model represented by an equivalent electrical circuit. The data analysis was done using THALES software from Zahner Elektrik. A three-electrode system was used for the EIS experiments. A Teflon tube with the inner diameter of 0.5 mm filled with 3 % agar in 0.15 M KCl with an Ag/AgCl wire was used as the working electrode, a platinum wire served as a counter electrode, and (Ag/AgCl, 3.5 M KCl) as the reference electrode. The thermostatically controlled measuring cuvette placed into a Faraday cage was used. All experiments were carried out at room temperature (21.0 ± 0.2) °C.

Results

Due to the suitable properties of agar surfaces to serve as a matrix for the formation of symmetrical lipid membranes, the EIS response of the bare agar-filled working electrode was studied first. Fig. 1 gives the Nyquist plot of the impedance spectrum with the inset of an equivalent electrical circuit for fitting the measured data. In the studied frequency range the spectrum is composed of an elongated semicircle at high frequencies prolonged by a curved line for low frequencies. The semicircle corresponds to the contribution of the resistance of the agar phase (R) in parallel with its capacitance represented with the constant phase element CPE due to the non-ideal capacitive response of the interface. CPE generates the impedance that can be expressed as follows:

$$Z_{\text{CPE}}(j\omega) = 1/Y_0(j\omega)^\alpha \quad (1)$$

where $0 < \alpha < 1$ is the CPE power and Y_0 is the CPE constant. ω is the angular frequency; j is the imaginary unit (McDonald 1987). The curved line should be ascribed to the CPE_d capacitance of the double-layer of the electrolyte/agar interface. This behavior is, basically, explained by diffusion charge transport that is dominant at lower frequencies. CPE_d behavior is intensified by

Table 1. Fitted data for the agar support after modification with PC and the addition of HDL

HDL ($\mu\text{g/ml}$)	C_d (pF)	R (M Ω)	C (pF)	R_e (k Ω)	C_s (pF)
0	320.3 \pm 57.9	82.2 \pm 23.7	50.7 \pm 4.5	11.1 \pm 0.1	45.0 \pm 0.3
20	345.8 \pm 75.9	136.9 \pm 49.0	46.1 \pm 4.6	11.1 \pm 0.2	45.5 \pm 0.3
40	430.3 \pm 104.6	157.3 \pm 57.4	42.1 \pm 4.1	11.2 \pm 0.1	45.6 \pm 0.4
60	436.7 \pm 108.4	199.0 \pm 60.9	38.7 \pm 2.1	11.1 \pm 0.1	45.4 \pm 0.5
80	504.9 \pm 74.8	242.1 \pm 48.7	38.6 \pm 2.6	11.4 \pm 0.3	45.3 \pm 0.2

inhomogeneities in the dimension and the shape of the capillary pores in agar. Resistance R_e in serial combination represents the contribution from the bulk electrolyte, contacts, and connections. The equivalent circuit was extended to take into account the capacitance C_s representing the stray capacitance of the measuring cell. Ideally, R_e and C_s are not affected by modification on the electrode surface as they represent the properties of the electrolyte solution and the measuring cell.

The quality of fitting was evaluated by the error value. By using the CPE, the quality of fitting had a decreased error value; the mean relative error of $|Z|$ did not exceed 0.1 %, the maximum error of φ did not overstep 0.3 deg. The fitting procedure yielded for the bare electrode a CPE capacitance value of $C = 53.4$ pF and a resistance value of $R = 85.4$ k Ω . The value of a CPE_d capacitance was determined as $C_d = 379.2$ nF. The disturbing capacitance for the entire circuit reached a value of $C_s = 44.5$ pF and an electrolyte resistance corresponding to the value of $R_e = 11.7$ k Ω .

The self-assembly of s-BLM was followed by means of monitoring the change of the modulus of the electrical impedance $|Z|$ and the phase shift angle φ of the system at 10 kHz sinusoidal voltage. The time for the lipid film stabilization was of the order of 30 min. This time is less than that obtained for Pt-supported membrane (Sabo *et al.* 1997), but it is twice as much as the stabilization of agar-thin film s-BLM described by Novotny *et al.* (1997). It should be mentioned that membrane formation strongly depended on the quality of the Teflon rim. If the rim was poorly cut, membrane formation was difficult and a rupture occurred within a few minutes. The lifetime of s-BLMs was rarely less than 24 h.

The lipid film changed the impedance spectra quite markedly, showing the formation of a densely packed lipid membrane. In Fig. 2 a typical spectrum of s-BLM is shown. Electrical parameters of s-BLM were

obtained from fitting the parameters of the equivalent circuit shown in Fig. 1 to the data. The circuit used is formally the same as that used for the bare agar electrode. It should be mentioned that now the CPE capacitance C of the supported system covered with lipid contained contributions of both the lipid layer capacitance C_m and the capacitance of the agar phase C_a of the bare electrode (Ding *et al.* 2002). The two capacitances were in series and hence the CPE capacitance C could be described by the following expression:

$$1/C = 1/C_m + 1/C_a \quad (2)$$

Similarly, the resistance R follows the expression:

$$R = R_m + R_a \quad (3)$$

As for the bare agar electrode CPE_d behavior represents the charge transfer at the agar surface, now restricted by the deposition of the lipid layer.

The mean relative error of $|Z|$ did not exceed 0.5 %, the mean error of φ did not overstep 0.2 deg for s-BLM spectra. The fitting procedure yielded a CPE capacitance value of $C = 50.7$ pF and a resistance value of $R = 82.2$ M Ω . The obtained value of CPE power α (about 0.8) demonstrates a deviation of the system from ideal capacitive response and confirms the suitability of CPE for modeling the electrolyte/electrode interfaces. The value of a CPE_d capacitance was determined as $C_d = 320.3$ pF. As expected, the disturbing capacitance for the entire circuit and an electrolyte resistance did not differ significantly from the values obtained for the bare agar surface. Simulated values of the main electrical elements in the equivalent circuit are given in Table 1 as means \pm SD ($n = 6$).

Following expression (2) the contribution from the membrane capacitance C_m calculated from the fitted values of the capacitance C and the capacitance of the

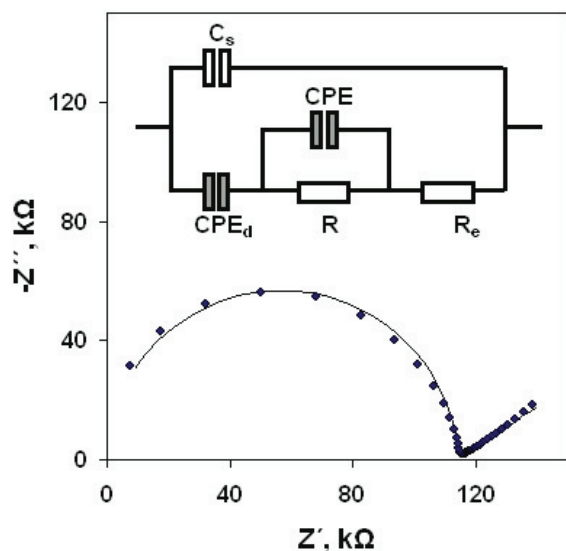


Fig. 1. The Nyquist plot of the impedance spectrum for the bare agar electrode. The solid line is the result of the fitting procedure using the equivalent circuit. Frequency range: 0.1 Hz–10 kHz. Amplitude of AC voltage: 20 mV. Inset: Equivalent circuit used to model impedance data.

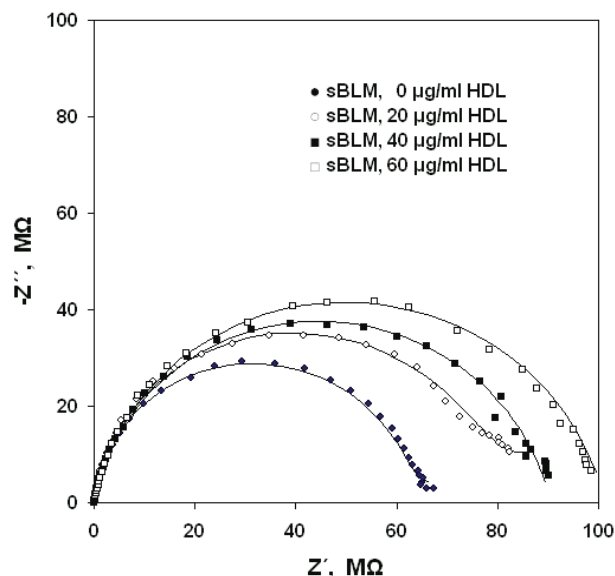


Fig. 2. Nyquist plots of the impedance spectra for the s-BLM on the agar support with the addition of HDL. The solid lines are the result of fitting the parameters of the equivalent circuit shown in Fig. 1. Frequency range: 0.1 Hz – 10 kHz. Amplitude of AC voltage: 20 mV.

agar phase of the bare agar electrode C_a comprises ≈ 880 pF, which represents the value corresponding to the well-developed lipid bilayer for the support area of $1.96 \times 10^{-3} \text{ cm}^2$.

HDL were used to investigate the functionality of s-BLM immobilized on agar surface. It is known that low HDL ($<35 \mu\text{g/ml}$) may be associated with the increased risk of the coronary atherosclerosis, while conversely high levels ($>55 \mu\text{g/ml}$) appear to have a protective effect. EIS response of agar s-BLM was observed in the concentration range from 20 to $80 \mu\text{g/ml}$ of HDL. Fig. 2 shows impedance spectra at different HDL concentrations. The Nyquist plots provide a more direct and easy understanding of the conduction processes involved. It can be noticed that the areas enlarge with increasing HDL.

Analysis of the fitted data (Table 1) revealed that the major changes were those in the capacitance C of the constant phase element CPE and the resistance R . The change of CPE_d capacitance C_d was observed as well. Addition of HDL resulted in a decrease of the capacitance C and an increase of the resistance R . Approximately 15 min after HDL addition the impedance spectra remained constant (not shown). The overall effect of HDL on the electrical parameters of s-BLM was more pronounced for the resistance R . When the concentration of HDL in the electrolyte reached $80 \mu\text{g/ml}$, the change in the resistance R (195 %) was much higher than that obtained for the

capacitance (33 %), indicating that R could be more effective in the detection of the HDL/s-BLM interaction compared with the CPE capacitance C .

As the change of the CPE capacitance C is indicative of the possible change of the thickness of the deposited membrane, the dependence in Fig. 3 shows the behavior of the s-BLM capacitance C_m over the studied HDL concentration range. A steep decrease of membrane capacitance observed for the low concentration was followed by a subtle change of the membrane capacitance over $60 \mu\text{g/ml}$.

For further illustration the meaning of the fitted impedance data, the calibration plots of the CPE capacitance C_m and the resistance R were made. Fig. 4 exhibits the calibration plot of the difference in the inverse capacitance C_m after and before HDL interacted with the lipid film, $\Delta(1/C_m)$, as a function of HDL concentration. While the plot shows for the lowest HDL concentrations a linear relationship, for higher concentrations the gradual saturation of the curve was observed. Fig. 5 shows the calibration plot of the difference in the resistance ΔR as a function of HDL concentration. Like for the capacitance, a deflection from linear dependence was observed for higher HDL concentrations. As revealed by the evaluation of the impedimetric data, the resistance signal is quite sensitive for the study of the HDL/s-BLM interaction as the calculated sensitivity reached the value of $1.5 \text{ M}\Omega/\mu\text{g/ml}$.

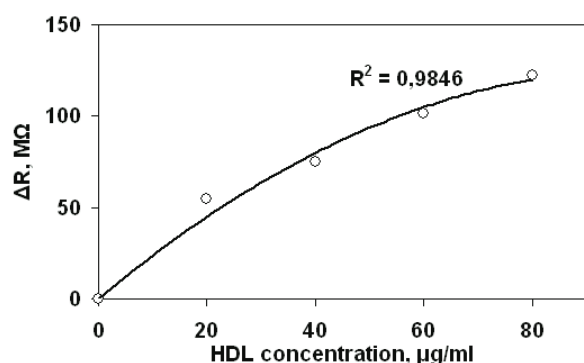


Fig. 3. Response of s-BLM capacitance C_m to HDL concentrations.

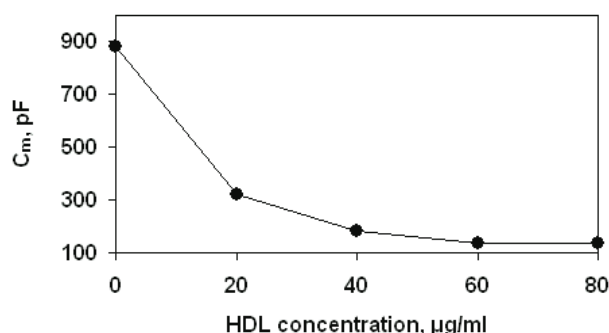


Fig. 4. Changes in the inverse CPE capacitance $\Delta(1/C_m) = 1/C_{mHDL} - 1/C_m$ of s-BLM as a function of HDL concentration.

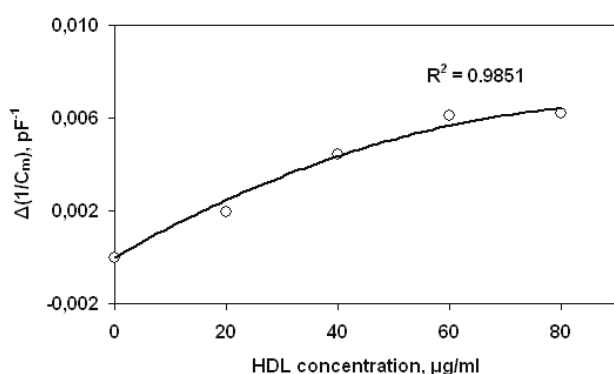


Fig. 5. Changes in the resistance $\Delta R = R_{HDL} - R$ of the lipid bilayer on the agar as a function of HDL concentration.

Discussion

Agar (agarose) intensively used recently as a support makes it possible to prepare the stable thin membrane separating two aqueous media that are viscous, fluid and polar enough possessing the electrical properties of the conventional BLM. If the major drawback of free-standing BLMs is their low long-term stability, usually not exceeding 8 h (Tien and Ottova 1999), s-BLMs reported here exhibited significantly higher mechanical and long-term robustness, lasting

rarely for less than 24 h. The subsequent rupture of s-BLM may be due to hydrolysis of the ester linkage of the glycerol residue of natural phospholipid in the presence of water (Costello *et al.* 1999). The products of the hydrolysis are soluble and diffuse into the agar gel.

The contribution from membrane capacitance C_m (≈ 880 pF) calculated from the fitted values of the capacitance C and the capacitance of the agar phase of the bare agar electrode C_a represents the value corresponding to the well-developed lipid bilayer for the support area of $1.96 \times 10^{-3} \text{ cm}^2$. It is close to those values of the specific membrane capacitance of BLM. The highest value of the specific capacitance of BLM reported is $1.5 \mu\text{F}/\text{cm}^2$, although most researchers use an upper limit of $1.0 \mu\text{F}/\text{cm}^2$. The lower limit of $0.3 \mu\text{F}/\text{cm}^2$, and values in this range, corresponds to bilayers that are highly expanded, probably containing organic solvent between the phospholipid tails (White 1985).

The analysis of impedance data led to the area-related membrane resistance of $\approx 0.16 \text{ M}\Omega \cdot \text{cm}^2$ that is comparable well with other types of s-BLMs reported (Bordi *et al.* 2002). However, it is lower than the specific membrane resistance of the free-standing planar lipid bilayers, which exhibit very high resistance of the order of $10^7 - 10^8 \Omega \cdot \text{cm}^2$ (Mueller and Rudin 1968).

Surface roughness and porosity of the electrode surface have been considered as important factors contributing to CPE behavior. It seems possible that the low value of CPE power α results from the increased roughness of the lipid film due to the disturbing effect of chains of the agarose polymer.

According to Wang *et al.* (2002), the CPE behavior is also due to diffusion, which is increased by inhomogeneities in the dimension and shape of the capillary pores. CPE_d values obtained for the bare agar electrode and s-BLM support the idea that the deposition of the membrane led to the substantial decrease of CPE_d capacitance.

Finally, it is worth mentioning that the equivalent circuit selected for fitting does not meet all requirements, especially those regarding the direct extraction of the s-BLM capacitance C_m and the resistance R_m and needs additional improvement.

The second aim of this work was to study the interaction of human plasma HDL with s-BLM free of cholesterol. The interaction showed a response of the system proportional to the concentration of HDL. A decrease of the CPE capacitance C and, at the same time, an increase of the resistance R was observed. It is

plausible that the increase of the membrane resistance or the decrease of a leakage current after addition of HDL could be conceivably caused by a thickening process. The possibility cannot be ruled out because the membrane system capacitance is observed simultaneously to decrease. The improved leakage properties at low HDL concentration can result from an annealing process, in which the holes in the bilayer are gradually filled in. A decrease in the capacitance at the higher HDL concentrations reaches a value that is indicative of an eventual adsorption of HDL on the bilayer creating multilayer structures.

The increased amount of water molecules displaced from the lipid layer due to the annealing process could give rise to the mean dielectric constant in the interspace. Then the change of CPE_d capacitance, due to the added HDL, can result from the change in the mean dielectric constant in the space between agar and the lipid layer.

It has been proposed that cholesterol transport from plasma membranes to acceptor HDL could be

modulated by transient anchoring of HDL to the plasma membrane through the association of amphipathic helices of apoA-I. Even if HDL binding proteins identified in the plasma membrane (Graham and Oram 1987) have been considered essential for HDL-membrane interaction, some studies indicate that HDL can interact with a protein-free lipid bilayer (Johnson *et al.* 1988). Tricerri *et al.* (1998) studying the influence of the recombinant HDL by measuring leakage on 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and POPC/cholesterol vesicles showed the ability of rHDL to interact even with a cholesterol-free lipid bilayer. The results presented here are also not in contradiction with the experiments of Vojčiková and Sabo (2001), which showed the tendency of HDL to increase the resistance and to decrease the capacitance of s-BLM deposited on Pt support.

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