Electrically insulating pore-suspending membranes on highly ordered porous alumina obtained from vesicle spreading

Eva K. Schmitt,* Mohammed Nurnabi, Richard J. Bushby and Claudia Steinem*

a Institut für Organische und Biomolekulare Chemie, Georg-August Universität, Tammanestr. 2, 37077 Göttingen, Germany
b Centre for Self-Organising Molecular Systems, University of Leeds, Leeds, LS2 9JT, United Kingdom

1. Materials and Methods

Materials. Aluminum substrates (thickness 0.5 mm, purity 99.999%) were obtained from Goodfellow (Huntington, UK). 1,2-Diphtanyloyl-sn-glycerol-3-phosphocholine (DPhPC), 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycerol-3-phosphate, mono sodium salt (DPPA) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Diethylether and Tris were from Carl Roth GmbH (Karlsruhe, Germany). Sodium chloride (NaCl) and potassium chloride (KCl) were from Merck (Darmstadt, Germany). n-Decane was from Sigma Aldrich (Taufkirchen, Germany). Cholesterylpolyethylenoxyl thiol (CPEO3) was synthesized following the procedure developed by Boden et al.1 Purple membranes were isolated according to standard procedures from Halobacterium salinarium.2 The water used was ion-exchanged and millipore-filtered (Milli-Q-System, Millipore, Molsheim, France; specific resistance $R > 18 \text{ M} \Omega \text{ cm}^{-1}$, pH 5.5).

Porous substrates. Porous alumina substrates were utilized with a mean pore diameter of 60 nm. The aluminum foils were cleaned in ethanol p.a., electropolished and anodized in aqueous oxalic acid solution (0.3 M) at $V = 40 \text{ V}$ and $T = 2 \text{ °C}$ in a two step process. In a first step, the aluminum substrates were anodized for 12 h, followed by an incubation in an aqueous solution of phosphoric acid (6 wt%) and chromium(VI)oxide (1.8 wt%) for 5 h at 70 °C to remove the porous alumina. This procedure generates a hexagonal pattern in the aluminum substrate serving as a mask for the second step, where the substrates were again anodized for 5 d to obtain a porous aluminum oxide layer of about 200 µm thickness. The resulting porous alumina substrates were then treated with a saturated HgCl2-solution to remove the underlying aluminum layer. To obtain a sieve-like structure, the pore bottoms were removed by chemical etching at $T = 30 \text{ °C}$ with 10 wt % phosphoric acid solution. The bottom surface of the porous alumina substrates was coated with a thin gold layer (25 nm) using a sputter coater with a thickness control unit (Cressington sputter coater 108auto, Cressington MTM-20, Elektronen-Optik-Service, Dortmund, Germany). The gold coated substrates were then functionalized with CPEO3 by incubation with a 1 mM n-propanol solution of CPEO3 for more than 12 hours. The obtained hydrophobic substrates were thoroughly rinsed with ethanol p.a. and dried under a stream of nitrogen.

Vesicle preparation. Lipid vesicles were prepared according to the extrusion method. A chloroform solution of DPhPC and DOPC in a molar ratio of 60:40 was added to a glass tube. The solvent was evaporated under a stream of nitrogen followed by several hours under vacuum at 30 °C resulting in a lipid film. After incubation of the lipid film with electrolyte
solution (0.1 M NaCl) for 30 min, the sample was vortexed for 30 s in three cycles at intervals of 5 min. The resulting suspension of multimamellar vesicles was extruded 31 times through a polycarbonate membrane with nominal pores of 1000 nm (Avestin, Mannheim, Germany) to obtain large unilamellar vesicles (LUVs) with an average vesicle diameter of (600 ± 20) nm. The final vesicle concentration was 1 mg/mL.

**Bacteriorhodopsin containing vesicles.** Unilamellar vesicles containing bacteriorhodopsin were prepared according to the reversed phase method. 1 mg of DPhPC/DOPC/DPPA in a molar ratio of 60:30:10 were dissolved in chloroform p.a., dried in a small round bottom flask under a stream of nitrogen followed by several hours under vacuum at 40 °C, which results in a lipid film. The film was dissolved in 0.5 ml diethylether. 51 µl of a 6.4 mg/mL purple membrane suspension were added resulting in a protein content of 1 mol%. After the sample was tip sonified for 2 min while cooling with ice, 449 µl buffer solution (10 mM Tris/HCl, 100 mM KCl, pH 7.4) were added to the yellow solution. The diethylether was removed at 40 °C using a rotary evaporator and another 500 µl buffer solution were added. The sample was again set under vacuum until residual organic solvent was removed yielding in a 1 mg/mL lipid concentration. The resulting suspension appeared purple indicating the successful incorporation of bacteriorhodopsin in the vesicles. Finally, the resulting suspension was extruded 31 times through a polycarbonate membrane with nominal pores of 1000 nm.

**Impedance spectroscopy.** The formation process of pore-spanning lipid bilayers and their electrical properties were characterized by means of a gain/phase analyzer SI 1260 combined with a dielectric interface SI 1296 (Solartron Instruments, Farnborough, UK), controlled by a personal computer. For AC impedance analysis, CPEO3-functionalized and dried porous alumina substrates were placed horizontally between two Teflon chambers equipped with two sealing rings (diameter 1.5 or 2.0 mm). Both chambers were filled with electrolyte solution (0.1 M NaCl). Prior to the addition of vesicle suspension the sealing ring region was primed with n-decane. Platinized platinum wires served as working and counter electrodes. The absolute value of the impedance $|Z| (f)$ and the phase angle $\phi (f)$ between voltage and current were recorded in a frequency range of $10^{-1}$-10$^6$ Hz, which took approximately 2 min per cycle. Some measurements were performed from $10^{-3}$-10$^6$ Hz, resulting in cycles of 40 min. For time dependent measurements, the magnitude of the impedance was monitored at a fixed frequency. Data recording and analysis was performed using the Zview 2.9c software package with Calc-Modulus data weighting.

**Photocurrent measurements.** The same setup as used for the impedance analysis was used for photocurrent measurements. The upper surface of the functionalized porous substrate was illuminated using a 250 W halogen lamp. The light was passed through a 515 nm cut-off filter and guided by an optical fiber into a darkened Faraday cage. A mechanical shutter operated by a wire release was used for switching the light on and off. The light induced current was measured with a current amplifier (Keithley 428, Keithley Instuments, Germering Germany). Data were transferred via a GPIB interface to a personal computer using an acquisition program written in Labview 6.0. The direction of the positive current corresponds to the translocation of protons from the cis- to the trans-compartment.

**2. Kinetics of vesicle spreading and fusion**

Since the process of bilayer formation takes rather a long time as concluded from our first impedance analysis, we performed a detailed study on the formation process by following the change in the electrical properties of the insulating layer after addition of vesicles in a time-resolved manner. Vesicles were added to the functionalized porous alumina substrate and
impedance spectra were recorded frequently. Figure S-1 shows the absolute value of the impedance $|Z| (f)$ and the phase shift $\phi (f)$ of a functionalized porous alumina substrate recorded between 4 and 22 hours after vesicle addition. During the process of bilayer formation, which is assumed to be based on vesicle adsorption, spreading and fusion, the impedance spectra are characterized by two dispersions. The dispersion at higher frequencies eventually vanishes resulting in spectra as depicted in Figure S-1 that do not significantly change within the next hours. While in most of the experiments, the resistance at low frequencies remains more or less constant or is slightly increased, in some preparations, the resistance even decreased for longer incubation times. We suggest that the dispersion at higher frequencies in the impedance spectra during the formation process might be a result of attached vesicles and probably hemifused vesicles, while part of the surface is already covered by planar pore-suspending bilayers. During the formation process, the attached vesicles, which may add to the overall resistance of the membrane system, can either spread to form highly insulating pore-suspending membranes or detach from the surface leading to a decrease in resistance.

3. Insertion of OmpF in pore-suspending membranes

We raised the question whether the newly established membrane system is suited to monitor the channel activity of a membrane protein. The outer membrane protein F (OmpF) of *E. coli* (a kindly gift from Biomade, Groningen, NL) was chosen to elucidate the possibility to insert the protein and monitor its channel activity in pore-suspending membranes by impedance analysis in an integral manner. Prior to the insertion experiment, impedance spectra were
recorded to determine the frequency range, which allows for the observation of the membrane resistance $R_{bl}$. 1 Hz turned out to be a reasonable frequency, since the absolute value of the impedance is frequency independent within a frequency range of 0.5-5 Hz. In Figure S-2, the magnitude of the impedance at 1 Hz is shown as a function of time. A value of around 850 kΩ is achieved, which represents $R_{bl}$, since $R_{el}$ contributes only very little to this large resistance. The protein OmpF is dissolved in an octyl-polyoxyethylene (o-POE) detergent solution. As a control, the detergent was first added to the pore-suspending membrane at the cis-side resulting in a decrease of $|Z|$ (1 Hz) by about two orders of magnitude, demonstrating that the detergent itself decreases the membrane resistance $R_{bl}$. However, upon rinsing with NaCl solution, the impedance regains to its original value. Performing the same experiment, but adding OmpF in o-POE (0.5 nM final concentration) again leads to a decrease of $|Z|$ (1 Hz) by about two orders of magnitude but only an increase upon rinsing with NaCl solution to 50 kΩ. The net drop in bilayer resistance $R_{bl}$ is a result of the formation of OmpF pores in the pore-suspending membrane demonstrating that proteins are functionally inserted into the established membrane system.

**Figure S-2.** Time-resolved change of the magnitude of the impedance $|Z|$ at a fixed frequency of 1 Hz of a pore-suspending membrane after the addition of o-POE with a final concentration of 0.0002 % (v/v) and rinsing with buffer followed by the addition of OmpF dissolved in o-POE to the cis-side leading to a final protein concentration of 0.5 nM in 0.0002 % (v/v) o-POE. Electrolyte: 0.1 M NaCl

**References**