Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioelectrochemistry 63 (2004) 161–167
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Tethered bilayer lipid membranes self-assembled on mercury electrodes

M.R. Moncelli[a,*], L. Becucci[a], S.M. Schiller[b]

[a] Chemistry Department, Laboratory of Bioelectrochemistry, University of Florence, Via della Lastruccia 3, 50019 Sesto Fiorentino Florence, Italy
[b] Max Planck Institute for Polymer Research, Ackermannweg 10, D-55128 Mainz, Germany

Received 23 June 2003; received in revised form 13 October 2003; accepted 16 October 2003

Abstract

In order to incorporate integral proteins in a functionally active state, metal-supported lipid bilayers must have a hydrophilic region interposed between the bilayer and the metal. This region is realized with a hydrophilic molecule terminating at one end with a sulfhydryl or disulfide group that anchors this ''hydrophilic spacer'' to the surface of a metal, such as gold or mercury. The other end of the hydrophilic spacer may be covalently linked to the polar head of a phospholipid molecule, giving rise to a supramolecule called ''thiolipid'' (TL). With respect to gold, mercury has the advantage of providing a defect-free and fluid surface to the self-assembling spacer. Hydrophilic spacers consisting of a polyethyleneoxy or a hexapeptide chain, as well as thiolipids derived from these spacers, were employed to fabricate mercury-supported lipid bilayers. The formation of a lipid bilayer on top of a self-assembled monolayer of a hydrophilic spacer, or of a single-lipid monolayer on top of a self-assembled monolayer of a thiolipid, was realized by simply immersing the coated mercury electrode into an aqueous solution across a lipid film previously spread on its surface at its spreading pressure. Particularly stable mercury-supported lipid bilayers were obtained by using thiolipids. The biomimetic properties of these lipid bilayers were tested by incorporating channel-forming polypeptides (gramicidin and melittin) and proteins (OmpF porin). The effect of the transmembrane potential on the function of these channels was estimated by using a simple electrostatic model of the mercury–solution interphase.

1. Introduction

Integral membrane proteins and channel-forming peptides may retain their functionally active state only if they are incorporated into lipid bilayers that (i) are in the liquid crystalline state, (ii) have an aqueous or hydrophilic medium on both sides, and (iii) are sufficiently free from pinholes and other defects that may provide preferential pathways for electron and ion transport across the bilayer [1]. In this respect, the interposition of a hydrophilic layer between a metal and a lipid bilayer is of utmost importance to create the hydrophilic environment required for the proper folding of the extramembrane sections of integral proteins; it is also fundamental to allow a flow of ions across metal-supported lipid bilayers incorporating channel-forming peptides or proteins.

Hydrophilic molecules consisting of polyethyleneoxy [2–6] or polypeptide chains [7], terminating at one end with a sulfhydryl or disulfide group for anchoring to a metal, have been frequently employed as ''hydrophilic spacers'' to be interposed between the metal and a lipid bilayer. Different procedures have been adopted to form a lipid bilayer on top of hydrophilic spacers self-assembled on gold [2–5] or on mercury [6,7]. Thiolipids are supramolecular structures obtained by covalently binding the polar head of a phospholipid molecule to one end of a hydrophilic ethylenoxy [2,3,8–12] or oligopeptide [13–15] chain terminating with an anchoring sulfhydryl group at the other end. Self-assembly of a thiolipid on a metal surface yields a lipid monolayer, on whose top a second monolayer may be self-assembled by spontaneous spreading and splitting of lipid vesicles. Hydrophilic spacers and thiolipids have been successfully anchored to gold and used to incorporate integral proteins by spreading, or by spreading and splitting, of proteoliposomes [13–18]. With respect to gold, mercury has the advantage of providing a defect-free, fluid and readily renewable surface to the self-assembling film, although it is not suitable for biosensor applications.

This note aims at describing the use of a hanging mercury drop electrode (HMDE) as a support for a spacer/(lipid bilayer) assembly. Phase-sensitive AC voltammetry and impedance spectroscopy were employed. The main

* Corresponding author. Tel.: +39-55-4573-100; fax: +39-55-4573-098.
E-mail address: moncelli@unifi.it (M.R. Moncelli).

1567-5394/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2003.10.017

objective of this note is to demonstrate the suitability of this biomimetic membrane for the incorporation of channel-forming peptides and proteins, such as gramicidin, melittin and OmpF porin. The role of the transmembrane potential on the function of these channels is estimated on the basis of a simple electrostatic model of the mercury/water interphase [19–21].

2. Materials and methods

The water used was obtained from light mineral water by distilling it once and by then distilling the water so obtained from alkaline permanganate. Merck suprapur® KCl was baked at 500 °C before use to remove any organic impurities. Dioleoylphosphatidylcholine (DOPC) was obtained from Lipid Products (South Nutfield, Surrey, England), while diphytanoylphosphatidylcholine (DphyPC) was purchased from Avanti Polar Lipids (Birmingham, AL). Valinomycin, melittin, gramicidin D and cholesterol were purchased from Sigma and used without further purification. An aqueous solution of 1.6 mg/ml OmpF porin in a pH 7.3 buffer containing 20 mM NaH_2PO_4 and 1% octyl-POE was a generous gift of Dr. G.L. Orris from Basel University. Triethyleneoxythiol (EO3) was provided by the Centre of Self-Organising Molecular Systems of Leeds University and synthesized as described elsewhere [22]. The thiolpeptide $HS-(CH_2)_2-[Aib-Glu(OTeg)]_2-Aib-Ala-OH$ (Aib = α-aminoisobutyric acid; Teg = triethyleneglycol monomethylether) (TP), synthesized as described in Ref. [7], and the thiolipid (TL) obtained by linking TP to the polar head of dimyristoylphosphatidylcholine (see Fig. 1) were provided by Prof. C. Toniolo from Padova University. The 2,3,di-*O*-phytanyl-*sn*-glycerol-1-tetraethylene-glycol-D,L-α lipoic acid ester lipid (DPTL) was synthesized as described in Ref. [23] (see Fig. 1). The other chemicals and solvents were commercially available and used as received.

DOPC and DphyPC solutions were prepared by diluting a proper amount of stock solutions of these phospholipids with pentane. Solutions of 1×10^{-3} M EO3 and 0.2 mg/ml DPTL in ethanol were prepared from a 0.6 M suspension of EO3 in ethanol and from a 2 mg/ml solution of DPTL in ethanol, while solutions of 1×10^{-3} M TP and 5×10^{-4} M TL in chloroform were prepared by diluting their 1×10^{-2} M stock solutions with the same solvent. Stock solutions of these spacers and thiolipids were stored at −18 °C. With the exception of DPTL, they were also stored under argon, to prevent the oxidation of these thiols to disulfides.

Stock solutions of 8×10^{-5} M valinomycin (VAL) were prepared in chloroform or ethanol, those of 0.2 mg/ml melittin in water were freshly prepared before use, porin was used as provided, and stock solutions of 1.5×10^{-4} M gramicidin D were prepared in ethanol. These solutions were stored at +4 °C.

All measurements were carried out in aqueous 0.1 M KCl. A home-made hanging mercury drop electrode (HMDE), described elsewhere [24], was employed. It allows accurate changes in drop area of as little as 0.04 mm^2. Use was made of a home-made glass capillary with a finely tapered tip, about 1 mm in outer diameter. Capillary

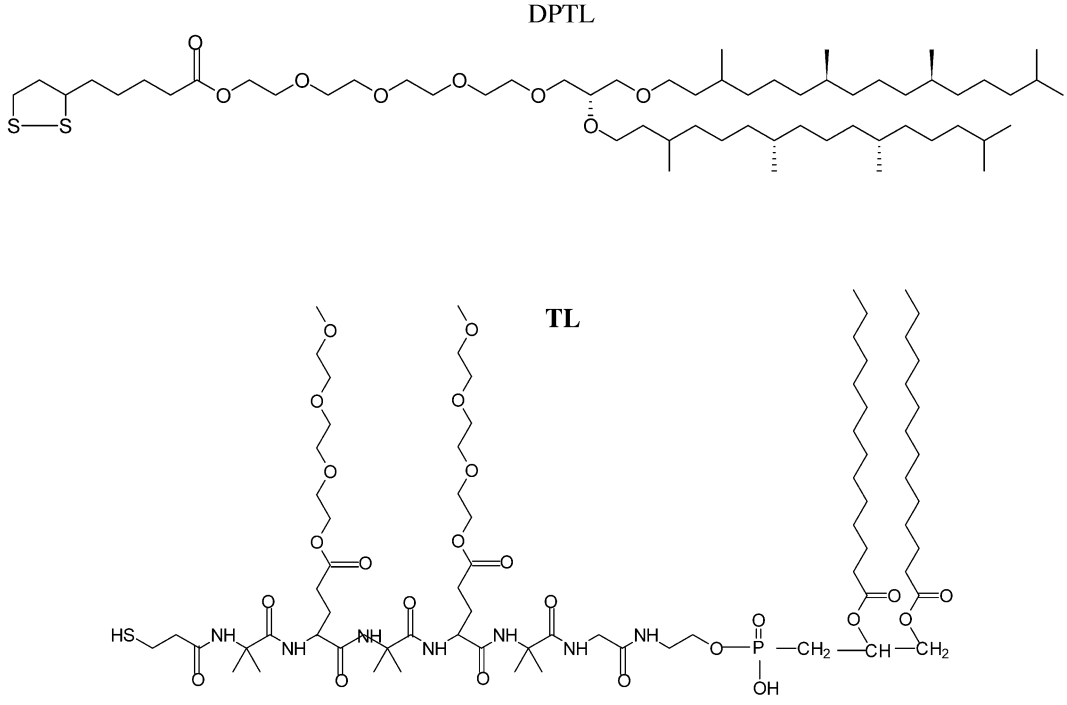

Fig. 1. Chemical structure of DPTL and TL.

and mercury reservoir were thermostated at 25 ± 0.1 °C by the use of a water-jacketed box to avoid any changes in drop area due to a change in temperature. Two glass electrolysis cells containing aqueous 0.1 M KCl and a small glass vessel containing the ethanol or the chloroform solution of the spacers were placed on a movable support inside the box [25]. The HMDE and the support were moved vertically and horizontally, respectively, by means of two oleodynamic systems that ensured the complete absence of vibrations.

AC voltammetry and impedance spectroscopy measurements were carried out with an Autolab instrument (Echo Chemie) supplied with FRA2 module for impedance measurements, SCAN-GEN scan generator and GPES3 software. Potentials were measured versus a Ag|AgCl (0.1 M KCl) reference electrode, but are referred to a saturated calomel electrode (SCE).

Monolayers of EO3, TP, TL and DPTL were self-assembled on the HMDE by keeping the mercury drop immersed in the small vessel containing the spacer solution for a period depending on the nature of the spacer, but not longer than 30 min. In the meantime, a pentane solution of DOPC or DphyPC was spread on the surface of the 0.1 M KCl aqueous solution contained in one of the two electrolysis cells, in an amount corresponding to five to six phospholipid monolayers, and the pentane was allowed to evaporate. Using the oleodynamic system, the spacer-coated HMDE was then extracted from the vessel, washed with the organic solvent to remove the excess of adsorbed spacer (for the case of thiolipids), and kept in an Ar atmosphere for the time strictly necessary to allow the solvent to evaporate. Immediately afterwards, the electrolysis cell containing the aqueous solution on whose surface the phospholipid had been previously spread, was brought below the HMDE and the latter was lowered so as to bring it into contact with the phospholipid film. With the EO3 and TP hydrophilic spacers, the drop was positioned in such a way as to keep the drop neck in contact with the lipid reservoir [6,7]; with the TL and DPTL thiolipids, the drop was completely immersed into the electrolyte solution. The applied potential was then repeatedly scanned over a potential range depending on the spacer nature, while continuously monitoring the curve of the differential capacity, C, vs. the applied potential, E, until a stable C vs. E curve was attained. The minimum differential capacity of the resulting functionalized electrode ranged from 0.5 to 1 μF cm^{-2}, with the exception of TL (see below), and was therefore close to, or even lower than the capacity, ~ 0.8 μF cm^{-2}, of a solvent-free black lipid membrane [26].

The resistance and capacity of the spacer/(lipid bilayer) films on Hg were estimated from electrochemical impedance spectra covering the frequency range from 0.1 to 10^5 Hz at an applied potential lying over the minimum capacitance region. For their interpretation, use was made of a simple equivalent circuit consisting of an RC mesh, which simulates the whole film, with, in series, the resistance R_Ω of the aqueous electrolyte. With a good tethered bilayer, the resistance was higher than 0.1 MΩ cm^2.

To evaluate the effect of the incorporation of the channel-forming peptides and proteins, their stock solutions were simply added to the aqueous subphase and stirred for a few minutes, while keeping the bilayer at an applied potential lying over the minimum capacitance region. With the TL and DPTL thiolipids, the addition of the peptides was made after transferring the mercury-supported tethered bilayer into the cell containing the 0.1 M KCl aqueous solution without the lipid film on its surface, to avoid any progressive loss of peptide molecules from the coated electrode into this lipid film. After this transfer, the reproducibility and stability of the bilayer were tested before any addition.

3. Results and discussion

3.1. Lipid bilayers on top of hydrophilic spacers

TP shows a high tendency to form a 3_{10}-helical structure. This thiolpeptide has two triethylenoxy side chains that impart it a satisfactory hydrophilicity and are intended to keep the anchored thiolpeptide chains sufficiently apart, so as to accommodate water molecules and inorganic ions and to create a suitable environment for the incorporation of integral proteins. On the other hand, EO3 has a linear structure. The mercury-supported lipid bilayers making use of the two hydrophilic spacers TP [7] and EO3 [6] were characterized by AC voltammetry with phase resolution, chronocoulometry and impedance spectroscopy. Their suitability as biomembrane models was tested by incorporating ubiquinone-10, valinomycin and, in the case of EO3 [6], the channel-forming peptide melittin; with this peptide, a conductance entirely analogous to that reported on black lipid membranes was recorded, with the appreciable advantage of a higher stability and resistance to disruption.

3.2. The absolute potential difference across a mercury-supported lipid bilayer

It is well known that the potential difference across a biomembrane (the so-called transmembrane potential) modulates the function of many biomolecules. The transmembrane potential is thermodynamically significant, because it is the potential difference between two phases which, being characterized by the same solvent (water), have a very similar composition. Analogous considerations apply to conventional bilayer lipid membranes. When dealing with a metal-supported lipid bilayer, it is important to make an extrathermodynamic estimate of the absolute potential difference across the bilayer.

The extrathermodynamic absolute potential difference, ϕ, across the interphase between a mercury electrode coated with a neutral phospholipid monolayer (say, DOPC) and the

adjacent solution can be approximately expressed by the equation [19,21]:

$$\phi = \sigma_M/C + \chi_l \quad (1)$$

Here, σ_M is the charge density on the metal, C is the differential capacity of the monolayer and χ_l is the surface dipole potential due to the lipid polar heads. The surface charge density σ_M at a HMDE coated with a self-assembled DOPC monolayer and immersed in aqueous 0.1 M tetramethylammonium chloride, was measured by a technique described elsewhere [27]. Briefly, σ_M was obtained by analogical integration of the capacitive current that flows at constant applied potential as a consequence of a slight contraction of the mercury drop. The lipid-coated mercury drop was contracted while keeping its neck in contact with the lipid film spread on the surface of the electrolytic solution. This procedure ensures that the monolayer maintains its properties, including its thickness, as the drop is expanded or compressed. The capacitive charge flowing during a change ΔA in drop area, once divided by ΔA, yields directly the charge density σ_M on the metal. At an applied potential $E = -450$ mV/SCE, σ_M is equal to -0.75 μC cm^{-2}, while C amounts to 1.8 μFcm^{-2}. The surface dipole potential, χ_l, of the polar heads of a phosphatidylcholine monolayer, as estimated by different extrathermodynamic procedures both on BLMs and on mercury, ranges from +150 to +250 mV and is positive toward the hydrocarbon tails [21]. Upon substituting these values into Eq. (1), we obtain an extrathermodynamic absolute potential difference ϕ across the mercury/solution interphase of about -200 mV when the applied potential E equals -450 mV/SCE. Therefore, an approximate ϕ value can be simply obtained by increasing the applied potential E vs. an SCE by 250 mV.

As distinct from phospholipids, thiols self-assemble on mercury through a covalent bond, which involves partial or total charge transfer from the sulfur atom to the mercury electrode. In this case, Eq. (1) no longer applies. As a rough approximation, the absolute potential difference ϕ between a thiol-coated mercury electrode and the adjacent aqueous solution can be expressed by the equation (for a more accurate expression, see Ref. [21]):

$$\phi = q_M/C + \chi_t \quad (2)$$

Here, χ_t is the surface dipole potential of the thiol monolayer, C is the differential capacitance of the interphase, and q_M is the charge experienced by the diffuse layer ions; this can be estimated by applying the Gouy–Chapman theory to the experimental dependence of the diffuse-layer capacity of a thiol-coated mercury electrode upon the electrolyte concentration of the adjacent aqueous solution. Because ϕ at any given applied potential E vs. an SCE can be estimated as described above, and q_M and C can be measured, Eq. (2) may be used to determine χ_t. By this procedure, a χ_t value of -200 mV was ascribed to a mercury-supported EO3 monolayer [6]. This value was then confirmed by incorporating valinomycin (VAL) in a DOPC bilayer self-assembled on top of the EO3 monolayer [21]. VAL is a K^+ selective ion carrier that may shuttle K^+ ions across the bilayer, thus increasing its conductivity. When q_M is positive, the K^+ ions are electrostatically repelled from the hydrophilic spacer EO3. Shifting the applied potential to more negative values, K^+ ions start penetrating into the spacer, and the conductance increases up to a limiting value. Under the simplifying assumption that the short-range interactions of K^+ with the spacer medium are comparable with those with the aqueous phase, it can be demonstrated that the inflection point of the curve of the conductance G vs. the applied potential E corresponds to a zero value of the transmembrane potential across the bilayer. Because a symmetrical lipid bilayer has a zero surface dipole potential, under these conditions, the absolute potential difference ϕ across the whole Hg/EO3/(DOPC bilayer) interphase practically coincides with the surface dipole potential χ_t of the spacer. The ϕ value at the inflection point of the G vs. E curve is about equal to -200 mV, as expected.

3.3. Lipid bilayers consisting of a lipid monolayer on top of a tethered thiolipid

In spite of some satisfactory results obtained with lipid bilayers self-assembled on top of the mercury-supported hydrophilic spacers EO3 [6] and TP [7], their use is rather uncomfortable, because the drop neck must be kept in contact with the lipid reservoir on the surface of the aqueous electrolyte during measurements. In fact, their complete immersion into the aqueous solution destabilizes them because of the nonnegligible solubility of the hydrophilic spacer. This inconvenience is removed with thiolipids, because their solubility in water is notably decreased by the phospholipid moiety. Thus, mercury-supported lipid bilayers formed with thiolipids are entirely stable when immersed in aqueous solutions, and can also be safely transferred from one solution to another.

Self-assembling a DOPC monolayer on top of a tethered TL thiolipid yields a biomimetic membrane that, albeit relatively stable in aqueous solution, is characterized by a differential capacity C as high as 1.8 μF cm^{-2}; this denotes a poor organization of the lipid bilayer, although the resistance of the film, $R = 0.1$ MΩ cm^2, is relatively high. Better results were obtained by self-assembling the second monolayer from a 10:1.4 (mol/mol) DOPC–cholesterol mixture spread on the surface of the aqueous electrolyte; the presence of cholesterol decreases C down to 1.3 μF cm^{-2} over a potential range of about 400 mV, while increasing R to some extent. This supported membrane allows the incorporation of gramicidin and alamethicin. However, a further increase in the percentage of cholesterol increases the rigidity of the lipid bilayer to such an extent as to prevent any reconstitution.

Particularly satisfactory results were obtained by self-assembling a DphyPC monolayer on top of a tethered DPTL

film. One advantageous feature of this thiolipid and of DphyPC is represented by the fact that the saturated phytanyl and phytanoyl chains are not oxidized by air and are in the liquid crystalline state at room temperature, having transition temperatures as low as −80 °C. Moreover, the anchoring disulfide group of the lipoic moiety is also not oxidized by air, and the ether linkages of the two phytanyl chains to the glycerol backbone are more stable than the usual ester linkages. The Hg/DPTL/DphyPC system exhibits a differential capacity of 0.6–0.7 μF cm^{-2} over the very broad potential range from −0.200 to −1.200 V/SCE and a resistance of 0.2 MΩ cm^2. The excellent fluidity of this tethered bilayer allows an easy incorporation of channel-forming peptides and of OmpF porin from their aqueous solutions, by mild stirring.

Fig. 2 shows the conductance of a Hg-supported DPTL/DphyPC film incorporating VAL against the applied potential *E*, as measured by the in-phase component, Y′, of the electrode admittance at 10 Hz. The inflection point of the sigmoidal curve of Y′ vs. *E* lies at about −0.500 V/SCE. This potential can be roughly regarded as the applied potential at which the potential difference across the lipid bilayer (the transmembrane potential) vanishes. It corresponds to an absolute potential difference ϕ across the whole mercury/solution interphase of about (−0.500+0.250) V=−0.250 V. This value is also a rough measure of the surface dipole potential of the hydrophilic spacer bound to the phospholipid moiety of the thiolipid, which consists primarily of a tetraethyleneoxy group. This value should be compared with the dipole potential, −0.200 V, estimated for the triethyleneoxythiol EO3 [6]. We may therefore tentatively ascribe a surface dipole potential of about −60÷−65 mV to each ethyleneoxy group.

Fig. 3 shows the in-phase component, Y′, of the electrode admittance for a Hg-supported DPTL/DphyPC film incorporating gramicidin D against the applied potential *E*. Gramicidin D is a channel-forming peptide that is voltage independent and alkaline-ion selective. As the applied potential is gradually shifted toward more negative values, the electric field inside the hydrophilic spacer, which is initially directed toward the solution, thus repelling the K^+ ions, decreases in magnitude and ultimately changes direction. This explains the rising section of the Y′ vs. *E* curve in Fig. 3, whose inflection point lies at about −0.450 V; this potential is about 50 mV more positive than that for the sigmoidal curve in Fig. 2, due to the VAL ion carrier. Proceeding toward more negative potentials, the conductance decreases, giving rise to a bell-shaped curve. This decrease is probably due to saturation of the hydrophilic

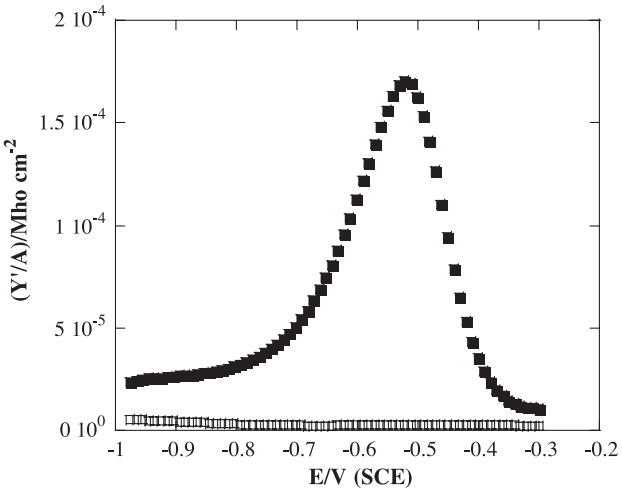

Fig. 3. Plot of the in-phase component, Y′, of a Hg-supported DPTL/DphyPC film bathed by aqueous 0.1 M KCl against the applied potential *E*, both in the absence (□) and in the presence (■) of 1×10^{-7} M gramicidin D. Frequency = 10 Hz.

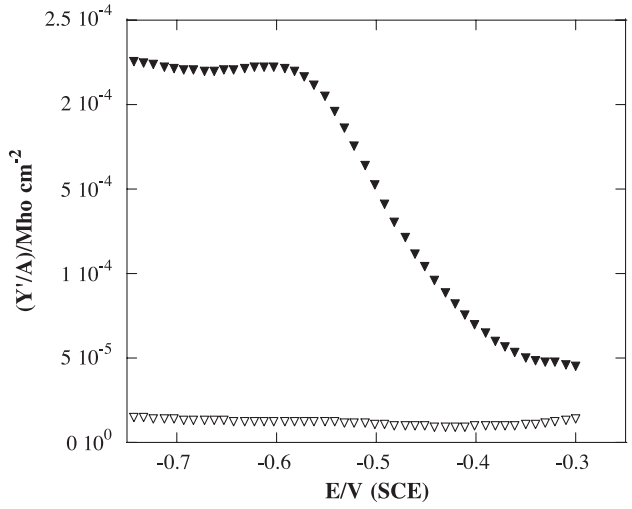

Fig. 2. Plot of the in-phase component, Y′, of a Hg-supported DPTL/DphyPC film bathed by aqueous 0.1 M KCl against the applied potential *E*, both in the absence (▽) and in the presence (▼) of 2×10^{-7} M valinomycin. Frequency = 10 Hz.

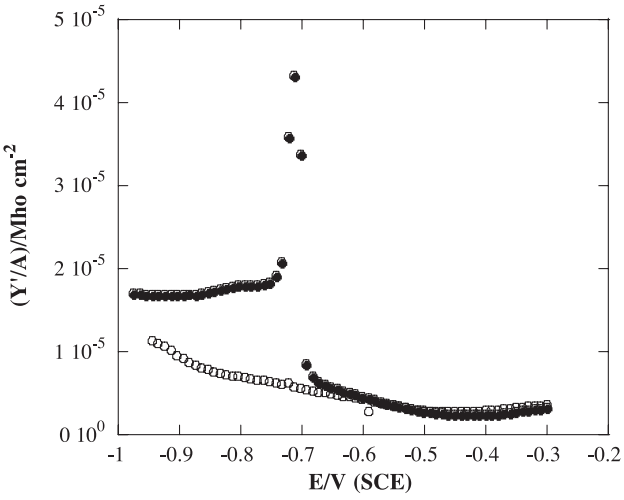

Fig. 4. Plot of the in-phase component, Y′, of a Hg-supported DPTL/DphyPC film bathed by aqueous 0.1 M KCl against the applied potential *E*, both in the absence (□) and in the presence (■) of 0.4 μg/ml melittin. Frequency = 10 Hz.

spacer by the K^+ ions translocated across the lipid bilayer along the gramicin channels. A quantitative interpretation of this behavior will be provided in a future note. The presence of a descending branch, not observed with VAL, can be tentatively ascribed to the number of K^+ ions transported by a single gramicidin channel per second being about three orders of magnitude higher than the turnover number of a single VAL molecule [28].

Melittin is a channel-forming peptide with very low ion selectivity, but strongly voltage dependent. Its conductance in conventional BLMs increases very rapidly as the transmembrane potential becomes negative on the side of the lipid bilayer opposite to the one where it is present [29]. The in-phase component of the electrode admittance for a Hg-supported DPTL/DphyPC film incorporating melittin from its 0.4 μg/ml aqueous solution is shown against the applied potential E in Fig. 4. Even in this supported bilayer, the conductance of melittin shows a very steep rise in the proximity of −0.700 V/SCE, followed by a rapid decrease due to the saturation of the hydrophilic spacer by the supporting cation K^+. In particular, a conductance of 5×10^{-6} S cm^{-2} is attained at −0.690 V/SCE. Considering that the potential difference across the present lipid bilayer assumes a zero value at about −0.500 V/SCE, the transmembrane potential at −0.690 V/SCE can be roughly estimated at −0.190 V. This value should be compared with the transmembrane potential at a conventional BLM, under otherwise identical conditions. At a BLM incorporating melittin from aqueous 1.8 M NaCl containing 0.4 μg/ml melittin, a conductance of 5×10^{-6} S cm^{-2} is attained for a transmembrane potential of −0.130 mV (see Fig. 4 of Ref. [29]). A decrease in the concentration of NaCl (or KCl) from 1.8 to 0.1 M is reported to cause a negative shift in the transmembrane potential at constant conductance and melittin concentration, which is given by $(2.3RT/1.1F)\ 0.5 \log(0.1/1.8) = -33$ mV. Therefore, the transmembrane potential of −0.190 mV at the supported lipid bilayer, estimated by extrathermodynamic considerations, should be compared with a transmembrane potential of −0.163 V, measured at a BLM. In view of the rough assumptions made in the extrathermodynamic approach, agreement is quite satisfactory.

OmpF porin is one of the major outer membrane proteins of *Escherichia coli*. When reconstituted in BLMs, this protein forms a channel comprising three monomers that are closed at high transmembrane potentials (130–200 mV), so that the single-channel conductance decreases in steps of approximately one-third [30–32]. Fig. 5 shows that the in-phase component of the electrode admittance for a Hg-supported DPTL/DphyPC film incorporating OmpF porin from its 130 ng/ml dispersion in 0.1 M KCl, is roughly constant over a potential range straddling −0.500 V/SCE, which corresponds to a zero transmembrane potential. At −0.825 mV, namely, at about −300 mV from the estimated zero transmembrane potential of the lipid bilayer, the conductance decreases by about two-thirds in two hardly distinguishable steps, while about one-third remains unsuppressed.

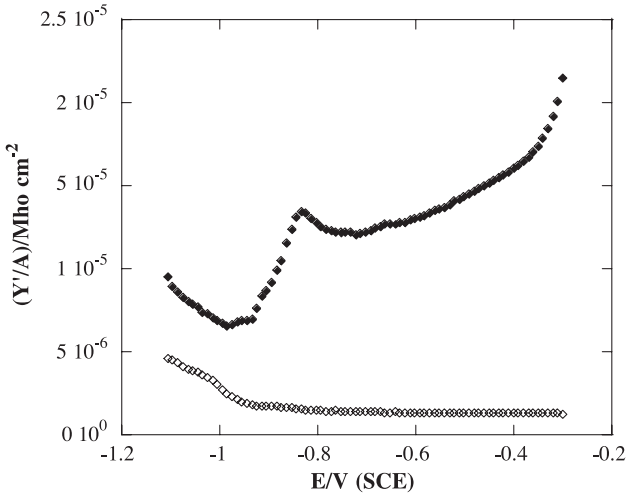

Fig. 5. Plot of the in-phase component, Y′, of a Hg-supported DPTL/DphyPC film bathed by aqueous 0.1 M KCl against the applied potential E, both in the absence (◇) and in the presence (◆) of a dispersion of 130 ng/ml OmpF. Frequency = 10 Hz.

The above results demonstrate the suitability of a Hg-supported DPTL/DphyPC tethered bilayer as a biomembrane model for reconstitution of channel-forming peptides and proteins.

Acknowledgements

The authors are grateful to Prof. Claudio Toniolo (Padova University) and to Dr. G. L. Orris (Basel University) for providing them with the TL thiolipid and with OmpF porin, respectively. Thanks are due to the Ente Cassa di Risparmio di Firenze and to MIUR (Ministero dell'Istruzione, dell'Universita' e della Ricerca) for financial support.

References

[1] R. Guidelli, G. Aloisi, L. Becucci, A. Dolfi, M.R. Moncelli, F. Tadini Buoninsegni, New directions and challenges in electrochemistry. Bioelectrochemistry at metal/water interfaces, J. Electroanal. Chem. 504 (2001) 1–28.

[2] L.M. Williams, S.D. Evans, T.M. Flynn, A. Marsh, P.F. Knowles, R.J. Bushby, N. Boden, Kinetics of the unrolling of small unilamellar phospholipid vesicles onto self-assembled monolayers, Langmuir 13 (1997) 751–757.

[3] L.M. Williams, S.D. Evans, T.M. Flynn, A. Marsh, P.F. Knowles, R.J. Bushby, N. Boden, Kinetics of formation of single phospholipid bilayers on self-assembled monolayer supports, as monitored by surface plasmon resonance, Supramol. Sci. 4 (1997) 513–517.

[4] A. Toby, A. Jenkins, R.J. Bushby, N. Boden, S.D. Evans, P.F. Knowles, Q. Liu, R.E. Miles, S.D. Ogier, Ion-selective lipid bilayers tethered to microcontact printed self-assembled monolayers containing cholesterol derivatives, Langmuir 14 (1998) 4675–4678.

[5] C. Steinem, A. Janshoff, W.P. Ulrich, M. Sieber, H.-J. Galla, Imped-

ance analysis of supported lipid bilayer membranes: a scrutiny of different preparation techniques, Biochim. Biophys. Acta 1279 (1996) 169–180.
[6] L. Becucci, R. Guidelli, Q. Liu, R.J. Bushby, S.D. Evans, A biomimetic membrane consisting of a polyethyleneoxythiol monolayer anchored to mercury with a phospholipid bilayer on top, J. Phys. Chem., B 106 (2002) 10410–10416.
[7] C. Peggion, F. Formaggio, C. Toniolo, L. Becucci, M.R. Moncelli, R. Guidelli, A peptide-tethered lipid bilayer on mercury as a biomimetic system, Langmuir 17 (2001) 6585–6592.
[8] H. Lang, C. Duschl, H. Vogel, A new class of thiolipids for the attachment of lipid bilayers on gold surfaces, Langmuir 10 (1994) 197–210.
[9] C. Duschl, M. Liley, G. Corradin, H. Vogel, Biologically addressable monolayer structures formed by templates of sulfur-bearing molecules, Biophys. J. 67 (1994) 1229–1237.
[10] C. Steinem, A. Janshoff, K. von dem Bruch, K. Reihs, J. Goossens, H.-J. Galla, Valinomycin-mediated transport of alkali cations through solid supported membranes, Bioelectrochem. Bioenerg. 45 (1998) 17–26.
[11] B. Raguse, V. Braach-Maksvytis, B.A. Cornell, L.G. King, P.D.J. Osman, R.J. Pace, L. Wieczorek, Tethered lipid bilayer membranes: formation and ionic reservoir characterization, Langmuir 14 (1998) 648–659.
[12] B.A. Cornell, V.L.B. Braach-Maksvytis, L.G. King, P.D.J. Osman, B. Raguse, L. Wieczorek, R.J. Pace, A biosensor that uses ion-channel switches, Nature 387 (1997) 580–583.
[13] R. Naumann, A. Jonczyk, R. Kopp, J. van Esch, H. Ringsdorf, W. Knoll, P. Gräber, Incorporation of membrane proteins in solid-supported lipid layers, Angew. Chem., Int. Ed. Engl. 34 (1995) 2056–2058.
[14] R. Naumann, A. Jonczyk, C. Hampel, H. Ringsdorf, W. Knoll, N. Bunjes, P. Gräber, Coupling of proton translocation through ATPase incorporated into supported lipid bilayers to an electrochemical process, Bioelectrochem. Bioenerg. 42 (1997) 241–247.
[15] N. Bunjes, E.K. Schmidt, A. Jonczyk, F. Rippmann, D. Beyer, H. Ringsdorf, P. Gräber, W. Knoll, R. Naumann, Thiopeptide-supported lipid layers on solid substrates, Langmuir 13 (1997) 6188–6194.
[16] S. Heyse, O.P. Ernst, Z. Dienes, K.P. Hofmann, H. Vogel, Incorporation of rhodopsin in laterally structured supported membranes: observation of transducin activation with spatially and time-resolved surface plasmon resonance, Biochemistry 37 (1998) 507–522.
[17] E.K. Schmidt, T. Liebermann, M. Kreiter, A. Jonczyk, R. Naumann, A. Offenhausser, E. Neumann, A. Kukol, A. Maelicke, W. Knoll, Incorporation of the acetylcholine receptor dimer from *Torpedo californica* in a peptide supported lipid membrane investigated by surface plasmon and fluorescence spectroscopy, Biosens. Bioelectron. 13 (1998) 585–591.
[18] R. Naumann, E.K. Schmidt, A. Jonczyk, K. Fendler, B. Kadenbach, T. Liebermann, A. Offenhäusser, W. Knoll, The peptide-tethered lipid membrane as a biomimetic system to incorporate cytochrome *c* oxidase in a functionally active form, Biosens. Bioelectron. 14 (1999) 651–662.
[19] F. Tadini Buoninsegni, L. Becucci, M.R. Moncelli, R. Guidelli, Total and free charge density on mercury coated with self-assembled phosphatidylcholine and octadecanethiol monolayers and octadecanethiol/phosphatidylcholine bilayers, J. Electroanal. Chem. 500 (2001) 395–407.
[20] A. Dolfi, G. Aloisi, R. Guidelli, Photoelectrical response of purple membrane fragments adsorbed on a lipid monolayer supported by mercury and characterization of the resulting interphase, Bioelectrochemistry 57 (2002) 155–166.
[21] L. Becucci, M.R. Moncelli, R. Guidelli, Ion carriers and channels in metal-supported lipid bilayers as probes of transmembrane and dipole potential, Langmuir 19 (2003) 3386–3392.
[22] N. Boden, R.J. Bushby, Q. Liu, S.D. Evans, P.F. Knowles, A. Marsh, The design and synthesis of simple molecular tethers for binding biomembranes to a gold surface, Tetrahedron 53 (1997) 10939–10952.
[23] S.M. Schiller, R. Naumann, K. Lovejoy, H. Kunz, W. Knoll, Archaea analogue thiolipids for tethered bilayer lipid membranes on ultrasmooth gold surfaces, Angew. Chem., Int. Ed. 42 (2003) 208–211.
[24] M.R. Moncelli, L. Becucci, A novel model of the hanging mercury drop electrode, J. Electroanal. Chem. 433 (1997) 91–96.
[25] F. Tadini Buoninsegni, R. Herrero, M.R. Moncelli, Alkanethiol monolayers and alkanethiol/phospholipid bilayers supported by mercury: an electrochemical characterization, J. Electroanal. Chem. 452 (1998) 33–42.
[26] M. Montal, P. Mueller, Formation of bimolecular membranes from lipid monolayers and study of their electrical properties, Proc. Natl. Acad. Sci. U. S. A. 69 (1972) 3561–3566.
[27] L. Becucci, M.R. Moncelli, R. Guidelli, Surface charge density measurements on mercury electrodes covered by phospholipid monolayers, Electroanal. Chem. 413 (1996) 187–193.
[28] E. Bamberg, P. Läuger, Channel formation kinetics of gramicidin A in lipid bilayer membranes, J. Membr. Biol. 11 (1973) 177–194.
[29] M. Pawlak, S. Stankowski, G. Schwarz, Melittin induced voltage-dependent conductance in DOPC lipid bilayers, Biochim. Biophys. Acta 1062 (1991) 94–102.
[30] H. Schindler, J.P. Rosenbusch, Matrix protein from *Escherichia coli* outer membranes forms voltage-controlled channels in lipid bilayers, Proc. Natl. Acad. Sci. U. S. A. 75 (1978) 3751–3755.
[31] A. Engel, A. Massalski, H. Schindler, D.L. Dorset, J.P. Rosenbusch, Porin channel triplets merge in *Escherichia coli* outer membranes, Nature 317 (1985) 643–645.
[32] T.K. Rostovtseva, E.M. Nestorovich, S.M. Bezrukov, Partitioning of differently sized poly(ethyleneglycol)s into OmpF porin, Biophys. J. 82 (2002) 160–169.