Stable insulating tethered bilayer lipid membranes

Inga K. Vockenroth and Christian Ohm
Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany

Joseph W. F. Robertson
Semiconductor Electronics Division, Electronics and Electrical Engineering Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899

Duncan J. McGillivray[a] and Mathias Lösche
Department of Physics, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213 and Center for Neutron Research, National Institute of Standards and Technology (NIST), 100 Bureau Drive, Gaithersburg, Maryland 20899

Ingo Köper[b]
Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany

(Received 14 January 2008; accepted 28 March 2008; published 7 May 2008)

Tethered bilayer lipid membranes have been shown to be an excellent model system for biological membranes. Coupling of a membrane to a solid supports creates a stable system that is accessible for various surface analytical tools. Good electrical sealing properties also enable the use of the membranes in practical sensing applications. The authors have shown that tethered membranes have extended lifetimes up to several months. Air-stability of the bilayer can be achieved by coating the membrane with a hydrogel. The structure of a monolayer and its stability under applied dc potentials have been investigated by neutron scattering. © *2008 American Vacuum Society.*
[DOI: 10.1116/1.2912097]

I. INTRODUCTION

Biological membranes are highly structured architectures that are in most cases too complex to be used in fundamental studies or practical applications. There have been several attempts to couple whole cells to transistors, however their functionality is still limited.[1–4] Therefore, different model systems have been investigated for their potential in biosensing applications, where embedded proteins would be used as sensing units in an otherwise inert and insulating membrane. For example, the use of alpha-hemolysin channels in a stochastic sensing approach, with the proteins being incorporated in a planar bilayer lipid membrane, has been widely discussed.[5–7] However, this platform has the disadvantage of being fragile and is thus not suited for applications that employ continuous monitoring or the use in portable devices.[8] Recent progress has been made by stabilizing such membrane architectures either by crystallization of S-layer proteins or deposition of hydrogel cushions or other polymer layers.[9–11] The membrane lifetime could be enhanced to days;[12–14] however the resulting lifetime improvement remains insufficient to allow for continuous monitoring over periods of months, which may be a requirement for some applications.

An alternative stabilization approach consists in the use of solid substrates to support the membrane.[15,16] The membrane can be placed either directly on the substrate, attached through physi- or chemisorption, or the interactions can be mediated via a soft polymer cushion.[17] Again, an increased stability has been reported, yet the obtained membranes often lack sufficient electrical sealing properties to allow measuring currents flowing through single proteins. An interesting approach is the use of porous substrates, which represent a hybrid system of planar and supported membranes. It yielded membranes with good electrical stability over several days.[18]

Tethered bilayer lipid membranes (tBLMs) are versatile model systems that provide a defined platform to study membrane related processes and membrane proteins.[19–23] They provide excellent stability by covalently linking the bilayer membrane to a solid support. First demonstrated by Cornell *et al.*, the inner leaflet of the membrane is bound to the solid support via a short spacer group.[19] Typically, thiol anchors are used to graft the membrane onto a gold electrode, which allows for electrical characterization of the membrane. Such systems provide excellent electrical sealing, especially when phytanyl-based anchor lipids are used. Similar systems have been shown to allow for the functional incorporation of various membrane proteins.[24–26] The molecular toolkit that forms the basis of such tBLMs has also been modified to be used on oxidic instead of gold substrates.[22]

Here we demonstrate the stability of the tBLM system with respect to its long-term stability and investigate its stability under the influence of an applied electrical potential. Finally, the stability of a membrane in air is shown, when protected by a hydrogel overcoating.

II. MATERIALS AND METHODS

A. Assembly of the system

tBLMs were typically assembled in a two step procedure. Lipid monolayers were formed via self-assembly by immer-

[a]Present address: Department of Chemistry, The University of Auckland, Private Bag 92019, Auckland, New Zealand.
[b]Author to whom correspondence should be addressed; electronic mail: koeper@mpip-mainz.mpg.de

sion of an ultra flat gold substrate into a diluted solution of the anchorlipid DPhyTL (0.2 mg/mL), which was synthesized as described previously.[21,27,28] DPhyTL consists of two phytanyl chains that are coupled via a glycerol linker to a tetraethylene oxide spacer. The molecules are grafted to the gold surface by sulfur-gold bonds via a lipoic acid moiety. In the second assembly step, the monolayers are completed to bilayers by fusion with freshly prepared small unilamellar DPhyPC (1,2-Diphytanoyl-sn-Glycero-3-Phosphocholine, Avanti Polar Lipids, 2 mg/mL in ultrapure water) vesicles (50 nm by extrusion).

In order to probe the functionality of the membrane, the peptide valinomycin (Sigma-Aldrich) was added in a final concentration of 36 μM to the adjacent buffer.

For the hydrogel coating, NIPAAM (*N*-Isopropylacylamide) and methylenbisacrylamide (25:1 100 mg/mL in 100mM KCl) were added to cover a preformed bilayer and were allowed to polymerize overnight. Thus, a hydrogel layer of several hundred micrometers in thickness has been formed.

B. Electrochemical impedance spectroscopy (EIS)

Measurements were conducted using an Autolab spectrometer PGSTAT 12 (Eco Chemie, Utrecht, Netherlands). Spectra were recorded for frequencies between 2 mHz and 100 kHz at 0 V bias potential with an ac modulation amplitude of 10 mV. Raw data were analyzed using the ZVIEW software package (Version 2.90, Scribner Associates, Inc.). Three-electrode measurements were performed with the substrate as the working electrode, a coiled platinum wire as the counter electrode, and a DRIREF-2 reference electrode (World Precision Instruments, Berlin, Germany), which has a potential of 0.2881 with reference to the NHE. The homebuilt Teflon cells had a buffer volume of 1 ml and an electrochemically active area on the substrates of about 0.28 cm^2.

The impedance data are presented in Bode plots, where the magnitude of the impedance and the phase angle are displayed as a function of the applied frequency. The graphs have been analyzed using a model equivalent circuit of resistors (R) and capacitors (C).[29] The different components can be attributed to the individual parts of the membrane architecture. We used a $R(RC)C_{SC}$-circuit consisting of a RC element describing the bilayer in series with a capacitor (C_{SC}) and an electrolyte resistance. C_{SC} represents the space charge capacitance due to the spacer region combined with the capacitive effects of the electrochemical double layer at the gold interface.[21,24] All electrochemical experiments were performed in 0.1 M KCl unless otherwise noted. For the long time measurements, the solution has been replaced before each measurement, ensuring that the membrane did not dry out.

C. Neutron reflectometry

Neutron reflection (NR) experiments were performed on the Advanced Neutron Diffractometer/Reflectometer (AND/R) at the NIST Center for Neutron Research (NCNR), Gaithersburg, MD.[30] For sample preparation, a thin layer of chromium was sputtered on 3 in. silicon wafers followed by ~150 Å of gold. This preparation ensures a flat gold surface.[23] Immediately after gold film deposition, wafers were immersed into solutions of DPhyTL in ethanol (0.2 mg/mL), and self-assembly of a monolayer (SAM) was allowed overnight. Samples were thoroughly rinsed with ethanol and placed in a fluid cell. All solutions were degassed prior to use. Electrical dc potentials were applied by using the gold coated backside of the cell as counter electrode and a DRIREF-2 reference electrode in the fluid outlet. NR experiments under potentiometric control were performed using a Solartron 1283 potentiostat/galvanostat equipped with a Solartron 1260 frequency response analyzer. Potential control and data collection were obtained with the Zplot/Zview software package (Scribner Associates, Inc.). During NR measurements, EIS spectra were collected to verify that there was no sample degradation.

NR results were modeled with the Parratt box model formalism[31] using software developed at the NCNR.[32]

III. RESULTS AND DISCUSSION

A. Long term stability

To investigate the electrical stability of a tBLM, five membranes were prepared in identical Teflon cells. The measurement cell provides an aqueous reservoir above the membranes. The electrical properties were measured using EIS (Fig. 1). In between the measurements, the cells were closed and stored at room temperature.

The resistance values for the different monolayers showed some variability, but they were all in the range of 3 -10 MΩ cm^2 and increased to $10-50$ MΩ cm^2 during vesicle fusion. The electrical parameters of the membranes thus correspond very well to values known from natural membrane as well as from planar bilayer experiments. Bilayer formation was also associated with a large drop in capacitance for all samples. Throughout the measurements, the bilayer resistance remained fairly stable for two of the samples (c and e). Two fluctuated but remained at a high level above 5 MΩ cm^2 (a and b), while one showed a decrease in resistance (d). In all cases, the resistance after 4 months is still higher than the initial monolayer resistance. Over the same time course, the bilayer capacitances increased steadily with time, but even after about 60 days the values remained so low (below the starting value of the monolayer), to be consistent with a tightly sealing membrane. From 60 days onward, two of the electrodes (b and e) showed a capacitance higher than the value of the monolayer, indicating a slow decay of the membrane sealing properties. Nevertheless, three of the samples were below 1 μF cm^{-2}, and all samples remained below 1.1 μF cm^{-2}.

The reason for the fluctuations is unclear, however, even after 3 months all membranes showed resistances above 10 MΩ cm^2, whereas the membrane capacitance showed a

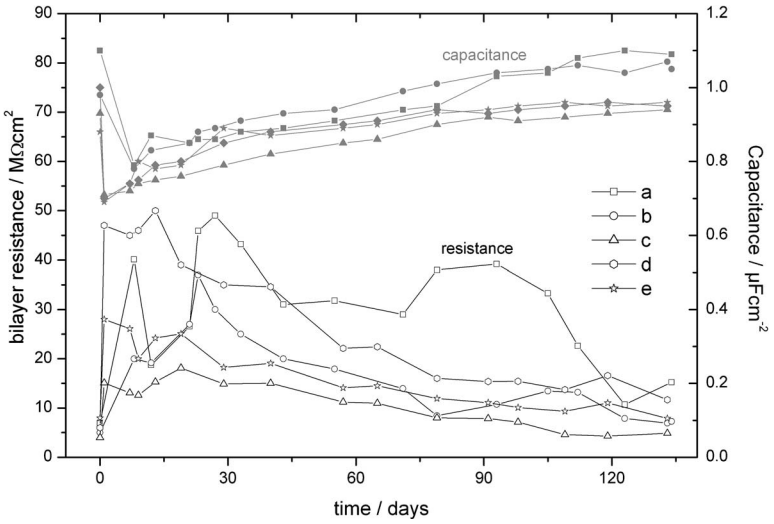

FIG. 1. Membrane resistance and capacitance of five tBLMs as a function of time. The membranes maintain their electrical sealing properties for several months.

slight and continuous increase over the whole time. This might be due to several processes. The electrolyte solutions were neither sterilized nor were any chemicals added to prevent biodegradation. There might have been slight biocontamination, e.g., with enzymes that slowly digest the membrane. At the same time, a small amount of lipids might have been dissolved in the electrolyte. The critical micelle concentration is small, but not negligible. Nevertheless, after 3 months all four membranes are still tightly sealing with capacitances around 1 μF cm^{-2}.

One of the membranes was stored at room temperature for a total duration of 228 days. After that period, the membrane resistance was still about 3 MΩ cm^2 (Fig. 2). In order to test whether the membrane was still functional, its response to incubation with valinomycin was investigated. Valinomycin is a small peptide that selectively transports potassium ions across membranes.[33] The addition of the peptide to the membrane led to a drastic decrease in the membrane resistance to 50 Ωcm^2. Replacing the KCl electrolyte with 100 mM NaCl led to an increase of the membrane resistance to almost its initial values (500 kΩ cm^2). The difference might be due to an incomplete exchange of the electrolyte in the static measurement cell. Even small traces of potassium can lead to a current flow and thus to a decrease in the membrane resistance.

B. Resilience in high electric fields

For some applications, it might be necessary to apply a voltage across the membrane, e.g., to address voltage gated proteins. Free-standing membranes are rather unstable and disruption of the membrane is a common problem. We used neutron reflectivity to study structural changes of the membrane architecture upon application of an external potential. NR results for a DPhyTL monolayer in contact with D_2O were to be analyzed using a box model (Fig. 3). This model can clearly distinguish between the hydrophobic alky chain region and the spacer region, which both showed the neutron scattering length densities (nSLDs) expected for the respective regions in the stratified structure.[23,34] The thickness of the spacer region is about 14 Å, while the hydrophobic part is about 25 Å, in good agreement with previous optical

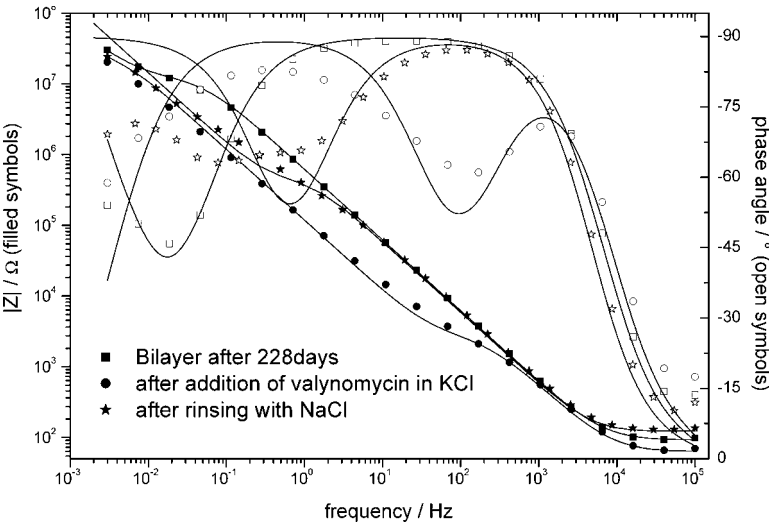

FIG. 2. Bode plot of a tBLM after 228 days. The experimental data were fitted using an $R(RC)C$ equivalent circuit, resulting in a membrane resistance of 3 MΩ cm^2. Addition of valinomycin leads to a decrease of the resistance to 50 Ωcm^2 [fitted with an $R(RC)(RC)$ circuit]. Subsequent rinsing with NaCl leads to a restoration of the membrane resistance to 500 kΩ cm^2.

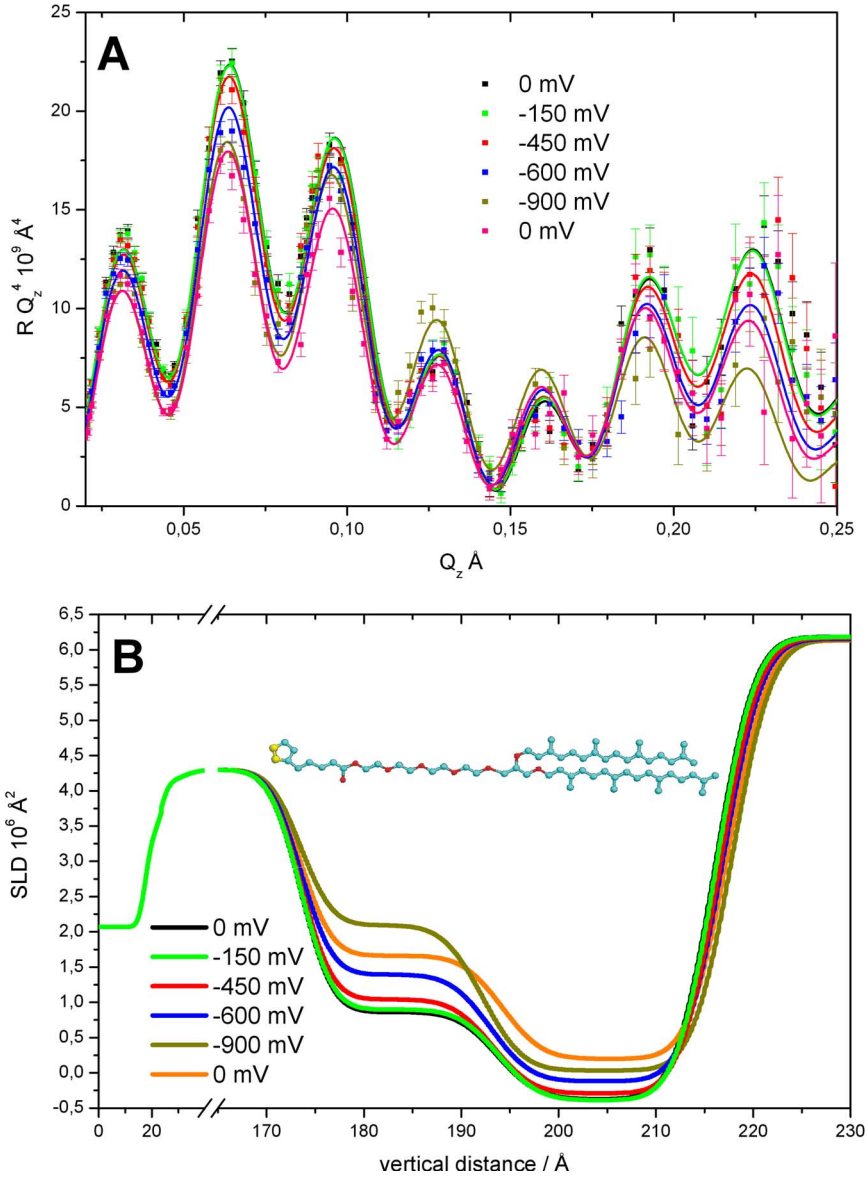

FIG. 3. (a) Neutron reflectivity data for a DPhyTL SAM under various potentials. The solid lines are the reflectivity fit of the experimental data to a box model shown in (b). (b) nSLD profiles of the models that are best fits to the data shown in (a). The model consisted of the following layers: Si substrate/SiO_x/Cr/Au/spacer region/lipid layer/electrolyte.

measurements.[20,21,25,27] NR spectra were recorded at different potentials; after each scan, a measurement was performed at zero potential to ensure reproducibility.

No significant changes in the membrane structure were observed up to −450 mV. Furthermore, results of the control experiments at 0 V were identical to those of the initial measurement (data not shown). The experiment at −600 mV showed a change in the spacer region, with a pronounced increase of the nSLD, which can be interpreted as an increased amount of water that is transferred into this region. Similarly, perturbations in the hydrophobic part can be observed. However, these differences relaxed to the initial structure, when the potential is released.

Irreversible changes were observed only at −900 mV. Apparently, the thiol bonds of the anchor are reduced and material is lost from the surface. When the potential is released, the structure relaxes only partially and a large amount of water is trapped in the spacer region. Nevertheless, compared with other model systems, the tBLMs shows a much higher stability in an external electric field.

C. Protective overcoatings

One disadvantage of almost all model membrane systems is their insufficient air-stability, because typically a membrane is destroyed when exposed to air. Recent progress has been made by encapsulating planar bilayers in agarose gels.[35,36] It has been demonstrated that membranes with embedded proteins could be stored for weeks and regain their function after rehydration. Similarly, hydrogels have been used to protect planar bilayers.[13] We followed a similar approach, by coating a tBLM with a poly(NIPAAM) hydrogel. A 2–3 mm thick layer was polymerized *in situ* on the mem-

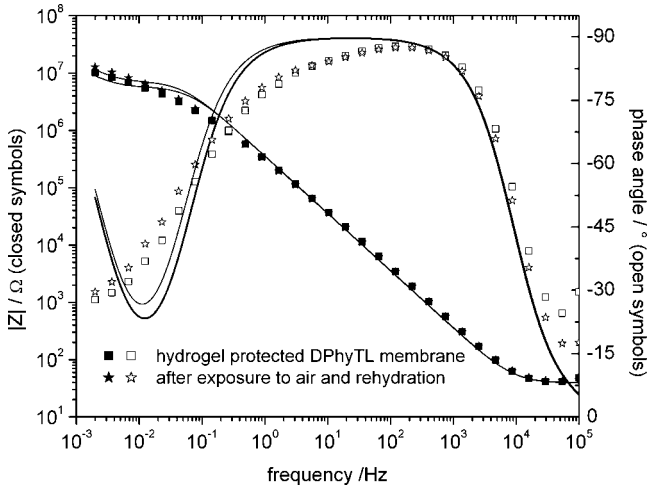

FIG. 4. Bode plot of a hydrogel coated tBLM after it has been left exposed to air for 24 h. Solid lines are obtained by fitting the data to an $R(RC)C$ equivalent circuit. The hydrogel coating leads to a lower initial membrane resistance. After exposure to air and rehydration, the hydrogel protected membrane regains the essential electrical properties of the original sample.

brane. Initially, the electrical properties of the membrane are perturbed. However, they recovered to values of about 1.5 $M\Omega\,cm^2$, which is slightly lower than for membranes without hydrogel coating (typically >10 $M\Omega\,cm^2$). However, the experimental data cannot be well described in an equivalent circuit model consisting of ideal capacitive and resistive elements. This might be due, for example, to a distribution of capacitive elements. Nevertheless, in order to perform a consistent comparison, we used the same equivalent circuit throughout. When an unprotected membrane is left to dry and is rehydrated after 24 h, the membrane is modified such that the electrical sealing properties are no longer present. In contrast, the hydrogel covered membrane could be dried for 24 h and regained its electrical properties after rehydration (Fig. 4). Apparently, the hydrogel keeps a sufficient amount of water in its network structure to keep the underlying membrane hydrated, and therefore functionally intact.

IV. CONCLUSION

Tethered bilayer lipid membranes have been shown to provide an electrically insulating model membrane platform for the study of membrane related processes and especially of the function of incorporated membrane proteins. For practical applications, the stability of the various model membrane systems has often been a serious concern. Most architectures fail to ensure stability over periods of days. We showed here that the DPhyTL-based tBLMs are sufficiently robust to provide electrical insulation for more than 3 months. For a membrane substantially older than half a year, we demonstrated the functional incorporation of the ion carrier valinomycin. The structure of the DPhyTL SAM is preserved under relatively high external electrical field and degrades only when the thiol anchors are electrochemically reduced. Protecting the membrane with a hydrogel enhances air-stability, an approach that we will further investigate in the future.

ACKNOWLEDGMENTS

This work was partially sponsored by DARPA through the Moldice program. J.W.F.R. acknowledges support through the National Research Counsel Research Associateship. M.L. acknowledges funding though the NSF (Grant No. CBET 0555201) and the NIH (Grant No. 1 R01 RR14812).

[1] N. Fertig, A. Tilke, R. H. Blick, J. P. Kotthaus, J. C. Behrends, and G. ten Bruggencate, Appl. Phys. Lett. **77**, 1218 (2000).
[2] G. Riquelme, E. Lopez, L. M. Garcia-Segura, J. A. Ferragut, and J. M. Gonzalez-Ros, Biochemistry **29**, 11215 (1990).
[3] C.-C. Shieh, Drug Discov. Today **9**, 551 (2004).
[4] N. Fertig, R. H. Blick, and J. C. Behrends, Biophys. J. **82**, 3056 (2002).
[5] H. Bayley and P. S. Cremer, Nature (London) **413**, 226 (2001).
[6] J. J. Kasianowicz, E. Brandin, D. Branton, and D. W. Deamer, Proc. Natl. Acad. Sci. U.S.A. **93**, 13770 (1996).
[7] S. M. Bezrukov and J. J. Kasianowicz, Phys. Rev. Lett. **70**, 2352 (1993).
[8] M. Winterhalter, Curr. Opin. Colloid Interface Sci. **5**, 250 (2000).
[9] B. Schuster, U. B. Sleytr, A. Diederich, G. Bähr, and M. Winterhalter, Eur. Biophys. J. **28**, 583 (1999).
[10] W. Meier, A. Graff, A. Diederich, and M. Winterhalter, Phys. Chem. Chem. Phys. **2**, 4559 (2000).
[11] F. Albertorio, A. J. Diaz, T. Yang, V. A. Chapa, S. Kataoka, E. T. Castellana, and P. S. Cremer, Langmuir **21**, 7476 (2005).
[12] R. F. Costello, I. R. Peterson, J. Heptinstall, and D. J. Walton, Biosens. Bioelectron. **14**, 265 (1999).
[13] T. J. Jeon, N. Malmstadt, and J. J. Schmidt, J. Am. Chem. Soc. **128**, 42 (2006).
[14] W. Ziegler, J. Gaburjáková, M. Gaburjáková, B. Sivák, V. Rehaček, V. Tvarozek, and T. Hianik, Colloids and Surfaces A **140**, 357 (1998).
[15] E. Sackmann, Science **271**, 43 (1996).
[16] M. Snejdarkova, M. Rehak, and M. Otto, Biosens. Bioelectron. **12**, 145 (1997).
[17] L. K. Tamm and H. M. McConnell, Biophys. J. **47**, 105 (1985).
[18] W. Römer and C. Steinem, Biophys. J. **86**, 955 (2004).
[19] B. A. Cornell, V. L. B. Braach-Maksvytis, L. G. King, P. D. J. Osman, B. Raguse, L. Wieczorek, and R. J. Pace, Nature (London) **387**, 580 (1997).
[20] I. Köper, Mol. Biosyst. **3**, 651 (2007).
[21] V. Atanasov, P. P. Atanasova, I. K. Vockenroth, N. Knorr, and I. Köper, Bioconjug. Chem. **17**, 631 (2006).
[22] V. Atanasov, N. Knorr, R. S. Duran, S. Ingebrandt, A. Offenhäusser, W. Knoll, and I. Köper, Biophys. J. **89**, 1780 (2005).
[23] D. J. McGillivray, G. Valincius, D. J. Vanderhah, W. Febo-Ayala, J. T. Woodward, F. Heinrich, J. J. Kasianowicz, and M. Losche, Biointerphases **2**, 21 (2007).
[24] R. Naumann *et al.*, Langmuir **19**, 5435 (2003).
[25] I. K. Vockenroth, P. P. Atanasova, J. R. Long, A. T. A. Jenkins, W. Knoll, and I. Köper, Biomembranes **1768**, 1114 (2007).
[26] M. R. Moncelli, L. Becucci, and S. M. Schiller, Bioelectrochemistry **63**, 161 (2004).
[27] S. M. Schiller, R. Naumann, K. Lovejoy, H. Kunz, and W. Knoll, Angew. Chem. **42**, 208 (2003).
[28] The same molecule has been described previously as DPTL, however, to be consistent with the common nomenclature for lipids, Phy is used for the phytanyl moiety.
[29] E. Barsoukov and J. R. Macdonald, *Impedance Spectroscopy—Theory, Experiment, and Applications. 2* (Wiley, Hoboken, NJ, 2005).
[30] J. A. Dura *et al.*, Rev. Sci. Instrum. **77**, 074301(2006).
[31] L. G. Parratt, Phys. Rev. **95**, 359 (1954).

[32]P. A. Kienzle, M. Doucet, D. J. McGillivray, K. V. O'Donnovan, N. F. Berk, and C. F. Majkrzak, http://www.ncnr.nist.gov/reflpak/garefl.html, 2000–2006.
[33]R. Naumann, D. Walz, S. M. Schiller, and W. Knoll, J. Electroanal. Chem. **550–551**, 241 (2003).
[34]G. Valincius, D. J. McGillivray, W. Febo-Ayala, D. J. Vanderah, J. J. Kasianowicz, and M. Lösche, J. Phys. Chem. B **110**, 10213 (2006).
[35]X.-F. Kang, S. Cheley, A. C. Rice-Ficht, and H. Bayley, J. Am. Chem. Soc. **129**, 4701 (2007).
[36]J. W. Shim and L. Q. Gu, Anal. Chem. **79**, 2207 (2007).