Spectroelectrochemistry

DOI: 10.1002/anie.201203214

Combined Electrochemistry and Surface-Enhanced Infrared Absorption Spectroscopy of Gramicidin A Incorporated into Tethered Bilayer Lipid Membranes**

Jacek Kozuch, Claudia Steinem, Peter Hildebrandt, and Diego Millo**

Membrane proteins exert a variety of fundamental functions such as electron and proton transfer, voltage-gated ion translocation, and enzymatic transformations. Since most of these processes can be monitored and controlled by electrochemical techniques, substantial efforts have been made to develop strategies for immobilizing membrane proteins on electrodes while preserving their native functions.[1–4] In one of the most interesting approaches tethered membranes are used to immobilize functional integral proteins on an electrode surface.[4–8] One strategy is to first attach a solubilized protein through a His tag to an appropriately functionalized metal surface, and then reconstitute a lipid bilayer around the fixed protein by detergent–lipid exchange.[5] In these protein-tethered lipid bilayers, however, protein mobility is severely restricted and the overall stability of the device is limited, in particular on non-ideal surfaces. Another more promising and versatile approach is based on lipid-tethered bilayer lipid membranes (tBLMs),[4–8] which consist of a self-assembled monolayer (SAM) of amphiphiles covalently attached to a metal electrode and a lipid layer on top of the SAM formed from fused unilamellar vesicles. This approach can be used to insert integral membrane proteins into the tBLM (Figure 1).[6,7] These devices closely mimic the natural environment of membrane-bound proteins, both with respect to the architecture and the excellent electrical properties, and display very good long-term stability.

[*] J. Kozuch, Prof. Dr. P. Hildebrandt
Institut für Chemie, Sekr. PC14, Technische Universität Berlin
Strasse des 17. Juni 135, 10623 Berlin (Germany)
E-mail: hildebrandt@chem.tu-berlin.de
Dr. D. Millo
Biomolecular Spectroscopy/LaserLaB Amsterdam
Vrije Universiteit Amsterdam
De Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)
E-mail: dmillo@vu.nl
Prof. Dr. C. Steinem
Institut für Organische und Biomolekulare Chemie
Universität Göttingen
Tammannstrasse 2, 37077 Göttingen (Germany)

[**] We thank Lars J. C. Jeuken for helpful discussions. This work was supported by the DFG (Cluster of Excellence "UniCat", P.H.; SFB 803, C.S.), the Alexander-von-Humboldt Foundation (D.M.), and the Netherlands Organisation for Scientific Research (NWO) (grant 722.011.003, D.M.). J.K. acknowledges a fellowship by the BIG-NSE. C.S. thanks the Brain Sustain Initiative of the University of Göttingen for granting a sabbatical leave and J. Gerber-Nolte for practical assistance.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201203214.

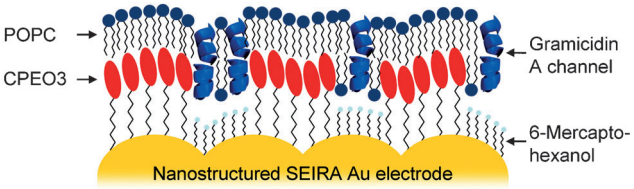

Figure 1. Schematic representation of the gramicidin A (gA)-doped tBLM assembled on the nanostructured Au electrode. The tBLM is composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) supported by a mixed monolayer of (cholesterylpolyethylenoxy)thiol (CPEO3) and 6-mercaptohexanol (6MH).

To date, the most common electrochemical method to characterize tBLMs is electrochemical impedance spectroscopy (EIS), which can be employed for monitoring the formation of the tBLM as well as for probing the function of the incorporated membrane protein. This technique has been widely used to investigate the behavior of ion channels.[8] However, EIS does not provide any structural information about the tBLM and the incorporated protein, which in turn requires appropriate spectroscopic techniques. So far, IR spectroscopic techniques were employed but these studies either referred to pure tBLMs or bilayers with peripherally attached proteins, or were restricted to devices in a non-electrochemical environment.[6,9] A combined electrochemical–spectroscopic approach suitable for in situ functional and structural studies of integral proteins in tBLMs in the same device has, as yet, not been established.

Herein we report on the combination of EIS with surface-enhanced infrared absorption (SEIRA) spectroscopy adapted to an electrochemical cell. This approach benefits from the enhanced IR absorption of molecules in close vicinity to a nanostructured Au film deposited on a silicon prism, which is used as the optical element in attenuated total reflection IR spectroscopy.[10] The Au film serves as both a signal amplifier and as working electrode in an electrochemical setup such that electrochemical and spectroscopic data can be obtained from the same sample. Because of the SEIRA selection rules, particularly strong signals are obtained for IR modes with dipole moment changes perpendicular to the surface. Spectral contributions from the species in the bulk solution are efficiently discriminated as a result of the distance-dependent attenuation of the enhancement. To date, SEIRA spectroscopy has been used to characterize peripheral and integral membrane proteins immobilized on various chemically modified surfaces, including protein-tethered bilayers.[11,12]

As a model membrane protein, we used gramicidin A (gA), a linear polypeptide that forms various right-handed β-

helical structures when introduced into phospholipid bilayers.[13,14] The conformation that is capable of transporting monovalent ions across the bilayer is a membrane-spanning head-to-head dimer denoted as the $\beta^{6,3}$ species. The magnitude of the conductivity depends on the size and the charge of the ion, and obeys the Hofmeister series. Anions are not transported across the membrane, and bivalent cations block the channel.

A mixed monolayer of (cholesterylpolyethylenoxy)thiol (CPEO3) and 6-mercaptohexanol (6MH) with a molar ratio of 1:1 in solution was deposited on the Au film.[15,16] Freshly prepared large unilamellar vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were added onto the mixed monolayer for 3–4 h to form the tBLM (see the Supporting Information). Prior to the incubation with gA, the buffer solution was exchanged for a Cs^+-containing buffer as the presence of Cs^+ ions favors the formation of the conducting state of the incorporated gA.[13]

Figure 2 displays the impedance data obtained from the mixed SAM and the tBLM. Owing to the nanostructured surface, all impedance spectra show two dispersions, one below 10 Hz and one in the frequency region of 10–1000 Hz.

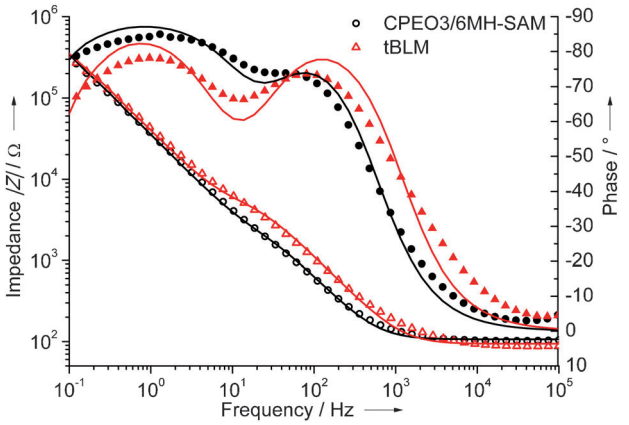

Figure 2. EIS spectra of the mixed CPEO3/6MH monolayer (black dots) and the tBLM after the spreading of POPC vesicles (red triangles); filled and empty symbols refer to the phase angle and the magnitude of the impedance, respectively. Lines represent the result of fitting the equivalent circuit $R_{Solution}(R_{Spacer}C_{Spacer})(R_{Bilayer}C_{Bilayer})$. Buffer: 20 mM Tris, pH 7.4, 100 mM NaCl.

The low-frequency component is ascribed to the spacer molecules of the SAM, that is, the 6MH molecule and the polyethylenoxy linker of CPEO3 (see the Supporting Information), whereas the high-frequency region is attributed to the electrical characteristics of the cholesteryl head groups of the mixed SAM and the bilayer. The equivalent circuit $R_{Solution}(R_{Spacer}C_{Spacer})(R_{Bilayer}C_{Bilayer})$, accounting for the contributions of the spacer and the bilayer, was fitted to the impedance data. The sum of the reciprocal capacitances C_{Spacer} and $C_{Bilayer}$ yielded the total capacitance of the tBLM (see the Supporting Information). The specific capacitance of the mixed SAM was determined to be 1.55 $\mu F\,cm^{-2}$ and it decreased to 0.86 $\mu F\,cm^{-2}$ for the tBLM. These values are in line with previous findings and are indicative of the formation of a tBLM.[17] The membrane resistance is smaller than that of tBLMs on planar gold surfaces, which reflects a larger number of defects owing to the roughness of the nanostructured gold surface.[18] Changing the solution to a buffer containing Cs^+ ions instead of Na^+ ions did not affect the bilayer properties even after extensive rinsing, proving the formation of a very stable tBLM. The addition of gA leads to the incorporation of the peptide into the tBLM, indicated by the increase of the capacitance to 0.96 $\mu F\,cm^{-2}$,[15] probably as a result of the thinning of the bilayer or the replacement of lipid molecules by the peptide (see the Supporting Information).

Formation of a tBLM by vesicle spreading and fusion on the mixed monolayer was monitored also by SEIRA spectroscopy. The spectra shown in Figure 3 were obtained by using the spectrum of the mixed SAM as a reference. In this way, positive and negative bands represent contributions of the

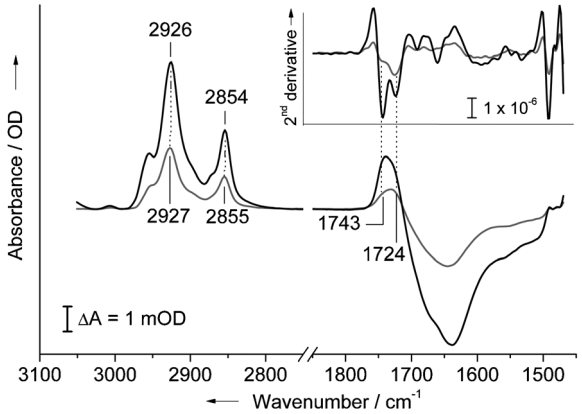

Figure 3. SEIRA difference spectra obtained during the spreading of POPC vesicles, recorded 3 min (gray) and 3 h (black) after incubation, and related to the SEIRA spectrum of the mixed monolayer. The inset shows the second-derivative spectra. Buffer: 20 mM Tris, pH 7.4, 100 mM NaCl.

species bound to and removed from the Au surface, respectively. The positive bands between 2800–3050 cm^{-1} and at 1733 cm^{-1} are assigned to C–H stretching modes and the C=O stretching of the ester groups of POPC, respectively. The broad negative band centered at 1645 cm^{-1} originates from the removal of water molecules close to the CPEO3/6MH SAM that have been replaced by POPC molecules. During tBLM formation, only minor shifts and no variations in the relative intensities of the bands in the C–H stretching region are observed. The only notable exception refers to the C=O stretching region, which displays significant time-dependent changes both in the position and the relative intensities of the bands involved. These changes, which are particularly evident in the second derivatives of the spectra (Figure 3, inset), indicate the existence of two distinct POPC components inside the tBLM. At an early stage of vesicle fusion, the C=O stretching region is dominated by a band at 1724 cm^{-1}, whereas at the end of the process the second band at 1743 cm^{-1} prevails. The kinetic analysis, which yields rise times of 2 and 480 min for the bands at 1724 and 1743 cm^{-1}, respectively (see the Supporting Information), indicates that

tBLM formation on the mixed CPEO3/6MH monolayer is a two-step process. Fast spreading and fusion of POPC vesicles onto the monolayer is followed by rather slow reorientation of some phospholipid molecules to fill the gaps in the mixed monolayer, a process that may involve a "flip over" of lipids with the head group facing the solution to an orientation with the head group pointing toward the electrode surface. Taking into account the lower frequency, the 1724 cm^{-1} band is attributed to the C=O stretching mode of the solvent-exposed (hydrogen-bonded) head groups in the upper layer, whereas the 1743 cm^{-1} band may then be ascribed to the respective mode of the buried, electrode-facing head groups in the bottom layer.

The incorporation of gA leads to a small negative band at 1741 cm^{-1} reflecting the replacement of a few POPC molecules by the peptide (Figure 4). The frequency is slightly lower than that of the lately prevailing POPC component (1743 cm^{-1}), which may be caused by both the presence of

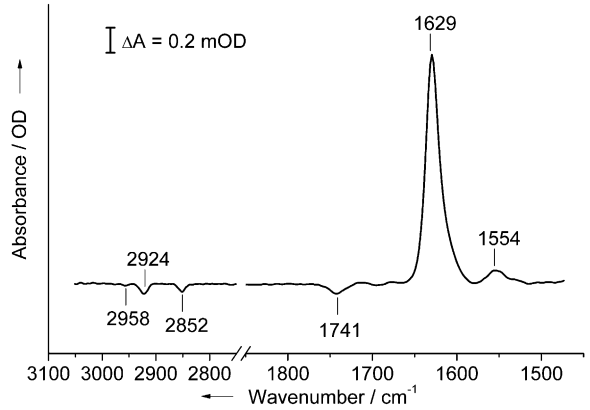

Figure 4. SEIRA spectrum of a tBLM after incorporation of gA, using the spectrum of the pure tBLM as the reference. Buffer: 20 mM Tris, pH 7.4, 100 mM CsCl.

Cs^+ ions instead of Na^+ ions in the solution and a tighter packing of the bilayer over time.[19] The amide I and amide II modes of the peptide are located at 1629 and 1554 cm^{-1}, respectively, which are indicative of the $\beta^{6,3}$ species.[20] The high amide I/amide II intensity ratio (ca. 16) is consistent with a perpendicular alignment of the gA β helix with respect to the electrode surface.[21] The time-dependent changes in the amide I intensity display biexponential behavior (see the Supporting Information) with a fast phase (7.2 min) attributed to the direct incorporation of gA into the upper lipid layer and a slow phase (111 min) that may reflect the translocation of the peptide from the upper into the lower layer of the tBLM.[22]

The functionality of the incorporated gA was studied by measuring SEIRA difference spectra of gA in the presence of Li^+, Na^+, K^+, Cs^+, and Tl^+, taking the spectrum of the Ba^{2+}-containing buffer as the reference (Figure 5 and Supporting Information). The observed amide I changes result from replacing Ba^{2+} ions at the binding site in the gA channel with the various monovalent cations. Thus, these spectral changes, which were found to be reversible, reflect an increase of the

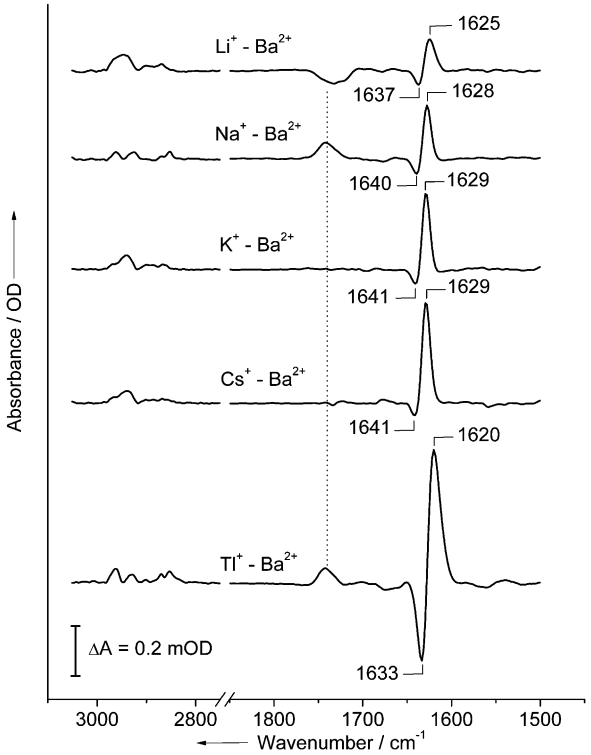

Figure 5. Baseline-corrected SEIRA difference spectra of gA incorporated into tBLMs in the presence of different monovalent cations, using the spectrum of the sample in the presence of Ba^{2+} as a reference. Buffer: 20 mM Tris, pH 7.4, 100 mM MCl_x with M = Ba, Li, Na, K, Cs; in the case of Tl the fluoride salt was used. Details on the baseline-correction procedure are reported in the Supporting Information (Figure SI5).

conducting state (i.e. the positive difference band between 1620 and 1629 cm^{-1}). At least in the case of Na^+, K^+ and Cs^+, this conducting state can be confidently ascribed to the $\beta^{6,3}$ conformation.[20] The amide I frequency increases with the size of the cation, except for Tl^+, which has a diameter similar to that of K^+. This discrepancy can be tentatively explained by considering the higher polarizability of the Tl^+ ion, which may influence the nature of the gA/tBLM interaction.

The spectral changes in the C=O stretching region (Figure 5, dotted line) can be interpreted on the basis of previously reported data on cation binding to POPC.[23,24] Accordingly, Li^+, Na^+, and Ba^{2+} ions bind very strongly to the POPC head groups such that exchanging Ba^{2+} by Li^+ or Na^+ leads to notable difference bands in the C=O stretching region, albeit of opposite sign. These findings may be attributed to a different vertical displacement or tilt angle of the Li^+- and Na^+-bound lipid head groups with respect to the surface, thereby accounting for the negative and positive signals in the difference spectrum. An analogous interpretation may hold for the binding of Tl^+ ions. In contrast to the strongly binding Li^+, Na^+, and Ba^{2+} ions, previous studies ruled out the direct interaction of K^+ and Cs^+ ions with the C=O groups of POPC,[22,23] which is consistent with the lack of difference bands in the present SEIRA spectra (Figure 5).

When EIS was employed to study gA-doped tBLM in the presence of different cations, the conductive state (i.e. Li^+,

Na^+, K^+, Cs^+, and Tl^+) was easily distinguished from the non-conductive Ba^{2+}-bound state that displays the highest impedance (Figure 6 and the Supporting Information). These data are correlated with the intensities of the positive difference bands in the amide I region (1620–1629 cm^{-1}) obtained for the corresponding cation-exchange experiments (Figure 6, left). Additionally, the electrochemical and spectroscopic data are consistent with the Hofmeister series except for the impedance value for Li^+. Probably owing to its small size, this cation is capable of diffusing through defects in the tBLM, causing a decrease of the impedance.

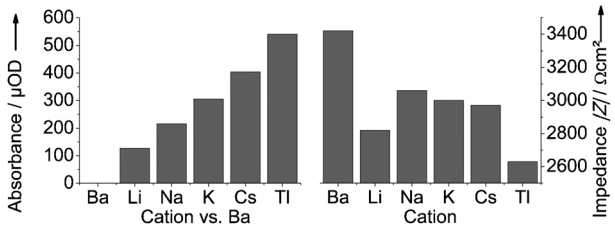

Figure 6. Left: Intensities of the positive amide I band (1620–1629 cm^{-1}) of the conductive state in the SEIRA difference spectra (Figure 5). Right: Impedance values at a frequency of 45 Hz of the tBLM with gA in the presence of different cations. The errors of the absorbance and the impedance values are within 10 and 5%, respectively.

In conclusion, we have successfully assembled a tBLM on a nanostructured Au electrode as demonstrated by EIS and SEIRA spectroscopy. Despite the rough surface structure of the Au film required for the surface enhancement of the IR signals, the tBLM displays satisfactory impedance (see the Supporting Information) and high mechanical and long-term stability (> 60 h), which is the prerequisite for the structural and functional characterization of proteins integrated in the tBLM. The present case study on tBLM-bound gA provides novel structural information about the molecular interactions of cations with the peptide and the membrane, and demonstrates the high potential of our approach for analyzing the functioning of membrane proteins on a molecular level.

Received: April 26, 2012
Published online: July 2, 2012

Keywords: bilayer lipid membranes · electrochemistry · gramicidin A · ion channels · SEIRA

[1] I. Willner, E. Katz, *Angew. Chem.* **2000**, *112*, 1230 – 1269; *Angew. Chem. Int. Ed.* **2000**, *39*, 1180 – 1218.
[2] M. Ferri, F. Mazzei, T. Ferri, *Anal. Bioanal. Chem.* **2010**, *398*, 1554 – 1564.
[3] A. Janshoff, C. Steinem, *Anal. Bioanal. Chem.* **2006**, *385*, 433 – 451.
[4] E. K. Sinner, S. Ritz, Y. Wang, J. Dostálek, U. Jonas, W. Knoll, *Mater. Today* **2010**, *13*, 46 – 55.
[5] R. L. C. Naumann, C. Nowak, W. Knoll, *Soft Matter* **2011**, *7*, 9535 – 9548, and references therein.
[6] L. J. C. Jeuken, *Nat. Prod. Rep.* **2009**, *26*, 1234 – 1240.
[7] Y. H. Chan, S. Boxer, *Curr. Opin. Chem. Biol.* **2007**, *11*, 581 – 587.
[8] C. Steinem, A. Janshoff, H.-J. Galla, M. Sieber, *Bioelectrochem. Bioenerg.* **1997**, *42*, 213 – 220.
[9] Y. Cheng, N. Boden, R. J. Bushby, S. Clarkson, S. D. Evans, P. F. Knowles, A. Marsh, R. E. Miles, *Langmuir* **1998**, *14*, 839 – 844.
[10] F. Siebert, P. Hildebrandt, *Vibrational Spectroscopy in Life Science*, Wiley-VCH, Weinheim, **2009**.
[11] K. Ataka, J. Heberle, *Biochem. Soc. Trans.* **2008**, *36*, 986 – 991.
[12] D. Millo, P. Hildebrandt, M.-E. Pandelia, W. Lubitz, I. Zebger, *Angew. Chem.* **2011**, *123*, 2680 – 2683; *Angew. Chem. Int. Ed.* **2011**, *50*, 2632 – 2634.
[13] B. A. Wallace, *Annu. Rev. Biophys. Biophys. Chem.* **1990**, *19*, 127 – 157.
[14] D. D. Busath, *Annu. Rev. Physiol.* **1993**, *55*, 473 – 501.
[15] J. K. R. Kendall, B. R. G. Johnson, P. H. Symonds, G. Imperato, R. J. Bushby, J. D. Gwyer, C. van Berkel, S. D. Evans, L. J. C. Jeuken, *ChemPhysChem* **2010**, *11*, 2191 – 2198.
[16] E. K. Schmitt, M. Numabi, R. J. Bushby, C. Steinem, *Soft Matter* **2008**, *4*, 250 – 253.
[17] A. Erbe, R. J. Bushby, S. D. Evans, L. J. C. Jeuken, *J. Phys. Chem. B* **2007**, *111*, 3515 – 3524.
[18] Ref. [15].
[19] C. W. Meuse, G. Niaura, M. L. Lewis, A. L. Plant, *Langmuir* **1998**, *14*, 1604 – 1611.
[20] S. V. Sychev, L. I. Barsukov, V. T. Ivanov, *Eur. Biophys. J.* **1993**, *22*, 279 – 288.
[21] X. Jiang, M. Engelhard, K. Ataka, J. Heberle, *J. Am. Chem. Soc.* **2010**, *132*, 10808 – 10815.
[22] E. Bamberg, H. J. Apell, H. Alpes, *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 2402 – 2406.
[23] B. Klasczyk, V. Knecht, R. Lipkowsky, R. Dimova, *Langmuir* **2010**, *26*, 18951 – 18958.
[24] A. Cordomí, O. Edholm, J. J. Perez, *J. Phys. Chem. B* **2008**, *112*, 1397 – 1408.

