Electrochimica Acta 53 (2008) 6680–6689

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Tethered bimolecular lipid membranes—A novel model membrane platform☆

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ARTICLE INFO

Article history:
Received 25 February 2008
Received in revised form 29 February 2008
Accepted 29 February 2008
Available online 20 March 2008

Keywords:
Model membranes
Membrane proteins
Solid-supported membranes
Tethered membranes
Impedance spectroscopy

ABSTRACT

This contribution summarizes some of our efforts in designing, synthesizing, assembling, and characterizing functional tethered bimolecular lipid membranes (tBLMs) as a novel platform for biophysical studies of and with artificial membranes or for sensor development employing, e.g., membrane integral receptor proteins. Chemical coupling schemes based on thiol groups for Au substrates or silanes used in the case of oxide surfaces allow for the covalent and, hence, chemically and mechanically robust attachment of anchor lipids to the solid support, stabilizing the proximal layer of a tethered membrane on the transducer surface. Surface plasmon optics, the quartz crystal microbalance, fluorescence- and IR spectroscopies, and electrochemical techniques are used to characterize the build-up of these complex supramolecular interfacial architectures. We demonstrate, in particular, that bilayers with a specific electrical resistance of better than 10 MΩ cm^2 can be achieved routinely with this approach.

The functionalization of the lipid membranes by the incorporation of peptides is demonstrated for the carrier valinomycin which shows in our tBLMs the expected discrimination by four orders of magnitude between the translocation of K^+- and Na^+-ions across the hydrophobic barrier. For the synthetic channel-forming peptide M2 the high electrical resistance of the bilayer with the correspondingly low background current allows for the recording of even single channel current fluctuations.

From the many membrane proteins that we reconstituted so far we describe results obtained with the redox-protein cytochrome *c* oxidase. Here, we also use a genetically modified mutant with a His-tag at either the C- or the N-terminus for the oriented attachment of the protein via the NTA/Ni^{2+} approach. With this strategy, we not only can control the density of the immobilized functional units, we introduce a completely new and alternative concept for the stabilization of lipid bilayers, i.e., the protein-tethered membrane.

Our efforts in experimentally characterizing the resulting membrane functions and correlating the data with the structural details of the bilayer architectures are complemented by theoretical studies modeling the electrical and electrochemical response of functional tethered lipid bilayer membranes by extended SPICE simulations.

1. Introduction

Recent years have seen a remarkable progress in the design and experimental realization of various novel model membrane architectures [1–3]. The most prominent and wide-spread examples are solid-supported lipid bilayers on plane [4] or nanostructured substrates [5], vesicles tethered to a solid support [6], and the tethered bimolecular lipid membranes (tBLMs) [7–9]. The strongly increased interest in this variety of novel model membrane systems originates from the need to provide an experimental platform that allows for the study of membrane properties and processes with simultaneous access to structural and functional data. This has been a serious limitation when dealing with other classical model membrane platforms, e.g., vesicles in dispersions are very well-suited for structural studies of the lipid bilayer organization by scattering techniques, of their (mixed) phase behavior by thermodynamic investigations, or of their dynamic properties, e.g., the lateral diffusion of lipids in the bilayer which is a smectic liquid–crystalline, hence, fluid leaflet. However, functional aspects, e.g., of the transport of ions or molecules across the hydrophobic barrier of the bilayer are barely accessible for this class of model membranes. Bimolecular (black) lipid membranes (BLMs), on the other hand, are perfectly suited for detailed studies of the electrical properties (e.g., capacitance and ionic currents across the membranes) of bare and ionophores- or protein-functionalized bilayers, however, the fragile nature of these thin fluid membranes prevents most tools for structural investigations from being applied.

☆ Plenary lecture.

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doi:10.1016/j.electacta.2008.02.121

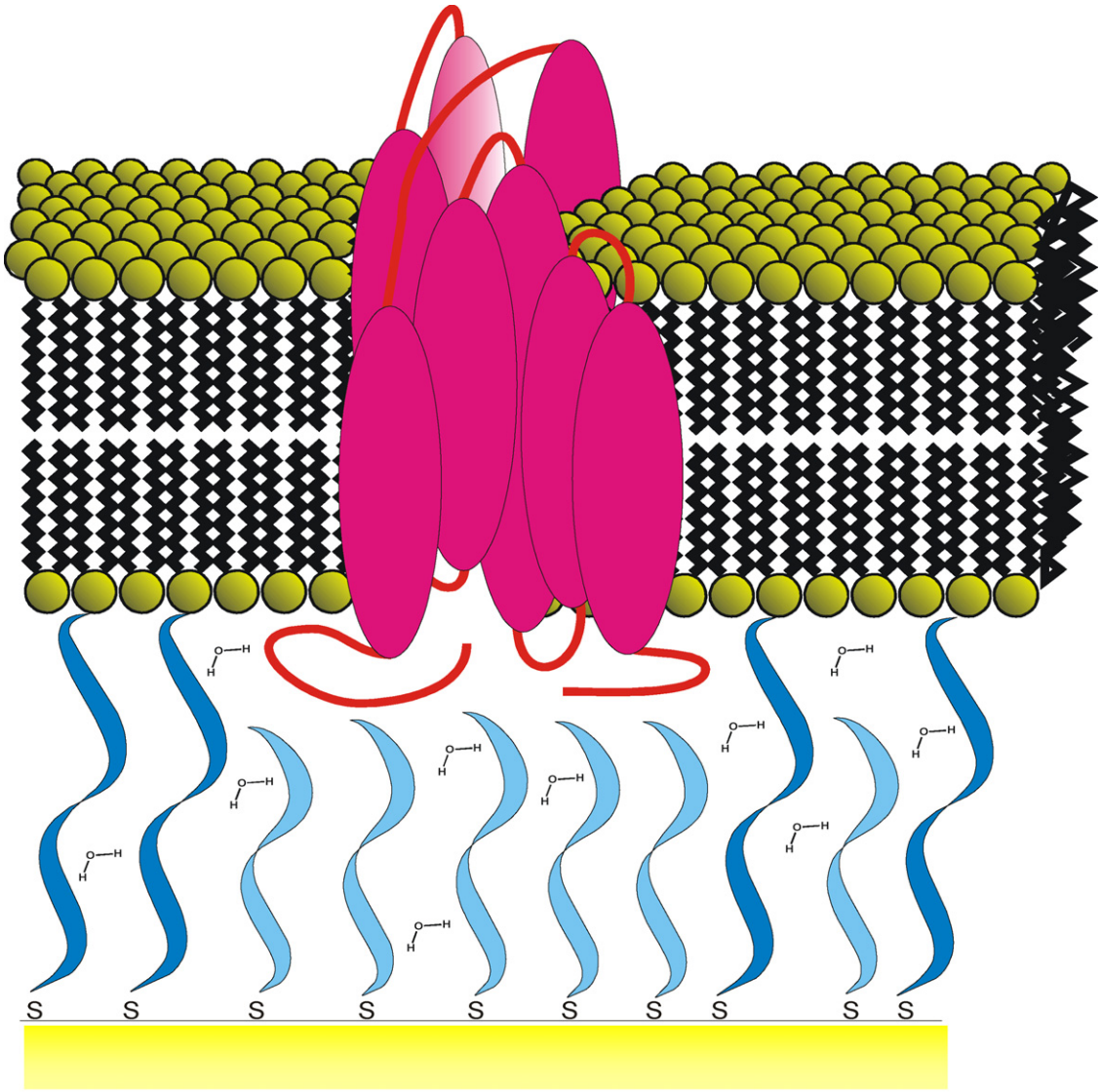

Fig. 1. The tethered bimolecular lipid membrane (tBLM) architecture.

In our work, we have mostly focused on the third concept, i.e., the construction of tethered bimolecular lipid membranes. The basic architecture of this novel model membrane system is given in Fig. 1. The lipid bilayer is covalently coupled to the solid support via a flexible spacer giving this system the mechanical and chemical stability and robustness that is needed should this platform ever become suitable for biosensor applications. The functional task of the tethering lipid molecules that act as anchor groups are at least twofold: on the one side, this (partial) covalent attachment of the membrane to the substrate prevents the whole architecture from being washed away upon mechanical or chemical (osmotic) stress during operation. The resulting stability was systematically tested and the electrical properties of the membrane were found to be unchanged over periods of up to several months [10]. On the other hand, the spacer prevents the lipid bilayer also from interacting too strongly with the solid support compromising, in particular, the dynamic properties of the lipid molecules in the bilayer, e.g., their lateral diffusion. Studies of the diffusion coefficient of individual lipids within the bilayer by fluorescence recovery after photo-bleaching measurements indicated the sufficiently high lateral mobility of these membrane constituents despite the proximity of the solid support [11]. Moreover, the spacer layer is also providing the space necessary to prevent membrane integral proteins from interacting with the substrate in an undesired way changing thereby, e.g., their natural functional properties or even denaturing completely.

The chemical variety of the anchor group has been extended recently from thiol- or disulphide groups designed for the covalent binding of the tether lipids to Au substrates to silane-based chemistry for reaction with the silanol groups of glass substrates or the non-metallized SiO_2-gate electrodes of transistors, and to phosphonate chemistry for alumina surfaces [12,13]. In addition to spacers of various lengths, the tool box also contains different architectures as far as the number of anchor groups or the number of amphiphiles coupled to the spacer moiety is concerned [14].

This variety of functional groups enables to covalently couple the membrane architecture to substrates of different types of materials. This opens a broad spectrum of surface analytical techniques to be applied for the structural and functional characterization of the formation process and the final property profiles of the resulting membranes [8,15], e.g., the Au substrate indicated in Fig. 1 allows for the surface plasmon optical monitoring of the assembly process

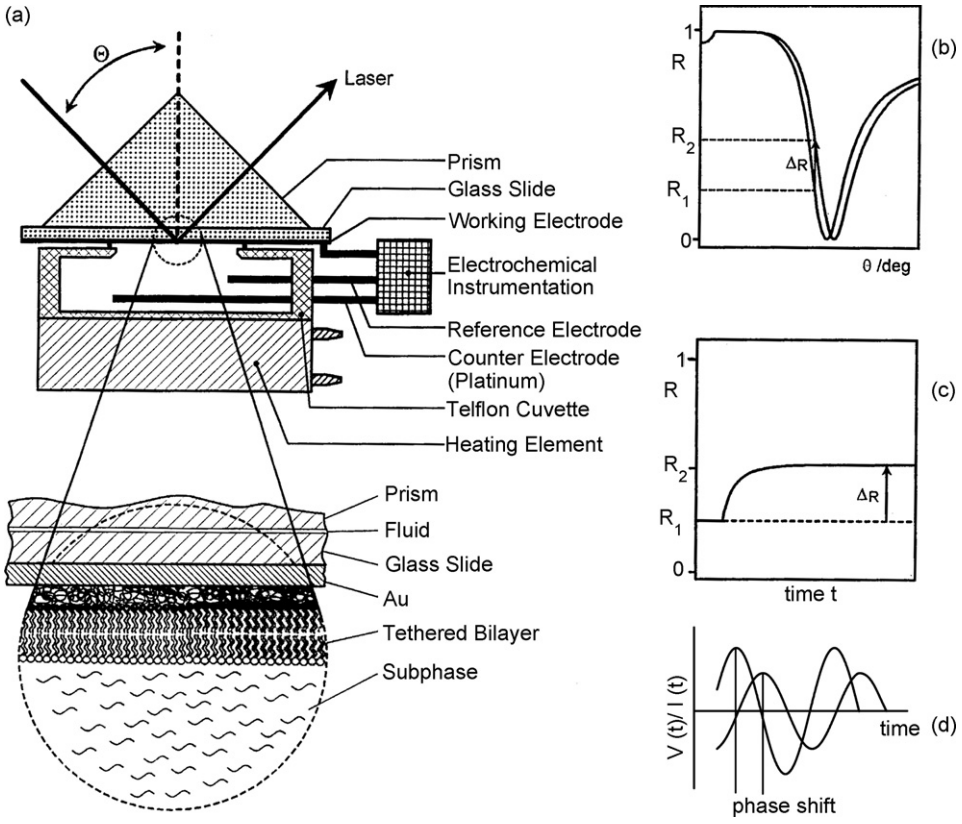

Fig. 2. (a) Experimental set-up based on a surface plasmon spectrometer combined with an electrochemical/impedance spectroscopic module for the simultaneous characterization of structural and functional features of tethered bimolecular lipid membranes. The enlargement shows the solid/solution interface with the thin Au layer used for surface plasmon excitation, the tethering layer and the lipid bilayer in contact to the aqueous buffer phase; (b) typical angular surface plasmon resonance scans before and after the formation of the distal lipid layer (on top of the self-assembled proximal tethered lipid monolayer) by vesicle fusion; (c) kinetic mode of the fusion process recorded by monitoring the change of the reflected intensity at a fixed angle of incidence (cf. (b)) as a function of time; (d) schematics of an impedance spectroscopic experiment, i.e., recording the (amplitude and phase of the) current response upon the application of a small-amplitude ac voltage.

of the tethered monolayer. Similarly, the following fusion process of vesicles from dispersion in contact with the tethered monolayer resulting in the spontaneous formation of the distal layer thus completing the membrane architecture can be monitored [11,16]. In addition, the incorporation of functional modules such as proteins can be followed in real time.

At the same time, these processes can be followed by electrochemical techniques, e.g., by impedance spectroscopy giving important information on the functional properties of the systems under study and their correlation with the structural integrity of the tethered membrane (cf. below). Many supported or tethered bilayer architectures look acceptable when studied by structural techniques only, however, still contain a significant level of structural defects generating non-specific channels for the translocation of ions across the bilayer, thus, screening any specific effect of particular ionophores.

2. Structural and functional characterization of tBLMs

A key role in the battery of analytical tools that we typically apply for the structural and functional characterization of tethered bimolecular lipid membranes is played by the combination set-up of a surface plasmon resonance (SPR) spectrometer and an electrochemistry/impedance spectroscopy instrument. The basic concept of this very powerful experimental tool is schematically given in Fig. 2(a). The classical SPR (here shown in the Kretschmann configuration) is complemented by a three-electrode electrochemistry instrument. The Au substrate that is the basis for the thiol-based self-assembly of the tethered monolayer is also used to guide the surface plasmon mode resonantly excited at the corresponding angle of incidence along the metal/buffer interface. Any change of the interfacial architecture that results in a modification of the refractive index that is probed by the surface plasmon mode, i.e., within its evanescent wave extending approximately 200 nm into the buffer phase can be monitored by the change of the resonance angle for surface plasmon excitation (Fig. 2(b)). If these changes are recorded at a fixed angle of incidence as a function of time, kinetic information can be derived, e.g., on the characteristic time scale of the self-assembly process or on the time required to complete the fusion process by vesicle fusion (Fig. 2(c)).

At the same time, the Au layer is used as a working electrode next to a separate counter and reference electrode for the electrochemical studies. Fig. 2(d) documents the concept of such an impedance spectroscopic experiment in which a sinusoidal ac voltage is applied between the working and the reference electrode and the resulting modulus and phase angle of the resulting current is monitored. These measurements, when evaluated classically on the basis of an equivalent circuit or by a recently introduced SPICE code for network analysis then yield data on the capacity and the resistance of the lipid bilayer. Both techniques complement each other in a most relevant way in that the structural data alone would not give the full picture of a lipid bilayer that also needs (at least for the translocation of ions that is considered here) to fulfil the requirement of being an effective barrier for the non-specific permeation of ionic charges across the membrane. Only the correlated analysis of structural information derived from the surface plasmon

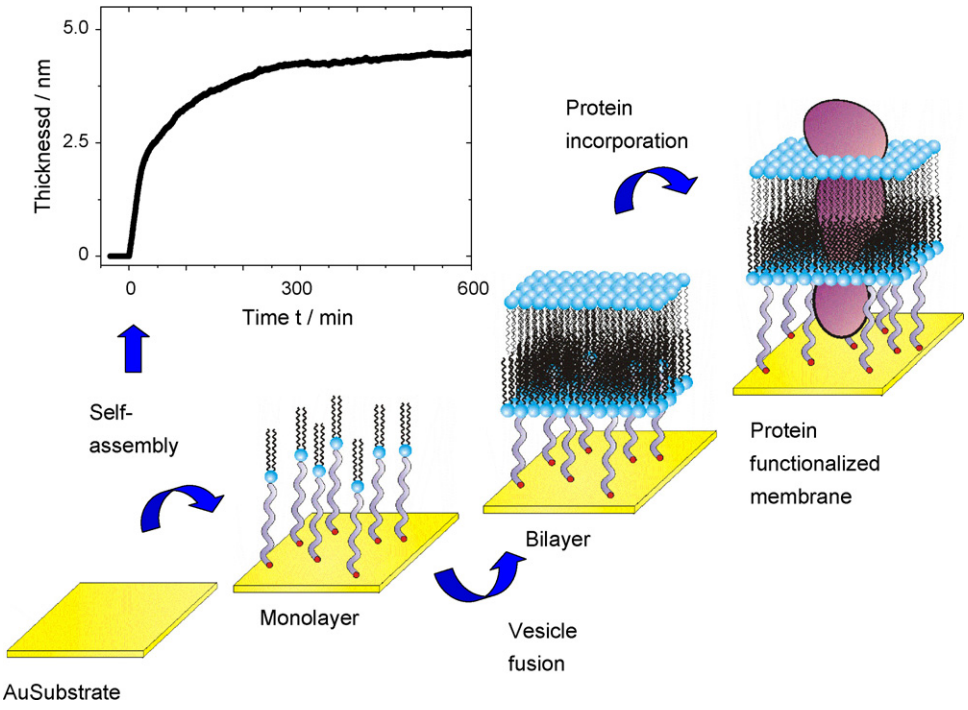

Fig. 3. The assembly process that leads to the formation of a tethered bimolecular lipid membrane: onto a clean Au substrate used for surface plasmon- and impedance spectroscopic characterization of the assembly process as well as of the final membrane properties the telechelic amphiphiles self-assemble to form a tethered monolayer. The inset shows the SPR kinetic data of this adsorption process of the tethered lipid molecules from solution to the Au substrate. The bilayer is then completed by vesicle fusion with a simultaneous (by fusion of proteo-liposomes) or sequential reconstitution of functional proteins into the tethered membrane.

experiments and the functional characteristics obtained from the impedance data allows one to optimize the architecture and document the specific function of an incorporated ion carrier or channel system [16,17].

The whole process of the functional membrane fabrication is schematically summarized in the carton given in Fig. 3. It starts with the telechelic molecules that are designed to assemble and covalently bind to the substrate while constituting the proximal monolayer of the final membrane bilayer. The fusion of vesicles results in the formation of the distal monolayer completing the lipid bilayer followed by the eventual incorporation of any functional structures such as peptides or proteins. The inset shows a kinetic surface plasmon optical recording. The change in reflected intensity monitored at a fixed angle of observation as a function of time is converted to a thickness increase of the interfacial layer with a final value of ca. 3.5 nm for the tethered monolayer. The whole assembly process takes several hours to be completed well in line with observations with other self-assembling thiols on gold.

Similar results were obtained with silane derivatives that assemble on quartz, glass [13], or ITO for various specific applications in which lipid bilayer membranes play a role. We should also mention that the information on the lipid bilayers formation and the structural properties of the resulting membranes by surface plasmon optics constitutes only one element of the range of data that are needed to fully characterize these systems. Typically, further structural information is obtained by X-ray and, in particular, by neutron reflectometry experiments, by investigations with the quartz crystal microbalance, by various forms of scanning probe microscopies, and by a whole range of other spectroscopic techniques including Raman scattering and IR spectroscopy, and more recently by surface plasmon fluorescence spectroscopy.

A very sensitive way to analyze the functional characteristics of incorporated ionophores, namely carrier systems or channel proteins is given by impedance spectroscopy. One of the first examples that we found to satisfy the needs of a membrane that imposes a real barrier against the mere passive permeation of ions across its hydrophobic barrier is given in Fig. 4. Shown are the impedance data in the form of a Bode plot. The analysis of the data based on the equivalent circuit shown in the inset results in the following values: the specific membrane capacitance is found to be $C_m = 0.52\ \mu F\,cm^{-2}$, in excellent agreement with values that were reported for black lipid membranes. The real breakthrough towards using tethered lipid bilayer membranes for ion translocation studies was the specific membrane resistance that was found for this

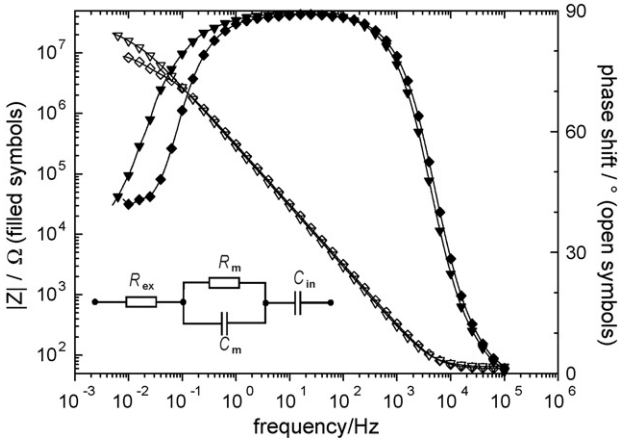

Fig. 4. Impedance spectroscopic characterization of a tethered bimolecular lipid membrane. The fit to the data of the Bode plot with the equivalent circuit shown in the inset results in a value for the area normalized membrane resistance of $R_m = 14.9\ M\Omega\,cm^2$. The resistance of the buffer solution can be obtained from the impedance data at high frequencies and for this example is about 100 Ω. The capacitance results in a specific value of $C_m = 0.52\ \mu F\,cm^{-2}$.

system: $R_m = 14.9\,M\Omega\,cm^2$. In order to realize what this value means let us assume a membrane area of, e.g., 100 μm × 100 μm. With the given resistance and upon the application of a potential of $U = 100$ mV, the current across the membrane corresponds to $I = 1$ pA, which has to be seen in comparison to the currents that typically cross a single channel in the open state, i.e., currents in the range of 10 pA. As we will show later, in fact, the electrical barrier of the unmodified tBLMs is good enough to monitor the current fluctuations induced by the opening and closing of single individual channels.

We should also mention that these excellent barrier properties are the result of an optimized molecular architecture of the self-assembling lipid molecule with a lipoic acid anchor group, a short EO unit as the spacer, and a (ether bond coupled) lipid analogue with two phytanoyl chains that improve the fluidic (hence, sealing) character of the hydrophobic core, and the use of an ultra-flat Au substrate prepared by the so-called template stripping [9,11]. The Au layer is evaporated onto a particularly smooth Si wafer, and then glued with its backside to the high index glass slide needed for the excitation of a surface plasmon in an aqueous environment. The template is stripped off the sandwich resulting in a fresh Au surface that exhibits the smoothness of the template it was first evaporated to. Other than with some of the substrates used in scanning probe microscopies which can have atomically flat surfaces, however, only over domain areas of a few hundreds or thousands of nanometers in diameter with deep trenches in between the domains, the template stripped Au substrates can be produced with areas as large as several cm^2. This makes this substrate attractive even when considering the needs for processing tethered membranes in large quantities for (commercial) biosensor applications.

An example for the simultaneous structural and functional characterization of the fusion of vesicles to a self-assembled tethered monolayer and the following incorporation of a toxin, α-hemolysin (α-HL), that 'drills holes' into the membrane is given in Fig. 5 [17]. The addition of the vesicle dispersion results in a substantial increase of the thickness of the interfacial architecture (given by the strong increase of the reflected intensity monitored in this experiment) and leads to a dramatic increase of the background resistance of the resulting lipid double layer. Upon addition of the α-HL, a slight increase of the thickness indicates the incorporation of the toxin into the membrane. It could, however, simply reflect some non-specific adsorption of the protein to the lipid head groups without functional incorporation, or both. The simultaneously measured impedance data clearly indicate that (at least

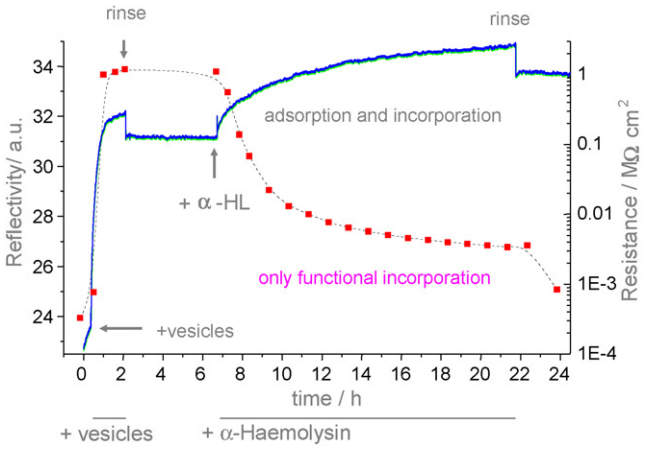

Fig. 5. Protocol of the formation of the complete lipid bilayer structure by the fusion of vesicles onto a tethered monolayer, followed by the incorporation of α-hemolysin a heptameric toxin derived from *Staphylococcus aureus* [17]. For details see text.

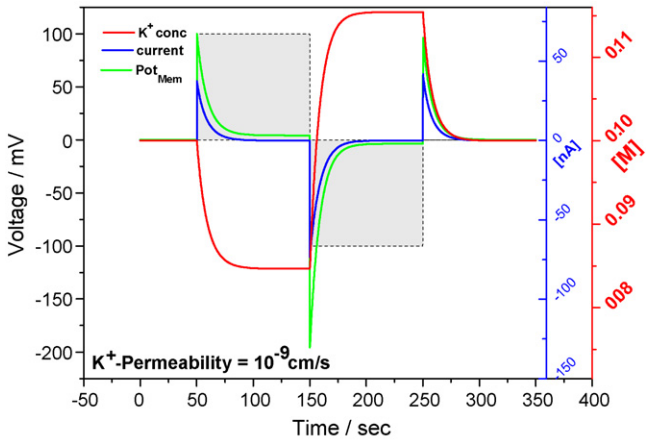

Fig. 6. Simulation data obtained by a SPICE code of the change in the K^+ concentration inside the cleft between the Au electrode and the tethered bilayer, the transient current that is caused by this rearrangement of ions, and the potential drop across the bilayer upon switching the voltage between the Au working electrode and the reference electrode in the electrolyte from 0 V to +100 mV (with the Au electrode being the positive side) then to −100 mV and then back again to 0 V. The time-dependent changes are calculated assuming a passive K^+ permeability of 10^{-9} cm/s (corresponding to a membrane resistance of 10 MΩ cm^2).

a substantial fraction of) the protein, in fact, is being incorporated into the tethered bilayer as can be deduced from the strong decrease of the membrane resistance by more than 3 orders of magnitude. As expected for this system, rinsing the flow cell with buffer removes some of the physisorbed protein resulting in a slight decrease of the layer thickness, the functional fraction inside the membrane, however, is not washed out (on the contrary, it might be even better organized by the flow field-induced shear stress).

A concern with respect to the proper performance of tethered membranes and the function of ionophores or proteins incorporated was related to the fact that the current architectures offer only a very narrow cleft between the lipid bilayer and the solid substrate. The question was whether this extremely limited volume will be sufficient to allow for any translocation of ions at all and how it would influence the current across the membranes. To this end we performed simulations based on the SPICE code originally developed for the analysis of complex networks. Further refinements of the algorithms are still an ongoing effort but already at the current level of simulations very interesting insights into the translocation of ions across tethered bilayers in general and the specific influence of the very limited aqueous reservoir on the substrate side of the membrane could be gained.

An example of such a simulation is given in Fig. 6: the evaluated scenario started with a tethered membrane in a 100 mM K^+-solution, assuming that only the K^+-ions can permeate across the lipid barrier with a permeability coefficient of 10^{-9} cm/s (corresponding to a specific) membrane resistance of 10 MΩ cm^2. At first, the potential difference between the substrate (the working electrode) and the reference electrode was 0 V, then switched to +100 mV (substrate electrode positive), then to −100 mV, and then back again to 0 V. Upon switching the potential of the working electrode to +100 mV, for the very first moment the full 100 mV drop across the lipid membrane because in the serial arrangement of electrode/buffer interface/tethering layer/lipid bilayer/bulk buffer phase/counter electrode the highest resistance has, in fact, the lipid bilayer. This potential drop across the bilayer drives K^+-ions though the membrane resulting in a transient current across the membrane. The accumulation of K^+-ions then leads to a concentration asymmetry and, hence, to an electrochemical gradient generating a

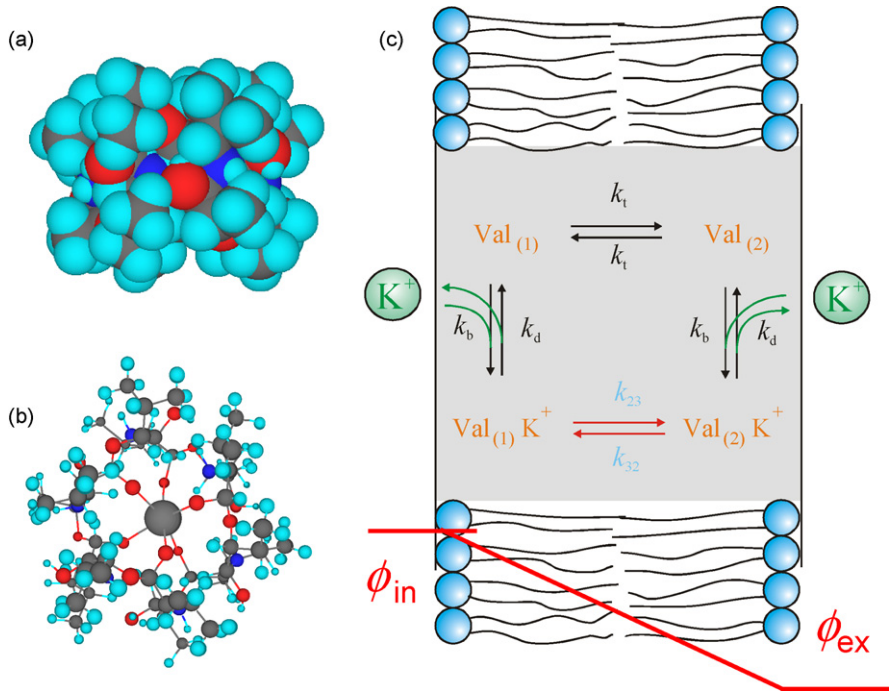

Fig. 7. Translocation of K^+-ions across the lipid bilayer by valinomycin (Val). (a) Molecular model of valinomycin showing the strong hydrophobic character of the outside of the cyclic peptide. (b) Simplified model indicating the complexation of alkali ions through the 6 carbonyl moieties defining a slightly negative cavity inside the peptide. (c) Transport mechanism of valinomycin shuttling K^+-ions across the lipid bilayer by association on one side of the membrane, translocation of the charged complex across the hydrophobic barrier, and release of the ion on the other side by dissociation and return of the empty shuttle. Each individual reaction step is quantified by rate constants.

voltage across the membrane that counteracts the applied external voltage. As a consequence the current drops until a new electrochemical equilibrium is reached: the current goes to zero, and the voltage across the lipid bilayer is only a small fraction of the voltage applied to the whole circuit, about $U = 10$ mV in our example. The concentration of K^+-ions in the cleft in this newly established equilibrium then amounts to 85 mM.

These simulations indicate that we need to be extremely careful when discussing membrane processes that depend on the applied potential, e.g., when studying voltage-gated channels. Without the full control over all elements in this serial arrangement of components of the equivalent circuit no conclusions can be drawn with respect to the trans-membrane voltage, the local ion concentration (including H^+-ions, hence local pH values), etc. However, once this control is established, the specific architecture of the membrane tethered to a solid support does not compromise the usefulness of this novel model membrane system.

In view of results discussed further down we should point to the time course needed to establish the new equilibrium. Strongly depending on the permeability coefficient which controls how fast this equilibrium can be reached we note that for the value of 10^{-9} cm/s used in these simulations it takes a few 10 s until the potential across the membrane drops to its final value. We will come back to this point later.

3. The translocation of ions across the tethered membrane by the carrier valinomycin

An excellent example for the proof of the concept of tethered lipid bilayer membranes can be given by measurements with the ion carrier valinomycin. This cyclic depsi-peptide has a rather hydrophobic outer surface (cf. Fig. 7(a)), which gives it a high partition coefficient into the lipid bilayer, with a cavity in the middle of the structure, which is composed of carbonyl groups (Fig. 7(b)) that can chelate alkali ions very effectively. Valinomycin transports K^+-ions 10^4 times better than Na^+-ions and, hence, allows for a clear differentiation between specific transport and mere passive permeation through defects in the membrane which would be much less ion specific—if at all.

The peptide operates according to shuttle mechanisms as schematically indicated in Fig. 7(c). Being located at the membrane/solution interface it can complex an alkali ion to form a charged complex that can transfer by a hopping process across the potential barrier of the hydrophobic membrane core to the other interface, release the ion there and return as an empty shuttle to the original side. As a net result an ion is transported across the membrane. The whole reaction cycle is described by rate constants, with only the translocation of the charged complex being voltage-dependent.

Fig. 8 shows the result of the corresponding experiment with valinomycin being incorporated into a tethered lipid bilayer membrane. The latter was assembled in a solution containing 100 mM NaCl. The resistance was found to be in the 10 MΩ cm^2 range. The addition of valinomycin reduced the barrier to about 1 MΩ cm^2 which might be indicative of some structural rearrangements in the lipid bilayer that has to accommodate the bulky valinomycin molecules. Upon the replacement of parts of the NaCl solution by KCl (keeping the ionic strength constant) one can induce a significant decrease of the membrane resistance, eventually by the expected 4 orders of magnitude once the majority of ions in the buffer solution are K^+-ions (at 20 mM) that are so much more efficiently transported than Na^+-ions.

If one reverses the addition of KCl and goes back to a pure NaCl solution the resistance again increases to almost the original level (the fact that in the example of Fig. 8 only a lower resistance is reached after the solution exchange merely reflects the poor liquid

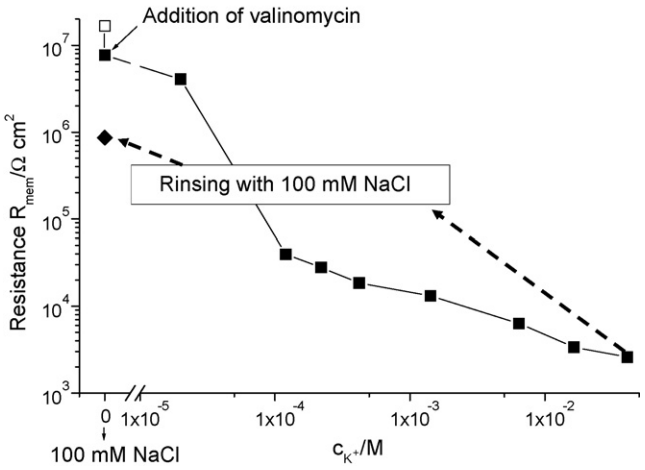

Fig. 8. Specific resistance of a valinomycin-doped tethered bilayer lipid membrane in 100 mM NaCl, and after partial ion exchange (at constant ionic strength) against a KCl solution. Note the (reversible) decrease of the membrane resistance by 4 orders of magnitude upon the replacement of Na^+-ions against K^+-ions. For more details, see text.

handling in our flow cell which probably still contains an appreciable amount of K^+-ions which could only be removed by extended rinsing). This proves that the decrease of the resistance is a specific feature of the valinomycin-mediated ion transport across this tethered membrane and has nothing to do with any defect structures which would not respond to the exchange of one specific ion by another one, at least not to the extend observed in the experiment. It is only the excellent sealing properties of our architectures that allow for such a clear discrimination of ion currents as a special feature of valinomycin, well known from BLM studies but not reported so far for tethered membranes.

4. Single channel current fluctuations

Another characteristic property of these tethered membranes with their very high background resistance is the correspondingly low ionic current in the absence of any specific ionophore or electrogenic protein. This allows then for the recording of current increments as they are observed upon the opening and closing of single channel proteins. This is shown in Fig. 9 for an experiment with a synthetic M2 peptide that mimics the trans-membrane part of the nicotinic acetylcholine receptor. The potential applied to the substrate, i.e., the working electrode was switched from −100 mV to 0 V and then to +100 mV with the current across the membrane monitored with a patch clamp amplifier located in the electrolyte solution above the tethered lipid bilayer membrane [18,19].

A few observations should be pointed out: the level of the background current is in the range of a few (10) pA, a consequence of the high sealing resistance of the membrane. Upon switching the applied voltage to 100 mV it drops after an initial value of roughly 40 pA to a much lower level of a few pA only, and does so with a time constant in the range of 10–20 s. Both observations are in full agreement with the simulation data presented in Fig. 6 which predict this drop of current as a result of the voltage-induced redistribution of ions across the bilayer and the concomitant build-up of a counter acting potential gradient.

On top of this time-dependent background current, however, one can clearly see the opening and closing of individual channels incorporated into this membrane, leading to the stochastic increase and decrease of the current as demonstrated in Fig. 9 [18]. These observations, for the first time document that tethered lipid bilayer membranes, when properly designed and assembled, can match or even surpass the performance of the former standard for ultra-sensitive current detection, i.e., the black lipid membranes (BLMs). However, the novel model membrane system presented here offers the additional advantage of allowing for a very comprehensive structural investigation in fundamental studies of the structure/function relation of membranes. Moreover, it promises

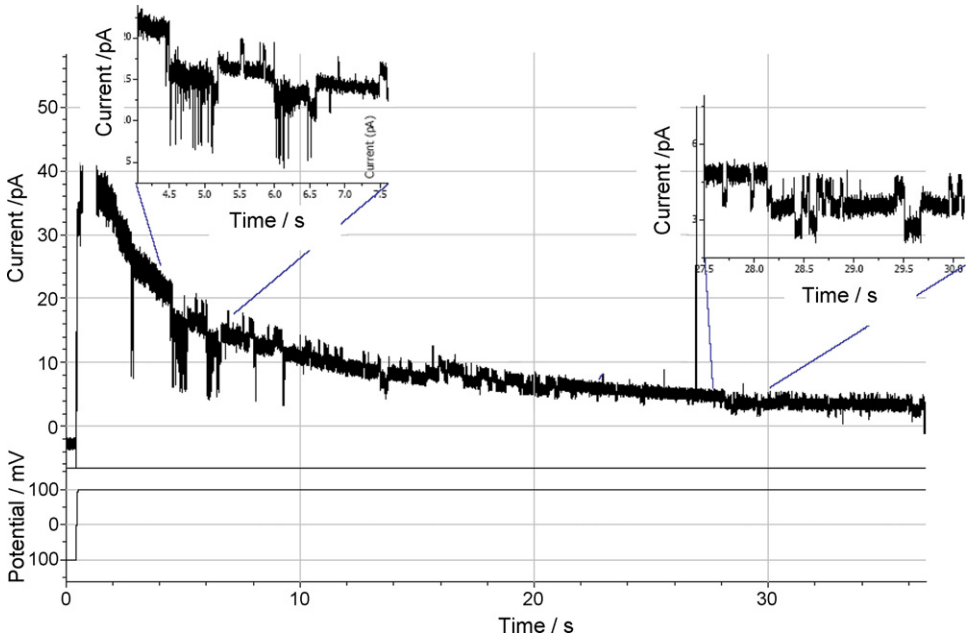

Fig. 9. Current trace measured with a patch clamp amplifier of a tethered bimolecular lipid membrane-doped with a small amount of synthetic M2, a pentameric peptide that mimics the channel part of the nicotinic acetylcholine receptor protein. Note the voltage change as indicated in the lower trace: the potential was switched from −100 mV to 0 V and then to +100 mV. Note further the gradual decay of the background current with the superimposed opening and closing of individual channels resulting in current increments at the few pA level.

to provide the stability and robustness needed for practical applications, e.g., in biosensor development.

5. The protein-tethered membrane: 'wiring' redox proteins to the Au electrode

In all the examples discussed so far, the membranes were tethered to the solid support via anchor lipids that stabilize the bilayer without compromising the local dynamics and without preventing the membrane constituents – at least to some extend – from laterally diffusing.

This approach results in very stable and electrically insulating membranes, however, the incorporation of proteins follows a rather stochastic mechanism: unless any specific asymmetry, e.g., a pronounced shape anisotropy with a large external part of the protein sticking out of the bilayer induces a preferred insertion, the orientation is not controlled. Moreover, the packing density of the reconstituted proteins in the lipid bilayer is not well controlled either. Hence, any correlation between their functional performance and their orientation and number density are not possible.

In some other cases it might additionally be desirable to have the lipids completely decoupled from the solid substrate and stabilize the membrane via the incorporated proteins that are attached to the support. Examples are redox proteins that one might want to connect electronically to the base electrode because they need to be 'wired' to the support in order to allow for efficient heterogeneous electron transfer between the external circuit and the redox center of the protein.

This has lead to the concept of protein-tethered lipid bilayer membranes schematically depicted in Fig. 10(a) [20,21]: individual membrane proteins, solubilized in a detergent micelle are first assembled at the electrode surface by adsorption from solution. In case one is dealing with genetically modified proteins that contain in addition to their native primary amino acid structure a so-called tag, e.g., a sequence of typically 6 histidines as 'His-tags', one can perfectly control the orientation of the assembled proteins: depending on whether the His-tag is engineered to one or the other side of the protein one can determine which way the protein adsorbs and, hence, will be incorporated into the final lipid membrane.

The corresponding assembly process can be followed on-line and *in situ* by, e.g., surface plasmon spectroscopy. An example is given in Fig. 10(b) for cytochrome *c* oxidase (CcO), a membrane protein from the respiratory chain. The blue curve shows the formation of the CcO monolayer, a process that could be controlled by dilution of the complex forming surface architecture [22] and, hence, would result in lower (controlled) protein coverage. Since in this case the His-tag was attached to subunit II of the CcO molecules are all assembled with their cytochrome *c* binding site facing to the substrate (cf. Fig. 11(a)) [23,24] allowing then finally the very efficient electron transfer from the electrode to the redox center.

The next important step is then the formation of a lipid bilayer 'surrounding' the tethered and oriented proteins by a continuous bimolecular leaflet through a process that is the surface equivalent of a classical dialysis step in model membrane studies: the sample is brought into contact with a dispersion of lipid micelles (containing the lipid that should constitute the final membrane) with the detergent molecules then being washed out through a dialysis membrane that seals the compartment containing the protein sample and the micelle dispersion off the dialysis buffer. In the on-line analysis of this process by surface plasmon spectroscopy one

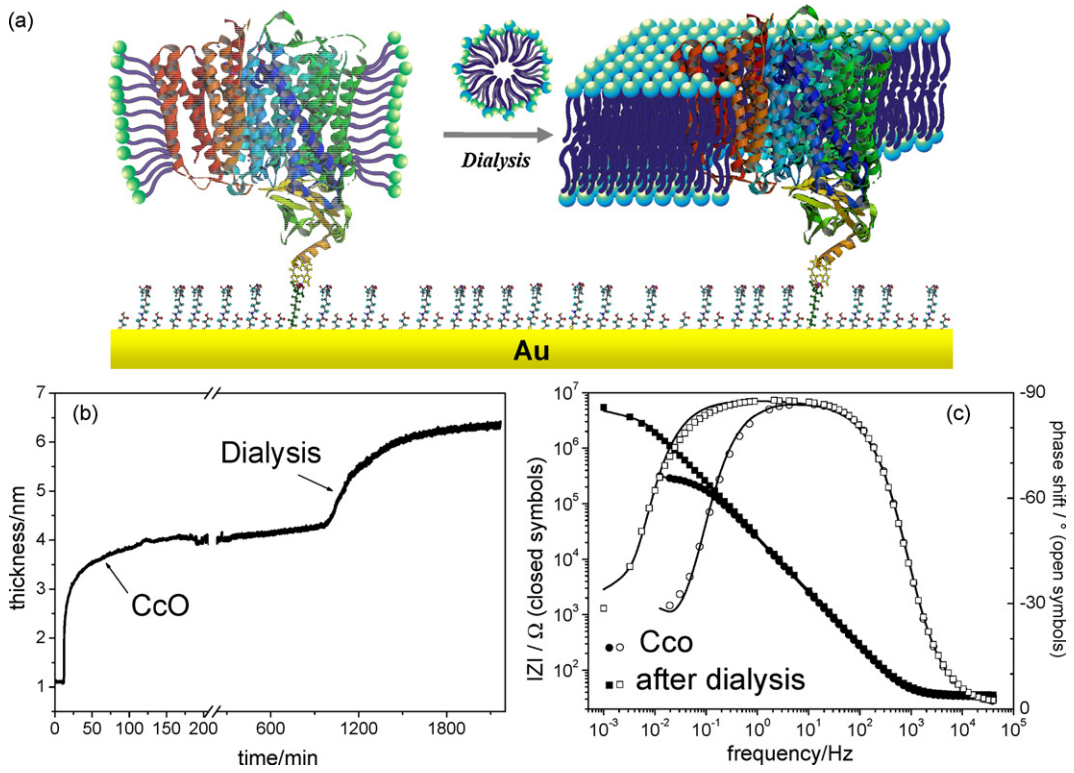

Fig. 10. (a) Tethering membranes via incorporated proteins: the solubilized membrane proteins are covalently attached to the Au electrode, e.g., via NTA/Ni^{2+}-coordination to the His tags of a genetically modified protein, followed by a surface dialysis step that generates a continuous membrane around the incorporated proteins. (b) Surface plasmon spectroscopic protocol of the attachment of the His-tagged protein, cytochrome *c* oxidase in this case and the following dialysis. (c) The corresponding impedance spectroscopic evidence of the dialysis step leading to an electrically well isolating membrane (cf. the change from the measurement taken after the immobilization of the solubilized protein ($R = 0.3\ M\Omega\ cm^2$) to the curve recorded after the membrane was assembled ($R = 12.1\ M\Omega\ cm^2$).

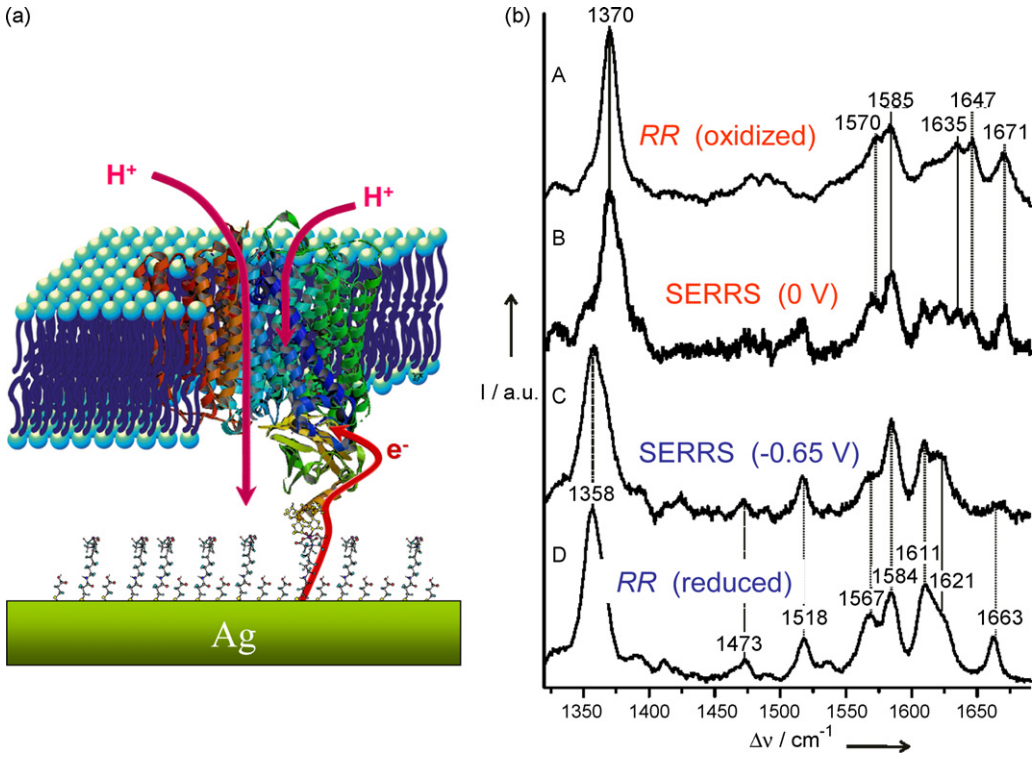

Fig. 11. Surface-enhanced resonance Raman scattering (SERRS) spectra of cytochrome *c* oxidase tethered to an Au electrode. (a) The His-tags used to 'wire' the protein through Ni^{2+} to a NTA/thiol derivative was genetically engineered to subunit II of the protein resulting in an orientation with the cytochrome *c* binding site facing to the substrate. This allows for an efficient electron transfer from the electrode to the active center of CycOx with the full control over the redox state of the protein. This can be seen in the surface-enhanced resonance Raman spectra given in (b): depending on the applied potential the protein can be tuned into its reduced or oxidized form, respectively, with the corresponding change in the Raman spectra as one can deduce from the comparison with Raman spectra taken from the protein in solution.

can see the dialysis step as a further increase of the thickness of the total interfacial architecture (Fig. 10(b)).

More importantly, however, is the impact of the dialysis on the electrical properties of the resulting protein-tethered membrane as it is seen in the impedance spectroscopic measurement shown in Fig. 10(c). Although at the high protein density used in these experiments already the mere assembled monolayer shows a relatively low conductivity (Fig. 10(c)) it is the final dialysis step that seals the membrane against unspecific translocation of ions through defects across the bilayer: the resulting specific resistance is found to be better than 10 $M\Omega\,cm^2$ which compares very well with a typical membrane tethered via its anchor lipids (cf. above).

The best evidence for the functional coupling of the redox proteins to the substrate and hence the successful 'wiring' of the redox centers to the electrode is given by the surface enhanced resonance Raman spectra presented in Fig. 11 for two different electrode potentials corresponding to the two states of the protein: the oxidized form at 0 V and the reduced form at −650 mV, respectively [23]. As schematically shown in Fig. 11(a) the CycOx was attached to the electrode via the His-tag attached to subunit II thus burying the binding domain for cytochrome *c* in the cleft between the membrane and the substrate, however, allowing for the direct electron transfer from the electrode to the protein. By comparing the spectra recorded for the tethered protein with those obtained from chemically oxidized or reduced protein in solution one can identify the spectral shift of various characteristic vibrational bands that clearly indicate the change in the redox state of the protein upon switching the external potential applied.

6. Outlook

One of our long-term strategic goals is the development of an artificial membrane system for the investigation of complex membrane proteins, such as G-protein coupled receptors (GPCRs). The famous family of GPCRs illustrates very well the 'bottle neck' of membrane protein research: GPCR species, isolated in functional form are practically not available. Despite the important role of GPCRs in nature, most of their regulatory, structural and functional details are still unknown. Combining material research, surface engineering and tools from molecular biology, we like to develop an experimental platform for the investigation of GPCRs, such as time resolved ligand binding and structure–function analysis (Fig. 12).

To this end, we employ *in vitro* synthesis of GPCRs using the protein synthesis machinery of a cell extract in order to encompass direct incorporation of the nascent protein into a membrane mimicking structure. Current methods of nanotechnology provide the sensitivity and specificity together with bio-inspired synthesis providing a hydrophobic matrix with defined composition for membrane protein insertion.

With the described composite membrane platform as a 'logic gate' for membrane protein research, one is now in a position to create membrane chips with defined molecular composition and to apply sensitive analytical methods for the read-out of membrane events, e.g., affinity binding, voltage gating, general signal transduction, etc. Ligand screening of orphan GPCRs resulting in new assignments may be obtained using this platform, which significantly extend the realm of synthetic biology towards medical applications.

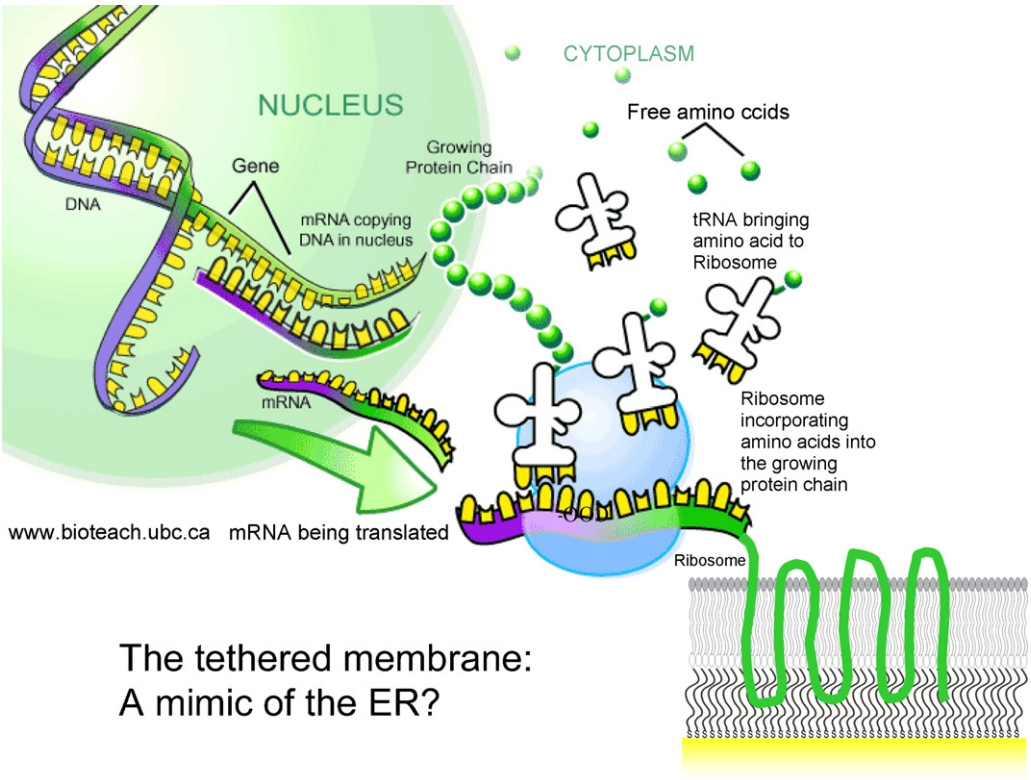

Fig. 12. The concept of cell-free expression of membrane proteins with their immediate insertion into the tethered lipid bilayer membrane: in vitro transcription and translation of, e.g., GPCRs [25].

Acknowledgements

Helpful discussion with many students working on the subject during their Ph.D. project in our group are gatefully acknowledged. We are particular thankful to Inga K. Vockenroth, Henk Kaiser, Randolph Duran, and Joana Long. Financial support came from DARPA trough the MOLDICE program and from the European Union, Specific targeted research project (STREP) FuSymem in the framework 6 program.

References

[1] Special issue on supported membranes. Biointerphases, 2008.
[2] M. Tanaka, E. Sackmann, Polymer-supported membranes as models of the cell surface, Nature 437 (7059) (2005) 656.
[3] Y.H.M. Chan, S.G. Boxer, Model membrane systems and their applications, Curr. Opin. Chem. Biol. 11 (6) (2007) 581.
[4] E. Sackmann, Supported membranes: scientific and practical applications, Science 271 (1996) 43.
[5] W. Römer, C. Steinem, Impedance analysis and single-channel recordings on nano-black lipid membranes based on porous alumina, Biophys. J. 86 (2004) 955.
[6] Y.H.M. Chan, P. Lenz, S.G. Boxer, Kinetics of DNA-mediated docking reactions between vesicles tethered to supported lipid bilayers, Proc. Natl. Acad. Sci. U.S.A. 104 (48) (2007) 18913.
[7] B.A. Cornell, et al., A biosensor that uses ion-channel switches, Nature 387 (1997) 580.
[8] I. Köper, Insulating tethered bilayer lipid membranes to study membrane proteins, Mol. Biosyst. 3 (10) (2007) 651.
[9] S.M. Schiller, et al., Archaea analogue thiolipids for tethered bilayer lipid membranes on ultrasmooth gold surfaces, Angew. Chem. 42 (2) (2003) 208.
[10] I.K. Vockenroth, et al., Stable insulating tethered bilayer lipid membranes. Biointerphases, in press.
[11] R. Naumann, et al., Tethered lipid bilayers on ultraflat gold surfaces, Langmuir 19 (2003) 5435.
[12] R.F. Roskamp, et al., A new surface for functional tBLMs: aluminum oxide, in preparation.
[13] V. Atanasov, et al., Membrane on a Chip. A functional tethered lipid bilayer membrane on silicon oxide surfaces, Biophys. J. 89 (3) (2005) 1780.
[14] V. Atanasov, et al., Highly insulating tethered bilayer membranes. A generic approach for various substrates, Bioconjug. Chem. 17 (2006) 631.
[15] I. Köper, et al., Functional tethered bimolecular lipid membranes (tBLMs), in: Advances in Planar Lipid Bilayers and Liposomes, Academic Press, 2006 (Chapter 2) p. 37.
[16] I.K. Vockenroth, et al., Functional incorporation of the pore forming segment of AChR M2 into tethered bilayer lipid membranes, Biochim. Biophys. Acta (BBA) -Biomembr. 1768 (5) (2007) 1114.
[17] I.K. Vockenroth, et al., Incorporation of alpha-hemolysin in different tethered bilayer lipid membrane architectures, Langmuir 24 (2) (2008) 496.
[18] M.K. Henk, et al., Functional ion channels in tethered bilayer membranes—implications for biosensors, Chembiochem 8 (11) (2007) 1246.
[19] M. Andersson, et al., Detection of single ion channel activity on a chip using tethered bilayer membranes, Langmuir 23 (6) (2007) 2924.
[20] F. Giess, et al., The protein-tethered lipid bilayer: a novel mimic of the biological membrane, Biophys. J. 87 (2004) 3213.
[21] K. Ataka, et al., Oriented attachment and membrane reconstitution of His-tagged cytochrome *c* oxidase to a gold electrode: in-situ monitoring by surface enhanced infrared adsorption spectroscopy, J. Am. Chem. Soc. 126 (2004) 16199.
[22] M.G. Friedrich, et al., Activity of membrane proteins immobilized on surfaces as a function of packing density, J. Phys. Chem. B 112 (10) (2008) 3193.
[23] M.G. Friedrich, et al., Active site structure and redox processes of cytochrome *c* oxidase immobilised in a novel biomimetic lipid membrane on an electrode, Chem. Commun. (2004) 2376.
[24] M.G. Friedrich, et al., Electronic wiring of a multi-redox site membrane protein in a biomimetic surface architecture, Biophys. J. 94 (2008) 3698–3705.
[25] R. Robelek, et al., Incorporation of in vitro synthesized GPCR into a tethered artificial lipid membrane system, Angew. Chem. -Int. Ed. 46 (4) (2007) 605.