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Structural Analysis of Tethered Bilayer Lipid Membranes

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Solid supported membrane systems have been established as biomimetic architectures, which allow for the systematic investigation of various membrane-related processes. Especially tethered bilayer lipid membranes have been a successful concept. They consist of a lipid bilayer that is covalently anchored to a solid substrate through a spacer group. The submembrane part, which is defined by the spacer group, is important especially for the biological activity of incorporated membrane proteins. Anchor lipids with different spacer and anchor groups have been synthesized, and the resulting membrane structures have been investigated by neutron reflectivity. The different molecular architectures had a significant effect on both the amount of water incorporated in the spacer region and the electrical properties of the bilayer. A detailed understanding of the structure−function relationship allows for an optimized design of the molecular architecture with respect to possible applications, for example an optimized protein incorporation.

Introduction

Model membrane systems have been developed in order to systematically investigate fundamental processes at lipid bilayer interfaces such as the functioning of membrane proteins, cell−cell signaling, or protein−membrane interactions.[1] Biomimetic model architectures should provide a simplified system, which allows for systematic investigation of the membrane while maintaining the essential membrane characteristics such as membrane fluidity or electrical sealing properties. Among these systems, solid supported bilayer membranes have been extensively used and characterized.[2] Additionally, these systems have been proposed for biosensing applications.[2] The decoupling of a lipid bilayer from the substrate, either by a small water layer, a polymeric cushion, or by specifically synthesized anchor lipids typically increases the stability of the bilayer when compared to other model membrane systems.[3−5] Furthermore, the solid support allows the use of various surface analytical techniques.[6]

Tethered bilayer lipid membranes (tBLMs) are solid supported membranes, where the inner leaflet of a lipid bilayer is covalently attached to a surface through a short spacer group (Figure 1).[7−9] This spacer group covers small surface roughness features and provides an ion and water reservoir underneath the membrane. Inspired by the properties of archaea bacteria membranes, typical lipids have branched hydrocarbon units, which are in a fluid phase at ambient conditions.[10] The spacer consists in most cases of a short oligomer and the anchor lipid can bind to substrates through suitable chemistry, e.g., sulfur−gold interactions (cf. Table 1).

Similar strategies have been reported from different groups showing that tBLMs can provide stable model membrane systems.[10−12] Especially the electrical properties in terms of membrane resistance and capacitance are close to values known from patch-clamp experiments on natural membranes. Electrical impedance spectroscopy (EIS) has been established as a valuable technique for the characterization of solid supported membranes.[13−16]

Typically, a tBLM is formed in a two-step process.[6] First, the anchor lipid is assembled onto a substrate by self-assembly. This monolayer is then exposed to small unilamelar vesicles, which fuse and form the distal layer of the bilayer. Alternatively, the monolayer can also be incubated in an ethanol−lipid solution, which is afterward exchanged by buffer. This process leads then to the spontaneous formation of a bilayer.[17]

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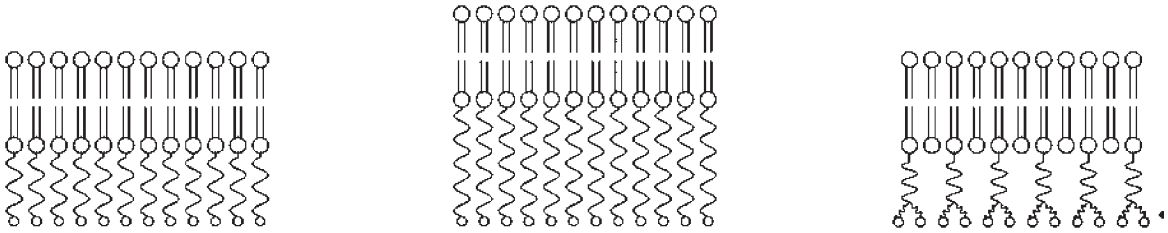

Figure 1. Schematic depiction of tBLM architectures based on different anchor lipids.

The type and the length of the spacer and the type of anchor have a significant influence on the properties of the bilayer membrane (Figure 1). Densely packed and short spacer units typically lead to very high membrane resistances; however, this can decrease the probability for a functional incorporation of proteins. Alternatives are the use of long polymeric spacer groups,[18] which then leads to decreased electrical properties, or the dilution of the inner leaflet of the membrane by introducing small surface active molecules.[19,20]

A series of anchor lipids has been developed with different spacer lengths and with anchor groups suitable for the assembly of the membrane on noble metals[11,21] or oxide substrates.[22,23]

Here, molecular architectures of molecules suitable for assembly on gold surfaces via thiol chemistry were studied (cf. Table 1). The common feature of the molecules was a diphytanoyl chain, which forms the hydrophobic part of the anchor lipid. The spacer part consisted of a short ethylene glycol chain of variable length and lipoic acid moieties or short thiols were used as anchor groups.

The different anchor lipids have been shown to have a significant influence on the electrical properties of the resulting membrane.[21] Additionally, it has been shown that the more relaxed structure of DPhyHDL significantly enhances the incorporation properties of the relatively large exotoxin α-hemolysin.[15] This special anchor lipid consists of two lipoic acid molecules which should, as an alternative to using dilution molecules,[19,20] create more space between the individual lipids.

So far, the detailed structure of the tBLMs, especially of DPhyTL-based architectures, has been deduced from surface plasmon resonance (SPR)[7] or infrared (IR) experiments.[24] Here, a detailed analysis using neutron reflectivity (NR) is presented. NR is an ideal tool to study buried interfaces, and thus it was possible to determine the structure of tBLMs formed by the different anchor lipids. Furthermore, the hydration of the spacer part was addressed.

Materials and Methods

For all preparation steps, ultrapure water, filtered with a Millipore device (Billerica, MA) was used. NR measurements were performed either in 0.1 M NaCl (Sigma-Aldrich, Munich, Germany, ≥99.5%) or in PBS (0.1 M, pH 7) prepared with Na_2HPO_4 (Fluka, Munich, Germany, ≥99%) and NaH_2PO_4 (Fluka, Munich, Germany, ≥99%).

Lipids. The anchor lipids were synthesized as described before.[21] The distal membrane leaflet was formed using DPhyPC lipids (Avanti Polar Lipids, Alabaster, AL).

Membrane Assembly. For neutron reflectivity measurements, 10 mm thick silicon wafers (Si-Mat, Landsberg/Lech, Germany) with a diameter of 76.2 mm were used, coated with 2.5 ± 0.5 nm chromium and 20 ± 0.5 nm gold. Lipid monolayers were self-assembled on the gold film by immersion of the substrate in a lipid solution (0.2 mg/mL in ethanol) for 24 h. Before use, samples were cleaned with ethanol (Sigma-Aldrich, Munich, Germany) and dried in a nitrogen stream. For NR measurements, monolayers were completed to bilayers by rapid solvent exchange. All experiments were carried out at room temperature (22 ± 1 °C).

Neutron Reflectivity. In a neutron reflectometry experiment, the intensity of the neutron beam reflected by a surface is measured as a function of the scattering vector $Q = 2 \sin \theta$. The reflectivity profile, i.e., the plotted intensity of reflected radiation R as a function of Q, provides information about the structure of the surface, including thickness, density, and roughness.

The position of the critical edge gives information about the composition of the substrate of the sample. The periodicity of the fringes is related to the layer thickness δ (approximately $2\pi/\delta$), and their amplitude can be connected to the nuclear contrast across the interface. The attenuation of the reflected intensity is dependent on the roughness of the surface. Additionally, these parameters are all depend on the sample substrate and the solvent. All those factors superimpose and result in a complicated reflectivity profile.

Generally speaking, thicker layers lead to smaller periods, films with smaller nuclear contrast to smaller amplitudes.

All neutron reflectivity investigations were carried out at the NIST Center for Neutron Research, Gaithersburg, MD, using the NG1, NG7, and AndR reflectometers.

Samples were placed in a silicon wet cell, mounted on the instrument, aligned, and slowly filled with buffer, carefully avoiding any air bubbles forming in the cell.

The different assembly steps of the system (monolayer, bilayer) were followed sequentially by performing NR measurements in two different contrasts, namely in D_2O and in an H_2O/D_2O mixture ($^2/_3D_2O + {}^1/_3H_2O$) with a SLD of about 4×10^{-6} Å^{-2} (CM4).

Using contrast matching or variation, we are able to increase the information obtained during the experiments significantly by using different ratios of H_2O/D_2O and highlight or blend out one element. Using different contrast also facilitates data analysis. Because of the loss of phase information with the inversion of experimental data into scattering length density values, a measured reflectivity curve cannot be uniquely described by exactly one scattering length density profile. If at least two sets of data, with different contrast, were obtained, the phase information can be recovered and a unique sld profile can be calculated.

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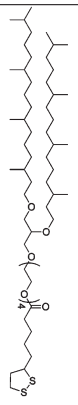
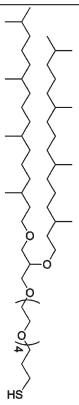
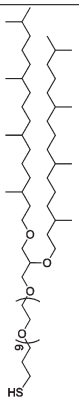
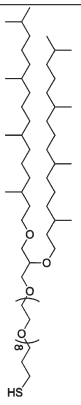
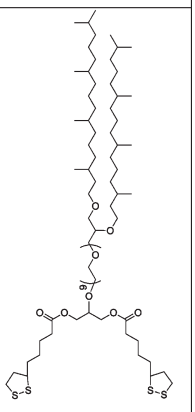
Table 1. Summarized Parameters of Five Different tBLM Architectures[a]

name	DPhyTL	DPhyTT	DPhyHT	DPhyOT	DPhyHDL
structure					
calculated length	5.0 nm	4.5 nm	5.2 nm	5.9 nm	5.6 nm
monolayer resistance/MΩcm^2	1 - 10	0.5 - 5	0.6 - 5	0.01-0.02	0.01 -0.3
monolayer capacitance/μFcm^{-2}	0.7 - 1.4	0.7 - 1.2	0.7 - 1.2	0.94-1.1	0.8 - 4
bilayer resistance/MΩcm^2	3 - 55	3 - 15	2 - 35	0.01-0.09	0.2 - 6.5
bilayer capacitance/μFcm^{-2}	0.6 - 1.1	0.6 - 0.85	0.6 - 0.85	0.8-0.9	0.6 - 0.8
monolayer thickness	3.9 nm	3.03 nm	3.31 nm	5.52 nm	5.6 nm
spacer length	2.2 nm	1.35 nm	1.43 nm	2.34 nm	2.7 nm
spacer sld [·10^{-6}Å^{-2}]	0.54	0.8	1.4	2.6	3.4
water incorporation (Spacer)	5%	13%	21%	40%	65%
Alkyl chain length	1.68 nm	1.68 nm	1.88 nm	3.18 nm	2.9 nm
Alkyl sld [·10^{-6}Å^{-2}]	0.32	0.1	0.42	1.27	2.03
Water incorporation (Alkyl chains)	1%	1.6%	6.4%	19.9%	28%
distal layer length	0.9 nm	1.03 nm	0.8 nm	2.29 nm	3.34 nm
distal layer sld/10^{-6}Å^{-2}	0.24	0.12	2.4	4.23	2.71
Water incorporation (distal layer)	3.5%	1.8%	73.2%	65.8%	28%
roughness/Å	7.0 Å	9.6 Å	11 Å	24 Å	14 Å

[a] The nomenclature of the lipids follows the following systematics: DPhy = diphytanoyl, (T, H, O) for the length of the spacer, (L, DL, T) for one or two lipoic acid groups or a thiol anchor, respectively. EIS data have been reported previously.[28] Thickness values are obtained by fitting the NR data to a box model.

Fitting of the Data. Analysis of the NR data was conducted in three steps using software from the NIST Center for Neutron Research.[25] Data reduction was performed using reflred. The data were then analyzed using reflfit and finally using GaRefl.

The theoretical scattering length density values have been determined using the scattering length density calculator (http://www.ncnr.nist.gov/resources/sldcalc.html) provided by the NIST Center for Neutron Research.

The NCNR "reflpak" reflectometry package is one of many different methods to fit reflectivity data. It uses Parratt's recursion relation to calculate reflectivity values from a model profile, and parameters are varied in a systematic way. In order to calculate the neutron reflectivity, the background intensity is first subtracted from the specular reflectivity, and the difference is divided by the incident beam intensity. This is called data reduction. The data is fitted by "Reflfit" to a model of neutron scattering length density profile along the axis perpendicular to the substrate surface.

The fitted sld profile is structurally interpreted in terms of chemically distinct layers. In case of a solid supported lipid bilayer, the following order of layers was used: the silicon substrate, the silicon oxide layer, the chromium layer, the gold layer, the spacer region, the inner headgroup layer, the outer headgroup layer, and the bulk solvent phase. As "Reflfit" only simulates single data sets, it was only used for a rough calculation of the parameters. These parameters were then fed into "GaRefl". This software is also part of the "refpak" package and allows to simultaneously fit multiple data sets with isotopically different bulk solvents.

"Ga refl" is a C++-based system for modeling reflectivity data using Paratt formalism and model refinement using a genetic algorithm. Sets of possible values of fitting parameters are created randomly; the reflectivity is calculated for each data set and then evaluated against a test function (χ^2). Each time the quality of a fit is better than the current best, the old data set is substituted and the new parameter values are stored. The sld and thickness of the hydrocarbon layer can be utilized to calculate the area per lipid molecule and the completeness of the lipid bilayer. From simultaneous fits of the data sets with isotopically different bulk solvent phases, the hydration of the various layers can be calculated in terms of volume fraction of the lipids. This is a generic function of the "Garefl" software. The values for the volume fraction have been converted to the amount of solvent present in the lipid structure and are further on referred to as "water incorporation".

When analyzing the data, one has to keep in mind that the studied system is not a perfect, defect-free crystal, and more than 20 parameters are simulated. However, in combination with other techniques and theoretical considerations, a reasonable model of the architecture can be generated.

Electrochemical Impedance Spectroscopy (EIS). EIS data were collected as described previously.[9,26] In short, spectra were recorded for frequencies between 2 mHz and 100 kHz at 0 V bias potential with an ac modulation amplitude of 10 mV using an AUTOLAB spectrometer (Eco Chemie, Utrecht, The Netherlands).

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). Data were analyzed using an equivalent circuit, where the bilayer is represented by a parallel resistor−capacitor element.

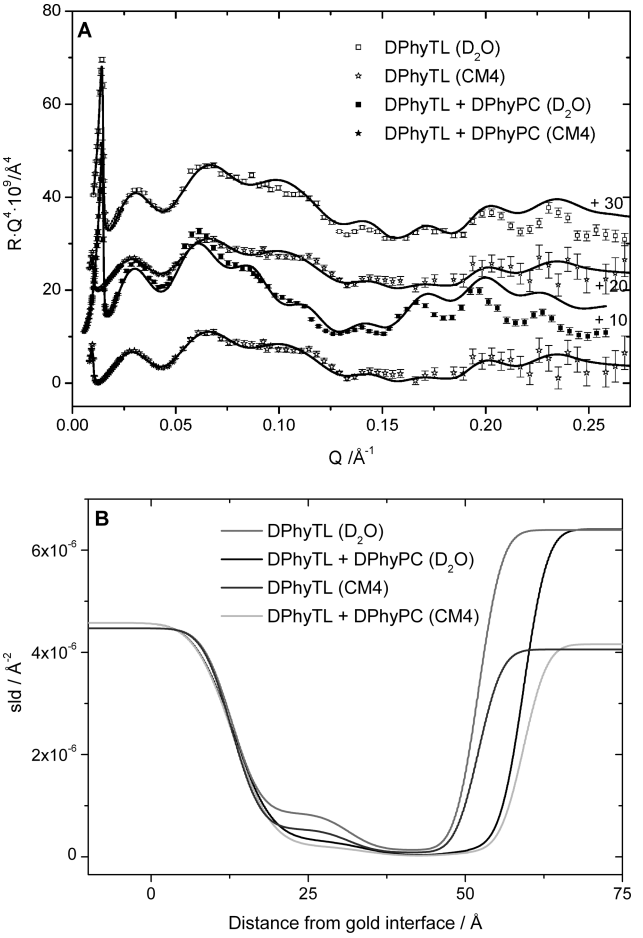


Figure 2. (A) Reflectivity plot of a DPyhTL-based mono- and bilayer. Data have been spread out for clarity. Numbers on the right-hand side show the added values. Solid lines represent the fit result using the profile shown in (B). One can distinguish between the spacer part, the inner and the outer hydrophobic layer. The data for the other anchorlipids are similar and can be found in the Supporting Information.

Results and Discussion

The molecular structures of the different membrane assemblies have been analyzed in terms of a multilayer system. While the scattering lengths densities (slds) for the solid substrate (silicon, silicon oxide, chromium, gold) were kept relatively fixed, allowing only slight deviations from the theoretical predicted slds and the layer thickness determined by the evaporation procedure, more variation was allowed for the parameters describing the membrane itself. The substrate parameters varied slightly due to variations in the sputtering process; not all samples could be prepared simultaneously. In fact, the results presented here were obtained during multiple runs at the NIST neutron facility.

The tBLMs could be described as the spacer part, the inner and the outer bilayer leaflet, each with an individual thickness and sld (Figure 2).

Monolayers and bilayers have been measured sequentially, but the presented values for inner and outer bilayer relate to the values in the complete bilayer system. "Open" inner bilayers with no outer bilayers on top are more hydrated than the inner bilayer in a complete bilayer system, but otherwise identical.

Theoretical molecular geometries were determined for fully stretched anchor molecules using Chem3D Ultra 6.0. These values were used as initial parameters for the analysis of the monolayer data, even if it has previously been shown that the anchor lipids do

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not adopt such a stretched conformation.[20,27] The obtained parameters were then used as starting parameters for the analysis of the bilayer data. The parameters obtained after fitting all data sets are summarized in Table 1. In addition, electrochemical parameters of the bilayers are also listed.[15,26,28] The amount of water in the different layers of the architecture was estimated form measurements performed in different contrasts, i.e., in D_2O and in mixtures of D_2O and H_2O.

All five different anchorlipids could be used to form tBLMs. In terms of their electrochemical properties, all molecules typically led to membranes with capacitance values of about 0.8 μF cm^{-2}, indicating complete bilayer coverage. However, the resistive properties varied significantly; i.e., anchor lipids with short chains of four EO units resulted in the highest bilayer resistances. DPhyHDL with the extended anchor group and especially DPhyOT with eight EO units yielded lower values, yet still within a reasonable range for lipid bilayers.

NR allows for a detailed study of the tBLM layer structure, analyzing the individual parts of the architecture in terms of a layer thickness and sld. In good agreement with SPR results, all anchor lipids formed a thinner proximal layer than predicted for fully stretched molecules.[7,21] The effective length of the spacer part increased with increasing number of ethylene oxide units. At the same time, also the sld of the spacer increased, indicating a more loosely packed architecture and therefore a higher water incorporation. DPhyTL and DPhyTT, both with four EO units, showed relatively low sld values, indicating a high packing density of the anchor lipids. This is also reflected in rather high resistance and low capacitance values, both for the monolayer and for the completed bilayer. Similarly, the incorporation of water in the alkyl part is negligible for both components. DPhyHT and DPhyOT, the structural analogous molecules to DPhyTT, showed an increased spacer length, however, with higher sld values corresponding to a less dense packing order. This can also be seen in reduced electrical properties and an increase in water uptake, both in the spacer and in the hydrophobic part. The more disordered packing led also to an increased roughness of the bilayer interfaces. Finally, DPhyHDL showed a slightly more ordered structure as DPhyOT, as judged by a lower sld and roughness. However, the structure allowed for a significant increase in water uptake, probably due to the extended submembrane space.

The obtained slds of the spacer region of DPhyTT, DPhyHT, and DPhyOT differed more from the theoretical value for a dense polymer layer than it did for DPhyTL. This is probably due to a less dense surface coverage. This also results in higher monolayer resistances for DPhyTL.[15,26] Additionally, the hydration of the ethylene glycol part of the molecule was 13% for DPhyTT, compared to 5% for DPhyTL, although the former molecule is slightly smaller and for the same chain packing similar water incorporation could be expected. In general, the hydration of the spacer part increased with increasing spacer length, probably due to both an increased volume and more loose packing.

The anchor lipids are bound on the surface through self-assembly. During self-assembly, molecules first adsorb very quickly and unordered to the surface, followed by a much slower reorganization.[29,30] These processes are influenced by a variety of parameters, including the hydrophobicity of the molecules and their interactions with the substrate. The molecular structure of DPhyTL seems to have an optimal ratio between hydrophobic and spacer part, which allows for an almost complete surface coverage.[6] Furthermore, the two sulfur atoms of the terminal lipoic acid moiety result in an optimal grafting of the molecule to the gold substrates. The other anchor lipids still form dense monolayer; however, their molecular structure leads to imperfections in the film formation, as also has been seen in experiments carried out at the air−water interface.[31]

DPhyHDL differs from the other molecules by having an extended anchoring unit. This resulted in a large submembrane reservoir with a high level of hydration. Yet, the molecule allowed for formation of sealing bilayers. The more relaxed structure allowed for a preferred incorporation of ion channel proteins.[15]

The different monolayer assemblies also had an effect on the bilayer properties. In general, a dense and well-packed monolayer led to a more compact distal leaflet. DPhyTL and DPhyTT showed very similar values for the sld and water incorporation, yet the membrane resistances were typically higher for DPhyTL. On the longer spacer molecules, the bilayer shows a higher defect rate and thus lower resistances and a high amount of incorporated water. Bilayer formed DPhyHDL, on the other hand, seemed to be relatively dense with high membrane resistances.

Conclusion

A series of five anchor lipids have been used to form tethered bilayer lipid membranes, and the resulting architectures have been investigated by neutron reflectivity as well as electrical impedance spectroscopy. All lipids had the same hydrophobic groups and varied in their spacer length and anchor groups. The structural and electrical parameters of the membranes depended strongly on the molecular geometry of the anchor lipids.

DPhyTL, with a short spacer and a lipoic acid anchor, led to the highest surface coverage, which also resulted in the highest membrane resistances. However, the molecule only allowed for little water incorporation in the spacer part,[27] which can hinder later protein incorporation and function.[15]

The thiol-based anchor lipids DPhyTT, DPhyHT, and DPhyOT showed an increase in water incorporation, however at a lower packing density and thus lower membrane resistance. Probably, interactions of the longer ethylene glycol chains inhibit the formation of a homogeneous and dense monolayer.

DPhyHDL, a molecule with a large anchor moiety, can provide an extended and well-hydrated submembrane reservoir at only slightly reduced resistive membrane properties when compared to DPhyTL. This is in good agreement with previously published EIS measurements that also demonstrate the importance of layer composition for artificial membranes and later on applications using them.

We could show that the architecture of the membrane has great significance on functionally incorporating α-hemolysin pores into tethered bilayer lipid membranes.[15] Modification of the membrane architecture on a molecular scale had a significant effect on the incorporation probability of the protein. Controlling the membrane assembly for an optimal incorporation of a desired

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protein can give useful information about the pathway a protein inserts into a membrane. This facilitates the incorporation of complex proteins that so far could not be reconstituted in other biomimetic systems.

In summary, a detailed analysis of the different membrane structures of a tBLM tool box[21] allows for an optimization of the membrane structure with respect to a selected applications. Complex membrane proteins will require a rather flexible architecture, even at the drawback of reduced electrical properties, while highly sealing membranes might be used for small or only few proteins, ideally for single channel measurements.[16]

Supporting Information Available: Reflectivity data and fit profiles for a DPhyTT, DPhyHT, DPhyOT, and DPhyHDL based membranes. This material is available free of charge via the Internet at http://pubs.acs.org.