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Impedance analysis of supported lipid bilayer membranes: a scrutiny of different preparation techniques

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Abstract

One topic of this study is the comparison of different preparation techniques to build up solid supported lipid bilayers onto gold substrates. The deposited lipid bilayers were investigated by a.c. impedance spectroscopy. Three different strategies were applied: (1) The gold surface was initially covered with a chemisorbed monolayer of octadecanethiol or 1,2-dimyristoyl-*sn*-glycero-3-phosphothioethanol (DMPTE). The second monolayer consisting of phospholipids was then deposited onto this hydrophobic surface by (i) the Langmuir-Schaefer-technique, (ii) from lipid solution in n-decane/isobutanol, (iii) by the lipid/detergent dilution technique or (iv) by fusion of vesicles. (2) Charged molecules carrying thiol-anchors for attachment to the gold surface by chemisorption were used. Negatively charged surfaces of 3-mercaptopropionic acid were found to be excellent substrates that allow the attachment of planar lipid bilayers by applying positively charged dimethyldioctadecylammoniumbromide (DODAB) vesicles or negatively charged 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol vesicles in the presence of chelating Ca^{2+}-ions. If positively charged first monolayers of mercaptoethylammoniumhydrochloride were used we were able to attach mixed 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol/1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine vesicles to form planar lipid bilayers via electrostatic interaction. (3) Direct deposition of lipid bilayers is possible from vesicles containing 1,2-dimyristoyl-*sn*-glycero-3-phosphothioethanol (DMPTE). A critical amount of more than 50 mol% of DMPTE was found to be necessary to form a solid supported lipid bilayer. Bilayers obtained with these different preparation techniques were scrutinized with respect to their capacitances, kinetics of formation and their long-term stabilities by impedance spectroscopy. The second feature of this paper is the application of the supported bilayers to study ion transport through channel-forming peptides. We used a DODAB-bilayer for the reconstitution of gramicidin D channels. By circular dichroism measurements we verified that the peptide is in its channel conformation. The ion transport of Cs^+-ions through the channels was recorded by impedance analysis.

Keywords: Lipid bilayer; Supported lipid bilayer; Impedance spectroscopy; Gramicidin D; Ion transport

1. Introduction

Supported lipid bilayers in addition to lipid vesicles are useful model systems to study basic interaction mechanisms that are responsible for the structure and function of biological membranes. The simple composition of an artificial bilayer in contrast to the complex mixture of lipids and proteins in biological membranes facilitates a detailed examination of a single membrane function, e.g. ion transport. Solid supported lipid bilayers in contrast to black lipid membranes have the advantages of ease and reproducibility of preparation, long-term-stability and the possibility to use an electrically conductive support [1–4]. The latter is interesting with respect to an application as molecular biosensors [1,5,6] whereby the most attractive goal is the incorporation of channel-forming proteins for the de-

Abbreviations: ODT, octadecanethiol; MPA, 3-mercaptopropionic acid; MEA, mercaptoethylammoniumhydrochloride; PC, phosphatidylcholine; DMPTE, 1,2-dimyristoyl-*sn*-glycero-3-phosphothioethanol; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DODAB, dimethyldioctadecylammoniumbromide; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetate; LUV, large unilamellar vesicles; CMC, critical micellar concentration; CD, circular dichroism.

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velopment of highly selective sensors based on ion transport through lipid membranes [4]. One possibility for the incorporation of channel proteins into solid supported lipid bilayers is their reconstitution into vesicles and their subsequent fusion onto the surface [7].

One topic of this paper is the characterization of several methods to build up solid supported lipid bilayers onto substrates using basically the well-known self assembly of thiols onto clean gold surfaces [8]. Three different ways to build up solid supported lipid bilayers were used:

(1) Separate deposition of the two monolayers whereby the first one is formed by self assembly of lipids with thiol-anchors at their headgroups [9]. The deposited monolayer then consists of closely packed hydrocarbon chains which make the gold surface hydrophobic and therefore well suited for the attachment of a second lipid monolayer to obtain a bilayer system. This second monolayer may be deposited onto the first one by fusion of vesicles, by the Langmuir-Schaefer technique [10], the painted lipid membrane technique [11] or by monolayer formation of phospholipids in the presence of detergents like 1-*O*-n-octyl-β-D-glucopyranoside [5].

(2) Coating the solid support initially with a charged monolayer [1], for example self assembled 3-mercaptopropionic acid (MPA). In a second step positively charged large unilamellar vesicles of dimethyldioctadecylammoniumbromide (DODAB) are spread spontaneously onto the negatively charged MPA-layer or negatively charged vesicles may be induced to fuse onto the surface in the presence of calcium ions. If the other way around positively charged monolayers of mercaptoethylammoniumhydrochloride (MEA) were initially deposited onto gold surfaces, the formation of a lipid bilayer may be obtained by fusion of vesicles containing negatively charged lipids.

(3) Adsorption and fusion of large unilamellar vesicles (LUV) on the surface of the substrate to build up a complete bilayer in one step. This is easily achieved by the use of vesicles containing phospholipids functionalized with thiol-anchors at the lipid headgroups.

The quality of the membranes was judged by their capacitances which are related to the bilayer thickness and the density of defects within the lipid bilayer. Moreover the kinetics of the bilayer formation and the stabilities of the bilayers were also investigated by a.c. impedance analysis.

To establish a solid supported system containing a simple channel-forming ionophore within the lipid bilayer matrix the best known channel-forming polypeptide, gramicidin D from *Bacillus brevis* was chosen. This linear pentadecapeptide consists in its active form of two antiparallel oriented monomers bound to each other by six hydrogen bonds [12]. The resulting dimer has a length of 26 Å, sufficient to span a phospholipid bilayer. In its active conformation which critically depends on solvent polarity [14] the peptide forms a pore of 4 Å diameter selective for monovalent cations. The sequence of conductivity for monovalent cations is $H^+ > NH_4^+ > Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ [13]. The ion transport through a supported lipid bilayer via the gramicidin dimer is demonstrated by a.c. impedance analysis.

2. Materials and methods

2.1. Materials

The phospholipids 1,2-dimyristoyl-*sn*-glycero-3-phosphothioethanol (DMPTE), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Water was purified first through a Millipore water purification system Milli Q RO 10 Plus and then finally with the Millipore ultrapure water system Milli Q Plus 185 (18 MΩ/cm). 1-*O*-n-Octyl-β-D-glucopyranoside was from Calbiochem (Bad Soden, Germany). Octadecanethiol (ODT) and dimethyldioctadecylammoniumbromide (DODAB) were purchased from Fluka (Neu Ulm, Germany). Mercaptoethylammoniumhydrochloride (MEA), 3-mercaptopropionic acid (MPA) and gramicidin D were obtained from Sigma (Deisenhofen, Germany). The gold used for the working electrodes was a generous gift from DEGUSSA (Hanau, Germany). The chromium was from Balzers (Balzers, Liechtenstein). Hellmanex used to clean the glass slides was purchased from Hellma (Müllheim, Germany).

2.2. Impedance measurements

Impedance spectra were recorded using a homemade spectrometer. The principle of the impedance measurement is illustrated in Fig. 1. Controlled with a personal computer a small sinusoidal a.c. voltage with a constant amplitude of 60 mV, obtained from a frequency generator, was applied to the resistor R_0 and the electrochemical system (ES) in series. The frequency ranged from 1 to 100000 Hz. One sweep containing 72 data-points subdivided in a logarithmic manner was recorded in about 10 min. The electrochemical system was composed of two equally designed working electrodes as shown in Fig. 1C. The mathematical analysis of the frequency dependent value of the impedance allows conclusions about the electrical properties of the system. Assuming an electrical network which represents the electrochemical system, the parameters of this equivalent circuit were determined by a non-linear-least-square-fit of the transfer function $|U_f/U_0(\nu)|$ or the phase angle $\Phi(\nu)$ between U_0 and U_f, respectively. The software

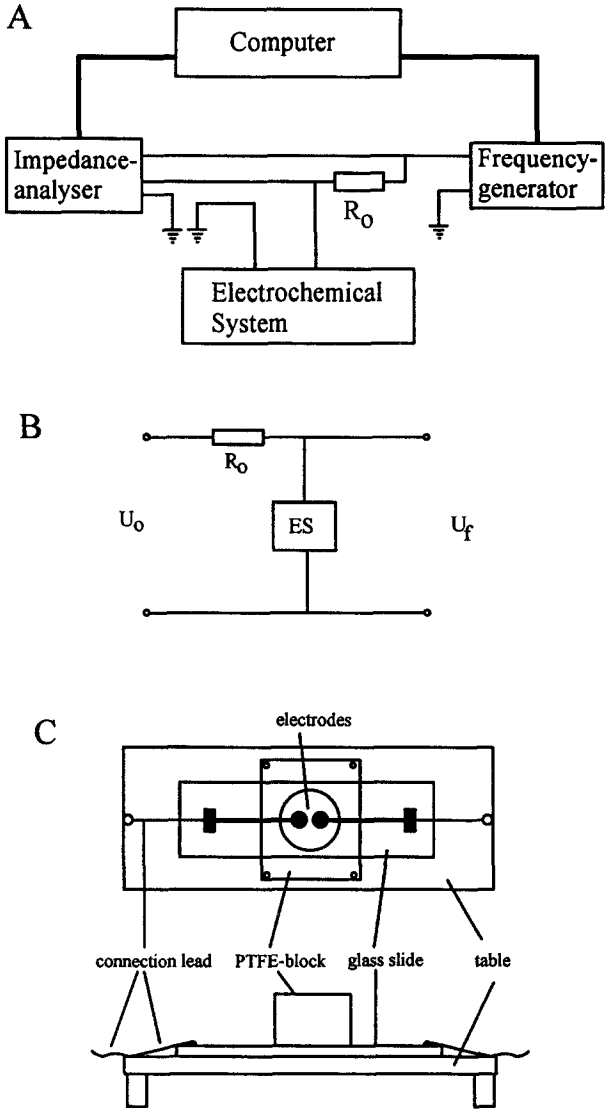

Fig. 1. (A) Schematic diagram of the impedance spectrometer used in the present work and (B) the principle of impedance measurement, (C) configuration of the experimental setup of the measuring cell and the gold electrodes used for the impedance spectroscopic analysis.

BWD [1] which includes a fitting procedure based on the Levenberg-Marquardt-algorithm [15] is a homemade program written in C.

2.3. Electrode preparation

Commercially available glass slides (26 × 76 mm) were cleaned by sonicating them for 30 min in a detergent solution containing 2% Hellmanex at 70°C. Afterwards the slides were rinsed extensively with ultrapure water to remove remaining detergent. The gold electrodes were manufactured by first evaporating a thin chromium layer of 10 nm thickness through a homemade mask on the glass substrates to increase the adhesion of the adjoining gold layer of 100 nm thickness. The metal coatings were carried out by an evaporation unit (E 605, Edwards, UK). Immediately before use, the gold electrodes were exposed to an argon plasma with high energy (plasma cleaner, Harrick, USA) for 10 min.

The electrodes were designed as demonstrated in Fig. 1C. This arrangement of two equally prepared working electrodes, each with an area of 0.13 cm^2, avoids an additional counter electrode. No significant differences in impedance spectra were observed compared to measurements using a classical two electrode setup of an Ag/AgCl-reference electrode and one working electrode.

2.4. Circular dichroism

CD-spectra of freshly prepared gramicidin D samples were recorded on a CD Dichrograph spectrometer from Jobin-Yvon (Paris, France) between 200–280 nm at room temperature. A 0.1 cm quartz cuvette from Hellma (Müllheim, Germany) was used.

2.5. Solid supported bilayers on a hydrophobic monolayer

2.5.1. Self assembly of thiols onto gold

ODT-monolayers were formed by placing the gold covered substrates into a 1 mM solution of octadecanethiol in chloroform for several hours. Impedance measurements revealed that the self assembly process was completed at least within 10 h. Directly before use the electrodes were rinsed thoroughly with chloroform and dried under a stream of nitrogen. The same procedure was applied to achieve phospholipid monolayers of DMPTE.

2.5.2. Formation of lipid bilayers by the Langmuir-Schaefer-technique

Similar to the well established Langmuir-Blodgett-technique [16] a phospholipid monolayer was spread on the aqueous surface using a film balance FW-2 from Lauda (Lauda-Königshofen, Germany). A constant film pressure of about 30 mN/m was adjusted in order to obtain a satisfying transfer ratio by breaking the hydrophobic substrate horizontally through the surface. Using phospholipid films it is very important not to pass the water surface twice, because otherwise the deposited phospholipid monolayer would be removed almost completely. The subphase contained a solution of 1 mM KCl to record impedance spectra directly after deposition of the phospholipid monolayer in the trough of the film balance.

2.5.3. Painted lipid membranes

20 μl of a solution containing 1 mg/ml of the phospholipid in decane/isobutanol (10:1, v/v) were placed onto the dry hydrophobic surface of the self assembled alkanethiol for at least 2 min. After this short incubation time the surface was vertically placed in the impedance

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cell filled with 10 mM KCl in order to remove remaining lipid.

2.5.4. Monolayer deposition in the presence of detergent

A clear micellar solution of phospholipid was prepared using 40 mM 1-*O*-n-octyl-β-D-glucopyranoside and 1 mM lipid dissolved in 10 mM Tris, pH 7.0. The phospholipid dispersion was sonicated in a bath-type sonicator (Bandelin sonorex RK 255 H, 2 × 320 W) until a clear micellar solution was formed. This solution was transferred to a octadecanethiol monolayer. Stepwise dilution of the detergent solution was repeated until the detergent was completely removed and constant impedance spectra of the lipid bilayer were obtained.

2.5.5. Fusion of large unilamellar vesicles on a hydrophobic monolayer

Large unilamellar vesicles (LUV) were prepared by the extrusion method [17] using a LiposoFast system from Avestin (Ottawa, USA). The solvent chloroform/methanol (1:1, v/v) of the lipid solution was removed under a stream of nitrogen and the yielded film was dried under vacuum for 3 h at 60°C. The preparation of LUV was performed above the main phase transition temperature of the corresponding phospholipid. Addition of buffer solution composed of 10 mM Tris, pH 7.0 to the dried lipid films and vortexing for 4 × 30 s resulted in multilamellar vesicles. These liposomes were converted to large unilamellar vesicles by extruding the sample through polycarbonate membranes with a pore size of 100 nm.

The bilayer was formed by adding the preformed vesicles to the hydrophobic monolayer consisting of DMPTE or octadecanethiol and keeping the temperature above the main phase transition temperature of the lipid. The lipid concentration was 0.5 mg/ml. The vesicle suspension was left in contact with the hydrophobic surface for a maximum of four hours. Excess vesicles were removed by rinsing the sample several times with buffer solution. The kinetics of the bilayer formation were detected by impedance spectroscopy.

2.6. Solid supported bilayers on a charged surface

2.6.1. Fusion of large unilamellar vesicles on negatively charged surfaces

Formation of supported lipid membranes on MPA-monolayers:

The gold covered electrodes were placed for 30 min in a 10 mM solution of 3-mercaptopropionic acid (MPA). After the incubation time the electrodes were rinsed with a buffer solution composed of 10 mM Tris, pH 8.6. Vesicles were prepared by the extrusion method [17] in the same buffer. The concentration of the lipid was 1.5 mg/ml.

(i) The addition of large unilamellar vesicles of dimethyldioctadecylammoniumbromide (DODAB) to the self assembled MPA-monolayer led to the formation of bilayer structures.

(ii) For the calcium controlled fusion of negatively charged vesicles the lipid DPPG was used. Addition of calcium ions in a final concentration of 2 mM to a solution of DPPG-LUV in the presence of the MPA-monolayer caused the vesicles to fuse on the surface and to build up a bilayer.

2.6.2. Fusion of large unilamellar vesicles on positively charged surfaces

The gold electrodes were placed for 30 min in a freshly prepared 10 mM solution of mercaptoethylammoniumhydrochloride (MEA). Then the electrodes were rinsed with a buffer solution of 5 mM Mes, pH 5.1. Large unilamellar vesicles consisting of a mixture of DMPG and DMPE (4:1) with a concentration of 1.5 mg/ml were prepared by the extrusion method in the same buffer before added to the MEA-monolayer.

2.7. Fusion of large unilamellar mixed DMPC / DMPTE vesicles onto gold

Vesicles with different DMPC/DMPTE ratios but with a constant lipid concentration of 1.5 mg/ml were prepared by the extrusion method in a 10 mM Bis-Tris-buffer at pH 5.5 and then exposed to freshly cleaned gold electrodes. The self assembly process was carried out at 50°C. Impedance spectra were recorded simultaneously. The vesicle suspension was removed when the capacitance remained constant.

2.8. Reconstitution of gramicidin D in DODAB-bilayers

The peptide was added from a stock solution in chloroform/methanol (1:1, v/v) to a DODAB solution in the same solvent (lipid to peptide ratio 100:1). LUV were prepared according to the procedure described in Section 2.6.1.

3. Results

3.1. Evaluation of the impedance spectra

A simple equivalent circuit was chosen (Fig. 2) that considers the most important electric properties of the different artificial membranes. The simplicity of this circuit reduces possible misinterpretations of the recorded data. The transfer function of this circuit is given by

$$\left|\frac{U_f}{U_o}\right| = \sqrt{\frac{(R_m + R_e)^2 + (\omega R_m R_e C_m)^2}{(R_o + R_m + R_e)^2 + \omega^2 (R_o + R_e)^2 R_m^2 C_m^2}} \quad (1)$$

Table 1
Summary of the results obtained from a.c. impedance analysis of the described supported lipid bilayers

	C_{ml1} ($\mu F/cm^2$)	C_{ml2} ($\mu F/cm^2$)	C_{bl} ($\mu F/cm^2$)	Time until the final capacitance was reached	Long-term stability
Method A	1.04 ± 0.05	1.26 ± 0.12	0.57 ± 0.02	immediately	1 d
Method B	1.04 ± 0.05	1.23 ± 0.08	0.56 ± 0.01	immediately	1 d
Method C	1.04 ± 0.05	1.13 ± 0.1	0.54 ± 0.02	immediately	1 d
Method D_1	1.04 ± 0.05	1.04 ± 0.09	0.52 ± 0.02	3 h	1 d
Method D_2	1.2 ± 0.1	1.1 ± 0.2	0.56 ± 0.02	3 h	1 d
Method E	–	–	0.89 ± 0.08	24 h	2–4 d
Method F	–	–	0.84 ± 0.03	1 h	2–4 d
Method G	–	–	0.66 ± 0.02	30 min	3 h
Method H	–	–	0.63 ± 0.02	6 h	2–4 d
Theoretical value	–	–	0.46	–	–
Biological membrane	–	–	2–7 [30]	–	–

The membranes are compared in terms of capacitance of the corresponding mono- and bilayer systems. If possible values for each monolayer are calculated ($C_{ml1,2}$) otherwise the double layer capacitance is given (C_{bl}). For the methods using a first hydrophobic monolayer the same phospholipid DPPC was used to get comparable results. Method A denotes the Langmuir-Schaefer technique, method B the bilayer preparation according to the painted lipid membranes, method C the bilayer formation with DPPC onto ODT in the presence of detergent, method D_1 the fusion of LUV onto ODT, method D_2 the fusion of LUV onto DMPTE, method E the fusion of DMPG/DMPE (80:20) vesicles (LUV) onto MEA, method F the fusion of DODAB vesicles onto MPA, method G the Ca^{2+}-ion induced fusion of DPPG-LUV onto MPA and method H the chemisorption of DMPTE/DMPC (60:40) vesicles onto gold. In the case of method E, F and G the capacitance of the bilayer includes the capacitance of the MPA- or MEA-monolayer respectively, which cannot be resolved from the lower capacitance of the upper bilayer. Average and standard deviation of the data are collected on three different preparations. For further information see text. The theoretical value is obtained from Eq. 2 assuming $\epsilon_r = 2.1$ for the dielectric permittivity and $d = 40$ Å for the bilayer thickness.

The capacitant and resistive behaviour of the membrane is represented by C_m and R_m. R_e is the resistance of the electrolyte solution. Considering the membrane as a plate condenser the capacitance is defined as

$$C_m = \epsilon_o \epsilon_r \frac{A}{d} \quad (2)$$

where A denotes the electrode area, d the thickness of the supported bilayer membranes and ϵ_o and ϵ_r are the permittivity of free space and the dielectric permittivity, respectively. The capacitance arising from the diffuse double layer (Gouy-Chapman-layer) was neglected because of its high value (~ 33 $\mu F/cm^2$) compared to the low capacitances of the adsorbed membranes (~ 0.8 $\mu F/cm^2$). In the case of the two step bilayer formation process it is possible to calculate the capacitance of the second phospholipid monolayer separately using the following equation:

$$C_{ml2} = \frac{1}{C_{bl} - 1C_{ml1}} \quad (3)$$

C_{ml2} denotes the capacitance of the second phospholipid monolayer, C_{bl} the capacitance of the whole bilayer and C_{ml1} the capacitance of the self assembled first monolayer. Eq. 3 is valid only if the membrane resistances of the two different monolayers are high enough to be neglected.

The resistance of the solid supported lipid membranes R_m was not exactly detectable by our device because of its very high value of around 1 MΩ. Therefore the membrane resistance was kept constant during the fitting procedure to a value of 1 MΩ. The resistance of the electrolyte R_e is assumed to be of ohmic nature and does not interfere with regard to membrane properties. Impedance spectra for pure lipid membranes were analyzed by the equivalent circuit shown in Fig. 2. Different equivalent circuits were applied in the case of membrane leakage and ion transport, what will be discussed later.

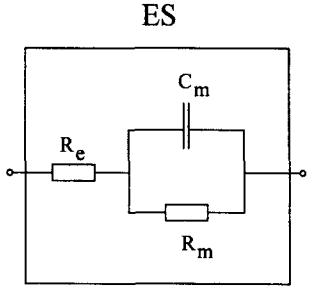

Fig. 2. Equivalent circuit representing the electrical properties of lipid bilayers (ES in Fig. 1B). R_m: membrane resistance, C_m: membrane capacitance, R_e: resistance of the electrolyte.

3.2. Solid supported bilayers on a hydrophobic monolayer

The first hydrophobic monolayer was deposited on the substrate by self assembly of octadecanethiol or DMPTE. The ODT-monolayer had a mean capacitance of 1.04 ± 0.05 $\mu F/cm^2$, the DMPTE-monolayer exhibited a mean value for the capacitance of 1.2 ± 0.1 $\mu F/cm^2$ (e.g. Table 1, values for C_{ml1}). The second layer was deposited on the

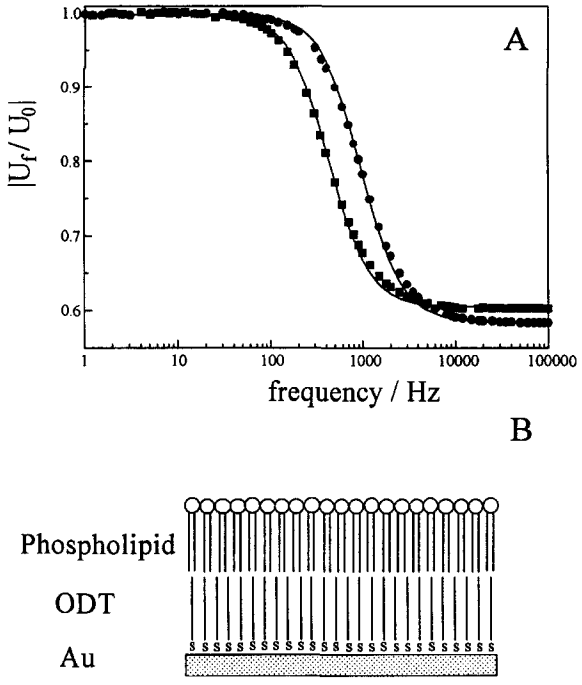

Fig. 3. (A) Impedance spectra (transfer function) of the ODT-monolayer (■) and the bilayer of an adsorbed DPPC-monolayer onto ODT (●). The spectra shown here are representative for results obtained by the different bilayer preparations on a hydrophobic ODT-monolayer (method A, B, C, D_1, D_2). (B) Schematic view of the solid supported bilayer.

hydrophobic monolayer by different techniques and the capacitance was determined. The results are summarized in Table 1. Two typical impedance spectra for a monolayer and a bilayer according to these methods are given in Fig. 3A.

3.2.1. Langmuir-Schaefer-technique (method A in Table 1)

This technique allows the deposition of a well defined second monolayer from the air-water-interface. Deposition of a liquid condensed DPPC-monolayer yields an average capacitance for the bilayer of $C_{bl} = 0.57 \pm 0.02$ $\mu F/cm^2$ which remained stable at least for 24 h. The capacitance of the deposited DPPC-monolayer was calculated by Eq. 3 to $C_{ml2} = 1.26 \pm 0.12$ $\mu F/cm^2$.

3.2.2. Painted lipid membranes (method B in Table 1)

Again the first layer was chemisorbed ODT but the lipid layer was deposited from a DPPC solution in decane/isobutanol as described under Section 2. Values obtained from impedance measurements are $C_{bl} = 0.56 \pm 0.01$ $\mu F/cm^2$ and $C_{ml2} = 1.23 \pm 0.08$ $\mu F/cm^2$ which are similar to the quality of the supported bilayer obtained by the Langmuir-Schaefer-technique.

3.2.3. Monolayer deposition from detergent solution (method C in Table 1)

Micellar solutions of DPPC in octylglucoside allow the formation of a lipid monolayer of low capacitance on ODT-monolayers. The capacitance decreased immediately after the addition of the lipid/detergent solution to the preformed ODT-monolayer although the detergent concentration was far above its critical micellar concentration of octylglucoside (CMC = 23.3 mM). This clearly shows that adsorption of lipids together with detergent onto the hydrophobic ODT-monolayer occurred. Dilution of the aqueous phase with buffer to detergent concentrations below the CMC caused a further decrease of the capacitance from $C = 0.90$ $\mu F/cm^2$ in the presence of detergent to $C_{bl} = 0.54 \pm 0.02$ $\mu F/cm^2$ after dilution of the detergent which corresponds to a DPPC-monolayer capacitance of $C_{ml2} = 1.13 \pm 0.1$ $\mu F/cm^2$. This value is somewhat lower but still close to the value obtained by the two other above mentioned techniques.

3.2.4. Fusion of large unilamellar vesicles on ODT-monolayers (method D_1 in Table 1)

This method avoids the use of organic solvents or detergents. We applied this preparation technique to a number of phosphatidylcholines differing in fatty acid chain length and degree of saturation. The final capacitances of the phosphatidylcholine monolayers strongly depended on the molecular structure of the lipid. The kinetics of the formation process, however, was found to be independent of the lipid. Bilayer formation was completed within at least 3 h which became apparent by a constant capacitance value. The results summarized in Table 2 clearly show a decreasing capacitance with increasing chain length if lipids with saturated hydrocarbon chains were used. The value for POPC carrying one saturated and one unsaturated chain is drastically increased to $C_{bl} = 0.68 \pm 0.02$ $\mu F/cm^2$ which results in a monolayer capacitance of $C_{ml1} = 1.96$ $\mu F/cm^2$ [2]. This effect is less pronounced in egg-PC which contains a fatty acid mixture of different chain lengths and degree of saturation.

From the experimentally obtained capacitances we calculated the fatty acid chain lengths d of the phosphatidylcholine monolayers for the saturated lipids by Eq. 2 assuming a dielectric permittivity for the alkyl chains of $\epsilon_r = 2.1$. Neglecting the contribution of the headgroup to the overall capacitance seems to be justified with regard to its high

Table 2
Influence of chain length and fatty acid saturation of different phosphatidylcholines adsorbed onto ODT (method D_1) on the capacitance C_m

Lipid	C_{bl} ($\mu F/cm^2$)	C_{ml2} ($\mu F/cm^2$)
DLPC	0.64 ± 0.03	1.65 ± 0.24
DMPC	0.56 ± 0.02	1.23 ± 0.12
DPPC	0.52 ± 0.02	1.04 ± 0.09
DSPC	0.51 ± 0.02	1.00 ± 0.09
POPC	0.68 ± 0.02	1.96 ± 0.23
egg-PC	0.61 ± 0.02	1.49 ± 0.15

C_{bl}: capacitance of the bilayer of ODT and phosphatidylcholine, C_{ml2}: capacitance of the physisorbed monolayer of phosphatidylcholine.

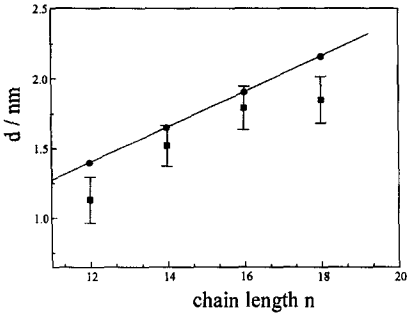

Fig. 4. Chain length dependence of the dielectric monolayer thickness for phosphatidylcholines with fatty acids between 12 and 18 carbonatoms per chain. The experimental values (■) were derived from the capacitances using Eq. 2 and a dielectric permittivity of $\epsilon_r = 2.1$. The corresponding calculated geometrical thicknesses (●) were obtained from the known C-C-bond length and angle assuming a fully extended chain with complete all-*trans* conformation.

value of about 30 $\mu F/cm^2$ [18]. This is in agreement with earlier reported values of the dielectric thickness of the bilayer obtained from capacitance measurements in contrast to the geometrical bilayer thickness measured by X-ray diffraction [19]. The values for the thickness d are given in Fig. 4 as function of lipid chain length together with theoretical values obtained from the number of C-C-bonds assuming 1.545 Å for the bond length and 110.5° for the sp^3-hybrid bond angle supposing an all-*trans* conformation with the chain oriented perpendicular to the surface [16]. The lipids with medium chain length, DMPC and DPPC nicely fit the theoretical monolayer thickness whereas short chain DLPC and long chain DSPC exhibit an unexpected considerably lower apparent monolayer thickness.

3.2.5. Fusion of large unilamellar vesicles on DMPTE-monolayers (method D_2 in Table 1)

A self assembled monolayer of DMPTE exhibits a mean value for the capacitance of 1.2 ± 0.1 $\mu F/cm^2$. As described in 3.2.4. different lipids could be used for the formation of a second monolayer. The final capacitance of $C_{ml2} = 1.1 \pm 0.2$ $\mu F/cm^2$ for a DPPC-monolayer was reached after 3 h. The double layer was stable after removal of the vesicle suspension for about 24 h at room temperature.

3.3. Solid supported bilayers on a charged surface

In method A to D_2 we used hydrophobic first monolayers on which a second lipid monolayer may be attached by hydrophobic forces. Now we report the coverage of the gold electrode by charged first monolayers using mercaptoethylammoniumhydrochloride (MEA, positively charged) or 3-mercaptopropionic acid (MPA, negatively charged) which allowed us crosswise to either fuse negatively or positively charged lipid vesicles onto the surface. The adsorption of negatively charged vesicles onto a negatively charged surface may be mediated by the addition of Ca^{2+}-ions.

3.3.1. Fusion of DMPG/DMPE vesicles on positively charged MEA-monolayers (method E in Table 1)

Mixed vesicles containing negatively charged DMPG and zwitterionic DMPE in a 4:1 molar ratio were fused onto a gold surface covered with a monolayer of positively charged MEA. Although the kinetics of the fusion of pure DMPG vesicles onto the MEA-monolayer was six times faster, the mixture of DMPG and DMPE gave the best results concerning the final capacitance in contrast to pure DMPG-bilayers which showed higher capacitances up to $C_{bl} = 1.3$ $\mu F/cm^2$. The fact that DMPE stabilizes planar bilayer membranes because of its molecular structure could be responsible for the lower capacitances. Since the driving force for the fusion process is the electrostatic interaction between the DMPG-headgroups and the aminogroups of mercaptoethylamine the fusion process is highly pH-dependent. In our experiments pH 5 was found to be best suited where the aminogroups are protonated and the phosphate groups of DMPG are deprotonated [20]. Values of the pH higher than 6 prevented the bilayer formation.

The self assembly process of mercaptoethylammoniumhydrochloride onto the gold substrate was finished after 30 min. The final capacitance of this monolayer varied between 10 and 16 $\mu F/cm^2$ and was dependent on the pH value [21–23]. After addition of the mixed DMPG/DMPE vesicles a bilayer formed but it took about 24 h to complete. The fusion process was easily observable by impedance spectroscopy (Fig. 5A). Although it is not possible to fit the intermediate spectra by the simple equivalent circuit shown in Fig. 2 the evolution of the bilayer is easy to follow by the shift to lower capacitances which qualitatively may be deduced from the shift of the transfer function to higher frequencies. The final spectrum was fitted and provided a capacitance for the bilayer of 0.89 ± 0.08 $\mu F/cm^2$. The bilayer was stable for several days.

3.3.2. Fusion of DODAB vesicles on negatively charged MPA-monolayers (method F in Table 1)

In contrast to the MEA/DMPG/DMPE-system in the case of fusion of positively charged DODAB vesicles on negatively charged MPA-monolayers only the charge of the MPA is influenced by the pH value of the bulk solution.

The self assembly of MPA onto the gold covered substrate was a very rapid process; after 5 min the final capacitance of 7–9 $\mu F/cm^2$, dependent on the pH value, was reached and did not change even if the incubation time was extended. In order to get deprotonated MPA a pH value of 8.6 was chosen. After addition of DODAB vesicles the capacitance decreased rapidly and reached a minimal value of $C_{bl} = 0.84 \pm 0.03$ $\mu F/cm^2$ after 1 h. The removal of residual vesicles by exchange of the electrolyte

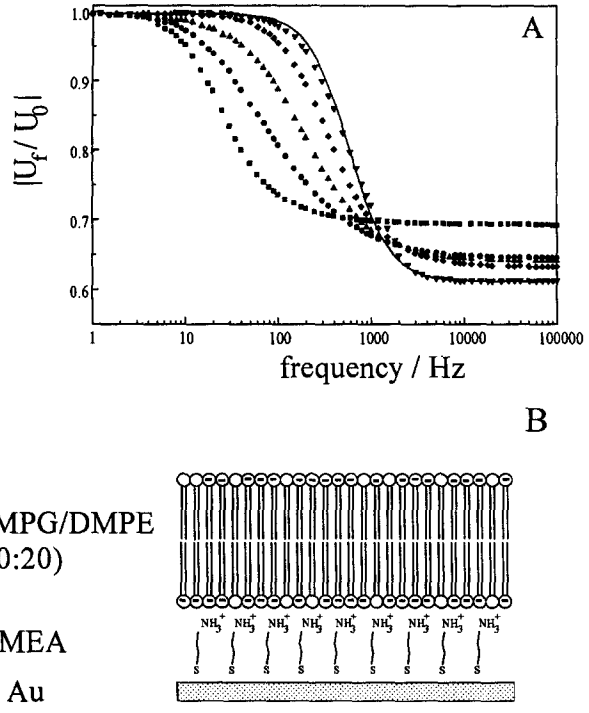

Fig. 5. (A) Impedance spectra recorded during the formation of a mixed lipid bilayer from vesicles containing 20 mol% DMPE and 80 mol% DMPG (method E). (■) 0 h, (●) 1 h, (▲) 8 h, (♦) 15 h, (▼) 24 h. The continuous line represents the fit according to the equivalent circuit given in Fig. 2 with $C_{bl} = 0.89$ $\mu F/cm^2$. (B) Schematic view of the supported bilayer system on a MEA-monolayer.

solution against pure buffer was not critical in terms of changes in the capacitance of the DODAB-bilayer. The lipid bilayer was stable for several days.

Addition of calcium ions with a concentration of 5 mM removed the DODAB-bilayer rapidly. The capacitance increased to 7 $\mu F/cm^2$ what corresponds to the capacitance of the pure MPA-monolayer. In contrast monovalent cations with a concentration of 40 mM did not effect the bilayer significantly.

3.3.3. Fusion of DPPG vesicles on MPA covered surfaces mediated by Ca^{2+}-ions (method G in Table 1)

It is also possible to fuse negatively charged vesicles of DPPG onto a negatively charged surface of MPA in the presence of calcium ions. By adding Ca^{2+} with a final concentration of 2 mM the self assembly process of the DPPG-bilayer was initiated and 30 min later a final capacitance of $C_{bl} = 0.66 \pm 0.02$ $\mu F/cm^2$ was recorded. Without calcium ions the capacitance of the whole system did not change in the presence of DPPG vesicles. In order to remove residual vesicles a buffer composition of 10 mM Tris, pH 8.6 with 2 mM Ca^{2+} has to be used because a buffer solution without calcium ions led to a complete disruption of the preformed bilayer. In contrast to the DODAB-bilayer the DPPG-layer was only stable for a maximum of 3 h. Addition of an EDTA-solution with a concentration of 2.5 mM in order to remove the calcium ions caused the removal of the bilayer within 10 min. Fig. 6A shows the impedance analysis of the DPPG-bilayer before and after the addition of EDTA. The admittance of the DPPG-bilayer increased due to a decrease of the membrane resistance R_m. Correspondingly the membrane capacitance C_m increased as can be seen from the fitting results. Further addition of Ca^{2+} ($c = 4$ mM) to overcompensate the EDTA concentration did not result in a complete regain of the DPPG-bilayer on the MPA-layer.

3.4. Direct adsorption of mixed DMPC / DMPTE vesicles on gold surfaces via chemisorption (method H in Table 1)

The formation of lipid bilayers using LUV consisting of a mixture of DMPTE and DMPC showed a strong dependence on the amount of DMPTE. Vesicles containing more than 50 mol% of DMPTE exhibited a decrease in capacitance down to $C_{bl} = 0.63 \pm 0.02$ $\mu F/cm^2$ within 6 h (Fig.

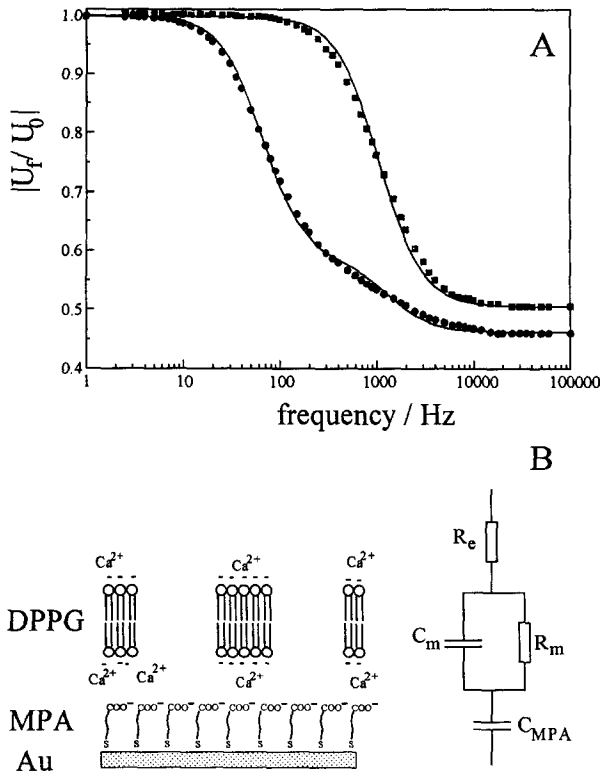

Fig. 6. (A) Influence of EDTA on a DPPG-bilayer immobilized via Ca^{2+}-bridges on a MPA-monolayer (method G). The impedance spectrum of the adsorbed DPPG-bilayer in the absence of EDTA (■) is fitted with the equivalent circuit from Fig. 2 yielding a capacitance of $C_{bl} = 0.66$ $\mu F/cm^2$. The decrease in impedance according to a higher membrane capacitance and a lower membrane resistance is clearly visible after EDTA addition (●). From the fitting procedure using the shown equivalent circuit including the capacitance representing the MPA-monolayer we obtained $C_m = 2.5$ $\mu F/cm^2$, $C_{MPA} = 8.8$ $\mu F/cm^2$ and $R_m = 70$ Ω cm^2. (B) shows a schematic view of the defect bilayer with its equivalent circuit which is representative in the presence of EDTA leading to a disruption of the previously completely covered electrode surface.

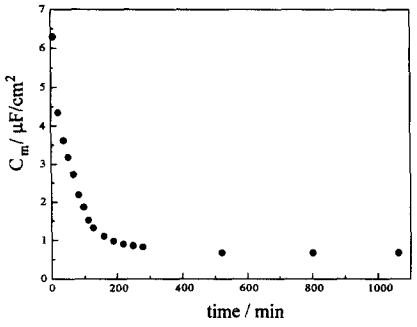

Fig. 7. Kinetics of adsorption of DMPC/DMPTE-LUV (40:60) onto gold (method H). The capacitances were obtained from fitting the impedance spectra using the equivalent circuit from Fig. 2.

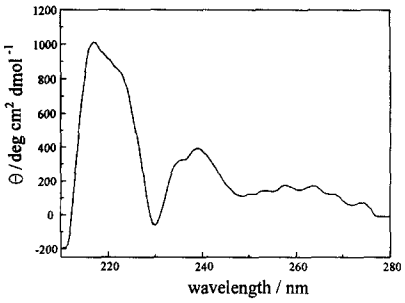

Fig. 8. Accumulation of five CD-spectra of DODAB vesicles with 1 mol% gramicidin D prepared in 10 mM Tris, pH 8.6 after baseline substraction obtained with pure buffer; the concentration of gramicidin D was 0.09 mg/ml; the lipid concentration was 0.3 mg/ml.

7). This critical amount of thiol-anchors was necessary to induce the formation of a bilayer system with high coverage. The final value did not change if the vesicle suspension was exchanged against pure buffer solution. The stability of the bilayer was maintained for several days at room temperature. Another important feature is that the formation process has to be carried out above the phase transition temperature of the lipids ($T_{DMPC} = 23°C$, $T_{DMPTE} = 22°C$), which were determined by differential scanning calorimetry using a Microcal MC-2 calorimeter (Bletchley, UK). At room temperature ($T = 20°C$) the capacitance of this self assembly process with the same lipid composition only reached a final value of $C = 6 \pm 1$ $\mu F/cm^2$.

3.5. Ion transport through gramicidin D channels incorporated into solid supported DODAB-bilayers

Gramicidin D has been used as a model channel to test the suitability of impedance spectroscopy for the study of ion permeability. Gramicidin D is known to incorporate into bilayers where it displays a conformation that is characteristic for a channel state. This conformation can be easily detected by CD-measurements with characteristic maxima at 217 nm and 237 nm and a minimum at 229 nm as well as a negative ellipticity below 205 nm. A pronounced negative ellipticity is characteristic for the non-conductive conformational state [14]. Fig. 8 represents the CD-spectrum of gramicidin D in DODAB-LUV. It clearly demonstrates that the peptide assumed the channel-forming state with a minor amount of non-channel conformation indicated by the slightly negative ellipticity at 229 nm.

Typical impedance spectra for DODAB bilayers on MPA containing 1 mol% gramicidin D are shown in Fig. 9A. Increasing amounts of CsCl were applied to demonstrate the ion flux through the bilayer. The same bilayer was used for all measurements by continuously increasing the Cs^+-concentration. The effect was completely reversible, removal of the Cs^+-ions yielded an impedance spectrum which was identical to the initial one. Addition of CsCl to DODAB-bilayers without gramicidin D did not influence the impedance spectrum significantly. To fit the data in Fig. 9A we had to introduce an additional subcircuit representing the ion transport. It consists of a resistance R_m which describes the ion flow through the membrane and a parallel RC-circuit (R_{pt}, C_{pt}) representing the situation at the interface. Increasing the Cs^+-concentration

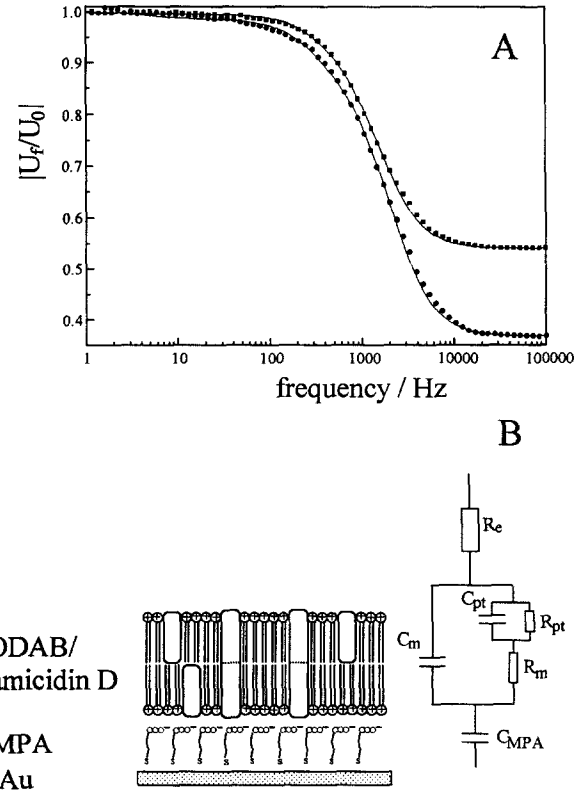

Fig. 9. (A) Impedance spectra (transfer function) of a gramicidin D (1 mol%) doped DODAB-bilayer immobilized electrostatically on a MPA-monolayer. The transfer functions in the presence of 13 mM CsCl (■) and 31 mM CsCl (●) are shown. The continuous lines are the results of the fitting procedure with the equivalent circuit from (B) which are summarized in Table 3. Impedance analysis was carried out at least two times without any changes in admittance. After removal of CsCl by exchange of the electrolyte solution against 10 mM Tris, pH 8.6 the transfer function of the unaffected bilayer fully regained. (B) Schematic view of the gramicidin D containing bilayer and the equivalent circuit representing the ion transport.

Table 3
Results of the fitting procedure using the equivalent circuit from Fig. 9B for increasing CsCl-concentrations

Concentration of CsCl (mM)	C_m (μF/cm^2)	R_m (Ω cm^2)	C_{pt} (μF/cm^2)	R_{pt} (Ω cm^2)
5	0.94±0.02	1900±100	0.27±0.02	28000±3000
13	0.95±0.02	500± 25	0.45±0.02	7100± 700
20	1.04±0.02	460± 20	0.73±0.03	4400± 400
26	1.14±0.02	420± 15	0.99±0.05	3200± 300
31	1.17±0.02	330± 10	1.15±0.05	2100± 200

C_{MPA} was kept constant to 8.8 μF/cm^2 during the fitting procedure.

increases the admittance of the subcircuit: the parameters are summarized in Table 3. R_m and R_{pt} decreased while C_{pt} increased with increasing Cs^+-concentration. Because of the increasing admittance due to the ion transport it was necessary to take the capacitance of the MPA-monolayer into account. This had been done by introducing C_{MPA} in series to the described network. Corresponding experiments have been performed with Sr^{2+}-ions which do not permeate through the gramicidin channel (data not shown). These ions did not influence C_{pt}, R_{pt}, C_m and R_m, but only contributed to R_e.

4. Discussion

4.1. Comparison of different preparation techniques

One topic of the present paper is to scrutinize different techniques for the preparation of solid supported lipid bilayers on gold surfaces. The reproducible and stable architecture of such a reconstituted lipid membrane system has become desirable in the last years because of a possible application as molecular biosensor devices. Such a device basically requires a lipid bilayer membrane of high resistances. Proteins that mediate ion conductivity preferentially by a ligand operating mechanism may be reconstituted into the lipid bilayer. The quality of the lipid bilayer however is the prerequisite for the successful application of lipid-protein-membranes to construct such a ligand selective sensor.

In this paper different techniques were used for the construction of supported lipid bilayers which are all based on the strong chemisorption of a first monolayer of thiols onto gold substrates but differ in the mode of attachment of the second monolayer or of a complete bilayer. Only method H does not make use of a first chemisorbed monolayer.

The results for method A–D, in which DPPC was used for the second monolayer, are summarized in Table 1. Independent of the mode of preparation the values of membrane capacitances obtained by a.c. impedance spectroscopy were found within a small range of $\Delta C_{bl} = 0.05$ μF/cm^2 around the mean value of $C_{bl} = 0.55$ μF/cm^2. This provides direct evidence that independent of the mode of formation starting from hydrophobic surfaces a second defined monolayer was deposited on top of the first one. The reduction of the hydrophobic surface due to the second monolayer is considered to be the driving force of bilayer formation [7].

In method D_1 monolayers of phosphatidylcholines differing in chain length and in degree of saturation were investigated. Saturated lipids with different chain lengths are expected to show a linear decrease in capacitance with increasing number of CH_2-segments which is equivalent to the increase of the monolayer thickness d considering Eq. 2. Since complete all-*trans* conformations have been assumed the calculated thickness is the upper limit for a fully extended chain which in reality will not be reached even below the lipid phase transition temperature. The lipids with medium chain lengths (C_{14} and C_{16}) nicely fit the theoretical values for the fatty acid chain lengths, however for the short chain- (C_{12}) and the long chain- (C_{18}) phosphatidylcholines the effective fatty acid chain length is lowered compared to the model assumption. This may be explained in two different ways. For the short chain lipids a less strong hydrophobic interaction can be expected which may be easily concluded from the very low phase transition temperature ($T_{DLPC} = 4.5$°C) between the crystalline and the fluid phase. Although all lipids were hold at temperatures above their main phase transition temperatures during the experiments one may assume, that short chain lipids are more susceptible for water in the hydrophobic membrane portion causing an increased permittivity of the lipid layer which is equivalent to a higher measured capacitance. Moreover, since short chain lipids tend to form micelles an incomplete electrode coverage has to be considered as well. For the long chain lipids with a high melting temperature ($T_{DSPC} = 55$°C) this argument does not hold. However, it is known that long chain lipids tend to create defects [24] which become noticeable as an uncomplete electrode coverage again increasing the capacitance of the system.

The high capacitance of POPC must be explained by a decrease of packing density. POPC, a phosphatidylcholine in which one chain is unsaturated is expected to exhibit a highly disturbed chain-chain-interaction providing the ability to incorporate water molecules into the monolayer. The structure of the film thus must exhibit an expanded monolayer with an increased dielectric permittivity. With egg-lecithin we obtained an intermediate value of $C_{ml2} = 1.49$ μF/cm^2 which nicely fits the expectation since egg-PC contains lipids with a variety of chain lengths and numbers of double bonds and moreover differing in position 1 and 2 at the glycerol backbone.

The situation gets more complicated if the second strategy of fusing charged vesicles on a charged surface is applied (method F and G in Table 1).The lipids DPPG and DODAB differ both in their headgroup charge and in their chain length. Nevertheless, it is interesting to compare the

values for DPPG-bilayers in the presence of calcium ions with those obtained for DODAB. DODAB which has by two C-atoms longer alkyl chains compared to DPPG exhibits a larger capacitance. This fact can again be explained in terms of differences in packing density of the two different bilayers and thus in terms of altered dielectric thickness [19]. From film balance measurements it is known that DODAB-monolayers do not exhibit a phase transition from the liquid expanded to the liquid condensed phase. At 30 mN/m a large molecular area of 62–66 $Å^2$/molecule was observed [25–27] which was interpreted by the electrostatic repulsion of the positively charged headgroups of DODAB. DPPG-monolayers show a completely different physical behaviour upon compression in the presence of Ca^{2+} in the subphase. A condensed state is reached with an area of 53 $Å^2$/molecule at 30 mN/m [28]. Considering the solid supported case where a bilayer exists a similar behaviour could be assumed. This would mean that the DODAB bilayer is packed less densely than the DPPG-bilayer what could allow water molecules to intercalate causing a higher dielectric permittivity and therefore a higher capacitance which is equivalent to a lowered dielectric bilayer thickness. The striking differences between bilayers prepared on hydrophobic or charged monolayers may be discussed in the same way. The generally lower capacitances of the bilayers prepared according to method A–D arise from the fact that the first monolayer of ODT or DMPTE contains only small amounts of water caused by their high packing density. These monolayers being directly bound to the gold surface represent the insulating behaviour of a nearly defect free monolayer causing their low capacitances.

Another feature of the two different strategies of lipid bilayer formation which has to be discussed is their stability on the solid support. Considering the DODAB-bilayer small amounts of calcium ions, an increase of the ionic strength, a decrease of the pH value or an increase of the temperature above the main phase transition of the lipid ($T_{DODAB} = 48°C$) are critical parameters which cause the disruption of the intermolecular forces between MPA and DODAB. These facts lead to the assumption that the immobilized DODAB-bilayer behaves homogeneous as one unit. The bilayer disruption in the presence of calcium ions is probably caused by the complexation of the carboxylic groups of MPA which causes the disruption of the electrostatic interaction between the MPA- and the DODAB-layer. The same results can be derived from experiments with the DPPG-bilayer connected via calcium ions on MPA. Destruction occurs in the presence of equimolar amounts of EDTA, whereby only small islands of DPPG remain on the surface. For the bilayers of DMPG/DMPE onto MEA pH value and temperature are the critical parameters.

In contrast to these considerations bilayers on the hydrophobic surfaces are not influenced by changes in ionic strength, temperature or pH in a usual range. Only if the bilayer is passed again through the air-water-interface the DPPC-monolayer on top of the chemisorbed monolayer is removed independent of the preparation technique. This is due to the fact that the second monolayer is connected weakly to the first monolayer via hydrophobic interactions whereas the first monolayer is strongly bound because of the chemisorption of the thiol-anchors on the gold surface.

An easy method to build up supported bilayer membranes is the use of vesicles that contain a thiol lipid like DMPTE. The advantage of this method is the direct deposition of the bilayer by fusing the lipids on the gold surface without a prior coverage with a chemisorbed monolayer. A critical aspect of this method is the relative amount of thiol lipid which is essential for the formation of the stable bilayer. In our experiments at least 60 mol% of the lipid had to be DMPTE in the vesicles which however may be different for thiol lipids differing in head group and chain length composition. The phase transition temperature of the lipid as well as phase separation phenomena have to be considered for a successful deposition of the bilayer.

4.2. Kinetics of bilayer formation

Kinetics recorded during the fusion of vesicles onto ODT- or DMPTE-monolayers were in the range of hours which is acceptable for practical use. The bilayers were at least stable for a day. The fusion of DMPG/DMPE vesicles onto MEA was the slowest process observed which may be caused by the reduced charge density in mixed DMPG/DMPE vesicles. However the stability of the lipid layers is satisfying. In contrast the Ca^{2+}-induced fusion of DPPG vesicles on MPA-monolayers is fast but the resulting bilayer is only stable up to 3 h. The most convenient methods with respect to easy handling, time until final capacitance is reached and long-term stability are the deposition of DODAB vesicles onto a MPA-monolayer and the deposition of thiol lipid containing vesicles onto the pure gold surface.

4.3. DODAB-bilayers containing gramicidin D channels

For the reconstitution of the channel-forming peptide gramicidin D we have chosen DODAB-bilayers because of several advantages. First of all gramicidin D reconstitutes nicely into DODAB vesicles maintaining the channel conformation what was shown by CD-measurements. Further the DODAB-bilayer formation is not influenced by the peptide and this method prevents the addition of Ca^{2+} which might interfere with the studied ion transport [29]. Therefore the system DPPG/Ca^{2+}/MPA is not applicable. The relatively high value of $C_{bl} = 0.84\ \mu F/cm^2$ for the DODAB-bilayer with reconstituted gramicidin was tolerable with regard to the studied ion transport.

The impedance spectra obtained in the presence of gramicidin D may be fitted neither by the simple equivalent circuit shown in Fig. 2 nor by the one given in Fig. 6B taking the MPA-monolayer into account. In Fig. 9B we

present an equivalent circuit that fits the data well including a proper physical meaning of the parameters. The impedance due to the ion transport consists of three elements, the resistance R_m due to ion flow through the membrane phase and an interfacial barrier represented by C_{pt} and R_{pt} (pt stands for phase transfer) forming a parallel RC-subcircuit. Mass transport in the water phase was not taken into account. The data obtained from the fits for increasing Cs^+-concentrations are given in Table 3 and clearly show the decreasing resistance R_m with increasing Cs^+-concentrations. The capacitance of the DODAB bilayer C_m increases perhaps due to a higher dielectric permittivity in the membrane phase arising from the cation within the lipid bilayer or processes concerned with the lipid/water interface.

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