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Characterization of complex gramicidin monolayers by light reflection and Fourier transform infrared spectroscopy

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The reflectivities of monomolecular films of water-insoluble fatty alcohols and fatty acid methyl esters are measured at the air/water interface. A correlation between chain length and reflectivity of the monofilm is established which agrees with calculated values derived from a theoretical model. The correlation is used to estimate thickness of a monolecular film of gramicidin A. Fourier transform infrared (FTIR) spectroscopy is applied to transferred mixed layers of ion-channel-forming gramicidin A and dioctadecyldimethylammonium bromide in order to evaluate the structure of gramicidin. Transfer conditions for these monofilms are elaborated. Results of the reflection method and FTIR spectroscopy demonstrate that gramicidin exists as double-stranded β-helix inside the monolayer at a lateral pressure similar to that found in biomembranes.

Introduction

Complex monolayers consisting of inert matrix molecules like long-chain fatty acids and amphiphilic dyes can be prepared by spreading a solution containing the water-insoluble components on a clean water surface. Upon compression by reducing the film area, the different molecules are organized into a condensed monolayer [1,2]. These monolayers have gained much interest in the field of photoinduced processes [3] and in the study of molecular association phenomena and other related effects [4].

The measurement of enhanced light reflection from the air/water interface in the presence of dye molecules is a recently developed technique [5]. Due to the phase relation between the incident light wave, the wave reflected at the interface and the waves emitted by the molecules located at the interface, the reflection is modified in the spectral region of the dye absorption band as compared with the reflection from the interface without dye molecules. It is known that the non-absorbing long-chain fatty acid monolayers themselves are transparent in the visible and near-ultraviolet region of the spectrum [6,7] but act as thin dielectric layers. These monolayers, with their refractive index being different from that of air and water and secondly by altering the water structure near the interface, change the characteristic reflectivity of the water surface in comparison to an uncovered water surface. The reflectivities, refractive indices and other related optical properties have been

Abbreviation: DOMA, dioctadecyldimethylammonium bromide; FTIR, Fourier transform infrared.

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measured for some long-chain fatty acids in transferred multilayers of these substances [6,7]. In this work, we present the results of reflectivity measurements obtained for monolayers at the air/water interface of a series of long-chain fatty alcohols and fatty acid methyl esters. We have further derived theoretical values of the reflectivities for the different long-chain monolayers by using Fresnel's equations [8] which have been derived by matching the E and H fields at the interface. These theoretically and experimentally obtained values were used to determine the thickness of spread monolayers. This new method also provides information on thickness changes upon compression of monolayers formed by substances with completely different structures from those of the long-chain fatty alcohols or fatty acid methyl esters, such as gramicidin D.

Gramicidin D is a mixture of three linear depsipeptides consisting of alternately connected D- and L-amino acids. This antibiotic isolated from *Bacillus brevis* has 15 amino acid residues. The main component is gramicidin A (80%), the other two components, gramicidin B and C, being present in very low amounts. They differ only by exchanges of a single amino acid from gramicidin A and for this reason the natural mixture of gramicidin D shall be referred to as gramicidin A in this study. Its main biological activity is the formation of ion-conducting channels specific for monovalent cations, especially alkali, NH_4^+, Tl^+ and H^+ ions [9] in bacterial cell membranes as well as synthetic vesicles [10]. The form active in ion conduction inside the membrane is probably a dimer consisting of two single-stranded β-helices [11]. In organic solvents, on the other hand, gramicidin adopts a double-helical structure consisting of an antiparallel β-helix with intermolecular hydrogen bonds. This change of conformation during insertion of gramicidin into lipid bilayers has been derived from circular dichroism measurements [12], X-ray crystallography [13] and vibrational spectroscopy [14].

As gramicidin A is able to form a monolayer at the air/water interface and its structure is very different from typical film-forming substances, Fourier transform infrared (FTIR) spectroscopy has been used to investigate mixed layers of gramicidin A and a typical monolayer-forming substance dioctadecyldimethylammonium bromide (DOMA). The application of this technique to mixed films is rather new because of the small amount of material available which causes sensitivity problems. Theoretical calculations on the probable conformation of gramicidin A at the air/water interface have been performed recently [15]. The deposition of multilayers of DOMA and mixed films of gramicidin and DOMA onto solid supports is necessary for the FTIR spectroscopy measurements. Therefore, deposition conditions for these monolayers were established. In general, the application of FTIR spectroscopy to mixed monolayers is of interest with respect to the characterization of the orientation of deposited molecules. Further this technique might give information about a possible change of conformation of gramicidin during insertion in the monolayer, a process similar to the insertion into biomembranes. In addition, mixed films of gramicidin and lipids deposited onto solid supports might be interesting with regard to ion specificity of gramicidin and may be used as a basis for sensing small concentrations of these ions in solution.

Experimental

Materials

The compounds used in this study were fatty alcohols with hydrocarbon chains ranging from 14 to 22 carbon atoms, fatty acid methyl esters with 14, 16, 18, 20 and 22 carbon atoms, DOMA and gramicidin A. They were obtained from Sigma Chemical Co. and used without further purification. The spreading solvent used was purified $CHCl_3$; deionised Milli-Q water (Millipore system) was used for the subphase.

Methods

Reflectivity measurements

A circular through made of polytetrafluoroethylene was used with a Wilhelmy balance for measuring the surface pressure [16]. The subphase was buffered with 5 mM Tris-HCl at pH 7.0 ± 0.05. Prior to spreading, the aqueous subsolution was swept. The films were swept at a constant rate of about 3 $Å^2 \cdot \text{molecule}^{-1} \cdot \text{min}^{-1}$ and held at a constant pressure of 35 mN/m. The reflection

spectrometer used for measurement of the normal incidence of light has been described earlier [5]. The through was provided with a reference compartment with clean water and its surface was used as a reflection standard in order to obtain absolute reflectivities. All measurements were made by fixing the monochromator at the wavelength of sodium D-line (589.3 nm).

The surface pressure-area (π-A) diagrams for DOMA, gramicidin A and mixtures of both in the molar ratios of DOMA/gramicidin A 2:1, 3:1, 4:1, 5:1 and 6:1 were determined by spreading aliquots of a 0.5 mM solution of gramicidin A in chloroform, or a 1 mM solution of DOMA or the appropriate mixed solutions in chloroform. The measurements were done at 20 °C and the compression rate was 21 $Å^2 \cdot molecule^{-1} \cdot min^{-1}$. The dipping machine used for monolayer transfer was a modified version of that described earlier [2]. Aliquots of substances to be transferred were spread and remained on the subphase for about 10 min. As solid support for film deposition silicon slides (12 × 30 × 1 mm), polished on both sides, were used. They were cleaned in chromic acid, rinsed in ultrapure water and dried in an oven. Slides were subsequently silanized by sonication in liquid $(CH_3)_2SiCl_2$ for 1 min, rinsed in isopropanol and dried in an argon stream. Each slide was used only twice as a deposition substrate. Using water as a subphase for a spread film, transfer of only one layer on a hydrophilic surface was possible. Attempts were made to transfer several layers of DOMA and DOMA/gramicidin A mixed films by addition of several ions to the subphase, which was buffered with 1–5 mM Tris-HCl giving a pH of 7.2. Anions HBO_3^{2-}, $(COO)_2^{2-}$, SO_4^{2-}, ClO_4^-, HPO_4^{2-} in the concentration range of 1–20 mM were tested to enable an efficient transfer of multilayers. During transfer, surface pressure was maintained constant between 20 and 35 mN/m.

FTIR spectroscopy was performed on a Nicolet DX-10 spectrometer with a resolution of 2.000 cm^{-1}. Spectra were recorded by taking 500 scans. Spectra of gramicidin A and DOMA in the solution state were recorded by applying three drops of a chloroform solution onto a sodium chloride disk after evaporation of the solvent. Spectra of transferred layers, the minimal number of which had to be six to obtain a good infrared absorption, were recorded in the transmission mode.

Results and Discussion

Evaluation of the data from reflection measurement

Fig. 1a shows the change in the reflection signal ΔR (difference in reflectivities of water surface covered with a monolayer and clean water surface) for some long-chain fatty alcohols measured upon compression at a constant rate to a surface pressure of 35 mN/m. The figure shows that between $t = 0$ and $t = 4$ min there is no rise in the reflection signal. This corresponds to the expanded state of the monolayer. Upon further compression, the reflection signal starts to rise and when the constant pressure of 35 mN/m is reached the signal reaches a steady value. It is seen that ΔR is a function of the chain length of the alcohols. Fig. 1b gives the plots of the square root of ΔR values obtained at 35 mN/m with monolayers of the different long-chain fatty alcohols and fatty acid methyl esters as a function of the chain length. The dotted line corresponds to the theoretical values of the reflectivities of the hydrocarbon chains ranging from 10 to 22 carbon atoms. These values were calculated for a three-layered structure consisting of air, monolayer and subphase using Fresnel's equations taking into account reflection of the light wave from both the monolayer/air and monolayer/water interfaces. The refractive index of water at 20 °C was assumed to be 1.330 and all the values have been normalised with respect to the refractive index of eicosanol ($n = 1.54$). This refractive index value was taken from calculations done on cadmium arachidate [6]. It is seen from Fig. 1b that the experimental reflectivity values obtained for the alcohols and fatty acid methyl esters cluster around the straight line from the theoretical values. The monolayer, by its intrinsic reflectivity (acting as a dielectric sheet) and by altering the water structure near the interface, changes the reflectivity of the water surface. A comparison of the experimental reflectivities of the fatty alcohols and the methyl esters with two different head groups shows that this modification of the head group region has no significant influence on the reflectivity. Further results from earlier optical measurements done on

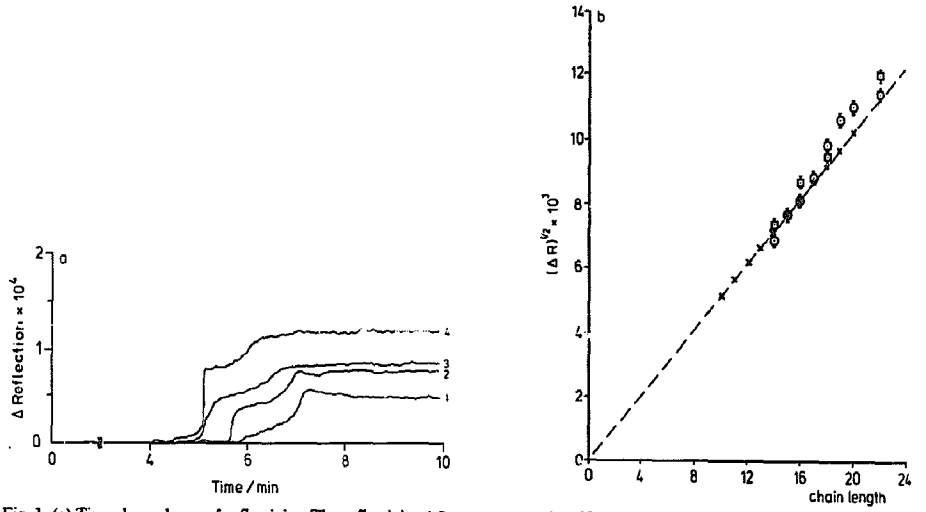

Fig. 1. (a) Time dependence of reflectivity. The reflectivity ΔR was measured at 589.3 nm for fatty alcohol monolayers with different numbers of carbon atoms at a surface pressure of 35 mN/m. Chain lengths were 14 (1), 16 (2), 17 (3) and 20 (4) carbon atoms. Subphase used was 5 mM Tris-HCl (pH 7.2). (b) Square root of reflectivity as a function of chain length. ΔR is plotted in relation to the chain length of the investigated fatty alcohols (circles) and fatty acid methyl esters (squares). The dotted line corresponds to calculated values of reflectivity using Fresnel's equations.

transferred multilayers of fatty acids seemed to indicate that they act as a birefringent medium [7]. In our theoretical estimation of the reflectivity values this aspect has not been considered and only the real component of the refractive index is used in the calculation. The correlation between the reflectivity and alkyl chain length may be used to predict thickness and nature of other monolayers at the air/water interface.

Estimation of the thickness of gramicidin at the air/water interface

In this work the thickness of monofilms of gramicidin A has been evaluated using the above correlation. Gramicidin A consists only of hydrophobic amino acids, the side chains of which are similar to lipid chains. Hence it may be assumed that the refractive index is similar to that of hydrocarbon chains. Gramicidin A, as mentioned earlier, could change its conformation from double-stranded helix to a single-stranded structure. From the reflectivity measurements indications about the structure of gramicidin A are obtained. Fig. 2 shows the surface pressure (π) and reflection (ΔR) measured as a function of molecular area of gramicidin A. The plateau seen around 15 mN/m has been attributed to a phase transition from a liquid to a condensed state and the conformation of gramicidin A might change from a single- to a double-helical structure at this pressure. This phase transition occurs between 305 and 235 Å^2/molecule and agrees well with the reported data [17,18]. A calculation of the thick-

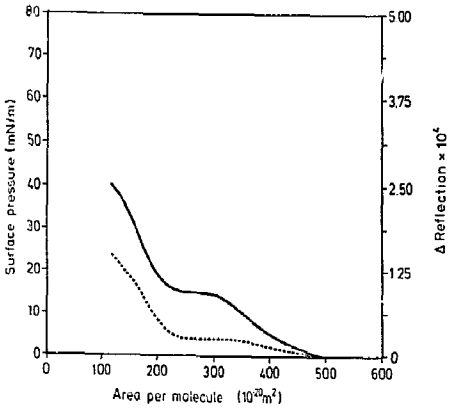

Fig. 2. Surface pressure π (——) and reflectivity ΔR (— — —) plotted as a function of molecular area of gramicidin A. A monomolecular film of gramicidin A was spread on a subphase containing 5 mM Tris-HCl (pH 7.2).

ness from the observed reflectivity values for gramicidin A using the straight line in Fig. 1b indicates that the thickness increases from an alkyl chain length of about 10 carbon atoms to 20 carbon atoms in going from $\pi = 15$ mN/m to $\pi = 30$ mN/m. This would mean a change in thickness from about 12.5 Å to 25 Å.

Conformational aspects of gramicidin A in complex monolayers evaluated by infrared spectroscopy

In order to obtain further information about the thickness and conformation of gramicidin A inside a complex monolayer, structural investigation by FTIR spectroscopy was used. Deposition of DOMA and mixed films of DOMA and gramicidin A on solid substrate was possible only in the presence of 10 mM potassium perchlorate and 1 mM Tris-HCl (pH 7.2). All other ions investigated failed to achieve an efficient monolayer transfer. Addition of potassium perchlorate to the subphase did not change the shape of the gramicidin A isotherm or those of mixed films of DOMA and gramicidin A. At 30 mN/m the transfer ratio was 1 for Y-deposition for DOMA; at 20 mN/m the ratio decreased after deposition of four layers. Deposition of mixed films also gave a transfer ratio of 1 for the first four layers at 30 mN/m which decreased during dipping step of

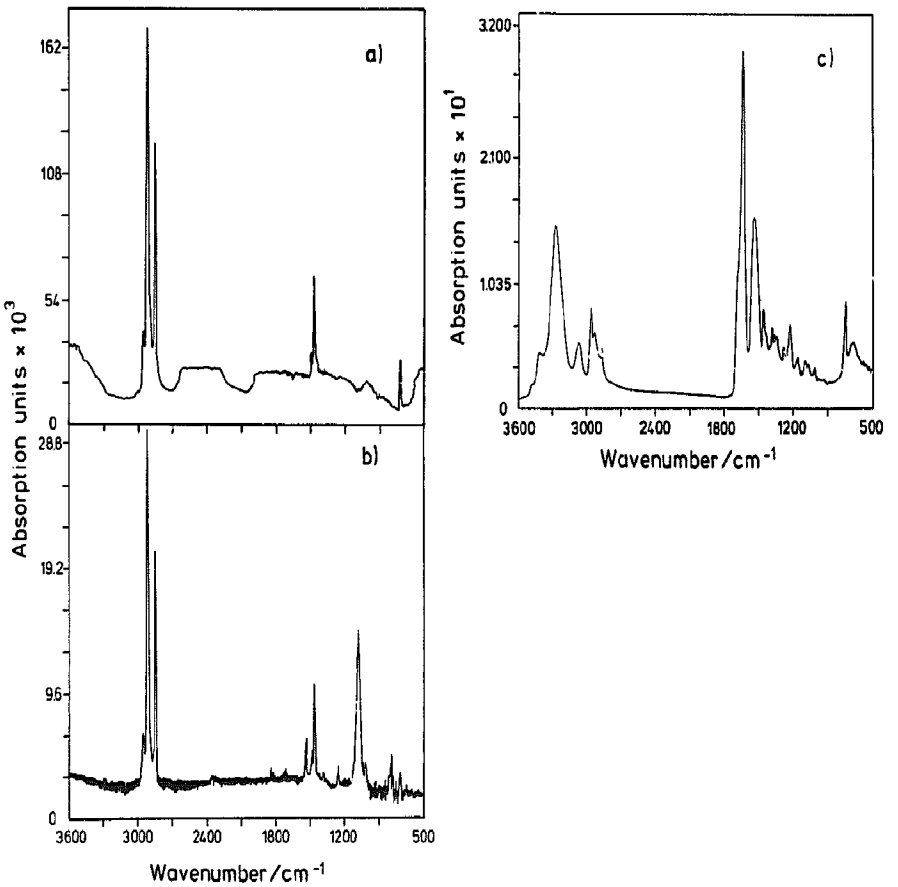

Fig. 3. FTIR spectra of DOMA and gramicidin A. About three droplets of a chloroform solution of each of the compounds were applied to NaCl disks, and spectra were recorded after evaporation of the solvent; (a) DOMA; (c) gramicidin A. The DOMA spectrum representing the solution state is compared to that of 10 transferred layers of DOMA shown in (b). Layers were transferred from a subphase containing 10 mM $KClO_4$ and 1 mM Tris-HCl (pH 7.2) at a surface pressure of 30 mN/m.

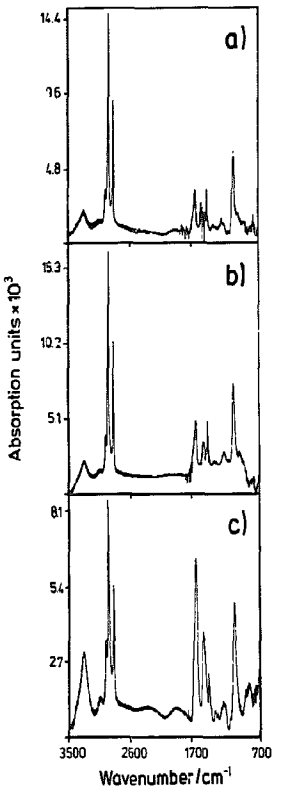

Fig. 4. Overview FTIR spectra of complex DOMA/gramicidin A films. Ten layers of a film with different mixing ratios were deposited from the subphase described in legend of Fig. 3 at a surface pressure of 30 mN/m. Molar ratios of DOMA/gramicidin A were 6:1 (a), 4:1 (b) and 2:1 (c).

further transfer cycles. For 2:1 and 3:1 molar ratios of DOMA/gramicidin, deposition was possible only with a transfer ratio smaller than 1.

Infrared spectra of gramicidin A and DOMA taken from dropping a solution on NaCl disks after evaporation of the solvent were compared with those of transferred layers of DOMA (Fig. 3). Spectra of mixed films shown in Fig. 4 reveal all bands observed for pure DOMA alone. The main bands of gramicidin are also observed in mixed films. These are the band of amide A at 3270 cm^{-1} derived from the N–H stretching vibration, the amide I at 1634 cm^{-1} (C = 0 stretch) and the amide II band at 1540 cm^{-1} (N–H bending vibration). The intensity of the amide I band, when plotted as a function of molar fraction of gramicidin A, follows a linear relationship (not shown). This fact clearly demonstrates the correlation of this band to the fraction of gramicidin present in the mixed layer.

Comparison of spectra of DOMA in the solution state and that of the transferred layers reveals a band near 1080 cm^{-1} present only for the deposited layer (Fig. 3b). This band is assigned to perchlorate anion [19] and not to the C–N stretching vibration. This indicates that the anion binds to the head group of DOMA and that this binding is the reason for dehydration of the head group layer allowing deposition of multilayers. The perchlorate ion itself is large but is surrounded by a small hydration shell resulting in a small total size of the hydrated ion allowing binding to head groups of DOMA. A similar effect has been observed in the group of halide ions interacting with DOMA films on the water surface [20].

The appearance of the amide I band at 1634 cm^{-1} and especially a shoulder near 1680 cm^{-1} is interpreted to be evidence for a double-helical structure found in the crystalline state [21,22]. As shown in Fig. 5, for two of the investigated mixtures of DOMA/gramicidin deposited at 30 mN/m the amide I band appears around 1644 cm^{-1}. Spectra of all five mixtures show a shoulder of amide I band between 1680 and 1684 cm^{-1}. Spectra recorded after time periods of days after transfer of mixed films to the solid support showed no qualitative change. Before transfer of the films, time intervals up to 2 h after spreading of the films did not cause changes in the recorded spectra. Complex formation between gramicidin and K^+ ions is not likely because the monolayer is water-insoluble and hence incorporation of ions in the channel is prohibited. The spectrum of gramicidin A in solution also shows a shoulder at 1685 and strong absorption of amide I at 1634 cm^{-1}. A similar band shape with a strong band near 1630 and a shoulder at 1690 cm^{-1} was found for poly(γ-benzyl-DL-glutamate) with a double-helical structure which was used as a model substance for gramicidin A [23]. For the model compound a shift of the amide I band to 1645 cm^{-1} was reported for a single-stranded β-helix. Single-stranded β-helix of gramicidin is indicated by an originally calculated frequency shift to 1656 cm^{-1} [24] while recent calculations predict values between 1643 and 1648 cm^{-1} [25] and, more im-

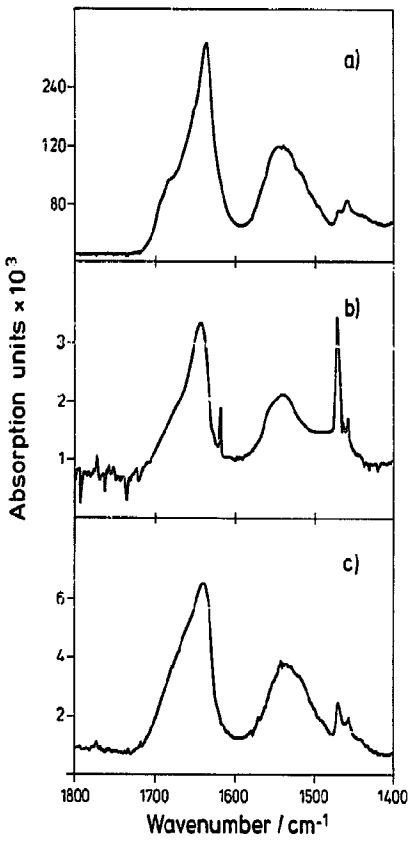

Fig. 5. Comparison of sections of FTIR spectra of gramicidin in solution state with those of mixed DOMA/gramicidin A films. (a) Spectrum of gramicidin in solution state, 10 layers of a DOMA/gramicidin A film with molar ratio 6:1 (b) and molar ratio 2:1 (c).

portant, the disappearance of the shoulder near 1680 cm^{-1}. Measured spectra revealed the disappearance of the shoulder for gramicidin incorporated in lipid vesicles, but the frequency of strong absorption of amide I was only shifted from 1633 cm^{-1} to 1638 cm^{-1} [26].

The presence or absence of the shoulder near 1680 cm^{-1} is more important than the shift of the amide I band in distinguishing between the two conformations. It is concluded that gramicidin incorporated in a monolayer of DOMA has a double-stranded structure because the shoulder at 1680 cm^{-1} is present for all the mixed films transferred at either 20 or 30 mN/m (see Fig. 5). The shift of strong absorption from 1634 to 1642–1644 cm^{-1} after incorporation into the monolayer might indicate the presence of a single-stranded helix. However, this is not sufficient evidence because of the uncertainties of calculated frequencies. It might indicate a stretching of the double-stranded β-helix due to the lateral pressure which would lead to a lengthening of the intermolecular hydrogen bonds resulting in a shift to higher frequency for C=O stretching vibration. Such a lengthening may also be concluded from the increase of the reflection signal above a surface pressure of 25 mN/m (for the molecular area below 180 $Å^2$ in Fig. 2). The fact that gramicidin retains the same structure adopted in solution after incorporation into the monolayer clearly indicates some differences between a monolayer and bilayers of lipid vesicles where a monomeric β-helix is formed [14]. These differences are reduced thickness of a monolayer and reduced mobility of molecules in a spread monolayer above phase transition. The reduced mobility results in a reduced ability of molecules to exchange between a monolayer and a subsolution. Especially the reduced thickness may prevent the conformational change from a double- to a single-helical structure.

Possible orientation of gramicidin at the air/water interface

An estimation of the thickness of gramicidin monolayers from the reflectivity near phase transition and at about 30 mN/m gave values of 12.5 Å and 25 Å, respectively. The value of 30 mN/m was chosen as it corresponds well to the pressure in biological membranes [27]. The values obtained for thickness agree with reported dimensions of a double-helical β-helix with an outer diameter of 15 Å and a length between 26 and 30 Å [13]. These dimensions for the double-helix represent that of the peptide backbone. The bulky tryptophan residues in gramicidin protrude from the long axis of the helix [28] and the calculated area requirement per molecule increases to 220 $Å^2$. Below and during the phase transition (Fig. 2) the molecule could exist as double-helix which is oriented with its long axis parallel to the water surface. Above the phase transition the helix may

become oriented perpendicular to the water surface. Alternative structure of a single-stranded monomeric helix would not give the observed thickness above phase transition. The results from FTIR spectroscopy clearly indicate a double-stranded structure. Below phase transition of gramicidin the possible formation of a single-stranded monomeric helix cannot be excluded. However, this structure seems improbable because two conformational changes during spreading of the organic solution (where gramicidin exists as a double-helix) and compression of the film would be necessary.

Our aim in this work was the characterization of a defined monolayer at the air/water interface. The approach was to use its reflectivity as an experimental tool, which has the advantage that it can be directly applied at the water surface. The reflection measurements were done with pure gramicidin monolayers whereas FTIR spectra were recorded for mixed films of DOMA and gramicidin A. As all of the examined mixtures show the same frequency and band shape of amide I band, it is concluded that no conformational differences exist for gramicidin in pure and mixed films. The calculated values of thickness for gramicidin A were taken from a model with a thin dielectric layer of homogeneous refractive index corresponding to that of hydrocarbons. The influence of the hydrophilic head group region on the interfacial water structure is assumed to be negligible. Although the peptide should show variations in refractive index due to the presence of channels, the contribution of these minor parts of the peptide cannot be evaluated from the reflection data.

In order to obtain improved calculations of reflectivity values, the exact contribution of the hydrophilic region will also be considered in future. Nevertheless, the model allows an estimation to be made about the hydrophobic region and also to evaluate qualitatively the thickness of monofilms of substances with complex structures at the air/water interface.

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