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Grazing incidence X-ray diffraction of highly aligned phospholipid membranes containing the antimicrobial peptide magainin 2

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Abstract We present the first study of grazing incidence X-ray diffraction on a model system of phospholipid membranes and antimicrobial peptides. For this purpose, highly oriented multilamellar samples have been prepared on solid substrates. By this technique, the short-range order of the lipid chains in the fluid L_α phase can be investigated quantitatively, including not only the mean distance between acyl chains, but also the associated correlation length. The short-range order in lecithin is found to be severely affected by the amphiphilic peptide magainin 2.

Key words Membrane structure · X-ray diffraction · Antibiotic peptides · Lipid-peptide interaction

Introduction

The interaction of phospholipid membranes with antimicrobial peptides is currently an active field of research relevant both to biological and pharmaceutical sciences (Bechinger 1997). While the peptide function in the natural organism is often well established, many of the underlying biochemical and structural mechanisms remain unknown. Valuable insight can be derived from simple model systems composed of only a few controlled molecular components, e.g. hydrated phospholipid membranes consisting of one or two lipid components and peptides at various concentrations. Structural information on such systems at the molecular level can be derived, for example, from spectroscopic techniques like magnetic resonance or from X-ray (Ludtke et al. 1995) and neutron scattering (Ludtke et al. 1996) experiments at in situ conditions of temperature and hydration. In the latter case, the amount of molecular information which can be derived is often limited as compared to, for example, crystallographic studies, since the membranes in the fluid L_α state lack long-range molecular order. As we show here, however, these limitations can be partially overcome by the preparation of highly aligned multilamellar stacks of membranes and the use of synchrotron radiation.

In particular, we demonstrate that detailed structural parameters of the acyl chain packing in multilamellar membranes of lipid-peptide model systems can be studied by grazing incidence X-ray diffraction (GID, see Fig. 1) on highly oriented membranes, apart from the average density profile along the membrane normal which is commonly assessed by reflectivity. In contrast to most previous studies on membranes in isotropic aqueous suspension or on supported membranes with a broader orientational distribution (mosaicity), the present method allows for a precise distinction between the momentum transfer parallel $q_\parallel$ and perpendicular q_z to the membranes. Indeed, the samples, prepared for this study exhibit a mosaicity below the instrumental resolution typically around 0.01° (see Fig. 2). Therefore, advanced interface sensitive scattering methods can be applied to such bilayer systems. Here we present a first study of this kind to a system of phospholipid membranes containing the membrane-active, antimicrobial peptide magainin 2.

Magainin 2 belongs to a family of immunogenic peptides expressed in the skin and intestines of frogs (Zasloff 1987; for a review see Matsuzaki 1998). Its broad bacteriocidal, fungicidal, and virucidal activities helps to protect the host organism from infection. Similar to other amphiphatic and antimicrobial peptides of many vertebrate species, magainins interact directly with the microbial cell membrane rather than with specific membrane proteins (Wade et al. 1990), subsequently causing an increase in membrane permeability and cell lysis.

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While random coiled in aqueous solution, magainins assume right-handed α-helical conformations in the presence of phospholipid bilayers or organic solvents (reviewed in Bechinger 1997). NMR spectroscopy of magainins which have been reconstituted into oriented phospholipid membranes indicates that the helix axis of the peptide is oriented parallel to the bilayer surface when the concentration of magainin 2 is in the range 0.8–3 mol% (Bechinger et al. 1996). Since helical wheel analysis of the 23-residue sequence GIGKFLHSAKKFGKAFVGEIMNS shows one side of the helix to be hydrophobic and positively charged (4–5 net charges per molecule at neutral pH) while the other is hydrophilic, a parallel binding state with the hydrophobic side groups indented into the lipid chain region seems highly plausible. For the present study of magainin 2 in neutral membranes of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidycholine (DMPC), we have probed the lateral structure of the membranes down to molecular scales in the DMPC/magainin 2 model system. We observe a strong decay in the ordering of the hydrocarbon chains and the respective lateral correlations with increasing peptide concentration.

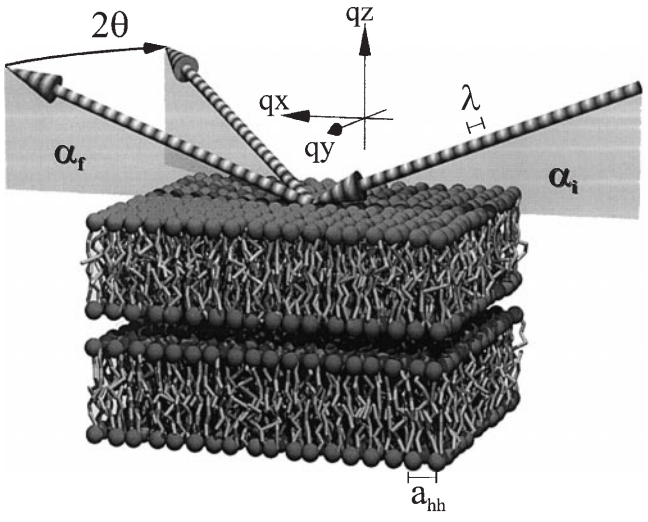

Fig. 1 Sketch of the grazing incidence X-ray diffraction (GID) experiment, where the diffracted beam is measured out of the plane of incidence at an angle 2θ while the angle of incidence α_i and exit α_f are kept close to the critical angle α_c

Materials and methods

Peptide synthesis

Magainin 2 amide (GIGKFLHSAKKFGKAFVGEIMNS) was prepared by solid-phase peptide synthesis on a Millipore 9030 automated peptide synthesizer using Fmoc chemistry. The peptide was purified by reversed phase HPLC using a Nucleosil 300-7 C4 preparative column (Macherey and Nagel, Düren, Germany) and a water/acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The identity and high purity of the peptides were further analysed by matrix-assisted laser desorption mass spectrometry.

Sample preparation

Multilamellar bilayers of DMPC (Avanti, Aao, USA) containing magainin 2 at varied concentrations were prepared on cleaned silicon or glass wafers by spreading

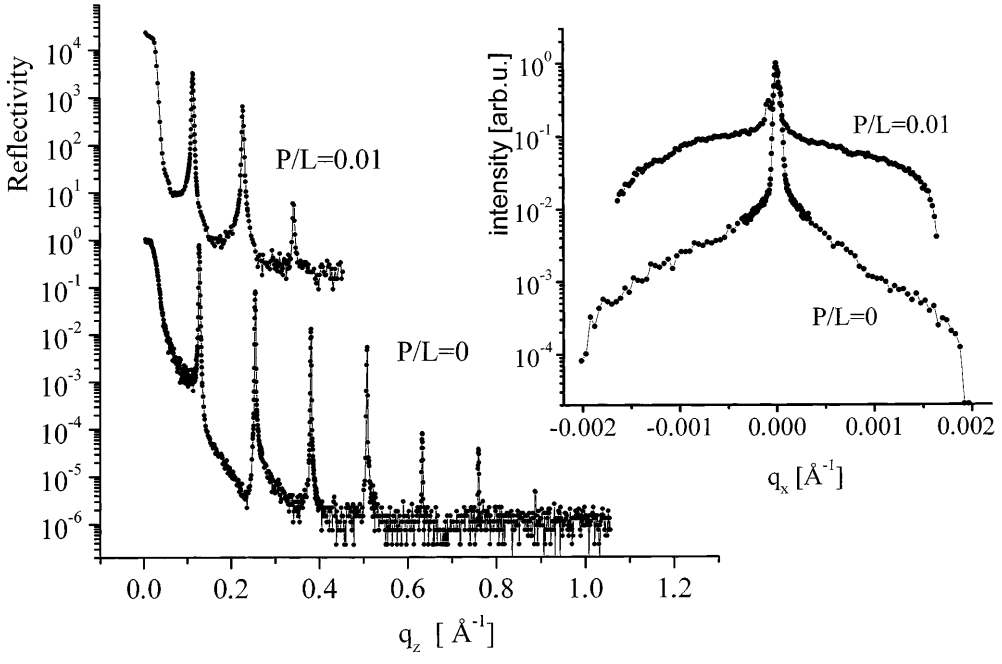

Fig. 2 The specular reflectivity of a sample at $P/L = 0.01$ (*top*) compared to pure DMPC (*bottom*), together with the corresponding rocking scans (*inset*) showing the sharp specular and the broad diffuse scattering, respectively. The disorder in the multilamellar stack clearly increases with peptide concentration

from organic solution, similar to the procedure first described by Seul and Sammon (1990). The important point is to simultaneously meet the solvation and wettability requirements. For sample deposition the substrates were cleaned by subsequent washing in methanol, twice with ultrapure water (specific resistivity ≥18 MΩ cm, Millipore, Bedford, Mass., USA), and one bath in trichloroethylene followed by extensive rinsing in ultrapure water. Finally, the substrates were rendered hydrophilic by washing in a 5 M solution of KOH in water (glass, quartz) and a saturated KOH solution in ethanol (silicon), respectively, for about a minute. Subsequently, they were rinsed several times in Millipore water. The lipid and peptide components were codissolved in the desired ratio (molar ratio P/L) in trifluoroethanol at total concentrations between 4 and 20 mg/ml, depending on the total mass to be deposited. A drop of 0.1 ml was then carefully spread onto well-leveled and cleaned substrates of typically 15×15 mm^2, yielding average film thicknesses of about $D \simeq 10$ μm. The spreaded solution was allowed to dry only very slowly in a closed chamber, with excess solvent placed close to the substrate to increase the ambient solvent vapor pressure. The slow evaporation process extending over more than 12 h prevented film rupture an dewetting and yielded a very uniform dry film, which was subsequently exposed to high vacuum over 24 h in order to remove all traces of solvent. The films were then rehydrated in a hydration chamber while tempering in successive temperature cycles around the main phase transition of the lipid to anneal defects. The high degree of membrane orientation with respect to the substrate of typically better than 0.02° was a prerequisite for quantitative specular and nonspecular (Sinha et al. 1988) reflectivity measurements.

For the X-ray experiments the films were kept in a closed, temperature-controlled chamber. The chamber consisted of two concentric aluminum cylinders, with kapton windows. The top and bottom of the inner cylinder were heated or cooled by a flow of oil, connected to a temperature-controlled reservoir (Julabo, Germany). The space between the two cylinders was evacuated to minimize heat transfer. The temperature was measured close to the sample holder by a Pt100 sensor, with a stability of better than 0.03 K over several hours. At the bottom of the inner cylinder a water bath was filled with salt-free Millipore water such that the sample was effectively facing a vapor phase of 100% relative humidity.

X-ray scattering

The samples were then routinely characterized by X-ray reflectivity at a high resolution in-house rotating anode reflectometer with a dynamic range of up to eight orders after correction of the diffuse background. The GID experiments were carried out at the experimental stations D4 (bending magnet) and BW2 (wiggler beamline of the synchrotron radiation source at HASYLAB/DESY using photon energies of 20 and 17 keV, respectively. In GID the X-ray beam impinges at a small α_i and is measured at small α_f after being scattered laterally out of the plane of incidence by an angle 2θ (Dosch 1992); see Fig. 1.

Results

The X-ray reflectivity profiles of two representative samples, shifted vertically for clarity, are displayed in Fig. 2 as a function of perpendicular momentum transfer q_z. $n = 7$ orders of extremely sharp, multilamellar Bragg peaks are recorded in the case of pure DMPC, indicating a high degree of translational order and a well-defined lamellar periodicity of $d = 4.96$ nm, with a membrane thickness of $\delta_m = 3.7$ nm, as determined from least-square fits to the structure factor, or alternatively Fourier synthesis from integrated peak intensities (Salditt et al. 1999). The relative humidity in the chamber was near 100%; however, the bilayers were swollen only up to a repeat distance of about $d = 50$ Å, in agreement with a phenomenon known as the vapor pressure paradoxon (Podgornik and Parsegian 1997). It is important to note that, at this partial hydration, fluctuations of the membranes are efficiently suppressed, and no broadening of the tails is observed for pure DMPC with increasing peak order, in contrast to isotropic aqueous suspensions of multilamellar vesicles (Zhang et al. 1994). Extensions of the lineshape models appropriate for partially hydrated, substrate-supported films are currently in preparation (Salditt et al. 1999).

Under the same humidity conditions, the sample at $P/L = 0.01$ has swollen to $d = 5.54$ nm, as an effect of electrostatic repulsion induced by the peptide charge. At the same time, the number of Bragg peaks has decreased to $n = 3$, indicating a drastic onset of disorder. Correspondingly, the rocking scans displayed in the inset show a much higher ratio of the diffuse to specular intensity. At the same time the scans prove the excellent alignment of the membranes with mosaicities below the instrumental resolution. It is important to note that the broad diffuse background does not stem from powder-like domains, since it shows the refraction effects typical for diffuse scattering of highly aligned systems. The data shown in Fig. 2 are part of a general trend, which is observed over the whole peptide concentration series: a decrease of specular, and an increase of nonspecular (diffuse), scattering with P/L. These effects illustrate the dramatic changes in lamellar ordering and elasticity induced by the peptides.

The lateral structure of the membranes on molecular length scales can be probed by measurements taken in the GID mode (see Fig. 1). This scattering method has been developed over the last decades as a powerful tool in surface and subsurface analysis of crystalline solids (Dosch 1992). The X-ray optics and refraction effects are used of efficiently discriminate against bulk background

signal and to gain depth resolution. Here we apply the method to a highly oriented stack of lethicin membranes to study the effect of magainin 2 on the lateral order. The scattering cross section as probed in GID can be written is distorted wave Born approximation as (Dosch, 1992, Helm et al. 1991)

$$\frac{d\sigma}{d\Omega} \propto |T_i(\alpha_i)T_f(\alpha_f)|^2 S(q_\parallel, q_z) \quad (1)$$

where $T_{i/f}$ denote the Fresnel transmission functions which lead to the characteristic Vinyard peaks when α_i or α_c equal the critical angle. In contrast to these optical effects, the structural information is contained in the structure factor

$$S(q_\parallel, q_z) = \left\langle \left| \int d\mathbf{r}\, \rho(\mathbf{r}) e^{i\mathbf{qr}} \right|^2 \right\rangle \quad (2)$$

where the vertical scattering depth along z can be tuned by α_i and α_f. Here, we present the first GID measurements of highly aligned mulilamellar membranes, which offer the advantage of much higher signals as compared to self-assembled monolayers, and furthermore allow the observation of biologically more relevant bilayer systems unperturbed by the substrate or the air/water interface.

Figure 3 shows the measured scattering distribution of pure DMPC at $T = 45$ °C as a function of $q_\parallel$, as measured at the D4 bending magnet station at 20 keV. The angle of incidence was set to $\alpha_i = 0.37°$, the exit angle to $\alpha_f = 0.23°$. In the humidity chamber the bilayers were swollen up to a repeat distance of about $d = 50$ Å. As is well known from isotropic solution scattering, the peak at $q_\parallel = 1.390$ Å^{-1} corresponds to the nearest neighbour distance $a = 4.52$ Å of individual acyl chains. Thus, the short-range lateral order of the lipid molecules in the liquid L_α state is probed. It is important to note that, contrary to the isotropic case, the present method not only allows for a precise determination of the peak position but also the associated lineshape. Indeed, the peak can be fitted to a Lorenztian of width 0.367 Å^{-1} (FWHM), corresponding to an exponential decay of the short-range liquid order with a decay length of $\xi = 5.4$ Å. Similarly, new details in the diffracted intensity distribution, like the broad shoulder or side peak at about $q_\parallel \simeq 0.8$ Å^{-1} corresponding to $a_{hh} = 7.8$ Å, are observable in this setup. Speculatively, one may be led to associate the shoulder with a correlation distance in the lipid headgroups region. Furthermore, the liquid correlation peak exhibits a sharp component at the position of the gel phase reflection (see arrow), indicating the presence of some gel phase domains even though the system is far away from the phase boundary. Possibly, these domains could be located close to the substrate. The inset of Fig. 3 shows the sharpening and increase of the reflection upon cooling the sample below the main phase transition. The curves are scaled according to the true intensity ratios (roughly 2.5 orders of magnitude between the peak intensities in the gel and liquid phases). The curves also reflect the ratio between the wide angle peaks and the diffuse scattering in the plane of incidence at $q_\parallel = 0$.

How does the scattering distribution change if the amphiphilic peptide magainin 2 is added? In Fig. 4(a)

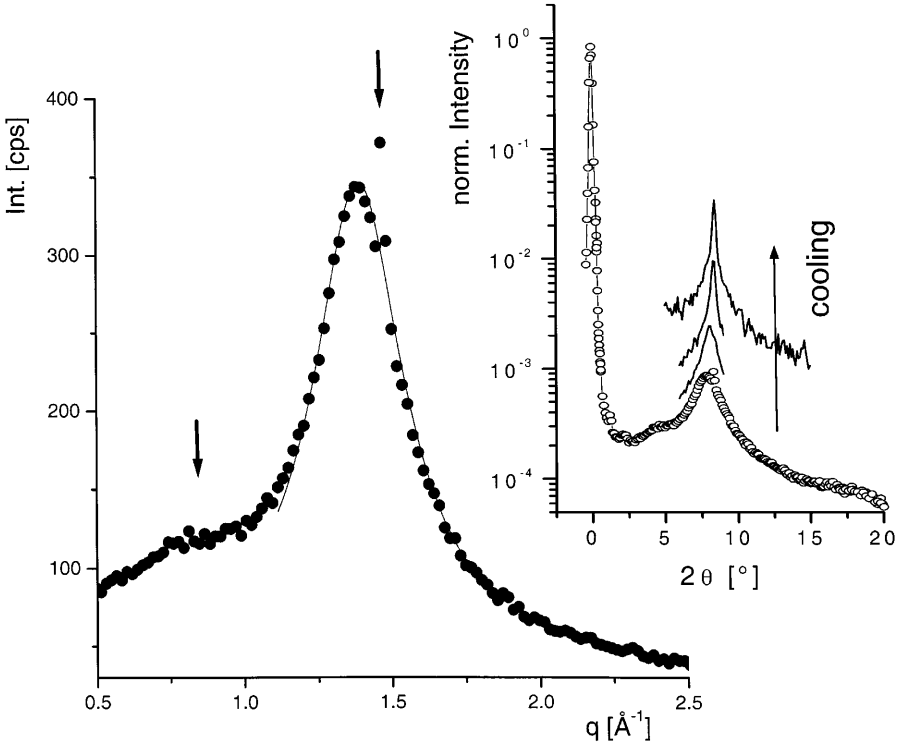

Fig. 3 GID on mixtures of DMPC at $T = 45$ °C. The strong and broad peak at $q_\parallel = 1.39$ Å^{-1} reflects the short-range order in the liquid L_α phase. The shoulder at lower q may reflect the less pronounced correlations between near-neighbor headgroups, not observable in powder data. The *inset* shows the change of the peak around the main phase transition upon cooling (*from bottom to top*)

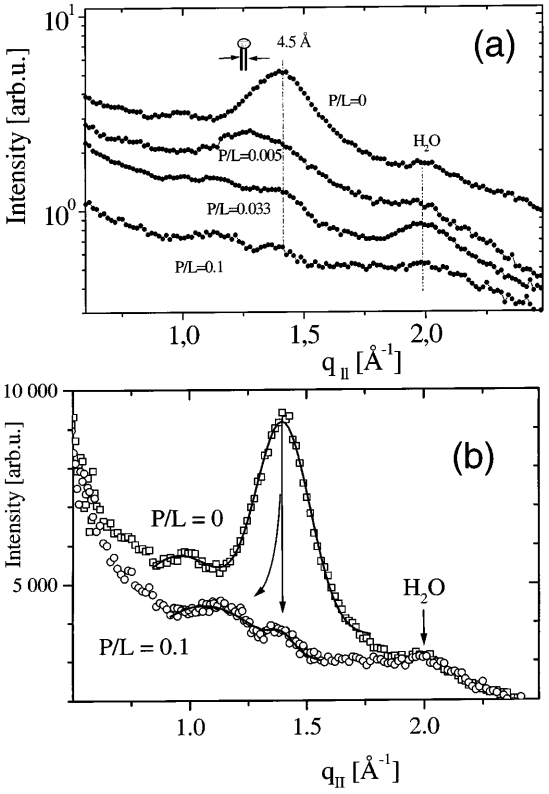

Fig. 4 **a** GID on mixtures of DMPC and magainin 2 on a logarithmic scale with the curves of different P/L values shifted vertically for clarity. **b** Comparison of a pure DMPC and a $P/L = 0.1$ sample on a linear scale with intensities normalized to the water peak at around 2 $Å^{-1}$. As can be clearly seen, the peptides induce a strong decay of the tail-tail correlations (see text)

several curves of increasing magainin concentration (from top to bottom, shifted vertically for clarity) are shown in a logarithmic representation. The scans were taken at the BW2 wiggler beamline at 17 keV photon energy and $\alpha_i = \alpha_f = 0.2° \simeq 2\alpha_c$. Magainin 2 clearly affects the ordering of the hydrophobic tails in a dramatic way, even at moderate concentrations. This may be taken as an indication that the peptides intercalate into the lipid chain region perturb the chain ordering rather than merely adsorbing onto the headgroups, which can be expected from their amphiphilic nature. For the $P/L = 0.1$ sample, the intensity distribution is shown in Fig. 4(b) on a linear scale in the correct intensity ratio with respect to the pure DMPC sample. The two broad peaks (simulation shown as solid lines could be attributed to two different populations of lipids, one at the ordinary nearest neighbor distance $a = 4.55$ Å and one at $a = 5.9$ Å in the vicinity of the peptides, where the lipophilic side groups of the helix indent into the chain region and may change the equilibrium conformation. This coexistence of lipids would occur on lateral length scales of the inter-peptide distance, and may not be confused with macroscopic phase separation, which can be excluded from the lamellar reflections. Furthermore, it is unlikely that the pitch of the α-helix of magainin may produce a maximum at this position, since the pitch is expected to be smaller. However, reciprocal space mapping in q_z and $q_\parallel$ could give further information and, most importantly, may also include the signature of the helical from factors. Such work is in progress.

While the changes in the acyl packing are most obvious, we found no evidence for peptide aggregation, pore forming, or significant lateral correlation. In particular, we did not observe a correlated liquid of densely packed channels, leading to intensity maxima at intermediate values of $q_\parallel$ as reported (Ludtke et al. 1996) for the case of charged bilayers. Of course, the present method is not sensitive to transient channels and the metastable conformation of minority populations. This point is investigated in more detail by ongoing synchrotron and neutron experiments, also in view of lipid specificity.

In summary, we have shown that the application of interface-sensitive scattering techniques, in particular GID, on highly oriented phospholipid membranes with and without antimicrobial peptides opens up a new experimental tool for the investigation of lateral membrane structure at high resolution under in situ conditions in the biologically relevant liquid L_α phase. In pure DMPC, the chain-chain correlation peak was monitored at thigh precision. At smaller parallel momentum transfer, diffuse intensity due to conformal membrane fluctuations was probed. The precise distinction between normal and lateral momentum transfer in the highly oriented samples can be used to develop refined models of structure and fluctuations from reciprocal space mappings. As a first example, the antimicrobial peptide magainin 2 was shown to promote significant disorder both in the lamellar stacking as well as the local molecular order in the membrane. Specifically, a decay of short-range order in the packing of the lipid acyl chains of DMPC was observed, probably resulting from a high degree of peptide intercalation. In future, these structural effects have to be investigated for different anionic and neutral lipids and put into perspective of the antibiotic function. The current scattering methods can be applied to numerous problems of correlations and structure in membranes in their L_α state, including other lipid/peptide model systems, native or reconstituted lipid/protein systems, and the effects of sterols and other additives on the membrane structure.

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