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X-ray scattering in the plane of membrane

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abstract.--We demonstrate a technique for measuring x-ray (or neutron) scattering with the momentum transfer oriented in the plane of a membrane, for the purpose of studying the lateral organization of proteins and peptides. As an example, the controversial question of whether gramicidin forms aggregates in membrane was investigated. We measured dilauroylphosphatidylcholine (DLPC) bilayers containing gramicidin in the molar ratio of 10:1. Very clear scattering curves reflecting gramicidin channel-channel correlation were obtained. Analysis of the data shows that the channels were randomly distributed in the membrane. We suggest that oriented proteins may provide substantial x-ray contrast against the lipid background without requiring heavy-atom labeling. This should open up many possible new experiments.

The biological functions of membrane-active peptides, such as alamethicin [1] and magainin [2], are related to the phenomena of lateral aggregation on the membrane. It has been shown [1, 2] that at low concentrations, the peptide molecules are adsorbed on the membrane surface. However, above a critical peptide to lipid molar ratio, the peptide undergoes a cooperative change of state that has been correlated to the cytolytic activity of the peptide [2]. It is reasonable to assume that the process of phase transition involves lateral aggregation of the peptide molecules. Large membrane proteins, such as bacteriorhodopsin and rhodopsin, are also known to aggregate, even in the absence of cytoskeletal interactions [3,4]. Such protein aggregations have been observed by freeze-fracture electron microscopy [3,4]. However the resolution of this technique is not fine enough to detect small peptides. Also the possibility of artifacts in the freeze-fracture process is difficult to assess. Atomic force microscopy is potentially a powerful tool for imaging membrane proteins. But again the membrane has to be fixed, otherwise the probing force would move the molecules, making imaging impossible [5]. Thus only the scattering method is applicable to the liquid (L_α) state of membranes. With suitably labeled samples, in-plane x-ray or neutron scattering provides a direct measurement of the lateral particle-particle correlation in the plane of the membrane. This idea is tested in the following experiment.

There have been reports of possible gramicidin organization in membrane [6]. We investigated this problem by performing x-ray in-plane scattering on hydrated DLPC bilayers containing gramicidin in the molar ratio of 10:1. This sample has been studied previously by x-ray lamellar diffraction to determine the binding sites for monovalent and divalent cations in the gramicidin channel [7, 8]. The water-intercalated multiple lipid bilayers were aligned between two SiO_2 -coated, polished Be plates [9]. In-plane scattering was carried out with a line source of $\text{Cu K}\alpha$ radiation, by θ - 2θ scan or by normal incidence--we call the latter geometry 0- 2θ scan. Within $\theta \leq 15^\circ$ the results of θ - 2θ scan and 0- 2θ scan

are essentially the same. In these measurements the momentum transfer of the photon scattering is parallel to the substrate surfaces; therefore, if the membranes are aligned homeotropically, the results are in-plane scatterings. However, if the sample contains smectic defects where the bilayers may be oriented perpendicular to the substrate surfaces, the results will contain lamellar diffraction. Lamellar diffraction of a multilayer sample is much stronger than in-plane scattering. Thus the technique for preparing a well-aligned multilayer sample [9] is essential for in-plane measurements. In Fig. 1, one notices a small peak at about 10° , corresponding to the first Bragg order of lamellar diffraction. The smallness of this peak indicates that the smectic multilayers were relatively free of defect. If a sample is not aligned well, that is, if it contains too many defects, the lamellar peaks would swamp the in-plane signal.

Fig. 1 shows the in-plane scattering of membranes containing gramicidin with or without thallium ions and that of pure membranes containing no gramicidin, after the background signal of beryllium plates and kapton windows was subtracted. The diffuse band near 10° , corresponding to the momentum transfer $q \sim 2\pi/(4.5 \text{ \AA})$, indicates that the lipid in each sample is in the liquid crystalline state [11, 12]. Outside of this liquid-paraffin peak, the in-plane scattering curve of a pure lipid bilayer is featureless. Samples containing gramicidin show additional peaks below 6° . Surprisingly, the gramicidin signal without thallium is very clear. As expected, two thallium ions bound inside each gramicidin channel increased the intensity of the gramicidin signal.

From the lamellar diffraction measurement [7], we know that gramicidin in our samples was in the channel form. The channel is approximately a cylinder of $26 \text{ \AA}$ long and $18 \text{ \AA}$ in diameter. The channel has a pore of $4 \text{ \AA}$ in diameter going through the cylindrical axis. Two symmetric Tl ion binding sites are located inside the pore $9.6 \text{ \AA}$ from the midpoint on each side [7, 8]. In comparison, the cross section of a DLPC molecule in the L_α phase is $\sim 52 \text{ \AA}^2$. Since the lipid is in the liquid state, the scattering curve of the gramicidin in a bilayer, I' , can be written as [10]

$$I' / I_e = NF^2(q) + NF^2(q) \int [n(r) - \bar{n}] J_0(qr) 2\pi r dr, \quad (1)$$

where I_e is the scattering intensity by a single free electron, N the total number of channels, $n(r)dA$ the number of channels in the area element dA at a position r relative to a channel, $\bar{n}$ the average density of the channels, J_0 the zeroth order Bessel function, and the integration is taken over the plane of the bilayer. F is the effective molecular form factor for a channel defined as

$$F(q) = \int [\rho_p(r) - \rho_l(r)] \exp[iq \cdot (r - r_a)] dV(r), \quad (2)$$

where r is the radial vector in the plane of the bilayer, r_a the position the channel axis, ρ_p the scattering density of gramicidin and ρ_l that of the lipid molecules. The radial distribution function $2\pi r n(r)$ is obtained from Eq. (1) by the Bessel transform,

$$2\pi r n(r) = 2\pi r \bar{n} + r \int \{I' / [NI_e F^2(q)] - 1\} J_0(qr) q dq. \quad (3)$$

Fig. 2a shows the form factor of a gramicidin channel. Fig. 2b shows the radial distribution function $2\pi r [n(r) - \bar{n}]$ obtained Eq. (3). The distribution function is qualitatively that of a liquid [13]. The first maximum of the radial distribution corresponds to the distance to the nearest neighbors; in this case it is $27 \text{ \AA}$.

To interpret the results obtained above, we performed computer simulation of harddisks in a plane. We let 600 harddisks of radius R diffuse randomly in a square plane with the periodic boundary condition and with the constraint that no two harddisks could overlap with each other. After the system

reached equilibrium, the scattering intensity $I_s = \left| \sum_j \exp(i\mathbf{q} \cdot \mathbf{r}_j) \right|^2$ was computed and averaged over time. The form factor of the disk is not included. Thus the simulated intensity is to be compared with the experimental curve divided by the form factor squared, $I'/F^2(q)$. The harddisk model is defined by two parameters, i.e., the radius R and the areal density of the disks—we use the corresponding peptide to lipid molar ratio P/L to represent the density. Fig. 3 shows the position of the first scattering peak and the distance to the nearest neighbors (from the first max. of $2\pi r[n(r) - \bar{n}]$) as a function of R and density. It is clear that the peak positions of the scattering curve and therefore the distance to the nearest neighbors are largely determined by the radius R and are relatively insensitive to the density. On the other hand, the peak amplitude and peak width are mainly determined by the density: the higher the density, the larger the peak amplitude and the narrower the width. The experimental data of gramicidin, $I'/F^2(q)$, were more or less reproduced by the harddisks of $R=12 \text{ \AA}$ at a density equivalent to $P/L=1/10$ (Fig. 4).

Thus gramicidin in a lipid bilayer is randomly distributed without significant aggregations. The effective hardcore-like repulsive radius is about $12 \text{ \AA}$, $3 \text{ \AA}$ larger than the size of the channel. That probably implies that there is always at least a lipid molecule between two gramicidin channels.

It is well known that the average bulk electron density of proteins ($0.33 \text{ e/\AA}^3$ in the case of gramicidin) is close to that of lipids ($0.35 \text{ e/\AA}^3$ in the case of DLPC). And one does not expect to see clear protein signal in lipid by x-ray scattering without heavy-atom labeling. For this reason, neutron scattering with deuterated samples is often preferred for studying protein correlation in membranes. In this experiment, the protein molecules are oriented in the membrane and the in-plane scattering depends on the areal electron density rather than the bulk electron density. If a protein is oriented, there may be areas of high and low electron densities in different parts of the cross section. For example the cylindrical shell of the gramicidin backbone discussed earlier has an areal electron density about twice that of the lipid background. That might explain why we could easily detect the gramicidin signal even without thallium ions. This discovery, that protein signal can be detected in membrane without labeling, should open up many possible new experiments.

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Fig. 1. -- a) In-plane scattering intensity of pure DLPC bilayers. The inset shows the two possible scattering geometry, θ - 2θ scan and 0 - 2θ scan. b) In-plane scattering intensities of DLPC bilayers containing gramicidin with thallium ions (molar ratio 10:1:1--dashed line) and without thallium (molar ratio 10:1:0--solid line). The samples were equilibrated in 100% RH. The background signal of the beryllium plates and kapton windows has been removed from the data. The sharp peaks near 1° are the first Bragg reflections of lamellar diffraction.

Fig. 2. -- a) The form factor for the gramicidin backbone in the channel form, normalized to one at $\theta = 0^\circ$. The scattering intensity of gramicidin (from Fig. 1b, dashed line) is multiplied by a constant factor to match the form factor at the high angle region. b) The radial distribution function $2\pi r [n(r) - \bar{n}]$, unnormalized.

Fig. 3. The result of computer simulation of the harddisk model. a) The position of the first scattering peak as a function of the disk radius. b) The distance to the nearest neighbor, obtained from the first maximum of $2\pi r[n(r) - \bar{n}]$, as a function of the disk radius. c) The position of the first scattering peak as a function of the disk areal density, expressed in the corresponding peptide to lipid molar ratio P/L . d) The distance to the nearest neighbors as a function of P/L .

Fig. 4. The experimental data (circles) of gramicidin, $I'/F^2(q)$, and the simulated scattering intensity (solid line) of harddisks of $R=12$ Å at a density equivalent to $P/L=1/10$