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Direct measurement of ion distributions between lipid membranes with X-ray diffraction

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Abstract

A new and simple method is introduced, which allows the direct measurement of the distribution of ions between lipid membranes with a conventional X-ray source. It is based on a difference method which is combined with a swelling experiment. The presented method is applied to unoriented powder samples of 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylglycerol in different ionic solutions of RbCl and $BaCl_2$. From these samples, results for the cation distributions with a resolution of 12 Å were obtained. Analysis of the experimentally obtained distributions shows that the simple Gouy-Chapman theory is probably not able to describe the experimental data consistently. Instead a better correspondence between experiment and theory is obtained with a generalized linear Gouy-Chapman model which takes into account the finite width of the lipid/electrolyte interface. Possible future improvements of the presented method with regard to the obtained resolution and the possibility to obtain ion densities on an absolute scale are discussed.

Keywords: X-ray diffraction; Ion distribution; Lipid; Gouy-Chapman theory; Membrane electrostatics

1. Introduction

Biological membranes are usually charged and located in an electrolytic environment. A so-called diffuse double-layer with a strongly position dependent ion distribution is formed in the aqueous medium near the membrane. The diffuse double-layer determines the electrostatic interactions between adjacent membranes and between membranes and charged particles. Further, biological processes at membranes that are sensitive to the presence of ions depend on the local ion concentrations and thus on the diffuse double-layer. Correspondingly it is an important goal of membrane biophysics to gain insight into the structure of the diffuse double-layer and how it depends on the composition of the ionic solution and/or the lipid membrane.

In contrast to its significance, membrane electrostatics is still largely unknown. Important quantities like the membrane charge and the spatial extension and the structure of the diffuse double-layer are difficult to measure. Usually indirect methods are applied ([1,2], and references, cited therein).

Another reason for experimental studies of ion distributions is to proof or disproof different theories. The probably most successful and most widely used theory of electrolytes at charged interfaces is the simple Gouy-Chapman theory [3]. This theory assumes a smooth, homogeneously charged interface and uses the 1-dimensional Poisson-Boltzman equation to calculate the spatial structure of the diffuse double-layer. Recently, it was shown by Peitsch et al. [4] in a thorough computational analysis, that the Gouy-Chapman theory is under certain conditions indeed a valid description of the diffuse double-layer near charged lipid membranes for solutions of monovalent electrolytes at relatively low concentrations. Discreteness of charge effects and the exact structure of the lipid head-groups seems to be of minor importance as long as the typical distances between the charges are smaller than about twice the Debye length. However, it is well known from several theoretical studies [1,5–8], that this simple theory should be wrong for divalent and higher charged ions. But even the more thorough statistical mechanical models of the diffuse double-layer depend on several simplifying assumptions. Thus, experimental methods are in demand, that can help to evaluate the different theories.

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To the best of my knowledge, at present only two experimental methods are available, which can probe the ion distribution near or between charged lipid membranes *directly*. One method [9] uses neutron diffraction and is limited in that it needs bulky organic 'model ions' which contain hydrogen that can be replaced by deuterium. The second method uses X-ray standing waves [10] and is only suited for low ion concentration ($\leq 10^{-3}$ M). Furthermore, the latter measurements can only be done at synchroton, because of the need of an extremely high incident beam intensity. A general disadvantage of both methods is therefore the high experimental effort. The method described in this paper, however, allows the direct measurement of ion distribution between charged lipid membranes with a conventional X-ray source. The resolution achieved in experiments with powder samples is approximately 12 Å and comparable to the resolution of the two more sophisticated methods mentioned above. An improvement of the obtained resolution up to approximately 5 Å to 6 Å should be possible in future experiments by using samples with oriented lipid multilayers. Further it will be possible to obtain the total number of ions in the interlamellar region by exploiting a changed experimental protocol.

2. Materials and methods

1,2-Dipalmitoyl-*sn*-glycero-3-phosphorylglycerol (DPPG) was from Avanti (Birmingham, AL) and used as obtained. $BaCl_2$ and RbCl (both purum, p.a.) were obtained from Merck (Darmstadt, Germany). Water was doubly distilled.

Samples for the swelling experiments were prepared gravimetrically by adding water to dry lipid. The swelling data were only used to obtain an interpolated structurefactor for the lipid/water system at a lamellar repeat distance that is identical to the lamellar repeat distance measured with a sample containing electrolyte. This interpolation is done by using the sampling theorem, as is explained in more detail in the theory section. The interpolated structurefactors of the lipid/water samples are normalized to the structurefactors of the lipid/electrolyte system on a *relative* scale. Knowledge of the absolute electron density of the system is not necessary in such an approach, and thus the exact amount of water was of no importance. Correspondingly, the exact amount of water of the samples for the swelling experiment was *not* monitored during the sample preparation. Samples containing electrolyte were prepared by adding a large amount ($\sim$ 2 ml) of electrolyte solution to a small amount ($\sim$ 30 mg) of lipid. All samples were allowed to equilibrate at room temperature for at least three days. In the case of the lipid/electrolyte samples the ion permeation through the membrane was enabled by a preceding 12 h equilibration at the chain melting temperature of the lipid ($\sim$ 41°C) before the final equilibration. Electrolyte concentrations used were: RbCL: 1 M, 2 M and 4 M; $BaCl_2$: 1 M.

All measurements were done at room temperature. For the X-ray experiments, the lipid samples were kept in a sealed sample holder between two mica windows, held 1 mm apart by a Teflon spacer. The lipid/electrolyte samples were filled into the sample holder with an excess amount of electrolyte. In independent experiments the sample holder was found be gas tight for at least one week, even at temperature well above room temperature. On the timescale of the measurements (typically 4–8 h) the experiments can thus be considered to be done at a constant sample composition.

A 1.5 kW X-ray tube with copper anode and a line focus was used. The Cu–$K_{\alpha 1}$ line was focused on a linear position sensitive detector with a Ge-(111)-monochromator. To obtain the positions and the integrated peak intensities, each of the observed peaks was fitted with a Cauchy line profile [1] [11], which was folded with the resolution function of the detector.

The relatively large entrance window and the finite volume of the gas filled detector chamber of the linear position sensitive detector causes a distortion of the ideal line profile of a Debye-Scherrer ring. This effect is further enhanced by the line focus of the X-ray source. However, all the smearing effects are of geometrical nature and can be calculated [12]. Such a calculation results in the following expression for the line profile (in the reciprocal space) as a function of the scattering vector, q,

$$I(q) = I_0 \int_{q_l}^{q_h} \frac{\gamma^2}{\left(\sqrt{q^2 + h^2} - q_0\right)^2 + \gamma^2} e^{-h^2/w^2} \, dh \qquad (1)$$

where q_0 is the position of the undistorted Debye-Scherrer line and γ its width. The integrated peak intensity is given by $I_{int}(q) \sim I_0 \gamma$. It should be noted, that q_0 and I_{int} are describing the 'real', undistorted Debye-Scherrer line as it would be seen by a fictitious detector with an entrance window of an infinite small height. Thus, the instrumental smearing is removed by analyzing the diffraction peaks with Eq. 1. The parameters q_l, q_h and w are instrumental parameters, describing the diffractometer. They were determined independently and are constant, as long as the experimental set up (sample to detector distance, slits) is unchanged. The exponential function in the integrand models the spatial variation of the incident intensity from the line focus.

The error in the lamellar repeat distance was always smaller than 0.1 Å. Because fitting the experimentally

[1] Both, a Gaussian and a Cauchy line profile can be used to model the profile of a Debye-Scherrer line [11]. Empirically, it was found, that a Cauchy profiles provides systematically a better fit than a Gaussian profile. An explanation is, that for a powder with a *distribution* in the 'grain size', the averaged interference function is better described by a Cauchy profile. This can easily be shown with numerical simulations (S. Kirchner: unpublished results) and was also already known long ago ([11], and Refs. 21, 22 cited in Chapter 9.1.3, p. 635).

observed line profile with Eq. 1 removes the distortion of the ideal Debye-Scherrer line profile, the usual corrections could be used to calculated the absolute value of the structurefactor from the integrated intensities, i.e., the structurefactors were calculated via $|F_h| = \sqrt{h^2 I_h}$, where h is the order of the reflex [13]. This relation corrects for the effect of the Lorentz factor and the distribution of the *total* diffracted intensity on a circle, the radius of which is proportional to the order of the Debye-Scherrer line. The results of the swelling experiments were normalized by the method of Blaurock [14,15].

3. Theory of the method

The simple idea of the method introduced in this paper is, to subtract the electron density of a lipid/water mixture from the electron density of a corresponding lipid/electrolyte mixture. The resulting electron density difference is directly proportional to the ion distribution in the electrolyte solution in the interlamellar region between the polar lipid headgroups.

The X-ray measurements are done in two steps. First, the lamellar repeat spacings and the integrated peak intensities of several lipid/water samples are measured with a conventional swelling experiment [14,16–20] at different water concentrations. Second, the lamellar repeat distance and the integrated intensities of the diffraction peaks of the same lipid is measured in the presence of excess electrolyte solution. The absolute values of the structurefactors (on an arbitrary scale) of these two sets of data are calculated from the integrated intensities as outlined in Section 2.

The data of the swelling series are used to calculate the continuous Fourier transform of the electron density profile of the membrane with the sampling theorem [21–23]. This approach is usually used to determine the phase factors (signs) of the structurefactors [18–20,24,25]. In contrast to this, here the sampling theorem is mainly used to construct interpolated electron density profiles at lamellar repeat distances of the lipid/water system, that match the measured lamellar repeat distances of the lipid/electrolyte samples. In the second step of the data analysis the normalization procedure of Wiener and White [26–28] is used to normalize the electron density profiles (or, equivalently, the structurefactors) of the lipid/electrolyte sample and the (interpolated) lipid/water reference sample to the same 'relative absolute' scale. After the normalization, the difference electron density profiles can be calculated from the two datasets.

The calculation of the difference electron density profile can be described more formally in the following way. The electron densities (in absolute units) of two periodic, centrosymmetric systems A, B, with a common repeat distance d can be calculated from the experimental X-ray structure factors as follows:

$$\rho_A(x) = \bar{\rho}_A + \frac{2}{k_A d} \sum_{h=1}^{h_{max}} F_A(h) \cos(2\pi hx/d) \quad (2)$$

$$\rho_B(x) = \bar{\rho}_B + \frac{2}{k_B d} \sum_{h=1}^{h_{max}} F_B(h) \cos(2\pi hx/d)$$

Here $\bar{\rho}$ is the average electron density of the system and k_A, k_B connect the experimentally determined structure factors, F_A, F_B, with the absolute structure factors F_A^*, F_B^* via $kF^* = F$.

A difference electron density profile can only be calculated from the experimental data, when the two individually measured electron density profiles are normalized to the same scale. For this purpose, not all of the quantities k_A, k_B, $\bar{\rho}_A$ and $\bar{\rho}_B$ in Eq. 2 need to be known. With the definitions

$$\Delta\tilde{\rho}(x) = k_A(\rho_A(x) - \rho_B(x)) \quad (3)$$

$$\Delta\rho_{eff} = k_A(\bar{\rho}_A - \bar{\rho}_B) \quad (4)$$

$$k_{eff} = k_A/k_B \quad (5)$$

it follows from Eq. 2 that

$$\Delta\tilde{\rho}(x) = \Delta\rho_{eff} + \frac{2}{d} \sum_{h=1}^{h_{max}} (F_A(h) - k_{eff} F_B(h)) \cos(2\pi hx/d) \quad (6)$$

Eq. 6 contains only two unknowns, $\Delta\rho_{eff}$ and k_{eff}. They can easily be determined if the difference of the two electron density profiles is known at two different points. For lipid bilayer in a lamellar phase this is straightforward, because the apolar hydrocarbon chain region of the lipid lamellae contains neither water nor ions. Thus, the electron densities are identical in this region.

From Eq. 6 it is clear, that the difference electron density profile, $\Delta\tilde{\rho}(x)$, is calculated from the difference of the two sets of normalized structure factors. Comparing Eqs. 2 and 6 reveals, however, that the calculation of the difference can equivalently be viewed to be done in real space, because one data set is simply normalized to the scale of the other. For simplicity, the latter point of view is chosen in the following, i.e., the calculation of $\Delta\tilde{\rho}(x)$ is simply called a 'subtraction' of electron density profiles.

The difference electron density profile $\Delta\tilde{\rho}(x)$ is directly proportional to the total ion density, when it is calculated from the data of a lipid/electrolyte and a corresponding lipid/water sample. In the case of ionic solutions where the atomic numbers of the anion and the cation are very different, this difference electron density is nearly identical to the distribution of the heavier ion. Thus, is it is possible to map directly the inter-membrane distribution of either the anion or the cation by choosing an appropriate combination.

It is clear that the approach presented in this paper rest on the same assumption as *every* difference method: the two structures to be compared must be isomorphous except of the presence of the interesting difference. This means for the present case, that the structure of the lipid bilayer must be assumed to be essentially unchanged by the presence of the ions. This question will be further addressed in Section 5.

4. Results

The experiments were done as described in Section 2 with the negatively charged phospholipid DPPG and solutions of RbCl and $BaCl_2$. In both cases, the atomic number of the cation was much higher than the atomic number of the anion. The measured electron density profiles are therefore nearly identical to the distribution of the counter-ions.

Structure factors of the lipid/water system as a function of the inverse lamellar repeat distance and the continuous Fourier transform are shown in Fig. 1. The results for the DPPG/electrolyte samples are also included. All the data shown in this figure were normalized according to the procedure of Blaurock [14,15]. The Fourier transform of the DPPG/water system was calculated by using the sampling theorem [21–23]. Clearly the results for the samples with and without electrolyte are different. This proves that the measurements are sensitive to the difference between both systems. However, the procedure of Blaurock can only be used to normalize lipid/solution systems which contain different amounts of the same solution but are otherwise identical. Thus, the difference between the structurefactors of the DPPG/water and the DPPG/electrolyte samples as shown in Fig. 1 is only qualitative and without any quantitative meaning. It is partly due to the fact, that the applied normalization is not adequate.

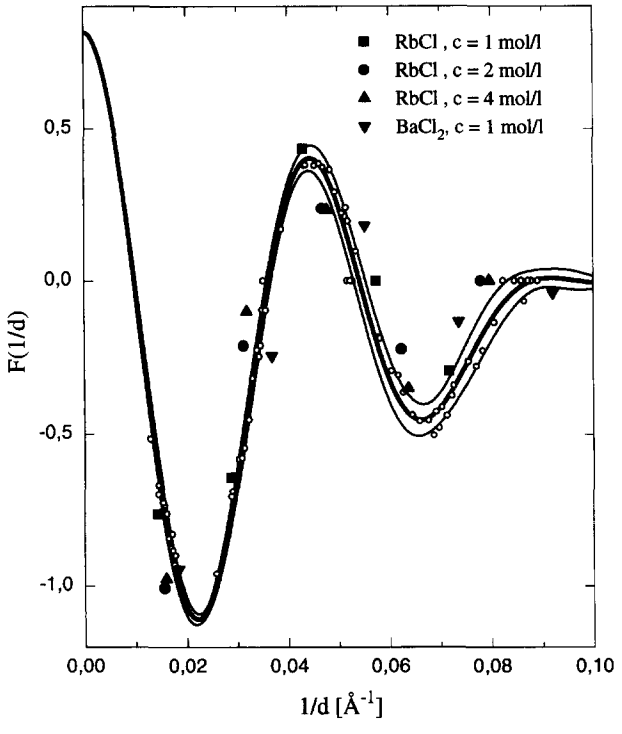

Fig. 1. Normalized structure factors as function of the inverse lamellar repeat distance. The results of the swelling experiments are shown as open circles. The thick line is the continuous Fourier transform which was reconstructed with the sampling theorem; the thin lines show the standard deviation.

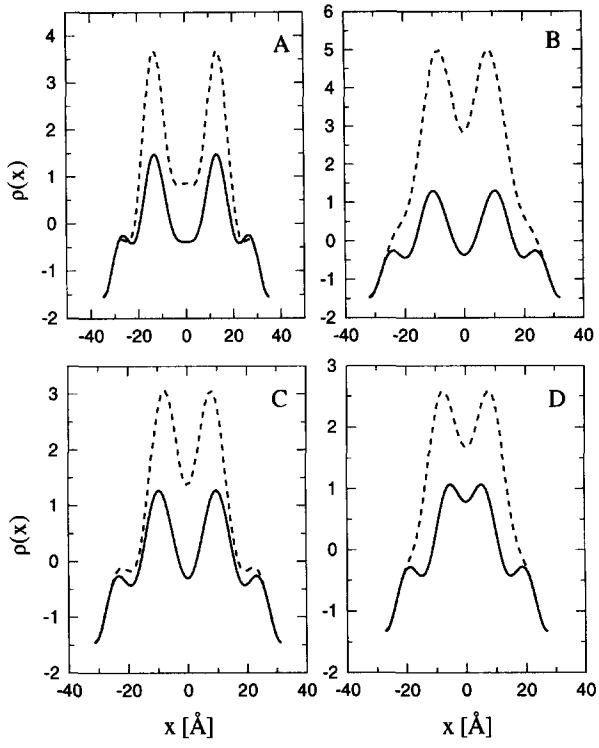

Fig. 2. Electron density profiles $\rho(x)$ (in arbitrary units) of the lipid/electrolyte (dashed lines) and the corresponding lipid/water (full lines) systems for (A) 1 M RbCl, (B) 2 M RbCl, (C) 4 M RbCl and (D) 1 M $BaCl_2$. The figures are drawn with the center of the solution layer at $x = 0$. The electron densities profiles in each panel are normalized to the same relative absolute scale.

The quantitative analysis of the data, i.e., normalization to the same relative absolute scale and the subsequent subtraction of the electron density profiles was done as described in the previous section. The results of such an analysis are shown in Figs. 2 and 3. Fig. 2 shows the normalized electron density profiles for the DPPG/electrolyte and the corresponding DPPG/water samples at a resolution of ca. 12 Å. From Fig. 2 it is immediately clear, that the two systems are indeed isomorphous at and near the center of apolar hydrocarbon chain region. The electron density difference profiles, that were calculated from the normalized electron densities are shown in Fig. 3. These profiles are directly proportional to the distribution of the cations in the lipid/electrolyte samples, because the

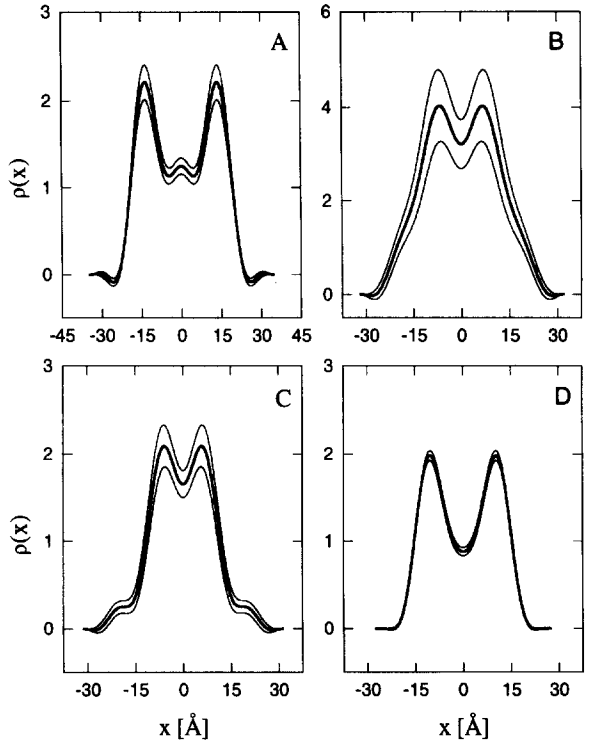

Fig. 3. Difference electron density profiles $\rho(x)$ (in arbitrary units) that were obtained from the experimental data of DPPG with electrolyte solution of (A) 1 M RbCl, (B) 2 M RbCl, (C) 4 M RbCl and (D) 1 M $BaCl_2$. The profiles were calculated from the data shown in Fig. 1 and Fig. 2 as described in the main body of the text. They are directly proportional to the distribution of the positively charged cations between the negatively charged headgroups of the DPPG.

number of electrons per ion of the Rb^+ and the Ba^{2+} cations is much larger then the corresponding number of the Cl^- anions. The obtained ion distributions are zero inside the membrane and show a pronounced maxima at the approximate position of the negatively charged lipid headgroups. Thus the results agree qualitatively with the expectation for the distribution of the counterions.

In all calculations, the phase combination (− − + − −) was used for both sets of data. For the DPPG/water system this agrees with previously published data for similar systems, such as dipalmitoylphosphatidylcholine [18], for example. For the DPPG/electrolyte samples other combinations of the phase factors were also tested. However, they gave no meaningful result. Testing the phase factors is necessary, because the ions in the aqueous compartment contribute significantly to the total electron density of the system (cf. Fig. 2). Because only datasets at a single lamellar repeat distance were measured with the DPPG/electrolyte samples, the sampling theorem could not be used to calculate their phasefactors. Correspondingly it is not clear a priori, that the phasefactors of the DPPG/electrolyte samples are identical with the phase factors of the DPPG/water samples.

For the calculation of the ion distribution it is necessary to choose two (arbitrary) points in the membrane interior the electron density difference of which is set to zero. The geometric center of the hydrocarbon chain region was always used as one of these points. To check the influence of the choice of the second point, the ion distributions were calculated for several such points. The average results of the calculations are shown as thick line in Fig. 3. The influence of the choice of the matching points is indicated by the thin lines around the average value. They represent the variation (standard deviation) in the electron density differences due to choice of different matching points. In all cases the presence of ions between the lipid bilayers is clearly visible. The main features of the distributions are not sensitive to the special choice of the matching points in the membrane interior.

5. Discussion

Difference methods are well known in crystallography and were also already used to investigate the binding of ions in the transmembrane channel gramicidin [29–31]. These methods are similar to the method presented in this paper, in that they obtain the desired information by calculating difference electron densities. However, they are usually applied to a situation with a marker atom at a well localized position. This is completely different from the current application, where the difference, which caused by the presence of a 'marker' atoms extends over a considerable part of the structure under investigation.

The method presented in this paper is further new, as it combines two different methods, the swelling technique and the normalization procedure of Wiener and White, to measure ion distributions between lipid membranes in a most direct way. The present work is distinct from experiments using a somewhat similar approach by the following two points: (1) the swelling experiment and its analysis with the sampling theorem are made in order to allow the interpolation of the structurefactors of the lipid/water system at values that are not directly measured. This allows (after normalization) the subtraction of the lipid/water-electron density profile with the 'correct', i.e., the identical lattice spacing from the lipid/electrolyte-electron density profile. Thus, it circumvents the difficulty to obtain a lipid/water sample which has the identical lattice spacing as the lipid/electrolyte system or the error prone procedure, to subtract the electron density of a sample, the lamellar repeat distance of which is similar but not identical to the one of the lipid/electrolyte sample. (2) A normalization procedure is used, that only normalizes the two data sets on the same scale and not on an absolute scale.

The normalization procedure of Wiener and White only relies on the assumption, that the electron densities of the two systems are identical in a certain region of the considered system. Thus, the most attractive feature of this method is, that it only relies on the actual datasets. In

contrast to that stays the usual difference method, as, for example, exploited by Huang and coworkers to locate ion-binding sites in the gramicidin channel [29–31]. These authors used the full normalization to an absolute scale (cf. Eq. 2). Such a normalization is not directly possible for the lipid/electrolyte systems, because it relies on certain assumptions concerning the composition of the system and molecular areas [30], in order to be able to calculate the normalization factors. These numbers are not available for lipid/electrolyte samples. Instead, the absolute number of ions found in the diffuse double-layer is one of the questions that is in need for a direct experimental answer without any model independent data treatment. It should further be noted, that the results obtained with a normalization on an absolute scale depend on the accuracy of the assumed numbers and the sensitivity of the calibration against errors in these. However, the obvious advantage of a normalization on an absolute scale is, that it gives the difference profiles in absolute units. At the end of this section, it is shown, how the same absolute normalization can be achieved for lipid/electrolyte systems *using* the approach presented in this paper.

The current application focuses on the *structure* of the ion density distribution at/between charged lipid membranes. This (periodic) structure can already be analyzed quantitatively, when its Fourier coefficients are known on an arbitrary scale, because for a comparison between experiment and theory an arbitrary scale factor and a possible offset are not important. Correspondingly, the difference profiles need not to be known on an absolute scale, and the normalization of Wiener and White could be applied directly.

The obtained ion distribution (cf. Fig. 3) are all zero at and near the center of the apolar part of the lipid membranes and show a pronounced maximum close to the position of the lipid headgroup. However, they also obviously extends into the apolar chain region. This can be explained by the limited resolution of the current experiment. Only 5 Bragg peaks were observed, which corresponds to a resolution of ca. 12 Å. Structural details on a smaller scale are contained in the higher Fourier coefficients of Eq. 6 and are experimentally invisible.

The electron density in the interlamellar region and the polar headgroup region is strongly increased by the presence of the electrolyte, as can be seen in Fig. 2. Such behavior is theoretically expected. Simple Gouy-Chapman theory, for example, predicts an excess surface concentration of counter ions (with respect to the ion concentration in the center of the interlamellar layer) of 12 M for an arbitrarily charged ion at a surface charge of 0.2 $C\,m^{-2}$ [32]. The interlamellar region constitutes ca. 50% of the total structure. Because low order Fourier components emphasize the gross features of this structure the structural details in the chain region, as, for example, the typical 'plateau region' in the electron density profile of lipid chains in the $L_{\beta'}$-gel phase, can be lost at low resolution. Thus, the results presented in Figs. 2 and 3 may be somewhat misleading when compared to more 'traditional' difference profiles [29,30], where the difference is usually only due to small amounts of an additional marker atom at a well localized position. In the case of the lipid/electrolyte system with many ions which are thermally distributed within the *broad* diffuse double-layer, the changes of the electron density is much more dramatic. However, this in an unavoidable feature of the system itself and also one of the reasons, why direct structural studies are difficult.

An important assumption for the presented method is, that the electron density in the chain region of the lipid molecules is the same in both, the lipid/water and the lipid/electrolyte mixtures, i.e., that the two lipid structures are isomorphous. This assumptions seems to be well satisfied at and near the center of the apolar hydrocarbon chain region. The subtraction method can only be applied, when the presence of the ions does not cause a phase transition of the lipid. As long as the lipid chains are in the same phase (gel or fluid), this assumption is surely true. A possible counterexample might be the case of the 1 M $BaCl_2$ solution, as is discussed in more detail below. However, even if no changes occur in the lipid chain region, the difference profile is still sensitive to changes of the headgroup conformation, which are known to be induced by ions [1,45]. The difference profile always reports the ion density which is caused by the charge distribution on the lipid headgroup. Thus, it is sensitive to the conformation of the lipid headgroup in the ionic solution. This might be a possible limitation of the proposed method in experiments with high resolution, because in this case artificial structures in the difference profile are possible, due to a mismatch of the headgroups in the two systems. This limitation, however, does not render the presented method useless for such cases. Instead, it can provide direct experimental evidence for ion induced structural changes. Such evidence is usually concluded more indirectly from spectroscopic results as, for example, NMR [1]. Further, the difference electron density profile can still be normalized to an absolute scale (see below) and the total number of ions in the interlamellar region can even in such an unfavorable situation still be reliably calculated, because the normalization ensures, that the total number of subtracted electrons is independent on a change in the structure.

In the case of the RbCl solution, the maxima of the ion distribution are slightly shifted away from the apolar chain region of the lipid membrane into the solution, when compared to the electron density profile of the lipid/water system (cf. Fig. 2A–C and Fig. 3A–C). This agrees well with the fact, that the negative charge of the DPPG headgroup is located at the phosphorous atom, which is also the origin of the electron density maximum for the lipid/water system. Several reasons may contribute to the small shift. First, the finite size of both, the Rb^+-ion and the phosphate headgroup prevents the Rb^+-ion from taking

Table 1
Results of χ^2-fitting of the different ion distributions compared to the experimental data

Salt	c (mol/l)	d_{exp} (Å)	d_e^{exp} (Å)	Theor. λ_D (Å)	Gouy-Chapman model			Gaussian model	
					σ (C/m^2)	λ_D (Å)	d_e (Å)	s (Å)	d_e (Å)
RbCl	1	69.7	26.2	3.04	0.1	2.0	36.0	8.95	29.0
RbCl	2	64.1	20.6	2.15	0.1	1.4	32.0	9.00	21.8
RbCl	4	62.8	19.3	1.52	0.12	2.9	32.0	9.00	18.2
$BaCl_2$	1	55.0	11.5	1.52	0.1	2.0	17.0	9.00	21.0

Experimental data: d_{exp}, experimental lamellar repeat distance. d_e^{exp}, thickness of the interlamellar electrolyte solution layer as determined from the electron density profile (peak-to-peak distance between the maxima of the electron density profiles). The corresponding thickness d_w of the interlamellar water layer of the corresponding lipid/water reference sample is usually larger (cf. Fig. 2). Theor. λ_D, theoretical Debye length of the ionic solution; $\lambda_D = (3.04/\sqrt{c})$ Å, c in mol/l [34].

For the Gouy-Chapman model, the ion distribution was fitted with $c(x) \sim \exp(Ze\psi(x)/kT)$, and $\psi(x) = (\sigma\lambda_D/\epsilon\epsilon_0)\cosh(x/\lambda_D)/\cosh(< d_e/2\lambda)$ [34]. The fitting function was assumed to be zero outside the interval $[-d_e/2, d_e/2]$. Fitparameter were the surface charge density σ, d_e and λ_D. The surface charge density of a fully ionized DPPG membrane is approximately 0.3 C/m^2. The fitted value of 0.1 C/m^2 is consistent with the fact, that DPPG is even at pH 8.5 not fully deprotonated [43].

For the Gaussian model, fitting was done with $c(x) = \exp(-(1/2)((x - d_e/2)/s)^2) + \exp(-(1/2)((x + d_e/2)/s)^2)$ in the range $[d_e/2 - s, d_e/2 + s]$. Fitting parameter were d_e and width s.

place at the exact location of the phosphorous atom. This effect might be amplified by the hydration of these two charged items. Second, the maximum of the (projected) 1-dimensional electron density profile is close to the location of the phosphorous atom, but does not necessarily agree with it (see Fig. 9A, B in [40], for example). Third, minor differences in the headgroup conformation of the two systems can not be completely excluded.

The difference electron density profile that was obtained from the data of DPPG in the $BaCl_2$ solution shows a pronounced shift of its maximum into the apolar chain region, when compared to the corresponding lipid/water system (cf. Fig. 2D and Fig. 3D). The position of this shifted maximum corresponds approximately to the position of the esterbond between the glycerol backbone and the alkane chains. Another observation is, that the lamellar repeat distance in the 1 M $BaCl_2$ solution ($d = 55.5$ Å) is much smaller than in the 4 M RbCl solution. Thus, the divalent Ba^{2+}-ion seems to behaves different than the monovalent Rb^+-ion. One reason may be a specific interaction between the lipid headgroup and the Ba^{2+}-ion and a pronounced change in the headgroup conformation. This would agree with the published data of the phase behavior of DPPG in the presence of divalent ions [33]. There it was found, that a divalent cation (Ca^{2+}, Mg^{2+} or Mg^{2+}) induces a stable dehydrated phase in DPPG at bound cation to lipid mole ratios equal or greater to 0.5:1. Another reason may be a Ba^{2+} induced partial change of the phase state in the lipid chain region. The electron density profile of the lipid/$BaCl_2$ system does not show the pronounced plateau region which is usually typical for lipids in the non-interdigitated gel phase. The presence of the Ba^{2+}-ion might therefore cause a more disordered state of the lipid chains as compared to a lipid/water system. This observation, can not be compared with published data [33], because they only report the effects of divalent ions other than Ba^{2+} at lower concentrations (0.1 M). In the case of an increased disorder of the lipid chain region the application of the normalization procedure of Wiener and White would be questionable if not forbidden, because different lipid states (gel state in lipid/water system and partially disordered gel state in lipid/1 M $BaCl_2$-solution system) with different densities are normalized onto each other. However, the resolution of the present data is too low to allow a definite conclusion.

For a further quantitative analysis the Fourier coefficients of the experimentally obtained ion distributions were fitted [2] with two different models: the counter-ion distribution resulting from Gouy-Chapman theory [34] and an empirical distribution, assuming two Gaussians. Qualitatively the use of the latter can be justified with a generalized linear Gouy-Chapman Ansatz [1,37–39,41,42] which includes the intrinsic width of the interface. For completeness, the analysis was done for all of the ionic solutions. However, the result obtained for the $BaCl_2$ solution might be misleading because of the reasons discussed above. This is mirrored in a partially 'anomalous' behavior as compared with the results for RbCl. The results of the analysis are given in Table 1. For both fitting models they are not very satisfactory.

In the case of the Gouy-Chapman model the fitted Debye length, λ_D, does not agree with the theoretically calculated one. This is a commonly found behavior [35,36]. More important, however, is, that the Debye length considered as a function of the salt composition is not consistent with the theory. Its value should decrease ($\sim c^{-1/2}$) with increasing salt concentration.

[2] Ideally, the ion density itself should be analyzed. However, only the first few Fourier coefficients are available. Fitting the Fourier coefficients of the model distribution to these removes as good as possible artifacts caused by the finite resolution.

The empirical Gaussian model gives a more consistent description of the data. In all cases the fitted Gaussian width is virtually identical. This agrees well with theoretical expectation for an interface which intrinsic width is larger than the Debye length [1,37–39]. For such a system, the generalized Gouy-Chapman theory predicts, that the width of the ion distribution is determined by the spatial smearing of the position of the charge (headgroup) and should thus be independent on the electrolyte concentration. In case of $BaCl_2$ the fitted thickness of the solution layer seems to be too high.

The fact that the experimental data can not be fitted well with distributions derived from simple mean-field models is not surprising. The theoretical shortcomings of the Gouy-Chapman theory are well known [1,5–8]. Notwithstanding the recent results of Peitsch et al. [4] for low ionic strength, discreteness of charge effects and the finite size of the charged headgroup region might become important at high concentrations even in the case of usually well behaved monovalent ions, because of the much smaller value of λ_D. The possibility to test different theories is an important reason for direct experimental measurements of ion distributions.

The obtained result demonstrate unequivocally, that the proposed method is able to provide information about the ion distribution between charged lipid membranes. In the present paper, only the distribution of the counter ions was considered, because they are of the main interest. However, it is in principal be possible, also to measure the distribution of the co-ions.

The method introduced in this paper can best be applied, when no changes occur in the lipid chain region and when changes of the headgroup conformation are small. This seems to be the case for the data that were obtained with different RbCl solutions, a conclusion that is also corroborated by the data reported by Watts et al. [46]. There, the hydrocarbon chains were found at 20°C to be always in the gel phase at different pH and at a salt concentrations of 1.5 M KCl. In case of the $BaCl_2$ solution, however, the maximum of the difference profile is shifted towards the apolar lipid interior. Thus a conformational change of the lipid headgroup or even in the chain region can not be excluded for this sample. However, it is important to realize, that even so-called high resolution X-ray pattern which can be obtained with oriented samples show usually only about 12 lamellar orders. The corresponding resolution is ca. 5 Å. Thus, even experiments with oriented samples should be relatively insensitive to minor changes of the lipid conformation.

The present experiments were done with highly concentrated electrolyte solutions. The reason was simply, to make sure that a difference profile can be obtained easily. No efforts were made to test the low concentration sensitivity limit of the method. This might be important in future experiments, especially with regard to the aforementioned problem of a possible ion-induced change of the lipid headgroup conformation. Such structural perturbations are expected to be smaller at smaller ion concentrations. It should also be noted, that the method presented in this paper can be considered as being complementary to the X-ray standing wave method [10], which is restricted to measurements at very low ionic concentrations.

It was noted above, that the normalization procedure of Wiener and White only provides a normalization on a relative scale, and that a direct normalization on an absolute scale as done in other work [29–31] is not possible, because the additional information is not available. However, the procedure presented in this paper offers the possibility to obtain such a normalization on an absolute scale *without* a priori knowledge of the necessary normalization constants for the lipid/electrolyte samples. All that is necessary is to determine the electron density of the lipid/water system on an absolute scale. This can be done by monitoring carefully the actual water content during the gravimetrical sample preparation. Because the procedure of Wiener and White normalizes two data sets on one scale, this information is sufficient to normalize the difference electron density profiles on an absolute scale. This difference electron density can than be directly converted into an ion density by using the known atomic numbers and the charges of the ions. Such an approach would allow to monitor the total amount of ions found in the diffused double-layer as a function of pH, lipid composition, lipid headgroup, etc., for example.

In the present paper only low resolution ion distributions profiles from powder samples were obtained. Thus, the statements made about the quality of different theoretical models are limited and not necessarily the final word. However, it will be possible to improve the experimental results by using ordered samples. In this case typically 10 to 12 or sometimes even more Bragg peaks can be measured [17,24,25,44,29–31]. The corresponding resolution is approximately 4–6 Å, depending on the lamellar repeat distance of the lipid and the number of Bragg peaks measured. Finally it should be pointed out that the method introduced in this paper can also be applied to uncharged membranes and to non lipid systems, as, for example, clays [47]. For the latter systems, the aforementioned problem of a possible structural change is probably of no importance.

6. Conclusions

A new method was introduced, which allows a direct measurement of the ion distribution between charged (and uncharged) lipid membranes with a comparatively low experimental effort. The feasibility of the method was demonstrated with measurements of the distributions of Rb^+ and Ba^{2+} cations between negatively charged lipid membranes made of DPPG. Although only unoriented samples were used, it was possible to achieve a resolution

of 12 Å. This is already comparable to the resolution of more sophisticated methods using neutron diffraction or X-ray standing waves. The experimental data for the obtained ion distributions were analyzed with model fitting. The result of the analysis suggests, that the experimental results can not be described quantitatively with the simple Gouy-Chapman theory. It was shown that instead a generalized Gouy-Chapman theory, which takes into account the finite width of the lipid/electrolyte interface gives a more consistent description of the experimental data. Possible experimental improvements of the presented method with regard to resolution and normalization of the electron densities onto an absolute scale (i.e., the possibility to obtain ion distributions on an absolute scale) and possible future application in more thorough studies of membrane electrostatics were discussed.

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