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Localization of dihalogenated phenols in vesicle systems determined by contrast variation X-ray scattering

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Localization of 2,4-dichloro- and 2,4-dibromophenol in multilamellar vesicles in a 1/1 dihalogenated phenol/lipid molar ratio was investigated by classical contrast variation X-ray scattering using the isomorphous replacement method. The results were compared with those obtained by anomalous small-angle X-ray scattering from a vesicle system doped with 2,4-dibromophenol. Dissimilarities in the results of the two methods are discussed, taking into account the advantages and disadvantages of both techniques in studying multilamellar systems.

1. Introduction

In the last few decades, studying the effect of different guest molecules on model membranes has turned to a high interest subject because of the biological relevance. Among the different model systems, the multilamellar vesicles constituted from synthetic phospholipids are frequently used to elucidate the features of the complex real membranes.

It is known that a large number of phenolic compounds act on the basic cellular functions (Escher *et al.*, 1999; Escher & Schwarzenbach, 1996; Sinclair *et al.*, 1999). These molecules can evolve toxic effects without chemical reactions, but only by second order interaction while penetrating into the bilayer. Sikkema and co-workers (Sikkema *et al.*, 1995; Csiszár *et al.*, 2003) revealed that these lipophilic molecules cause lateral heterogeneity and cluster formation in the membrane lipids. The first goal of this work is to determine the localization of 2,4-dichloro- (DCP) and 2,4-dibromophenol (DBP) in a vesicle (or liposome, MLV) system made of 1,2-dipalmitoyl-*sn-glycero*-3-phosphatidylcholine (DPPC) and water in a 1/1 dihalogenated phenol/lipid molar ratio. The phenolic compounds studied are widely used in industry as flame retardants, biocides and wood treatment agents, and they have toxic effects on living organisms (Zhang *et al.*, 2004; Olsen *et al.*, 2002; Hassenklöver *et al.*, 2006).

To achieve the aim of this work, we have used contrast variation X-ray scattering by means of isomorphous replacement, which is a well established technique in wide-angle diffraction, but far less common in small-angle scattering. The basis of this method is the assumption that the two studied guest molecules are embedded in the surrounding media in the same way, but their diffraction patterns vary according to the different scattering densities of the guest molecules. By analysing the difference between the resulting electron density maps reconstructed from the X-ray data of the two systems, one can directly observe the displacement of the third component, in our case the dihalogenated phenols in a lipid/water system. Since this approach involves the assumption of the same effect of DCP and DBP on vesicle systems, we compare the results with our previous anomalous small-angle X-ray scattering (ASAXS) study on the DBP/DPPC system (Varga *et al.*, 2006).

2. Experimental

The structure of multilamellar systems is widely studied by small-angle scattering methods, since their characteristic distance is in the size range of several nanometres. According to the one dimensional order in these systems, Bragg peaks can be observed in the scattering patterns, whereby structural information can be deduced. However, the localization of guest molecules can hardly be followed by using traditional evaluation techniques of small-angle scattering, even if the periodicity is not destroyed, because the scattering of the guest molecules can not be separated from that of the whole system.

In the above mentioned problem, the use of contrast variation can bring success. The main difference between the two compared methods is that the previous one uses an appropriate modification of the investigated system to change the scattering density of the molecule in question, while the latter reaches the contrast effect by changing the used X-ray energy near to the absorption edge of the proper atom of the guest molecule.

Before we go into detail on the specification of these techniques we should summarize the basic theory of X-ray scattering, especially the reconstruction of the bilayer electron density (ED) profile of a vesicle system. The scattering pattern of an unoriented multibilayer system exhibits a series of discrete diffractions I_k (k = 1, 2, ...), which are related to the ED profile normal to the surface, $\rho(z)$, as follows (Blaurock & Worthington, 1966; Richardsen *et al.*, 1996):

$$\rho(z) = \frac{\text{const}}{d\sum_k k^2 I_k} \sum_{k=1}^{k_{\max}} \text{sig}[I_k]\sqrt{k^2 I_k}\cos\left(\frac{2\pi k z}{d}\right), \qquad (1)$$

where d is the lamellar repeating unit, and sig$[I_k]$ is the phase factor that is either +1 or −1 because of the symmetry of the vesicle.

In isomorphous replacement one uses two, slightly modified, guest molecules. Consequently, two different electron density profiles can be calculated from their scattering patterns. With the assumption of the same perturbation effect of the two derivatives on the pure lipid/water system, their localization can be determined from the difference of the two electron densities. The replacement of chlorine by bromine atoms yielded significant effects in the SAXS curves as it may be followed in our previously reported work (Bóta *et al.*, 2002).

The method of the isomorphous replacement has already been applied in other liquid crystalline systems too; the structural elucidation was executed on a second generation monodendron system for the determination of a cubic liquid crystalline phase and a counterion distribution (Dukeson *et al.*, 2003). Here we present this method adapted for studying the localization of guest molecules in a lamellar liquid crystal system.

In the anomalous scattering the situation is nearly the same as in isomorphous replacement, except that in ASAXS the variation of the scattering length density causes differences in the diffraction patterns, measured at different energies near the absorption edge of the resonant atom of the guest molecule. More detailed descriptions about ASAXS can be found elsewhere (Phillips *et al.*, 1977; Stuhrmann, 1980; Goerigk *et al.*, 2004; Goerigk & Williamson, 2006).

We should mention that the availability of the theory described by equation (1) can be used only if the scattering pattern exhibits at least four Bragg peaks, because the resolution of the electron density reconstruction will only be acceptable in that case. If this criterion is not fulfilled, either because of the destroyed periodicity or because the higher order Bragg peaks can not be observed, model electron density profiles should be used, and the parameters of them should be fitted to reproduce the measured scattering curve (Wiener *et al.*, 1989; Pabst *et al.*, 2000; Pabst *et al.*, 2003). In our previous ASAXS study, we have used a Gaussian model to describe the electron density profile, since in the pure resonant scattering curve (which is the separated scattering of bromine atoms) only two Bragg reflections were observable (Varga *et al.*, 2006).

2.1. Materials and methods

Synthetic high-purity 1,2-dipalmitoyl-*sn-glycero*-3-phosphatidylcholine (DPPC) and the halogenated phenols [2,4-dichlorophenol (DCP), 2,4-dibromophenol (DBP)] were obtained from Avanti Polar Lipids (USA) and from Sigma (Germany), respectively. The chemicals were used without further purification. The pure DPPC was mixed with crystallized dihalogenated phenol (DCP or DBP) and then deionized, triple quartz-distilled water was added to the system to gain a lipid concentration of 20 *w*/*w*%. The mixtures were kept at 303 K and vortexed intensively then quenched to 277 K. This process was repeated forty times to obtain an homogeneous system. For the X-ray measurements, the samples were transferred into thin-walled (Plexiglas) sample holders, the temperature was adjusted precisely with water flow in front of the walls. All the measurements in this paper were executed at 293 K.

The small-angle X-ray scattering (SAXS) measurements were carried out on the Jusifa (B1) beamline (Haubold *et al.*, 1989) at HASYLAB (DESY, Hamburg) in the modulus of scattering vector ($q = 4\pi\sin\theta/\lambda$, where θ is half the scattering angle and λ is the wavelength of the incident radiation) regime of 0.02–0.6 Å^{-1}. All measurements were made using a two-dimensional detector (multiwire proportional counter). The net scattering data, collected at 12790 eV X-ray energy, were normalized to the primary beam intensity and corrected for transmission. Finally the scattering curves have been calibrated to absolute intensity (e.u. nm^{-3}).

3. Results and discussion

Fig. 1 shows the scattering curve of the pure DPPC/water system after background correction. It exhibits five Bragg peaks in compliance with the well ordered multilayer structure (in the so called L_β gel phase at 293 K). In the inset of Fig. 1 the reconstructed ED profile of the pure system is shown, which is in agreement with the published one (Nagle *et al.*, 1996). The phase factors were chosen to be sig[I_k] = (- - + - -), according to a previously reported data (Nagle *et al.*, 1996). By adding the DCP molecules to the pure system, the correlation between the lamellae is decreased, so only two significant Bragg peaks can be observed in the scattering curves. However, if the concentration reaches the molar ratio of guest molecule/lipid = 0.6, the well ordered layer arrangement is recombined as Bragg peaks in higher order reappear. The positions of the reflections shifted to larger q values indicating the existence of the interdigitated phase (L_I), where the chain regions of the layers are embedded into each other. The scattering curves of the systems in a 1/1 dihalogenated phenol/lipid ratio are shown in Fig. 2. By replacing the DCP by DBP the positions of the peaks are not changed, *i.e.* the lamellar repeating units of these systems are the same d = 51.2 Å. The latter was calculated from peak positions as $d = 2\pi m/\sum_{k=1}^{k_{max}} q_k/k$, where m is the number of the observable Bragg peaks and q_k is the position of the k^{th} peak. Since the periodicity is very sensitive to the perturbation effect of the guest molecules, we can conclude that the localization of the two dihalogenated phenols is the same. Previous differential scanning calorimetry measurements also confirm this assumption (Bóta *et al.*, 2002).

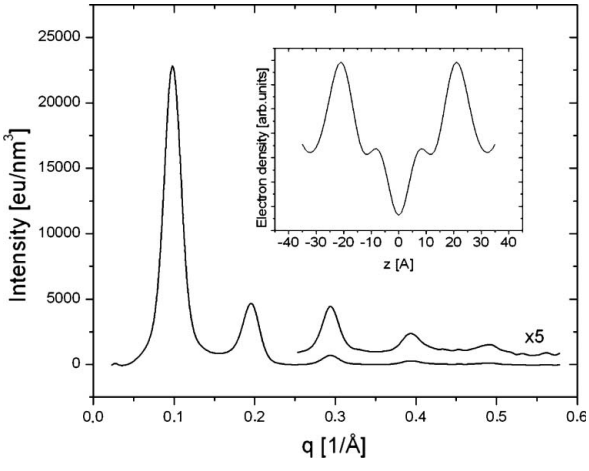

Figure 1
SAXS pattern of the pure DPPC/water system. The inset shows the reconstructed electron density profile.

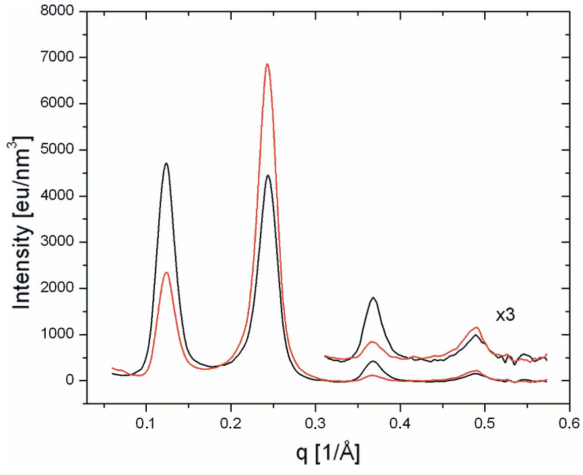

Figure 2
SAXS curves obtained from the DCP/DPPC (black) and DBP/DPPC (red) systems.

The electron density profiles corresponding to the systems doped with DCP and DBP are shown in Fig. 3*a* and Fig. 3*b*, respectively. The first conspicuous thing on these curves is that the typical minimum of the alkyl chains in the case of the pure system has vanished, which is the consequence of the formation of the interdigitated phase. The positions of the maxima of the ED profile indicate a shorter headgroup–headgroup distance in agreement with that mentioned above. The difference between these curves appears in the border of the headgroup and the chain region (around 10 Å from the bilayer centre), where a shoulder is increasing by replacing DCP by DBP.

The difference of the two electron density profiles is shown in Fig. 4, which can be identified with the distribution of the guest molecules along the bilayer normal if the assumption about the same localization is true. More precisely, the profiles give information only about the distribution of the halogen atoms of these molecules, which can be different from that of the centre of the whole molecule, if they are located in an oriented order. One can see a maximum at 9.5 Å from the bilayer centre, and a shoulder at the position of the headgroup region (around 15 Å from the bilayer centre).

In Fig. 4 the result of our previous ASAXS study on DBP/DPPC system is also shown (Varga *et al.*, 2006), which differs from the new results significantly. The anomalous method gives a narrow maximum at the position of the headgroup region, while the contrast variation due to the isomorphous replacement yields a broadened distribution of the halogen atoms. According to this difference in the character of the distribution we can only state, that the studied dihalogenated phenols are located near the headgroup region of the bilayer.

The contrast variation by replacing DCP by DBP results in higher contrast than the anomalous effect, but this advantage can not be exploited entirely. Namely, the electron density profiles, or rather their differences, are not precise enough because of the limited four orders of the Bragg reflections. Moreover, the assumption of the same location of both halogen atoms, consequently for the same layer structures, remains to be a query.

4. Conclusions

The localization of 2,4-dichloro- and 2,4-dibromophenol molecules in DPPC/water vesicles was studied by contrast variation by means of isomorphous replacement and results were compared with our previous ASXAS results (Varga *et al.*, 2006). The detailed study of the location of these halogenated phenols has given slightly different results, which can be attributed to the assumptions required by the two methods.

Both methods indicate that the guest molecules are embedded near the headgroup of the lipids, in the double layers. The formation of the interdigitated phase (L_I) is strongly connected to the localization of these molecules, which makes possible the fusion of the opposite chains.

The advantage of the anomalous scattering is clear: the system can be studied by itself, and the scattering from the guest molecules can be exactly separated from that of the whole system. The main drawback of the method has been mentioned already, namely, the problem of the weak effect. During the evaluation of the ASAXS curves, the distribution of the guest molecules, more precisely their resonant atoms, was modelled with one Gaussian function per lipid layer, so this model is not able to reproduce such a profile that results from isomorphous replacement. Neither did the increase in the number of Gaussian functions lead to better results, because then the fitting procedure became under defined. The other possible bottleneck of the evaluation of the ASAXS curves is the question of the anomalous scattering factors. In the literature usually theoretically determined values are used, which can sometimes differ from the real scattering factors since they depend on the neighbourhood of the resonant atom.

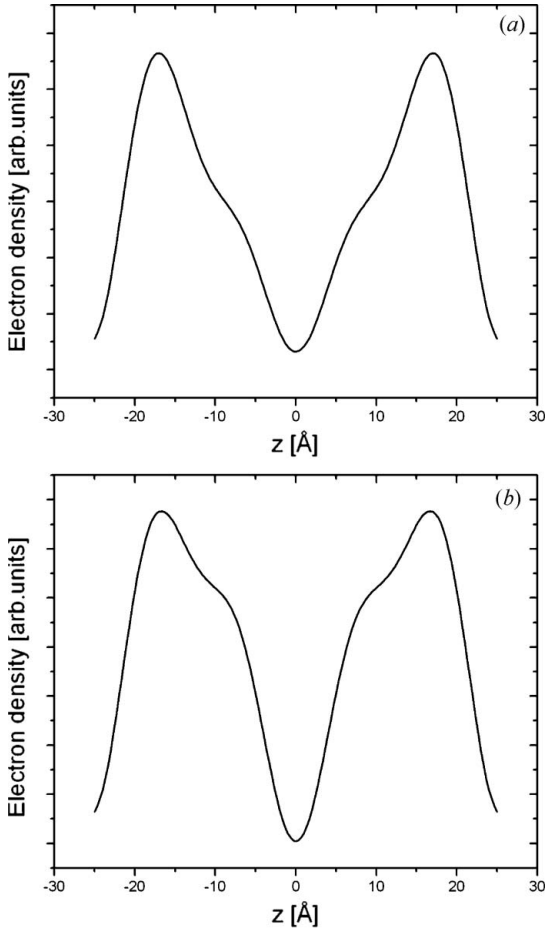

Figure 3
The reconstructed electron density profiles along the bilayer normal of the systems doped with (*a*) DCP and (*b*) DBP.

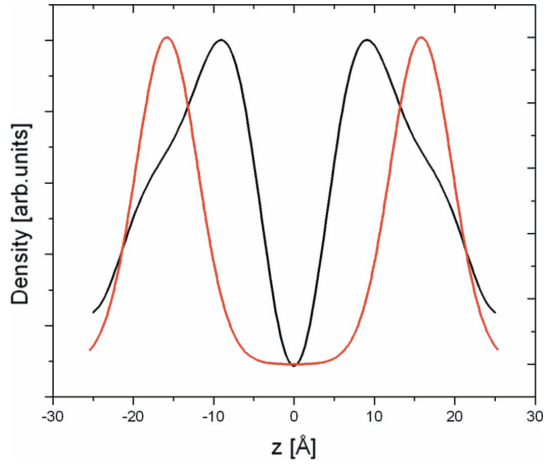

Figure 4
The distribution of the halogen atoms of the guest molecules along the bilayer normal obtained by isomorphous replacement (black) and by ASAXS (red).

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