Small angle X-ray scattering study on effect of replacement of hydrogen oxide (H$_2$O) by deuterium oxide (D$_2$O) on anionic phospholipid bilayers

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Abstract. In order to clarify the effect of replacement of hydrogen oxide (H$_2$O) by deuterium oxide (D$_2$O) on the structure of phospholipid bilayers, we carried out small angle X-ray scattering (SAXS) measurements of dimyristoylphosphatidylglycerol (DMPG) bilayers in H$_2$O and D$_2$O. DMPG is an anionic phospholipid and spontaneously forms unilamellar vesicles in water. The obtained SAXS intensity curves were analyzed by using a structural model. The analysis showed that the replacement of H$_2$O by D$_2$O induces structural change at interfacial regions of the DMPG bilayers.

1. Introduction

Owing to their amphiphilic nature, lipids form various molecular self-assembly structures in water; micelle, bilayer, bicontinuous cubic structure, etc. In these structures, the bilayer structure has been extensively studied because the bilayers formed mainly by phospholipids are essential building blocks of biomembranes [1]. Lipids can form so-called Langmuir monolayers on water surface, which are also excellent model systems for biomembranes, because a biomembrane can be considered as two weakly coupled monolayers [2].

Neutron or X-ray scattering (diffraction) and reflectivity measurements are widely used to investigate the structure of such systems. For samples in water or on water, one of advantages of the use of neutron beam is that a contrast variation method without any chemical composition changes can be applied by using replacement of hydrogen oxide (H$_2$O) by deuterium oxide (D$_2$O). In X-ray beam, the chemical composition change must be needed to carry out the contrast variation method, for example, adding salt or sugar to the solvent, binding of heavy metal to the sample, etc. The chemical composition change induces structural changes of the samples.

It cannot be, however, immediately concluded that the replacement of H$_2$O by D$_2$O does not induce any structural changes in biological samples, judging from following facts. A variety of effects of D$_2$O on living things has been known [3-5]. Furthermore, effects of D$_2$O on the physical properties of biological molecules also have been reported for various systems. D$_2$O increases the stability of proteins against thermal, guanidine hydrochloride (GuHCl), and urea-induced denaturation [6 and references therein]. D$_2$O increases the gel-fluid phase transition temperatures by approximately 2°C for
several phospholipid bilayers [7-10]. Therefore, we must carefully discuss a possibility that the replacement of H\textsubscript{2}O by D\textsubscript{2}O induces some structural changes of biological molecules or biological molecular assembly systems in water.

By means of small-angle X-ray scattering (SAXS) measurements, Kobayashi and Fukuda [11] have reported that the replacement of H\textsubscript{2}O by D\textsubscript{2}O decreases the lamellar repeat distances by approximately 0.1nm of neutral phospholipids, phosphatidylycholines (PCs), multilamellar vesicles and that the reduction of the lamellar repeat distances is due to the decrease of water layer thickness. In other words, the replacement of H\textsubscript{2}O by D\textsubscript{2}O does not affect the PC bilayer thickness. They [11] determined the bilayer thickness by so-called Luzzati method [12] in which the bilayer thickness is estimated by the combination of the data of the lamellar repeat distances and lipid volume fraction. However, this method does not account for water penetrating into lipid head group regions and has been questioned [13, 14]. Thus, it requires further studies based upon more precise and direct measurements.

In order to clarify the effect of the replacement of H\textsubscript{2}O by D\textsubscript{2}O on the structure of phospholipid bilayers, we tried to get detailed structural information by analyzing SAXS intensity curves obtained from unilamellar phospholipid vesicles in H\textsubscript{2}O and D\textsubscript{2}O, using a structural model. In this study, we measured the vesicle sample of an anionic phospholipid, dimyristoylphosphatidyglycerol (DMPG). It has been revealed that, under low ionic strength conditions, DMPG spontaneously forms unilamellar vesicles, owing to electrostatic repulsion interaction [15]. Here we report that the replacement of H\textsubscript{2}O by D\textsubscript{2}O induces a structural change at interfacial region of the DMPG bilayers.

2. Experimental

1,2-Dimyristoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (Sodium Salt) (DMPG) was purchased from Avanti Polar Lipids (Alabaster, AL). The DMPG had a purity >99% and were used without further purification. Water (H\textsubscript{2}O) used in this study was prepared with a Mill-Q system (Millipore Corp., Bedford, USA). Deuterium oxide (D\textsubscript{2}O) (99.8 atom % D) was purchased from Aldrich (Milwaukee, USA). The lipids were dispersed in pure water or pure heavy water to be 50 mM lipid concentration. The dispersions were incubated at around 40°C for about 10 min and vortexed after incubation.

Differential scanning calorimetry (DSC) measurements were performed with a DSC6100-EXSTRA6000 thermal analysis system (Seiko Instruments Inc., Chiba, Japan), by using a scan rate of 2.0 °C min\textsuperscript{-1}.

Small angle X-ray scattering (SAXS) measurements were carried out at RIKEN Structural Biology Beamline I (BL45XU) [16] at SPring-8, 8 GeV synchrotron radiation source (Hyogo, Japan). The X-ray wavelength used was 0.09 nm. The sample-to-detector distance calibrated by the diffraction from silver behenate was 847.4 mm. The sample temperature was controlled within 0.01°C by using a high-precision thermoelectric device. Typical exposure time was 0.5 s. SAXS data were recorded with a large-aperture (150 mm in diameter) TV-type detector consisting of a beryllium-windowed X-ray image intensifier and a charge-coupled device (CCD) image sensor (Hamamatsu Photonics, Japan) [17]. The image distortion and nonuniform response of the detector were corrected according to the method proposed by Ito et al. [18]. Two-dimensional data on CCD X-ray detector were transformed into one-dimensional data, i.e., intensity vs. scattering vector magnitude \(q = 4\pi\sin(\theta)/\lambda\) (2\(\theta\) is the scattering angle), by radial integration with FIT2D software written by Dr. A. Hammersley (http://www.esrf.fr/computing/scientific/FIT2D/).

3. Results and Discussion

At first, by means of DSC we investigated the effect of the replacement of H\textsubscript{2}O by D\textsubscript{2}O on the phase behavior of DMPG dispersed into pure water. Because sodium salt form lipid was used and the lipid concentration was 50 mM, there were Na\textsuperscript{+} ions dissociated from the lipid sample in the aqueous region but the ion strength must be less than 50 mM. This corresponds to the low ionic conditions under which DMPG spontaneously forms unilamellar vesicles [15]. A broad transition peak was observed
for both DSC thermograms of the samples in H\(_2\)O and D\(_2\)O (DSC profiles not shown), agreeing with previous studies [15,19] which have revealed that at low ionic strength, DMPG presents a large gel-fluid transition region, ranging from 18\(^\circ\)C to 35\(^\circ\)C.

Although the shapes of both DSC curve profiles of DMPG in D\(_2\)O and DMPG in H\(_2\)O were almost identical, the peak temperatures differed from each other. The temperature of DMPG in D\(_2\)O was \(~24\) \(^\circ\)C and was \(~2\) \(^\circ\)C higher than that of DMPG in H\(_2\)O. This is good agreement with the results obtained for other kinds of phospholipids [7-10].

The increasing of the transition temperature by the replacement of H\(_2\)O by D\(_2\)O was also confirmed from SAXS measurements. Figure 1 shows temperature dependence of SAXS profiles for 50 mM DMPG dispersion in H\(_2\)O (A) and 50 mM DMPG in D\(_2\)O (B). The SAXS profiles were recorded at 5\(^\circ\)C intervals during heating. At low temperatures where the DMPG bilayers are in a gel phase, the SAXS curves have distinct three bumps. By contrast, the bumps in the higher \(q\) region are unclear at high temperatures where DMPG bilayers are in a fluid phase. From the shape change of SAXS curves, it can be concluded that the DMPG bilayers in H\(_2\)O are almost in the fluid phase at 25 \(^\circ\)C, and that, on the other hand, the DMPG bilayers in D\(_2\)O are still in the gel phase at 25 \(^\circ\)C.

To get detailed structural information from the observed SAXS profiles, we carried out fitting analysis, using a model electron density contrast (relative electron density profile) of the lipid bilayers (see Appendix). Figure 2 represents relative electron density profiles with the best fit parameters determined by the fitting analysis. In the profiles, two peaks correspond to polar head groups of the DMPG bilayers. It can be seen from the profiles that the thickness of bilayers decreases to 77 \% of that in the gel phase, as a result of the chain melting transition. This is good agreement with a previous estimation (77 \%) from SAXS data analysis using different type of model [19]. Except for the transition temperature range (25\(^\circ\)C and 30\(^\circ\)C), the replacement of H\(_2\)O by D\(_2\)O induces no change of the bilayer thickness. This is identical with the case of PC bilayers [11]. However, the difference can be clearly recognized for the head group regions. By contrast, any significant differences cannot be seen for the hydrocarbon chain regions.
The fitting between the experimental and theoretical values at temperatures above the chain melting transition is not so better than that of the low temperatures (Fig. 3). It has been reported that the size of DMPG bilayers vesicles of the fluid phase under low ionic strength conditions is smaller than that of the gel phase [15]. The vesicle size effect on the SXAS curves is assumed to be negligible in the present analysis (see Appendix). This assumption might not be reasonable for the fluid phase. However, judging from the goodness of the fitting, it can be concluded that the difference induced by the replacement of H$_2$O by D$_2$O is not an experimental error for the gel phase at least.

The difference of relative electron density profiles possibly suggests that the replacement of H$_2$O by D$_2$O affects the head group packing mode or sodium ion binding at the interface regions of DMPG bilayers. In addition, it is likely that the depth of penetration of water into the headgroup regions is different between D$_2$O and H$_2$O. The origin of the difference observed in the relative electron density profiles is unclear at present.

Finally, it should be noted that it must be necessary to consider the possibility that the replacement of H$_2$O by D$_2$O affects the interfacial structures of lipid membranes when D$_2$O is used in neutron scattering or refractivity measurements, as indicated from the present study.

**FIGURE 2** Relative electron density profiles (relative contrast $\Delta \rho(x)$) obtained from the best fit to the observed SAXS intensity data of DMPG in H$_2$O (solid line) and D$_2$O (dot line).
4. Appendix: Scattering curve fitting procedure

If the size of vesicles is much larger than the bilayer thickness, we can assume that the scattering intensities observed in the \( q \) range from \( \sim 0.5 \text{ nm}^{-1} \) to \( \sim 5 \text{ nm}^{-1} \) mainly reflect from flat bilayers. The thickness of phospholipid bilayers is about 5 nm. For DMPG under low ionic conditions, it has been revealed by cryo-transmission electron microscopy that large unilamellar vesicles with mean diameter around 500-1,000 nm are formed at temperatures below the melting transition [15]. Thus, here we treat the observed SAXS intensities as those scattered from randomly orientated infinity flat bilayer sheets.

The SAXS intensity for a single flat particle (lipid bilayer in this case) averaged over all orientation in three-dimensional space is given by

\[
I(q) = A \frac{2\pi}{q^2} |F(q)|^2
\]

where \( A \) is the area of the flat membrane and \( F(q) \) is the form factor of the bilayer [20]. The form factor is given by the Fourier transform of the electron density profile in the direction parallel to the bilayer normal. This expression is given as

\[
F(q) = 2\int_0^\infty \Delta \rho(x) \cos(qx) dx
\]

where \( \Delta \rho(x) \) is the electron density contrast, i.e., the difference between the electron density as a function of distance \( x \) along the perpendicular to the bilayer (\( \rho_{\text{lipid}}(x) \)) and the electron density of water (\( \rho_{\text{water}} \)).

Although the absolute SAXS intensity value cannot be calculated because of unknown value of \( A \), the relative value can be calculated from a model contrast using Eqs. (1) and (2). Thereby, we can get structural information by fitting the observed SAXS data with theoretical intensity data calculated from a model contrast containing several adjustable parameters. Many different model electron density profiles of lipid bilayers have been proposed, such as strip models, models based upon the crystal structure of lipids, Gaussian models etc. A strip model containing eight parameters has been used in a previous study of DMPG bilayers [19]. In this study, we used a three-Gaussian model, in which the electron density of lipid bilayers represents a combination of three different Gaussian functions. The reason to choose the model is that relatively small numbers of parameters are necessary. The number is four as described below. This model was originally used for the analysis of multilamellar vesicles [21]. Recently, this has been also successfully applied for the analysis of unilamellar vesicles [22,23].

The \( \Delta \rho(x) = \rho_{\text{chain}}(x) - \rho_{\text{water}} \) in three-Gaussian model is given by

\[
\Delta \rho(x) = \exp \left( - \frac{(x - x_{\text{head}})^2}{\sigma^2_{\text{head}}} \right) + \exp \left( - \frac{(x + x_{\text{head}})^2}{\sigma^2_{\text{head}}} \right) - \rho_{\text{rel}} \exp \left( - \frac{x^2}{\sigma^2_{\text{chain}}} \right)
\]

where two Gaussians of width \( \sigma_{\text{head}} \) centered at \( \pm x_{\text{head}} \) correspond to the head groups, another Gaussian of width \( \sigma_{\text{chain}} \) at the center of the bilayer corresponds to the hydrocarbon chains, and \( \rho_{\text{rel}} = (\rho_{\text{chain}} - \rho_{\text{water}})/\rho_{\text{head}} \) is the ratio of the electron density of hydrocarbon chains (\( \rho_{\text{chain}} \)) to that of head group (\( \rho_{\text{head}} \)) when the electron density of water (\( \rho_{\text{water}} \)) is set to zero. Total numbers of the parameters are four, i.e., \( \rho_{\text{rel}}, \sigma_{\text{head}}, x_{\text{head}}, \sigma_{\text{chain}} \).

The data fitting analysis was performed using a standard user-defined curve fitting function of IGOR Pro software packing (WaveMetrics, Inc. USA). From a technical reason, actual fittings were done for \( I(q)q^2 \) not but \( I(q) \). Figure 3 shows typical examples of comparison between theoretical curves calculated from the model with best fitted parameters and experimental ones.
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FIGURE 3 Comparison between theoretical values calculated from best fit parameters (solid line) and experimental values (crosses). (A) DMPG in H2O at 10°C, (B) DMPG in H2O at 40°C.

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