Neutron Diffraction with an Excess-Water Cell

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Abstract. As part of a study of the molecular basis of membrane fusion by enveloped viruses, we have used neutron diffraction to study the lamellar (L_α) to inverse hexagonal (H_{II}) phase transition in the phospholipid N-methylated dioleoylphosphatidylethanolamine. This lipid was chosen because its phase transitions are particularly sensitive to the presence of agents that have been demonstrated to promote or inhibit membrane fusion. Two different geometries of neutron diffraction were used: small angle scattering (SANS) and a membrane diffractometer. The SANS measurements were carried out on the SWAN instrument at KEK, Japan, using dispersions of multilamellar vesicles (MLVs). The diffractometer measurements used the V1 instrument at BeNSC-HMI, Germany, with a specially-constructed cell that holds a stack of lipid bilayers in an excess-water state. The two approaches are compared and discussed. Although the diffractometer takes considerably longer to collect the data, it records much higher resolution than the SANS instrument. The samples recorded in the excess-water cell were shown to be well aligned, despite the lipids being fully hydrated, allowing for the production of high-resolution data. Trial measurements performed have demonstrated that sample alignment is preserved throughout the L_α to H_{II} phase transition, thereby opening up possibilities for obtaining high-resolution data from non-lamellar phases.

Key words: phospholipids, phase transitions, neutron diffraction, inverse hexagonal phase, lamellar phase, cubic phase

Abbreviations: DOPE-Me: N-methylated dioleoylphosphatidylethanolamine; DOPC: dioleoylphosphatidylcholine; H_{II} : inverse hexagonal phase phospholipid; L_α : lamellar liquid crystal phase phospholipid; MLV: multilamellar vesicle; q : $2\pi/d$; SANS: small angle neutron scattering; SAXS: small angle X-ray scattering

Introduction

Enveloped virus particles use membrane fusion to introduce their infective nuclear material into host cells [1]. Each virus employs specialized “fusion proteins” to promote the merging of two originally distinct membranes into one. It is now known that peptides derived from the N-terminus of these fusion proteins exhibit

Figure 1. The lamellar (L_{α} – a) and inverse hexagonal (H_{II} – b) phases of phospholipids. The L_{α} phase is composed of a stack of sheets, each one comprising a single phospholipid bilayer. In the H_{II} phase, cylinders of phospholipid, each with a water-filled core, are stacked in a hexagonal arrangement.

similar membrane fusion activities. We have been studying the relationship between structure and function in a number of these fusion peptides, including those from feline leukaemia virus [2–5] and simian immunodeficiency virus [6, 7].

The precise molecular events that occur during peptide-induced membrane fusion are still unclear. Viral fusion peptides are thought to promote membrane fusion by destabilizing the L_{α} (lamellar) phase of phospholipids and promoting the formation of non-bilayer structures, such as the inverted hexagonal (H_{II}) phase (see Figure 1), by increasing negative curvature strain [8]. This would bend the individual leaflets of both the viral and cell membranes into more tightly curved structures than normally encountered, eventually leading to the formation of non-lamellar structures prior to the formation of the fusion pore [9]. This theory can be tested by observing changes in the phase behaviour of a suitable phospholipid and altering the bilayer composition by including asymmetrical agents, such as peptides [4, 10–13], lysolipids [4, 5, 14–16] or other molecules that have contrasting effects on membrane curvature strain [4, 5, 8, 17, 18].

The phospholipid N-methylated dioleoylphosphatidylethanolamine (DOPE-Me) has often been chosen for these studies because its phase transitions are particularly sensitive to the presence of agents that have been demonstrated to promote or inhibit fusion. Using temperature-scan X-ray diffraction, we have previously characterised the phase behaviour of DOPE-Me in the presence of fusion peptides and/or fusion inhibitors [3–5]. Theory predicts that, if the non-lamellar lipid phases share structural intermediates with the fusion pathway, then systematic changes of phase behaviour should give insights into the fusion process. Indeed, from our X-ray data we have postulated that fusion inhibitors work against the action of fusion

peptides by inhibiting inverted phases in general and the H_{II} phase in particular [4, 5].

The detailed structures of the phase-transition intermediates are still not known. Problems arising from structural analysis of the X-ray data include the lack of high resolution information, difficulties of separating overlapping peaks and an inability to differentiate between type I and type II (inverted) phases. For example, our X-ray work has observed transitory bilayer states with modified d -repeats (the thickness of one repeating unit, comprising one bilayer and one water layer, in a multi-bilayer stack or a multi-lamellar vesicle, see Figure 1). The fusion peptide transiently reduces the d -repeat by 4 Å [3], while a fusion inhibitor increases it by up to 6 Å [4]. With X-ray diffraction alone, it is not possible to tell whether this is caused by changes in the thickness of the water layer or the phospholipid bilayer itself. Another possibility, which cannot be easily tested using lipid dispersions, is that the modified d -repeat structure is actually a rippled bilayer phase, as observed by us previously [19, 20] in a pure lipid preparation. Additionally, a study of PE phospholipids [21] has demonstrated that very different H_{II} structures can produce the same d -spacings. In other words, there is no simple relationship between membrane curvature and (hexagonal) d -repeat. Since the principal information gained from X-ray studies is usually the d -repeat, this information, alone, is not sufficient to generate molecular models and understand the effects of fusion-active agents on monolayer curvature strain. Such uncertainties could be resolved using neutron diffraction with $^2\text{H}_2\text{O}$ – H_2O exchange to differentiate between the water and phospholipid components of the structure.

This paper describes a study of the lamellar to hexagonal phase transition of DOPE-Me using two different types of neutron scattering, namely: small angle neutron scattering (SANS) from multilamellar vesicles (MLVs) and lamellar neutron diffraction from multibilayer stacks in excess-water conditions. The two approaches are compared and contrasted, in terms of the information they report and their resolution.

Materials and Methods

SMALL ANGLE NEUTRON SCATTERING

SANS data on multi-lamellar vesicles of phospholipid (MLVs) were taken at the SWAN (Small and Wide ANgle) detector at the KENS at KEK (Tsukuba, Japan). DOPE-Me was purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Approximately 250 mg of lipid, dissolved in chloroform, were dispensed into a Pyrex test-tube. The solvent was removed under a stream of oxygen-free nitrogen to leave a film of lipid at the bottom of the Pyrex tube, which was then placed under vacuum to remove trace amounts of solvent. Each film was resuspended with water of one of four isotopic compositions (8% $^2\text{H}_2\text{O}$: 92% H_2O , 50% $^2\text{H}_2\text{O}$: 50% H_2O or 100% $^2\text{H}_2\text{O}$) and vortexed thoroughly at room

temperature. All samples had a low lipid concentration of 100 mM, equivalent to 7.57% (*w/v*), thereby ensuring an excess-water condition. The lipid dispersions were then subjected to five freeze-thaw cycles as previously described [22] to ensure that the lipid was in the fully hydrated L_α phase, regardless of the thermal history of the lipid.

MLV suspensions were placed into quartz cuvettes (10 mm $\times$ 30 mm $\times$ 2 mm) and loaded into an automatic sample changer three at a time. The sample changer was positioned in the SWAN instrument and connected to a programmable circulating water bath. Data were simultaneously collected at small and wide angles as a function of temperature. The temperature was raised from 20 °C to 80 °C in 5 °C steps. After each temperature increase, all three samples were scanned in sequence before the temperature was raised again.

Corrections for detector efficiency were applied and the time-of-flight data converted into intensity versus q ($\text{\AA}^{-1}$). The wide-angle data were scaled to match the small angle data in the region of overlap.

LAMELLAR NEUTRON DIFFRACTION

The V1 instrument at the Berlin Neutron Scattering Center at the Hahn-Meitner Institut (BeNSC-HMI), was used to collect neutron diffraction data from aligned multi-bilayers of DOPE-Me. 20 mg of lipid was dissolved in chloroform and deposited by airbrush onto a thin quartz glass plate (approximately 40 mm $\times$ 20 mm $\times$ 0.5 mm). The plate was placed under vacuum for twelve hours to remove all traces of the solvent. This simple technique produces highly aligned multi-layers in a standard manner [23].

An aluminium holder for the slide had been constructed with raised edges at the top and bottom, on which the slide rests (see Figure 2). These raised edges leave a small (0.1 mm) gap between slide and aluminium for the excess water. Water (150 μ l, equivalent to approximately 300 waters per phospholipid molecule) was deposited in drops into the groove between the raised edges, and the slide placed lipid-side-down on the holder. Capillary action distributed the water evenly across the surface of the slide.

The sample holder was placed in an aluminium can (Figure 2), constructed to have a slot in the top just large enough to accept the slide and holder, without exerting too much pressure. The can was secured upright onto a standard goniometer head, positioned so the incident beam passed through the slide side of the sample. The sample can was connected to a circulating water bath for temperature control.

The V1 instrument used a neutron wavelength of 4.53 $\text{\AA}$. The sample to detector distance was approximately 1 m. The area detector was set to move in steps of approximately 2.5 ° in 2θ , starting as close as possible to the main beam. At each detector angle (2θ), the sample was rocked through a range centred on half the detector angle (θ). Data were collected as a function of temperature, from 20 °C

Figure 2. The “excess-water” sample cell used in the measurements on the V1 membrane diffractometer at BeNSC-HMI. The top of the chamber has been removed to show the sample chamber. Mounted on a standard goniometer head, the centre of the sample cell is a flat, vertical chamber that holds the quartz side and an aluminium space (bottom). The lefthand side of the cell is cut away for the incident and diffracted neutrons. The righthand side contains channels for thermostatically-controlled water, to maintain a fixed temperature in the cell.

to 80 °C in 5 °C steps. The data were corrected for detector response and sample absorbance then integrated to give intensity as a function of q ($\text{\AA}^{-1}$).

Results and Discussion

In previous studies, we have demonstrated the usefulness of neutron diffraction of aligned lipid multilayers. In these previous studies, the samples were hydrated from water vapour in a controlled, high humidity environment. This technique greatly

decreases sample mosaicity and increases long-range order, thereby improving the resolution of the reconstructed bilayer scattering density profile. There has been some concern over the observation that these types of samples do not swell with water uptake from a humid atmosphere in the same manner as lipid in contact with excess water [24]. This point was put to rest when an excess-water cell for aligned samples was used to show equivalence between bulk water and water vapour hydration [25]. Nevertheless, it cannot be assumed that this principle holds for non-lamellar phases, where movement of bulk water in or out of the lipid assembly may be restricted. The non-lamellar phase behaviour of lipids, and therefore the validity of modelling the fusion process, could be greatly affected by the type of sample used.

Against this background, we wanted to test an approach that maintains the lipid in excess-water conditions, while providing useful resolution structural data. Fortunately, an advantage of neutron diffraction is that we can sacrifice information content in terms of fewer diffracted peaks, but then recover it in the information provided from the use of hydrogen-deuterium contrast variation. We have tested two approaches to this problem. The first is small-angle powder diffraction method, using multi-lamellar vesicles of phospholipid. The second uses aligned bilayer stacks in contact with excess water.

Figure 3 shows a comparison of the data produced by the two approaches. Both clearly start in the same lamellar phase, as expected. At 30 °C and 100% $^2\text{H}_2\text{O}$

Figure 3. Comparison of neutron scattering profiles from the two types of neutron measurement: (a) Vertically-integrated data from multi-bilayer stacks of phospholipid, using the V1 membrane diffractometer at BeNSC-HMI; (b) Radially-averaged spectra from multi-lamellar vesicles of phospholipid, using the SWAN instrument at KEK. Both sets of data shown contained DOPE-Me and 100% $^2\text{H}_2\text{O}$.

(8% and 50% data not shown), the strongest feature is the first order lamellar peak, which appears in exactly the same position in both sets of data ($q = 0.106 \text{ \AA}^{-1}$), corresponding to a d -repeat of 59.6 Å, which is in close agreement with the literature [26]. The aligned data are, at first sight, qualitatively similar to our previous small-angle X-ray data, which uses the same preparation protocol as the SANS MLV data. From our SANS experience, we expect the sample mosaicity of MLVs to be high. Therefore, the decrease in instrumental resolution in neutron diffraction is compensated by the alignment of the bilayers. The aligned data at this point are most encouraging.

A commonly noted property of lipid lamellar diffraction is that only four Bragg orders are necessary to reconstruct the bilayer scattering density profile to sufficient resolution to locate large macromolecules such as peptides [27]. A strong second order lamellar peak was recorded on V1, but not on SWAN. Furthermore, this second order remains strong in the aligned sample data as the temperature is increased. Data were collected out to $q = 0.35 \text{ \AA}^{-1}$ on V1 and $q = 0.24 \text{ \AA}^{-1}$ on SWAN. With the addition of deuterium labels and heavy water contrast matching, the current signal could be further enhanced allowing higher resolution information to be gained.

Although the d -repeat of the MLV sample remains reasonably constant as the temperature rises, the d -repeat of the aligned sample clearly decreases (see Figure 4). It appears that d -spacing changes are more readily accessible in the aligned sample, despite the planar constraints on the system imposed by the solid sample support. A possible explanation is that the removal of water from the sample, necessitated by the decrease in d -repeat, is more easily achieved in the aligned

Figure 4. A full temperature-scan series of data from multi-bilayer stacks of DOPE-Me and 100% $^2\text{H}_2\text{O}$, using the V1 membrane diffractometer at BeNSC-HMI.

Figure 5. Calculated diffraction pattern from H_{II} phase phospholipid. The top panel shows the reciprocal lattice points of the H_{II} phase. The dotted lines, at constant radius, show how some of the peaks become superimposed when powder diffraction or radial averaging is used. The lower panel shows the reciprocal lattice points in radial-averaging geometry. The line emphasises the rapid decrease in intensity with q , when powder diffraction is used to study the H_{II} phase.

sample. Water movements along the bilayers could be quite rapid, especially when compared to the MLV sample in which water compressed out from between the inner bilayers of the onion-like vesicles would have to cross the outer bilayers in order to reach the bulk solvent. Kaasgaard and co-workers have demonstrated that removal of this trapped water causes structural rearrangements in MLVs [28].

The transition to non-lamellar phases is marked by the appearance of new diffraction peaks (see Figure 5). The SANS geometry is consistent with recording these off-axis peaks, in contrast to the diffractometer which is configured to scan only along one axis of diffraction. However, these new peaks tend to be rather weak and are either not recorded at all in the MLV data, because of spherical averaging of

Figure 6. (a) Non-lamellar peak appearing as an “ear” at the side of the second order of lamellar diffraction from an oriented sample of DOPE-Me in 67% $^2\text{H}_2\text{O}$. (b) Similar non-lamellar reflection, in a mixed DOPC-DOPE bilayer, previously interpreted as evidence of a rippled transition phase [19, 20].

the data, or are inseparable from other reflections. Figure 6 shows such a reflection, appearing as an “ear” on the side of the second order of lamellar diffraction in the V1 data. These reflections were first observed by us some years ago [19, 20], and interpreted as indications of a ripple phase.

Against the increased resolution and detail of the aligned sample approach, there is also an increased data collection time. With modern SANS instruments, a complete frame of data can be obtained in an hour or less. In comparison, the diffractometer used to collect data from the aligned sample needs to be reconfigured many times for every set of data. It should be noted that, although the measurements described here comprised a $\theta - 2\theta$ scan, in order to collect a full set of data it would be necessary to scan in two dimensions, not one. Fortunately, the damage inflicted on samples by neutrons is very much reduced in comparison to X-rays, and several days of continuous data collection from one sample should not be a problem.

The low- q region of the MLV data shows some interesting features that are not represented in the aligned data. These are relatively large structures (80–105 Å),

but it is not yet known what they are. They do not appear to be present in the aligned sample data, possibly indicating that they represent large liposome or cubic phase structures, which are not able to form in the constrained space of the excess-water cell.

Conclusions

This paper describes a novel neutron diffraction approach for the study of phospholipid phase transitions. The “excess-water” cell has all the advantages of multibilayer stacks (small sample size, high degree of alignment and corresponding high resolution of data) while retaining the physiological-relevance of experiments carried out in fully-hydrated conditions. In the experiment reported here, we have performed trial measurements with an excess-water cell, developed at BeNSC-HMI. The results show that the phase transition behaviour of a phospholipid in one of these cells is comparable to that previously observed from a lipid dispersion, but sample alignment is preserved throughout the transition, resulting in considerable improvements in resolution over previous SANS methods.

Having successfully demonstrated that the new cell does not affect the phase transition temperature of phospholipids, nor introduce artefactual structures into the phospholipid phase transition, we are now in the position to use the cell to obtain high-resolution data from non-bilayer phases. The high degree of alignment ($\geq 0.5^\circ$ mosaic spread) will allow high-resolution studies ($\geq 0.5 \text{ \AA}$) with suitable deuterium labels. Furthermore, it will allow us to locate viral fusion peptides in non-lamellar phases of phospholipids, to complement previous data on this peptide in lamellar-phase lipids [6].

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