Lamellar diffraction from lipid bilayers on MIRA, a triple axis spectrometer at the MLZ

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Abstract. Diffraction used in conjunction with molecular deuteration provides a model independent means to examine detailed structural and compositional information of model and real biological membranes in the lamellar phase. The technique provides specific information of localization of molecules and smaller units with respect to the unit cell. Deuteration is used to provide specific labelling and to provide phasing for the crystallographic reconstruction. Typical data sets consist of a series of lamellar diffraction peaks, usually collected under conditions of 3 contrasts of the water gas phase. Accurately integrated the diffraction peaks can be used for a Fourier reconstruction of the composition of the lamellar unit cell in real space. Each diffraction peak which can be integrated for 3 contrasts contributes to an additional Fourier term in the reconstruction and optimization of the number of peaks enhances the spatial resolution of the crystallographic reconstruction. Here we report on the use of the flexibly configurable cold triple-axis spectrometer, MIRA, at the Heinz Maier-Leibnitz Zentrum (Garching, Germany) for investigations of different lamellar systems using this approach. The data sets are acquired from lamellar stacks in an sample environment with humidity and temperature control with exceptionally low background. We discuss further enhancements of the instrument and sample environment which will provide information on the composition and equilibration of the sample.

1 Introduction

The low intrinsic curvature of most cell membranes ensure that highly oriented bilayers are excellent model systems for membrane biophysics. For this reason specular reflectivity[1] and lamellar diffraction[2, 3], which both provide compositional information in a direction normal to the lipid bilayer, are important tools to reveal the time averaged locus of molecules and their compositional fragments within the bilayer (Figure 1). This understanding is an important paradigm for a range of biophysical and biochemical problems but also forms an important and direct experimental reference point for computational membrane biophysics[4].

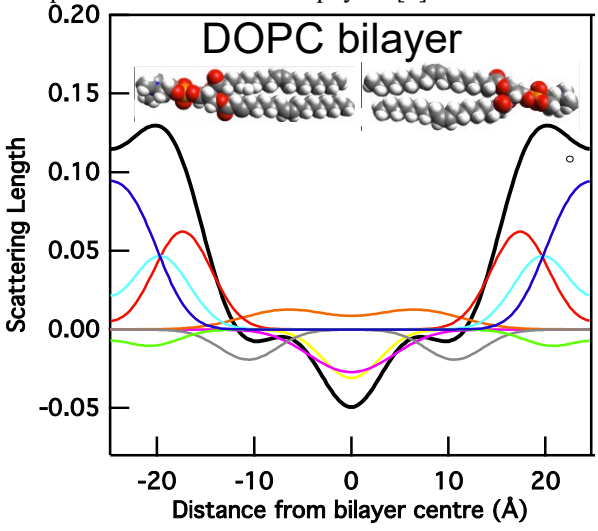

Fig. 1. Two 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) molecules forming a bilayer are shown at the top, giving a reference for the hydrocarbon tails, headgroups and water. A scattering length density profile determined from diffraction data. This profile (thick black line) has been decomposed into individual molecular fragments: 1. terminal methyl group (yellow); 2. –(CH2)7- group (magenta); 3. double bond (orange); 4. –(CH2)7- groups (grey); 5. glycerol (red); 6. phosphatidyl (light blue); 7. choline (green); and 8. water (dark blue).

Here we describe the use of lamellar neutron diffraction in the context of the problems in membrane biophysics to find the average location of molecules in the bilayer structure shown in Figure 1. It is also important to place this technique in the context of the complimentary neutron technique, specular neutron reflectivity. Both techniques are used to determine compositional information of the bilayer structures, however the demands of beamtime and samples are quite different. Both techniques can make use of deuterated molecules, which provided certain caveats are observed[5], provide a convenient means of labelling specific aspects/regions of the SLD profile shown in Figure 1.

Bilayer sample preparation for neutron reflectivity is extremely demanding. The real-space analysis is achieved by modelling of composition slabs, requires deconvolution the structure of interest from the overall scattering length density profile. The quality of data is largely limited by the ability to prepare molecularly smooth samples, a single bilayer, over the area of the reflected neutron beam (~cm^2) with appropriate contrast over a liquid sub-phase. The information content, and thus uniqueness of SLD modelling from reflectivity data, can be improved by selective labelling with deuterium, or contrast variation with the water phase or magnetic methods[6]. While sample preparation can be a fundamental limitation of the technique the success of

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neutron reflectometers is evident in the number of instruments and their facility support.

Lamellar neutron diffraction utilises multiple stacks of bilayers self-assembled on a solid substrate and data is analysed by a direct reconstruction of the scattering length density profile[7]. Cylindrical geometry of membranes may also be usefully exploited[8]. Samples are placed in an environment of controlled humidity. This environment is used for gas phase exchange and control of the contrast of the sorbed water between bilayers. The ease of control of humidity and ability to study dehydration processes has enabled successful studies related to cryobiology and anhydrobiology[9-11]. Understanding of molecular packing of lipid molecules which form important barrier in the skin's stratum corneum layer has been also been enabled by the technique[12, 13]. When samples consist of whole plasma membranes biophysically important aspects of the composition profile may be revealed[14, 15]. More generally the technique has revealed high resolution and important details of the packing of many small molecules into self-assembled bilayer stacks.

The Fourier reconstruction of the unit cell (Figure 1) is made from the variation of peak intensity with contrast which recovers that phase information that is lacking from simple diffraction peak intensity measurements. The mathematical formalism for the Fourier reconstruction of SLD is that same as that adopted elsewhere[3]. The structure factor contribution, *f(h),* of diffraction peak of order h, is

$$f(h) = \sqrt{I(h)A(h)\sin\theta} \quad (1)$$

Where *I(h)* is the measured peak integral, *A(h)* is the adsorption correction and is the Lorentz correction at angle θ. Each measured *f(h)* and its phasing is a contribution in the Fourier series to the overall variation of the scattering length density in the unit cell, $\rho^*(z)$ in the real space co-ordinate, *z*, per lipid molecule:

$$\rho^*(z) = \rho_0{}^* - \left(\frac{2}{dk}\right)\sum_{h=1}^{h_{max}} cos\left(\frac{2\pi hz}{d}\right) f(h) \quad (2)$$

where $\rho_o{}^*$ is the product of the average scattering length density per unit cell and the area per lipid molecule, *d* is the repeat spacing of the unit cell and *k* is an instrumental scaling constant. Determination of the phasing of each *f(h)* is made from plots of $\pm\sqrt{f(h)}$ versus % D_2O.

2 Methods and Materials

2.1 Sample Preparation

Orientated lamellar stacks with good registry with the substrate surface are most easily prepared by spraying all sample components from a suitable solvent mixture[9, 10] using a commercial airbrush on to a quartz microscope slide. Other sample strategies may be adopted depending on the amount of available material and/or a suitable solvent systems[7]. The area of sample should be of the order cm^2, containing ~ 5 mg of materials. Thicker samples, while diffracting more strongly, particularly for lipids in the gel phase[13], provide challenges in terms of formation of well ordered lamellae, equilibration times for D_2O exchange and efficient use of neutron beamtime. After initial deposition solvent can be removed in a vacuum oven, the layer can be annealed towards improved lamellar order with the high humidity, overnight at high humidity (e.g. 97 % RH) and/or temperature at above gel to fluid transition. Sample quality, particularly the number of lamellar reflections orders and mosaic spread may be monitored with laboratory X-ray diffraction in grazing incidence or Bragg-Brentano geometry (Figure 2). Typically a mosaic spread of the lamellar phase ± 0.05° may be expected.

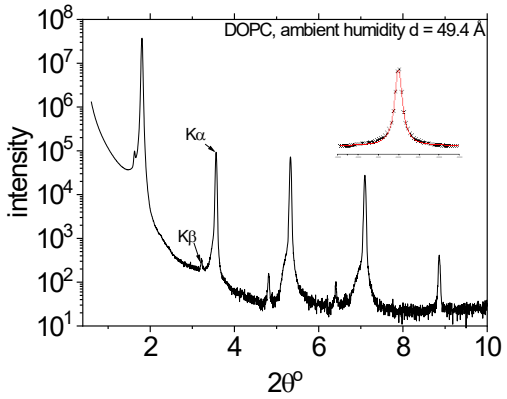

Fig. 2. Laboratory X-ray diffraction from successfully prepared sample of DOPC bilayers in ambient conditions showing well resolved Bragg peaks and the clear effects of the polychromaticity of Cu X-ray source, Kα and Kβ lines (labelled). The inset, shows a rocking curve around the first order diffraction peak.

2.2 MIRA – As two circle cold diffractometer and sample environment

The cold triple axis spectrometer MIRA (now known as LaDiff), can be utilized in elastic mode where the analyser axis is still in place but tuned to the same energy as the monochromator. This enables a much cleaner beam, with a very low background, and is ideal for measurement of normalized diffracted intensity from thin lipid lamellar films on flat substrates. The low background allows the measurement of many orders of lamellar diffraction, and most importantly accurate integration of the peak intensity. Each peak integrated for the 3 contrasts contributes an extra term to the Fourier series of equation 2. Samples are mounted in an environment with controlled humidity and temperature. Two systems are available for humidity and temperature control (10- 60°C):

1. Thermostated cylindrical aluminium cans which are equipped with a reservoir for a saturated salt solution. The humidity generated is determined by the salt identity. A range of salts is available with varying in the humidity and temperature dependence of the humidity[16].
2. 3D-printed metal cells also offer temperature control. This adaptation of the flexiProb[17] sample environment offers control of humidity and D_2O/H_2O contrast by use of a flow through system. There also

exists the possibility of introducing other volatile molecules to samples for gas phase adsorption with this system.

Using the sample environments above, a typical experiment consists of the measurement of peak integrals at 3 contrasts on the same sample for comparison of the peak intensities. For this reason it is critical to position the sample identically for each contrast and samples are typically cycled through the 3 contrasts in the one of 3 duplicate sample cans with locating pins ensuring identical sample positioning with respect to the incident neutron beam.

The triple-axis spectrometer MIRA has access to an appropriate range of scattering vectors to interrogate the integral intensity of each diffraction peak with variation of the contrast of the sorbed water phase. Additionally it has the ability to significantly reduce the background signal from the sample[18]. After the sample is first aligned with a laser illuminating the sample position along the direction of the neutron beam an iterative procedure between a θ/2θ scan and θ scan on the first order Bragg peak is used to align the sample and calibrate the θ/2θ geometry via an offset (Figure 3).

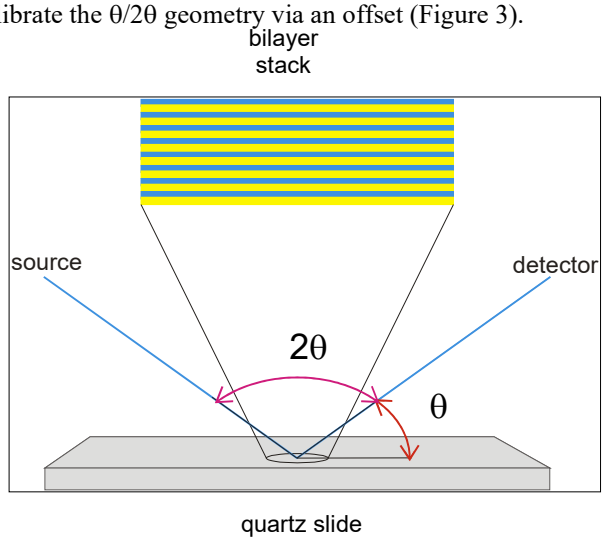

Fig. 3. Top schematic view of the measurement geometry with respect to the sample, a bilayer stack with excellent registry to the quartz substrate. With the source fixed with respect the sample, the θ/2θ measurement is a achieved by detector movement around the sample (2θ), and sample rotation, θ.

A typical measurement series consists 3 complete scans at contrasts of 50, 20 and 8 % (null water) D_2O in H_2O. Depending on the number of observable peaks, with each higher order peaks measured with 3 contrasts contributing an additional Fourier term to the reconstruction, measurement times have varied between 2-8 hours. While higher order peaks, which require longer acquisition times, are clearly desirable in order to avoid truncation artefacts of the Fourier series (equation 2) care must be exercised that these artefacts/features are smaller than features of interest in the SLD profile.

An important consideration is the equilibration time for samples. The time for equilibration between contrast changes is of the order several hours, but can be longer for samples in the gel phase.

3 Results

Figure 4 shows a data typical set from MIRA (DOPC 75 % relative humidity, 100% D_2O, 25°C) utilising 5 Å neutrons in the θ/2θ geometry. Together with the low background the instrument configuration is sufficient to obtain 5 orders of lamellar diffraction from DOPC in the lamellar fluid phase in approximately 3 hours for each contrast. The accessible minimum angle is sufficient for accurate integration of the first order peak. The maximum angle for the scan measured was determined by the available counting time. In the case of the profile shown as part of Figure 1 the profile has been reconstructed from 5 Bragg peaks at 3 different contrasts. This profile is consistent with those determined in the literature[19].

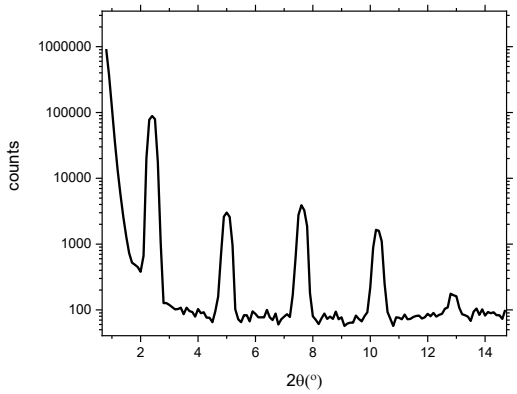

Fig. 4. Neutron diffraction from a oriented lamellar sample of DOPC on a quartz substrate at 25°C, 75 % relative humidity in 100 % D_2O. The humidity was produced by a saturated solution of NaCl in D_2O.

The overall profile was deconvolved into different Gaussian functions representing the average position of the molecule or molecular fragment and the contribution of each fragment to the scattering length density profile. The Fourier reconstruction and deconvolution of the resulting profile were achieved using specific macros for the program IgorPro (Wavemetrics, Oswego, USA) written by Dr Thomas Hauβ (Helmholtz Zentrum Berlin).

4 Conclusions and Outlook

Neutron diffraction is a technique which produces model independent information which is quite similar to that produced by the modelling of neutron reflectometry. However the technique is suitable for quite different problems in membrane biophysics, particularly those at reduced and variable hydrations, complex but ordered biological systems and lipid mixtures.

MIRA and its sample environment are quite suitable for making quantitative lamellar diffraction measurements. Sample preparation facilities and initial characterization are provided onsite at MLZ.

Current developments around the instrument include:

1. An *in situ* Raman measurement system. This development is aimed at quantify gas phase adsorption into the sample and details on the packing of molecules into the membrane stack.
2. An upgrade to the detector on MIRA (LaDIFF). The current point detector of MIRA is being replaced with detector bank around the sample. The 2D detector will be optimized for a lower background and will enable measurements of data sets which include sample rocking curves in measurement times currently unfeasible with a point detector.
3. Development of python based in house open source software for data processing, Fourier reconstruction and deconvolution of SLD profiles.

We would like to acknowledge the contributions of Dr Thomas Hauβ (Helmholtz Zentrum Berlin) for the IgorPro macros used for SLD profile reconstruction (available on request), Dr Ben Kent (School of Chemistry, University of New South Wales) and Professor Gary Bryant (RMIT University) to this work.

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