Neutron Diffraction Analysis of the Structure of a Lecithin/Cholesterol Bilayer Membrane using the Advanced Neutron Diffractometer/Reflectometer (AND/R)

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I. Introduction

Bimolecular layers of lipids are the basic structural element of biological membranes. The hydrophobic core of such bilayers provides the main permeability barrier to solutes such as salts, sugars and other metabolites. Phospholipids and cholesterol are main components of the plasma membranes of animal cells and bilayers of these compounds are extensively studied by physical-chemical techniques, recently including molecular dynamics simulations.

Many lipids spontaneously form smectic phases on evaporation of solvent from solutions. Oriented multilayer samples are readily formed on glass or silicon substrates and can be several thousand layers thick. Multilayers of just a few layers can be formed by Langmuir-Blodgett techniques. The samples are well ordered in one dimension, perpendicular to the layers, but disordered in the plane of the layers. Diffraction is thus one-dimensional and relates to molecular structure across the layers. By introducing deuterium into particular components and using difference analysis, structural details and information on dynamics can be obtained from limited diffraction data. Information obtained includes thickness of the hydrocarbon and polar group regions, molecular order and packing in the hydrocarbon region, hydration of the surface layers, locations and Debye-Waller parameters of added components, especially deuterium.

This experiment measures neutron diffraction from bilayers of dimyristoyl phosphatidylcholine and cholesterol using the Advanced Neutron Diffractometer/ Reflectometer (AND/R). This instrument will be used in the diffraction (crystallography) mode. This mode of measurement is preferable (for ease of data collection) to the reflectometry mode when there are many layers and the intensities are concentrated into sharp peaks at the Bragg positions. Samples usually consist of 2-3000 layers. Bragg peaks are already quite sharp with about 20 layers. Dimyristoyl-phosphatidylcholine consists of two 14-carbon fatty acid chains esterified to the glycerol group of glycero-phosphatidylcholine.

Objectives of the Experiment are:
1) To determine the repeat spacing (d) of the multilayers using the Bragg equation: $\mathbf{n\lambda = 2d\sin\theta}$.
2) To determine the magnitude of the one-dimensional structure factors from the Integrated Intensities of the diffraction peaks I(n).
3) To determine the phases of the structure factors by D_2O/H_2O exchange.
4) To calculate the one-dimensional scattering length density profile across a bilayer membrane using the results of 2) and 3) as a Fourier transform of the structure factors.
5) To calculate a difference profile (D_2O-H_2O) to determine the distribution of water across the bilayers.

II. Planning the Experiment

1. Sample size and thickness.

Samples are formed as thin layers on thin glass substrates (microscope slide cover slips). This choice has the disadvantage of being fragile, but provides minimum background since the amount of glass in the beam is small. Glass gives significant scatter which increases at high Q. Glass is preferable to silicon because of better adhesion of the lipid. Samples are mounted vertically and flow of lipid on silicon has sometimes been observed.

Diffraction intensities are attenuated due to incoherent scattering from hydrogen, of which there is much in the samples. This requires absorption corrections to the intensities. The correction is largest for the 1st order peak, where the incident angle is smallest according to the Bragg equation. To keep these corrections small and to produce well-oriented layers (mosaic spreads of 0.5 degrees or less) the lipid samples must be thin and 1-3 milligrams/cm^2 have been found to work well. Corresponding thicknesses are 10-30 microns.

The height and width of the sample needs to correspond to the beam at the instrument. The beam width is limited to about 2cm. Measuring to $\theta = 30$ degrees would allow a sample of 4cm width. Widths of 2-3cm are most frequently used since measurements can go beyond 30 degrees. Sample heights are up to about 4cm. Thus, amount of lipid is usually 16-36milligrams. Samples down to 4mg have been measured.

Question: If the membrane repeat spacing is 55 Angstroms and the instrument wavelength is 5 Angstroms, how many Bragg orders would be covered in a scan to $\theta = 30$ degrees?

2. Resolution and Background Considerations.

For a typical lipid bilayer repeat spacing of 55 Angstroms, the instrument wavelength of 5 Angstroms gives approximately 5.2 degrees between peaks in the scattering angle (2θ). Angular collimation before the sample of less than a degree is therefore desirable. Using only a pencil detector rather than a position sensitive area detector makes it preferable to have samples with small mosaic spread and angular determinations by sample rotation (θ) rather than detector rotation (2θ).

Collimation consists of two slits before the sample (S1 and S2, controlled by motors 1 and 2) and two slits after the sample (S3 and S4, controlled by motors 5 and 6). Sample and detector rotations are motors 3 and 4 respectively. In the crystallography mode of data collection, the first slit, S1 (226cm upstream from the sample, inside the monochromator shielding) is kept fixed, for example at 12mm width, giving a beam divergence at the sample of 0.3 degree. The second slit, S2, must be opened enough so that the sample sees all the 12mm wide source, but is used as a guard slit to reduce background. It is therefore opened stepwise during the scan so that all parts of the rotating sample see all the source. After the sample, slits S3 and S4 are opened to accept all the Bragg diffraction. They can be 20mm wide, nearly the width of the pencil detector.

In some cases the 1st slit after the sample (S3) is gradually opened to reduce background scattering near the beam.

The crystallography mode is different from the reflectometry mode in the control of the slits. In reflectometry, all slits start at small values and are gradually opened, the first two slits keeping a constant area of the rotating sample exposed to neutrons. The beam intensity on sample therefore changes and must be separately measured giving Reflectivity as the ratio of scattered intensity to the incident intensity. In the crystallography mode the incident beam intensity is kept constant, giving the relative intensities of the Bragg orders, but the absolute reflectivity is usually not determined. This can be done however, and is especially easy for flat, uniform samples (see Neutron Optics by V. Sears).

The scattered intensities are concentrated into the Bragg peaks. Therefore the background from incoherent scattering is the intensity between peaks and is easily subtracted without requiring a separate measurement.

III. Data Collection.

1. Instrument Configuration.

The sample should be clamped in position on the sample table, salt solution for relative humidity control added to the Teflon buckets and the Aluminum hat put on to enclose the sample environment. The sample angle will be close to correct but needs checking for each sample so that θ and 2θ are coupled correctly. The 1st slit (inside the monochromator shielding) is set to 12mm. Slit 2 (38cm upstream from the sample) is set at 2.2mm for 0 degrees and opens 0.4mm per degree change in theta. The slit just after the sample (S3, motor 5) is set at 3.2mm for 0 degrees and opens 1.2mm per degree change in theta.. It can also be set at 20mm and kept fixed if background at low angles permits. The slit just before the detector (S4, motor 6) is kept fixed at 20mm.

A θ-2θ scan is now made to find a peak. Then a check on θ-2θ coupling is made by setting the detector on at a peak and scanning θ to find the peak position and next setting θ at the peak and scanning 2θ to find the peak position with S3 set at 2mm or thereabouts. Theta is then reset if necessary to half the 2θ value at the peak. Sample tilt (motor 8) should be checked with the 1st order peak and S3 = 1mm, then the tilt set to give the maximum counts.

2. Measurement.

A θ-2θ scan for data collection can now proceed. Scan from 2θ of 3 degrees to start outside the direct beam. The 1st 4-5 orders are strong and can be scanned in about an hour (200K monitor/ point). Higher orders take longer. Scans are typically made in equal angular steps, say 0.05 degree steps in θ. The step size should be such as to have at least ten points on each peak and peak shape can be checked. Asymmetric peaks can be a sign that θ-2θ coupling needs attention (note that this is because of the small acceptance of the pencil detector and is not a problem with an area detector). Counting times will depend on counting rates in the Bragg peaks (depends on sample). If integrated intensities

with 1% counting statistics are desired then at least 10,000 integrated counts will be needed in the peak.

After data is collected for H_2O humidity, change to 20% D_2O salt solution. This increases the scattering length density of the water in the sample and is used to determine phases of the structure factors, which are 0 or π for the centrosymmetric structure.

IV. Data Reduction.

There are three steps to data reduction:
1) Integrating peak counts and subtracting background.
2) Correcting integrated intensities for absorption and geometric factors.
3) Calculating structure factors as square root of the corrected intensities.

Integrating the peaks for scans of constant angular steps is easily done by integrating in θ and is just a sum of the counts (constant $d\theta$). Crystallographers usually integrate intensities this way. When peaks need to be integrated in Reflectometry, this is usually done in Q, but since Q steps aren't constant with constant angular steps ($dQ=4\pi(\cos\theta)d\theta/\lambda$), the integration is less simple and dQ values must be applied. The $\cos\theta$ term in dQ accounts for a difference in geometric factors used in crystallography compared to reflectometry.

Geometric factors arise because of the different rates at which the rotating sample passes through the diffraction condition of Bragg's law. Another way of stating this is that the lattice points on the reciprocal lattice pass through the sphere of reflection (Ewald sphere) at different rates as the sample is rotated at constant angular velocity. In crystallography the correction is called the Lorentz factor. For equatorial diffraction such as measured here and in reflectometry, intensities are corrected by a Lorentz factor of $\sin2\theta$. Note that $\sin2\theta$ for the Lorentz factor requires integrating peaks in θ. If integrated in Q, the Lorentz factor is $\sin\theta$, since $\sin2\theta = 2\sin\theta\cos\theta$ and the $\cos\theta$ part is already applied by the dQ values in the integration.

The absorption correction requires the assumptions that the sample is of uniform thickness, no edge effects and uniform diffracting power throughout the sample. Because of these assumptions, samples are best kept thin so that the corrections are not too large. The absorption and incoherent scattering cross sections of the atoms in the sample provide a characteristic length (t_o) for 1/e absorption in the lipid (about 1.6mm). The path length through the sample depends on θ and also the depth in the sample where the diffraction occurs. Integrating across the sample thickness gives a correction factor of

$$\mathbf{C_{abs}(n) = z/(1-exp(-z))} \quad \text{where} \quad \mathbf{z = 2t/t_o\sin\theta}$$

and t is the sample thickness determined from lipid weight, area and density.

Structure Factors (magnitude only) are given by

$$\mathbf{|\,F(n)\,| = \{\, I(n)C_{abs}(n)\sin2\theta\}^{1/2}}$$

This may be compared with the Reflectivity formula in the kinematical approximation:

$$R = I/i_{inc} = 16\pi^2 \mid F \mid^2 / Q^2$$

Where $\sin\theta$ terms are contained in Q, one arising from the changing incident beam intensity (i_{inc}), which doesn't occur in the crystallographic mode. The other $\sin\theta$ factor is equivalent to the Lorentz term in crystallography with peaks integrated in Q rather than θ, as discussed in the second paragraph of this section.

V. Data Analysis and Fourier Profiles

Bragg spacings can be calculated using peak angles and the Bragg equation. Note however that the instrument software also gives Q values at each data point and Q is $2\pi n/d$. Higher order peaks give more accuracy for d if counts are sufficient. If θ is not zeroed exactly, the small error can be avoided by plotting n versus Q. The slope is $d/2\pi$. This way all the peaks are used to determine d. It provides the best accuracy and also a check that all peaks are in expected positions.

The $\mid F(n)\mid$ can only be used to determine a profile of the scattering length density if the relative phases of the structure factors $\mid F(n)\mid$ are known. For centrosymmetric structures these phases are restricted to 0 or π, corresponding (by trigonometric identity) to +/- signs for $\mid F(n) \mid$. Densities are given by

$$\text{Density} = \sum_n \mid F(n)\mid \cos(2\pi nx/d - \phi_n) \qquad \text{where } \phi_n = 0 \text{ or } \pi.$$

To determine ϕ_n, note that the water in the layers should be localized at a center of symmetry (there are two centers of symmetry, one between bilayers and one at the center of a bilayer, the hydrocarbon region). If the water location is chosen as the origin and the density is increased there by adding deuterium (as D_2O), a positive value is added to each structure factor. Positive structure factors will therefore increase in magnitude with deuterium addition and negative ones will decrease. Thus, just by inspection of whether intensities increase or decrease, phases are determined. Difficulty can arise for smaller structure factors if the added positive value is too large, giving a change of sign. This ambiguity can be removed by measuring in at least three H_2O/D_2O mixtures. A plot of structure factor versus % D_2O must be linear (centrosymmetric structure) and the ambiguity is removed because changes of sign are identified.

This phasing procedure is essentially that of the heavy atom method in macromolecular crystallography. Protein structures by X-ray diffraction depend on such methods in one form or another (multiple heavy atom replacement or multiple anomalous dispersion-MAD). Good data is needed for non-centrosymmetric structures since phases can be anything from 0 to 2π. In all cases at least three sets of data are needed and the change of scattering strength must be done in an isomorphous way. This phasing procedure is also the same as the "insertion method" of Reflectometry.

Phasing by using the water distribution assumes that the distribution is localized. The extreme of localization is the Dirac delta function. The Fourier transform of a Dirac delta function is just a constant. If the water distribution is Gaussian, the Fourier

transform is also Gaussian and so is always positive but eventually gets too small in the higher orders to change the structure factors and provide phases. If the water distribution is such as to have negative structure factors, then this must be known or phasing errors result.

Introducing deuterium into specific parts of the lipid molecules by chemical synthesis is another way of solving the phase problem. Since the deuterium is covalently bound it is usually better localized than the water distribution and thus gives phase information to higher orders. Measurements of at least two samples are needed and determination of phases is less straightforward than for water since the deuterium is usually not at a center of symmetry. Nevertheless this procedure works extrememly well and deuterium labeled cholesterol (5 deuterium near the hydroxyl group) has been used extensively.

As the final part of the experiment, calculate both the bilayer profile in H_2O and the water profile by Fourier difference using the D_2O data. Is the water distribution reasonably Gaussian?

References.

1) V. Sears, (1989). Neutron Optics. Oxford University Press. See especially Chapter 5.

2) U.W. Arndt and B.T.M. Willis, (1966). Single Crystal Diffractometry. Cambridge University Press. Chapter 11 gives a clear derivation of the crystallographic Lorentz factor.

3) K.E. van Holde, W.C. Johnson and P. S. Ho, (1998). Principles of Physical Biochemistry. Prentice Hall. Chapter 6 provides a good introduction to crystallography of macromolecules and methods for solving the phase problem. Chapter 7 treats X-ray and neutron scattering generally.

Neutron studies in structural biology, including membranes, are extensively treated in the following:

4) B.P. Schoenborn and R.B. Knott, editors, (1996). Neutrons in Biology. Plenum Press.

5) J. Katsaras et al., (in press). Neutrons in Biology.