Studying Interactions with Biological Membranes using Neutron Scattering

Duncan J. McGillivray

Chemistry Department, The University of Auckland, PB 92019, Auckland 1142
(e-mail: *d.mcgillivray@auckland.ac.nz*)

Introduction

Biological membranes, especially cellular membranes, play an exceptionally important role in most physiological processes. They function as the primary gate-keeper for the cell, and through their interaction with proteins or other biological molecules are essential in regulating the relationship between a cell and its surroundings and display a rich variety of structure and function. However, a cell membrane is a difficult entity to characterize at the molecular level. The cellular membrane is extremely complex and consists of a mixture of lipids, proteins and sugars. It has interacting functions that are not fully understood as illustrated by the complexity of cholesterol behaviour in lipid membranes.[1] Moreover, the cellular membrane is an intrinsically fluid and highly disordered system, which relies on this disorder for its physiological functioning. Thus, it is not well suited to crystallography studies. Indeed, the highly asymmetric environment of a cellular membrane, characterized by a strongly hydrophobic interior with fully hydrated hydrophilic headgroups forming the exterior, is difficult to reproduce in a crystal. Of the many proteins known to be integral to cellular membranes only a small number have been crystallized, and this number is growing much more slowly than for soluble proteins.[2]

Biological membranes are also fragile and consist of a very small percentage of the total mass in a cellular system, so surface sensitive techniques are essential. There has been considerable development of techniques capable of measuring bilayer membrane model systems, including Atomic Force Microscopy (AFM), electrochemical methods (especially Electrical Impedance Spectroscopy – EIS), ellipsometry, Quartz Crystal Microbalances, reflectance IR measurements, or through neutron and X-ray scattering.

Of these, neutron scattering is particularly well-suited to the investigation of the structures of membrane systems, and is currently being developed internationally to take advantage of a range of new, brighter neutron sources and instruments. To date, the principal obstacle to neutron scattering has been the limited access to neutron sources. The two major types of neutron sources are restricted to large-scale experimental centres. The first type is a reactor-based neutron source, where the operation of a research reactor (of much lower power output than a typical nuclear power station) is optimized so that the normal neutron radiation is maximized, while heat production is minimized. The other type of source is a spallation neutron source, in which pulses of particles (typically protons) are accelerated into a heavy metal target, literally chipping (*spalling* in mining parlance) neutrons free. Europe has traditionally dominated neutron-scattering based science, and the current two brightest sources of neutrons are there – the ILL (a reactor in the south of France)[3] and ISIS (a spallation source near Oxford, UK).[4] Recently, however, there has been a surge in the development of neutron sources, with new facilities recently opened or opening in the US, Japan, Germany and, more locally, in Sydney.[5] The proximity of the new research reactor in Sydney, which goes into user operation for most of its instruments early in 2009, makes a sustained program of research into biological membrane interactions using neutrons much more feasible in New Zealand.[5,6]

Neutron Scattering

The principles of neutron scattering are relatively straight forward, and similar to those of light or X-ray scattering. An intense beam of highly collimated neutrons shines upon a sample, and the fluctuations in the intensity of the scattered beam is measured as a function of either the scattering angle or the energy of the incoming beam (Fig. 1). In reflection mode, the scattering is from a surface flat and smooth enough to give rise to specular reflection. The reflectivity gives information about the nature of the interfacial region, specifically picking up on scattering length density changes perpendicular to the surface (which can be related to physical density changes).[7] In small angle scattering mode, on the other hand, the scattering is from a bulk sample and information is gained about density fluctuations in the bulk – such as the number, structure and shape of particles in solution. Extracting the information from the measured scattering is well-developed for both techniques,[8] and shares much with light and X-ray measurements. That said, there are a number of particular features of neutron scattering that make it well-suited to probing biological questions.

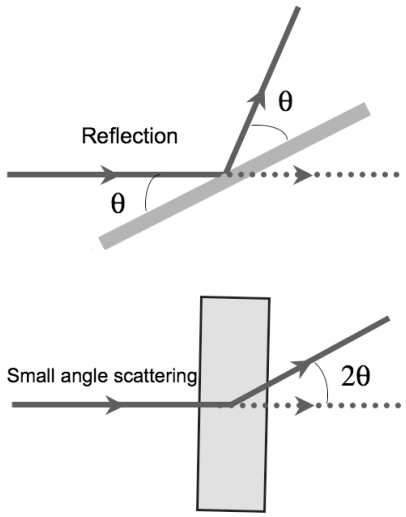

Fig. 1. Scattering geometries commonly used in neutron and X-ray scattering

The first is that the measurement is sensitive to interactions between the nuclei of atoms in the material probed

and the neutrons, rather than the electron cloud (as is the case for X-rays). The strength of this interaction is measured through a quantity known as the *scattering length* of the nucleus, which varies in an erratic fashion across the periodic table and may differ significantly even for isotopes of the same element. This measure is difficult to predict from first principles, but has been well measured for most common isotopes of biological interest.[9] Table 1 shows that the isotopes of hydrogen interact strongly with neutrons relative to other biologically relevant elements – this means that hydrogen atoms in biological structures tend to be well-defined by neutron scattering, in sharp contrast to X-ray scattering where the scattering from H is very weak. But perhaps the most important feature is that ^{1}H and ^{2}H (D) have very different scattering lengths. The scattering length difference between ^{1}H and ^{2}H give rise to the possibility of using deuterium labelling to highlight features of interest in biological structures, a particularly non-invasive and simple labelling method. The H/D scattering length difference also enables measurements of a single substrate that differ only in the scattering contrast due to the solvent isotopic composition, *e.g.* measurements made in H_2O and D_2O. By constrained modelling of these multiple measurements the unavoidable ambiguity in the measurements owing to the loss of scattering phases, common to all scattering techniques, can be reduced.

Table 1. Bound coherent scattering lengths (*b*) for some common isotopes of interest to biology[a]

Isotope	^{1}H	^{2}H (D)	^{12}C	^{14}N	^{16}O
$b/10^{-5}$Å	–3.74	6.67	^{12}C	9.37	5.81

[a]Data taken from ref. 9.

Other advantages of neutron scattering are based on the rather weak interaction of neutrons with matter. This means that the neutron beams are highly penetrating, and are able to be used in situations that involve elaborate sample environments. Examples are measurements made at the solid/liquid interface, where the neutron beam must pass through thick silicon substrates; or in cases when samples are subjected to high pressures, magnetic fields or temperatures. This also means that the neutron beams do not perturb or damage the samples – which is of particular importance in delicate biological systems.

Neutron Reflection from Model Membrane Systems

The complexity of the natural cellular membrane, and consequent difficulty of making suitable measurements on it, have led many research groups to develop suitable model membrane systems that mimic biological ones (biomimetic membranes), current methods of which have been reviewed.[10] These can be broadly characterized as vesicle-based bulk systems, or some form of flat lipid mono- or bi-layer.

As mentioned above, neutron reflection is particularly well suited to the study of flat and smooth surfaces. This includes systems such as Langmuir monolayers on water that have long been studied using X-ray reflectometry. Another type of model system that has grown significantly over recent years is the use of solid supported bilayer membranes.[11] Solid support lends robustness to model membrane systems. This enables long-term or sequential measurements to be made on a single surface, and ultimately is necessary for the development of biosensor applications. In turn, the development of good model membrane systems allows the study of the interactions between biological membranes and other proteins/peptides or other chemical interactions to be better characterized in physiologically relevant and non-crystalline conditions. Solid-supported membranes can be prepared in several different ways, including vesicle rupture on a clean surface, through Langmuir/Blodgett and/or Langmuir/Schaeffer film deposition, or through use of a suitable molecule anchor which tethers the bilayer to the substrate. Such a tether gives added stability to the model membrane, but comes at the cost of reducing the mobility of the molecules in the inner and outer membrane leaflets, and may reduce the free space available between the membrane and the solid surface.[11]

Our tethered bilayer system is an example of the power of neutron reflectometry to give structural information about such a system.[12] It is based on a synthetic lipid, constructed of two alkyl tails connected to a hydrophilic hexaethylene oxide *spacer* which is functionalized with a thiol to permit the formation of self-assembled monolayers on gold (Fig. 2). A complete bilayer membrane was formed by rapid solvent exchange, which involves the deposition of a concentrated lipid solution in ethanol flushed away rapidly by aqueous buffer.[13] Neutron reflectometry showed not only that the layer was complete, and gave parameters of the bilayer thickness and separation from the surface, but also showed that the tether molecules could be reduced to as little as 20% of the inner leaflet of the membrane without compromising the completeness of the layer. This reduction in the tethering density was found to be necessary to hydrate the inner leaflet of the membrane. Previously, this hydration had been inferred but not determined by electrochemical measurements – the use of isotopic labelling of the water permits the location and density of the water in the tether region to be directly measured.[14]

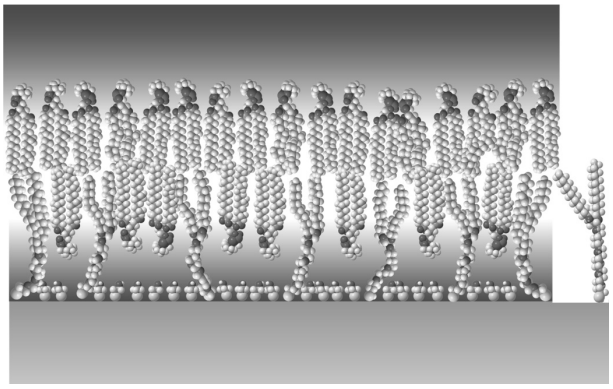

Fig. 2. A membrane system tethered to a flat and smooth gold surface suitable for characterisation by neutron reflectometry, with the synthetic tethering lipid pictured to the right. The synthetic lipid consists of two 14-carbon alkane chains, joined through ether linkages to a glycerol molecule functionalized in the 1-position with a hexaethylene glycol that is attached to the gold surface through a thiol. The spacing between tethers in the inner leaflet is controlled by the co-adsorption of small mercaptoethanol molecules to the surface. The bilayer membrane is completed with phospholipid molecules (reprinted with permission from ref. 12. Copyright American Institute of Physics 2007).

This membrane now has been used to test the membrane interactions with pore-forming and toxin proteins[15] and to give insight into the mechanism of Alzheimer's β-amyloid oligomer toxicity.[16] Each of these cases takes advantage of the ability of neutron scattering to use multiple solution isotopic contrasts to reduce ambiguity, and to sequentially measure change in the membrane structure after in situ changes to the membrane environment, *e.g.* pH, temperature.

Neutron Diffraction from Biomembrane Stacks

A special case of neutron reflection occurs when there is a semi-crystalline membrane bilayer stack formed on the solid-support (Fig. 3). These stacks are formed relatively simply through the slow evaporation of lipid-containing solvent solutions on the solid surface. They consist of thousands of bilayer repeats aligned on the surface; they show crystalline periodicity perpendicular to the interface, but usually remain disordered laterally. These stacks can be hydrated in highly humid atmospheres, and are measured in the absence of solvent, allowing X-ray and neutron measurements to be made. The constructive interference resulting from the scattering from multiple layers gives Bragg peaks, capable of providing detailed information about the perpendicular structure of the phospholipid bilayer within each bilayer repeat, to sub-nanometre resolution.

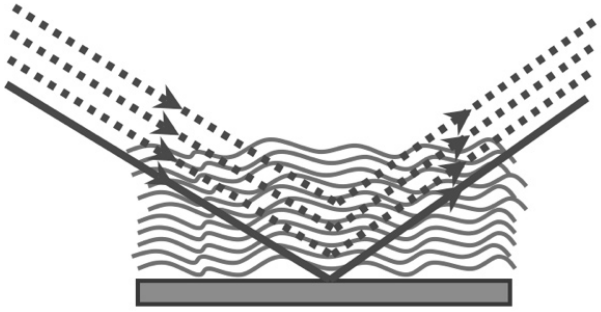

Fig. 3. Diffraction arising from a stack of bilayer membranes aligned on a solid support. The constructive interference from scattering from each of the membranes gives rise to Bragg peaks. Note that the membranes are still fluid, and laterally inhomogeneous even if they display crystallinity perpendicular to the surface.

This method has been used to great effect in understanding the behaviour of the lipids themselves in biological membranes, including their native thickness, degrees of interdigitation, and phase transitions with temperature.[17] The method also allows the resolution of the position and orientation of small additives to the biological membrane, such as cholesterol in biological membranes or fragments of membrane active proteins.[17] An unfortunate weakness of the method, however, is that there is very limited space between bilayer repeats (*ca*. 1 nm), meaning that it is not possible to incorporate larger membrane proteins that may extend beyond the phospholipid surface. It is also extremely difficult to achieve 100 % relative humidity around the bilayer to properly simulate physiological conditions.

Small Angle Neutron Scattering

The final major method of gaining structural information about model membranes using neutron scattering is small angle neutron scattering (SANS). The method, which probes the scattering from particles in bulk solution, is widely used for studying the structures of soluble proteins, and protein assemblies and is normally used in a complementary fashion to X-ray small-angle scattering (SAXS).[18] Biological membrane models used for SANS are typically phospholipid vesicles,[19] although some limited work has also focussed on the formation of planar *bicelles* formed from the mixing of a long and short-chain phospholipid (which allows experimental overlap with NMR).[20] The size resolution of SANS/SAXS is ~1–100 nm, meaning that experiments focus on the membrane rather than on the whole vesicle. An extension to SANS – ultra-small angle neutron scattering, USANS – is currently proposed for the Sydney reactor that will be able to resolve much larger structures to address this issue. As this is a solution measurement, there is no steric limitation to the size of proteins incorporated to the membrane, as in the case of neutron diffraction; and the samples are again relatively simple to form. The curvature of the vesicle also gives rise to features, such as asymmetric lipid distribution across the membrane, that are inhibited in planar geometries.

Conclusions

The strengths of neutron scattering in studying biological membrane systems, coupled with the forthcoming availability of neutrons at the OPAL research reactor in Sydney, means that the application of neutron methods to biological problems in NZ should significantly increase over the coming years. This work can build on the extensive international efforts to develop biomimetic membrane systems appropriate for structural characterization using neutrons and X-rays, and is expected to augment NZ's existing strengths in biological membrane systems.

Acknowledgement

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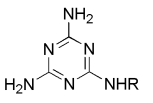
Melamine Food Contamination

It is impossible to know how many children have been affected by the melamine found in milk powder that occurred in 2008. Melamine ingestion is not a disease so the Chinese government is not required to notify how many have suffered. The BBC in October said more than 53,000 children had become ill from the contaminated milk powder. Other media reports suggest the number could be as great as 300,000.

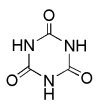
This is not the first melamine contamination problem to arise from China. In 2007, melamine was found in Chinese wheat gluten sold to the USA as a pet food thickener. This contamination led to cats and dogs becoming ill in the United States and Canada.

Melamine is not used by the body and is a very small molecule. In small amounts it is absorbed through the intestinal tract, goes to the kidney and is passed out in the urine. However in higher concentrations it can crystallise into kidney stones. This is what happened to some of the infants fed the milk and some of the pets in the 2007 case.

During the pet food contamination cyanuric acid, ammelin and ammelide were also found in the wheat gluten. Cyanuric acid can cause crystals to develop in the kidney tubules. At the end of October, Reuters reported some of the infants, who had drunk the melamine contaminated milk, were found to have developed crystals in their kidney tubules and these appeared to be cyanuric acid.



melamine: R = H
cyromazine:
R = cyclopropyl



cyanuric acid

After the first story broke about the milk powder contamination, many countries carried out extensive testing on other Chinese products. Among a number of reports, Canada reported melamine present in Mengniu Strawberry Flavour Sour Milk, Vietnam reported melamine present in Singapore's Pokka corporation Melon Milk, Cappuccino Coffee and Milk Coffee Europe and Gold Nutritionals Master Gaine Powdered Milk. This product uses milk powder from New Zealand but also has additives from Thailand. In mid-October Malaysian authorities found melamine above the safety standard, in ammonium bicarbonate imported from China. The high levels were first found in two brands of biscuits, the contamination was traced to ammonium bicarbonate (used as a raising agent). In late October, The New York Times reported that Hong Kong had found high levels of melamine in eggs imported from China. The levels were above the safety standards but not as high as that found in the contaminated milk.

Allowable concentrations of melamine in food products varies around the world. Vietnamese regulations require no melamine to be present in food. New Zealand regulations allow for 2.5 ppm in food products and 1 ppm in infant formulas. The allowance for some melamine in food products is because of possible leaching from food packaging or processing. New Zealand's Tatua Cooperative suspended exports in late 2008 of lactoferrin protein powder after low levels of melamine were found in the product. The levels were under the safety standards but Tatua were working to find the source. Low levels were also found in Westland Milk Product's lactoferrin. Westland used the same processing technology as Tatua.

The US FDA has published an interim safety/risk assessment on melamine and structural analogues and for melamine has established a tolerable daily intake (TDI) of 0.63 mg per kg of body weight per day. The European Food Safety Authority has published a provisional statement for melamine of a TDI of 0.5 mg per kg of body weight per day.

Why was melamine in the Chinese milk powder in such large concentrations in the first place? Melamine (2,4,6 triamino-*s*-triazine), contains six nitrogen atoms. If total nitrogen content measurement is used to equate to protein content, adding a high nitrogen substance, like melamine, appears to increase protein content. This means the product can be diluted but appear undiluted if the protein content is still within the range expected. At this stage it is hard to know when the contamination occurred. There are a number of theories as well as the dilution theory, another possibility is the milk came from undernourished cows so would not have passed the protein content test. According to the Wall Street Journal some Chinese farmers say that for years *protein powder* has been used to help milk from undernourished cows meet quality checks. It is also possible for melamine to enter the food chain from insecticides containing cyromazine used by farmers.

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