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The study of liposomes, lamellae and membranes using neutrons and X-rays

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Abstract

Advances in colloid and interface science have stimulated a renewed interest in the study of lipid–water systems. In recent years, much progress has been achieved in the domains of sample preparation and sample environments, offering the unique possibility of studying these systems under physiologically relevant conditions. In the case of neutron reflectometry, new experimental protocols allow for the unique structural determination of one-dimensional membrane profiles, while the advantages offered by synchrotron radiation (e.g., high flux and spatial resolution) make X-rays an excellent tool for addressing questions pertaining to membrane interactions. Most recently, holographic techniques are evolving so that one day they may be able to resolve, to atomic resolution, the structure of poorly crystallized membrane associated proteins.

1. Introduction

Neutron and X-ray scattering have over the years proven to be two of the most widely used techniques in structural biology, biophysics and materials science [1]. Many water soluble proteins have been crystallized and studied with atomic detail. On the other hand, due to the intrinsic disorder present in biomimetic samples – a disorder possibly important for the proper function of biology systems – many membrane samples are far from being perfect crystals. In such cases, less than atomic resolution structures are best described by broad statistical distributions, rather than sharp delta functions typical of perfect crystals. We will review recent advances in the scattering techniques used to study the structure of non-crystalline biomaterials with a focus on the lipid membrane.

Structural biophysics has successfully applied crystallographic methods to the study of planar membrane arrays, a system analogous to a one-dimensional (1D) crystal. This approach works well with either gel phase lipid bilayers or partially dehydrated liquid crystalline (L_α) bilayers. Discrete Bragg reflections collected in reciprocal space (q [$= 4\pi \sin(\theta/2)/\lambda$], where λ is the wavelength and θ is the scattering angle) correspond in real space to the 1D bilayer scattering density profile (SDP), which is routinely obtained by either simple Fourier reconstruction of the data, or by successive fits of the data to a model [2]. This approach, however, is not ideally suited to L_α bilayers where undulations preclude the measurement of the higher order Bragg peaks needed for resolving fine structural details. Instead, data analysis has recently focused on the diffuse, continuous scattering [3•,4•].

Neutron and X-ray reflectometry have emerged as powerful surface/interface probes used to characterize the structures of materials on solid and fluid planar surfaces. The most common approach is that of specular reflectivity, which measures the SDP perpendicular to the adsorbed surface. However, compared to X-ray reflectometry, neutron reflectometry possesses several advantages, such as the fact that neutrons are highly penetrating, enabling them to probe samples in complex sample environments [5]. Neutrons are also capable of locating low atomic number atoms among heavy atoms, and through judicious substitution of ^2H for ^1H provide a powerful method for selectively tuning the "contrast" of a given macromolecule. Over the years, neutron reflectometry has proven to be a powerful tool for the study of biologically relevant samples at the air/liquid [6] and liquid/solid [7,8•] interfaces.

Small-angle scattering (SAS) is another popular technique for the study of biological materials as it provides information

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on the size, shape and conformation of molecular assemblies in solution. In addition, neutron scattering has the capability to accentuate, or nullify, the scattering from individual parts of a macromolecular complex with its unique ability to distinguish between hydrogen and its isotopes. For example, by specific deuterium-labeling, it is possible to measure bilayer conformational changes and organization in both the perpendicular and lateral directions [9•,10].

2. Scattering from unilamellar structures

From a biological perspective, the most appealing model of a cell membrane is a unilamellar vesicle (ULV), a hollow sphere with a single lipid bilayer demarcating the inside and outside aqueous environments. Because of their cell-like topology and biologically derived composition, ULV are considered to be a promising morphology suitable for the engineering of biocompatible medical systems. For example, they are used to study the effects that different physiological conditions and additives have on the membrane and its associated proteins [11]. Although ULV exist in solution and their isotropic structural dimensions are typically characterized by low-resolution data, improved structural data can be achieved by utilizing the powerful neutron scattering method of contrast variation.

A new and innovative advance in the domain of scattering theory and ULV demonstrates how data analysis is capable of providing not only structural information in the direction perpendicular to the plane of the bilayer, but also information regarding a membrane's in-plane organization e.g. lateral lipid segregation (see Fig. 1). The comprehensive work of Pencer et al. [12••] outlines a model independent method for separating SAS into contributions from radial and lateral membrane heterogeneities. Under suitable contrast conditions, the analysis can effectively detect the presence of nanoscopic lateral segregation (e.g., lipid rafts) as well as the asymmetric distribution of lipid species within the bilayer [13•]. However, the size and domain composition can only be determined by calculating the appropriate form factor. Presently, a theory is being developed by Pencer et al., which will utilize SAS to detect domains as small as ~ 100 Å in diameter (personal communication).

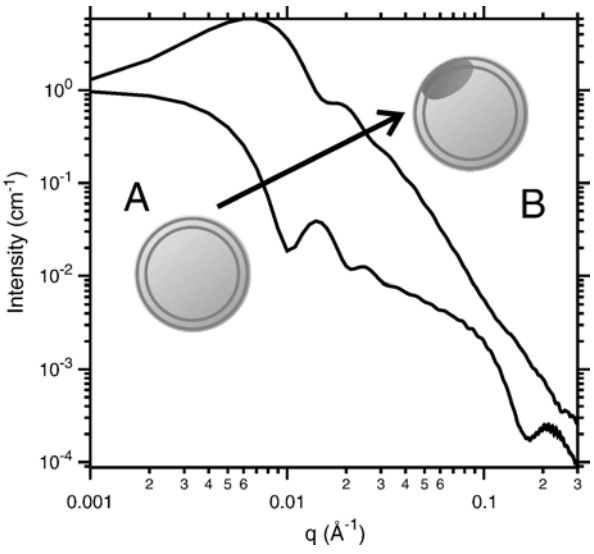

Fig. 1. Schematic representation of lateral segregation in ULV as detected by SANS (adapted from [8•]). (A) A cartoon of a contrast matched ULV and (B) of a ULV with laterally segregated domains and their expected scattering curves.

Additional structural details can be obtained through the use of aligned, solid-supported bilayers, which are well suited to interface sensitive scattering techniques such as reflectometry [14]. The geometry of these samples allows for the unambiguous determination of in-plane and out-of-plane contributions. However, a question regarding this sample preparation is the role of the substrate, whose interaction can modify the behavior of molecular adsorption [15]. To address this concern, a number of membrane support systems have been fabricated which are "decoupled" from the substrate [16] thus allowing the membrane to undergo unrestricted motion, and for water to penetrate all the way up to the substrate [17,18], simulating more biologically realistic conditions.

As is the case for all scattering, knowledge of both the amplitude and phase of the scattered intensity are needed for the unambiguous construction of an SDP. However, as the phase component is not recorded we are faced with the infamous "phase problem". Typical solutions to this problem involve model fitting, which can result in multiple and sometimes, equally plausible solutions. However, using polarized neutrons and reference layers of Cu, Ni, and Mo, Majkrzak et al. [19••] have measured both the real (amplitude) and imaginary (phase) parts of the complex reflection, allowing them to directly invert these data to produce a unique scattering density profile.

Conceptually, a similar approach for solving the phase problem can be used for X-ray scattering. Here, the SDP is obtained from measurements both near and at the absorption edge of a heavy atom present in the system by varying the energy of the X-rays, something easily carried-out at synchrotron sources. Despite the difficulties arising from inherently disordered lipid systems, this method of X-ray multi-wavelength anomalous dispersion has been successfully applied in characterizing Langmuir monolayers [20•] as well as, multilamellar structures [21,22].

2.1. Diffraction from multilamellar structures

Typical multilamellar samples are in the form of either large spherical objects (multilamellar vesicles, MLV) or aligned arrays of planar bilayers. Both samples exhibit similar physical behavior [23••] as any effects which can be attributed to the substrate are limited only to the first few bilayers. However, aligned samples, although generally easy to fabricate, have over the years proven difficult to fully hydrate from water vapor. Therefore, sample environments with precise temperature and humidity control are necessary for experiments where multibilayer stacks are hydrated from water vapor. After demonstrating that the so-called vapor pressure paradox was the result of bilayers being routinely hydrated in relative humidity (RH) conditions less than 100%, and not due to some intrinsic

difference between MLV and supported multibilayer stacks [23••,24], there has been a greater effort expended in the design of sample environments capable of achieving 100% RH [25,5].

Using a sample environment suitable for an aligned stack of bilayers, neutron scattering experiments have recently shown the location of cholesterol's hydroxyl group. While, normally it is found residing near the lipid/water interface of commonly studied model membranes (e.g. di-14:0 PC), in polyunsaturated lipid bilayer i.e. di-20:4 PC, it was observed sequestered in the center of the bilayer [26••] (Fig. 2). This dramatic change in cholesterol location was attributed to the inherent high disorder of polyunsaturated fatty acids. Moreover, the intrinsic properties of polyunsaturated lipids were also implicated in studies by Mihailescu and Gawrisch [27], where they consistently found the terminal methyl groups of the highly flexible polyunsaturated fatty acid chains to reside away from the bilayer center, thus created voids filled by the phospholipid's saturated chains. The unique role of polyunsaturated lipids in bilayers has also been demonstrated, among many other methods, using high-brilliance synchrotron X-rays [28•,29].

2.2. Diffuse scattering from fluctuating multilayers

Fully hydrated liquid crystalline samples are generally assumed to best mimic physiologically relevant conditions. However, these disordered bilayers do not diffract well (i.e. limited number of Bragg maxima) and as such, do not lend themselves ideally for crystallographic analysis. On the other hand, the scattering patterns from these thermally fluctuating bilayers contain diffuse scattering, which can be analyzed to reveal previously hard to obtain information regarding bilayer structure and interactions. This new method of gaining access to the physical properties of membranes allows for the understanding of the effective forces between mesoscale structures.

In recent years, much attention has been paid to the phenomenon of anomalous swelling taking place in multi-

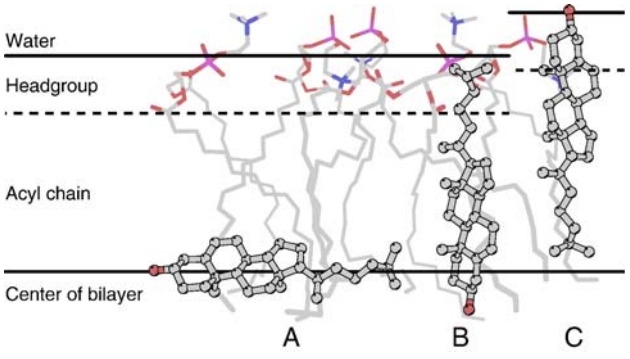

Fig. 2. Schematic of the calculated locations and orientations of cholesterol in a phospholipid membrane (adapted from [26••]). For 20:4–20:4 PC bilayers (A and B) the steroid label is found at the center of the bilayer and is shown in two possible orientations; lying flat within the bilayer center or inverted from its typical configuration. The thickness of 20:4–20:4 bilayers, indicated by the horizontal lines, is considerably smaller than the thickness measured in 16:0–18:1 PC, 18:1–18:1 PC, and 18:0–20:4 PC bilayers (C). This part represents the canonical location and orientation of cholesterol in a bilayer, where the "top" of the steroid ring is located 16 Å from the bilayer center.

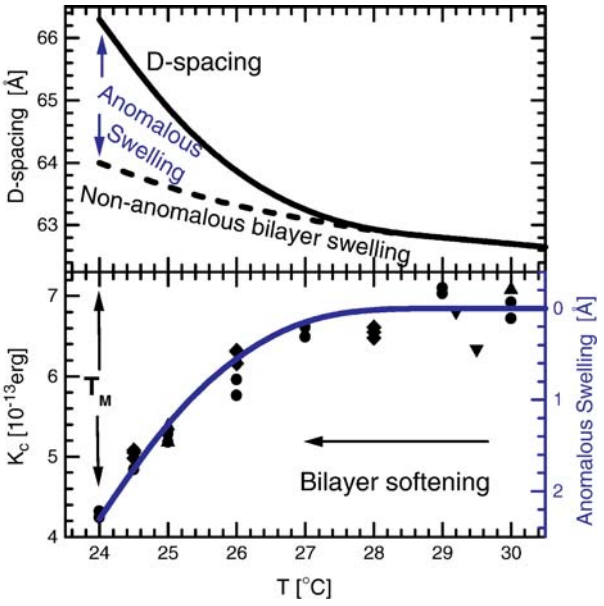

Fig. 3. Temperature dependence of DMPC's lamellar repeat spacing (D-spacing, solid line) and the experimentally obtained non-anomalous swelling (broken line) attributed to changes in the bilayer thickness, only (top panel). Here, anomalous swelling is defined as the difference between the two. Bottom panel shows the bilayer's bending modulus as a function of temperature (symbols), which is qualitatively similar to the anomalous swelling resulting from the water layer. Figure adapted from [32•].

bilayer systems near the main transition temperature T_M (Fig. 3). As the temperature decreases towards T_M, the repeat spacing in liquid crystalline bilayers increases. However, only half of this increase was attributed to a change in bilayer thickness. Pabst et al. [30] studied the temperature dependence of the bilayer's elastic properties by analyzing the diffuse scattering from DMPC MLV and obtaining the bilayer's bending modulus (K_c) from the measured Caillé fluctuation parameter. However, since this parameter is the product of the interbilayer compression (B) and in-plane bending moduli, additional osmotic pressure experiments were needed to estimate the individual K_c and B contributions. In the meantime, Lyatskaya et al. [31••] recognized the potential of aligned, fully hydrated samples in determining these two parameters, individually. Analysis of the diffuse scattering from such samples then revealed that the anomalous swelling taking place in a certain class of lipid bilayers was the result of bilayer "softening" [32•].

Thermally induced bilayer undulations play a critical role in fully hydrated multilamellar systems. While the superposition of repulsive (i.e. hydration and undulation) and attractive (i.e. van der Waals) forces result, in the case of gel phase bilayer, in a finite interbilayer distance, unbinding transitions have been reported in L_α bilayers. For example, Gordeliy et al. [33•] studied intermembrane interactions using DMPC MLV (2 wt.%) over a range of temperatures. Based on complementary SANS and high-resolution X-ray diffraction data, they reported an increase in bilayer fluctuations followed by an unbinding transition. This finding contradicted the previous result by Vogel et al. [34] in which the authors used highly aligned,

supported bilayers. In their case, the bilayers underwent a discontinuous unbinding transition without any indication of increased bilayer undulations. However, as was pointed out by Pabst et al. [35], oriented samples may not be well suited for the study of unbinding transitions as there exists the real possibility for the sample to desorbe from the substrate, which can be mistakenly interpreted as an unbinding transition.

2.3. Structure of lipid bilayers

In addition to the bilayer's elastic properties and interbilayer interactions, which are described by a structure factor, analysis of diffuse scattering offers the possibility of extracting the bilayer form factor. Instead of a few discrete Bragg maxima, diffuse scattering results in a continuous form factor with a substantially greater range of the scattering vector's perpendicular component. Presently, there are a number of groups which are taking advantage of the unique possibilities offered by diffuse scattering [36••,37–40].

By combining diffuse scattering data from aligned multilayers and ULV, Kučerka et al. have introduced a self-consistent global analysis to accurately determine the bilayer's structure [41•]. Providing increased intrabilayer information, analysis applied on different lipids revealed the effect of a fatty acid chain's double bond on the lipid's lateral area (Fig. 4) [42]. Of course, this type of analysis could not work if the bilayers from the two different sample preparations were not equivalent. An asymmetry in the SLD profile of a charged lipid bilayer was observed in ~ 1000 Å diameter ULV [43], while in zwitterionic lipid ULV this effect was absent (Fig. 5) [44]. Comparison of the data suggested that the asymmetry in the charged bilayers is most likely attributed to the interplay between the electrostatic interactions and a curvature effect, rather than bilayer curvature alone.

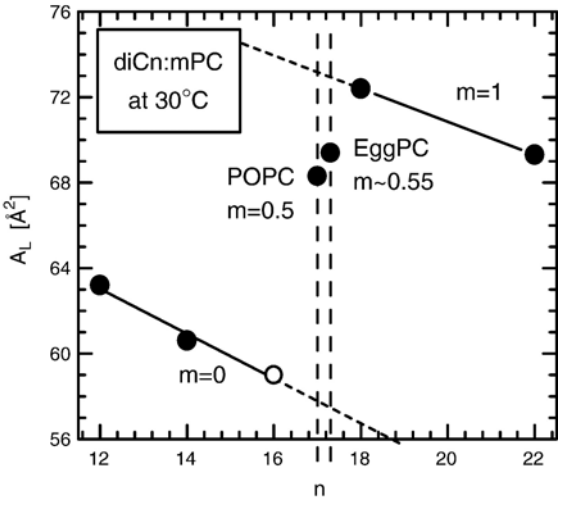

Fig. 4. Dependence of lipid area (A_L) as a function of chain length n and number of double bonds m at 30 °C (adapted from [42]). Saturated lipids have $m=0$ while di-monounsaturated have $m=1$. The figure suggests that the effect of the first double bond on bilayer structure – e.g. going from $m=0$ to $m=0.5$ – is much greater than the effect of subsequent double bonds e.g. $m=0.5$ to $m=1$.

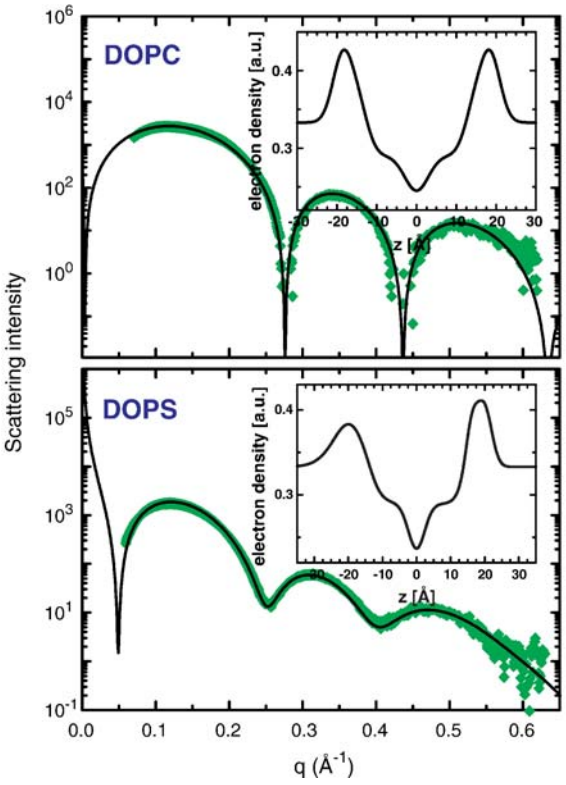

Fig. 5. X-ray scattering curves adapted from [44], corresponding to the scattering from ULV made from neutral DOPC bilayers (top) and charged DOPS bilayers (bottom). The scattering curves are visibly different in the low q region and in the fact that the scattering function from asymmetric DOPS bilayers does not have minima with zero scattered intensity, as is the case for symmetric DOPC bilayers. The insets to the figures contain the 1D electron density profiles. The asymmetry seen in DOPS bilayers is most likely due to the combined effect of bilayer curvature and charge.

2.4. Towards atomic resolution structures

Although membrane associated proteins constitute approximately one third of all known proteins, fewer than 100 structures have been elucidated to date. The primary reason for this scarcity of membrane protein structures is the lack of high quality single crystals, a strict requirement for traditional scattering techniques. However, in the case of atomic structure holography the only requirement is that the sample possess orientational order; translational order is not a necessary condition. Thus, some protein crystals unsuitable for traditional crystallography – due to translational disorder among its subunits – can potentially be solved to atomic resolution using holographic techniques.

Atomic resolution holography can trace its beginnings to Bragg's X-ray work [45] and Gabor's electron interference microscope [46]. In the last 15 years, or so, there have been an increasing number of publications on atomic resolution holography using either electrons [47,48] or hard x-rays [49]. The feasibility of neutron holography was recently demonstrated using the so-called "inside source" and [50•,51] and "inside detector" concepts [52]. More importantly, Sur et al. [53•] have developed a kinematical formulation for the diffraction pattern of monochromatic plane waves scattering from a mixed

incoherent (e.g., H atoms) and coherent scattering length distribution, which was shown to be analogous to that of spontaneous sources of isotropic radiation, or spherical waves. The formulation demonstrates the conditions under which one can reconstruct thermal neutron holographic data from samples with either a single or numerous incoherent scatterers per unit cell, as is the case for biologically relevant materials. There exists, therefore, the real possibility of solving to atomic resolution the three-dimensional structure of proteins from less than perfect three-dimensional crystals (i.e. contain translational disorder), or two-dimensional crystals.

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