Structure from substrate supported lipid bilayers (Review)

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Highly aligned, substrate supported membranes have made it possible for physical techniques to extract unambiguous structural information previously not accessible from commonly available membrane dispersions, or so-called powder samples. This review will highlight some of the major breakthroughs in model membrane research that have taken place as a result of substrate supported samples. © *2008 American Vacuum Society.* [DOI: 10.1116/1.2992133]

I. INTRODUCTION

The amount of information accessible from a neutron or x-ray diffraction experiment can be correlated to the sample's degree of alignment. Compared to powder samples, highly aligned membrane systems have in many instances enabled the determination of unambiguous structural information.[1–13] Specifically, aligned samples allow for the clear differentiation between in-plane and out-of-plane structures. From a practical point of view, because the signal is anisotropic, compared to powder or liposomal preparations, data acquisition is accelerated. In the case of powder samples, scattered intensity decreases precipitously as a function of scattering angle.

The most common method of fabricating aligned samples, consisting of hundreds of bilayers, is from evaporation of the organic solvent from a concentrated membrane solution deposited either on a flat[1,3,10,12,13] or a cylindrical solid support.[2,4–7,9,11,14] Less commonly used fabrication methods are Langmuir-Blodgett (LB) films[15–18] and centrifugation techniques.[19] It should be noted, however, that the LB method of fabrication is ubiquitous for single bilayer and monolayer samples used in neutron and x-ray reflectometry studies.[20–23] Nevertheless, despite their obvious utility aligned samples suffered from what eventually came to be known as the vapor pressure paradox,[24] where samples (either aligned or powders) hydrated from water vapor exhibited lamellar repeat spacings (*d*-spacings), particularly in the biologically relevant liquid crystalline phase, consistently smaller than those same samples dispersed in bulk water.[14,25–27] As the chemical potentials of bulk water and water vapor at 100% relative humidity (RH) are the same, membranes immersed in water (fully hydrated) should behave no different than those exposed to 100% RH. Attempts to immerse aligned multibilayers in excess water resulted in lipid bilayers becoming disordered.[28] It was not until the early 1990s that highly aligned gel phase multibilayers were fully hydrated through the condensation of water on samples,[2,29] however, not for liquid crystalline bilayers.

According to Helfrich,[30] undulating bilayers experience an effective entropic force causing them to repel and swell, effectively increasing their *d*-spacing. However, if bilayer undulations are suppressed, for example, as a result of surfaces being under tension (e.g., vapor/multibilayer or multibilayer/substrate interface), then these "rigid" bilayers exhibit lesser amounts of swelling. Evans and Parsegian[31] examined the effect of fluctuations that enhance the repulsion of parallel sheets of bilayers, and to some extent, could explain some of the experimental results.[14,25–27] Later on Podgornik and Parsegian[32] revisited the problem and concluded that in surfaces under tension, bilayer undulations are suppressed and the resultant attractive forces are communicated over the entire multibilayer stack. The Podgornik and Parsegian theoretical result was then corroborated by Tristram-Nagle *at al.*[33] using x-ray diffraction and aligned multibilayers of dimyristoyl phosphatidylcholine (di-14:0 PC, DMPC) and dipalmitoyl phosphatidylcholine (di-16:0 PC, DPPC).

The simplest explanation for the vapor pressure paradox is that 100% RH was never achieved in any of the reported experiments, as its attainment is not a trivial matter. For example, lowering RH from 100% to 99.9% results in almost a 30% decrease in the *d*-spacing of egg PC bilayers.[24] This 0.1% decrease in RH can be attributed to the presence of a temperature gradient of only 0.01 °C in the sample chamber.[24] To overcome this seemingly impossible task of

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constructing a sample environment with temperature gradients $\ll 0.01$ °C, Katsaras[34] constructed an aluminum (a material transparent to thermal neutrons) sample chamber whereby a sample aligned on a silicon substrate could be immersed in bulk water, while retaining its orientational order.

The "excess water" condition is met when fully hydrated lipid bilayers coexist with bulk water. In this condition, lipid bilayers such as DMPC and DPPC can assume a number of lamellar phases, which on increasing temperature are as follows: gel ($L_{\beta'}$), ripple ($P_{\beta'}$), and liquid crystalline (L_α).[35] Under certain sample conditions, however, there is the appearance of the so-called $L_{c'}$ phase (incubation of DPPC bilayers for a few days at ~0 °C), first observed by Chen *et al.*,[36] whose structure, as determined by x-ray diffraction, was characterized by two lattices—a molecular superlattice and a hydrocarbon chain sublattice.[4,6,7] Katsaras[34] demonstrated that the transition temperatures and *d*-spacings of aligned (<0.5° mosaic spread) DPPC and dihexadecyl phosphatidylethanolamine multibilayers on silicon substrates immersed in water, were in excellent agreement with liposomal preparations. This result was seemingly in contradiction with Podgornik and Parsegian[32] who postulated that the enforced "stiffening" of the bilayer in contact with the substrate is communicated over macroscopic length scales to the remaining multibilayers, thereby limiting their water uptake and resulting in a concomitant reduced *d*-spacing. However, as the samples were prepared in a so-called silicon "sandwich" with 0.005 cm Teflon spacers, it was possible for one of the multibilayer's stack surface to be in contact with bulk water, thus allowing the bilayers to undulate, resulting in the bilayer adhered to the other silicon substrate to "lift-off" from the substrate. In this scenario, the silicon substrates acted in confining the multibilayer stack, behaving in a manner similar to liposomes in bulk water.[34] This study was subsequently complemented by experiments using aligned DMPC multibilayer stacks aligned on mica substrates, which were hydrated from water vapor.[37] These experiments put to rest the notion of substrate suppressed undulations and the vapor pressure paradox, providing a method of directly comparing the physical properties of aligned samples with their counterparts dispersed in excess water.[13,38] Subsequently, Constantin *et al.*[39] provided measurements and detailed calculations of how the bilayer fluctuation amplitude changes from the substrate to the free boundary of the multibilayer stack.

Since then, the availability of fully hydrated aligned samples have enabled scattering techniques to resolve a number of outstanding issues and the development of a technique capable of determining the bilayer bending (K_c) and interbilayer interaction (B) moduli.[11]

II. RIPPLED BILAYERS

Hydrated multibilayer stacks made up of disaturated PC lipids (e.g., DMPC and DPPC) form a thermodynamic phase commonly referred to as the $P_{\beta'}$ or ripple phase. For over 30 years the structure of these bilayers has been researched by freeze-fracture electron microscopy[40–42] (FFEM) and x-ray diffraction.[43–51] Despite extensive studies, there was debate as to whether or not these rippled bilayers were symmetric (i.e., simple sinusoidal shape) or asymmetric (i.e., sawtooth). In addition, there was observation of an additional ripple morphology, as imaged by FFEM (Refs. 40 and 42) and deduced from powder x-ray diffraction.[48–50] After considerable uncertainty lasting over a decade regarding the conditions for observing different ripple morphologies, it was noted that the $P_{\beta'}$ phase can differ depending on whether it is formed on cooling from the L_α phase, or on heating from the $L_{\beta'}$ phase. The consensus, at least in the case of DPPC bilayers, was that upon heating from the $L_{\beta'}$ phase, the $P_{\beta'}$ phase consisted of short wavelength ($\lambda \sim 145$ Å) asymmetric ripples, while the ripple phase formed upon cooling from the L_α phase was a mixture of long wavelength ($\lambda \sim 260$ Å) ripples coexisting with the commonly observed short wavelength ripples.[46,52] However, coexistence over an extended range of temperature generally violates the Gibbs phase rule. Moreover, coexistence was disputed when previously supporting x-ray data were reanalyzed, and along with neutron powder diffraction data concluded that the analysis was consistent with pure long wavelength rippled bilayers.[53]

The solution of the vapor pressure paradox[37] allowed to unambiguously answer the question as to whether or not DPPC bilayers formed, upon cooling, a mixture of short and long wavelength rippled bilayers, or simply a single population long wavelength rippled bilayers.[48,49,53] To tackle this problem a sample environment suitable for x-ray scattering was constructed to interrogate fully hydrated DPPC bilayers (Fig. 1).[9] From the two-dimensional (2D) x-ray diffraction patterns (Fig. 2) it was clear that upon heating, both chiral and racemic DPPC bilayers form one population of rippled bilayers, as all reflections could be indexed to a unique unit cell.[9] However, upon cooling the situation was found to be very different. The data directly supported the commonly accepted notions of Yao *et al.*[48] and Matuoka *et al.*,[49] in that there are two distinct coexisting populations of ripples differing in *d*-spacing, ripple wavelength, and ripple symmetry.

III. DETERMINATION OF THE BILAYER BENDING AND INTERBILAYER INTERACTION MODULI

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 quasi-Bragg peaks), and as such do not lend themselves ideally for traditional crystallographic analysis. On the other hand, the scattering patterns from these thermally fluctuating bilayers contain diffuse scattering which can be successfully analyzed to reveal previously hard to obtain information regarding bilayer structure and interactions. In short, 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 multibilayer systems near the main transition temperature T_M. As the temperature decreases toward T_M, the *d*-spacing in liquid crys-

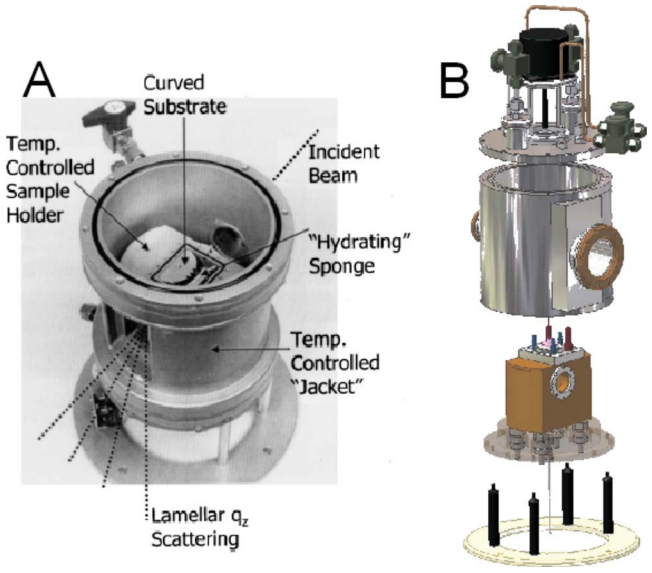

FIG. 1. Perspective photograph of the 100% RH x-ray oven. (A) The inner sample chamber is a sealed unit—shown with sealing panel removed—and contains the water saturated evaporative sponge used in hydrating the sample and the cylindrical substrate enabling the simultaneous collection of multiple Bragg reflections. The sample chamber is thermally isolated from the outer jacket via the use of an acrylic base. (B) Exploded view of the x-ray sample oven. Figure adapted from Ref. 9.

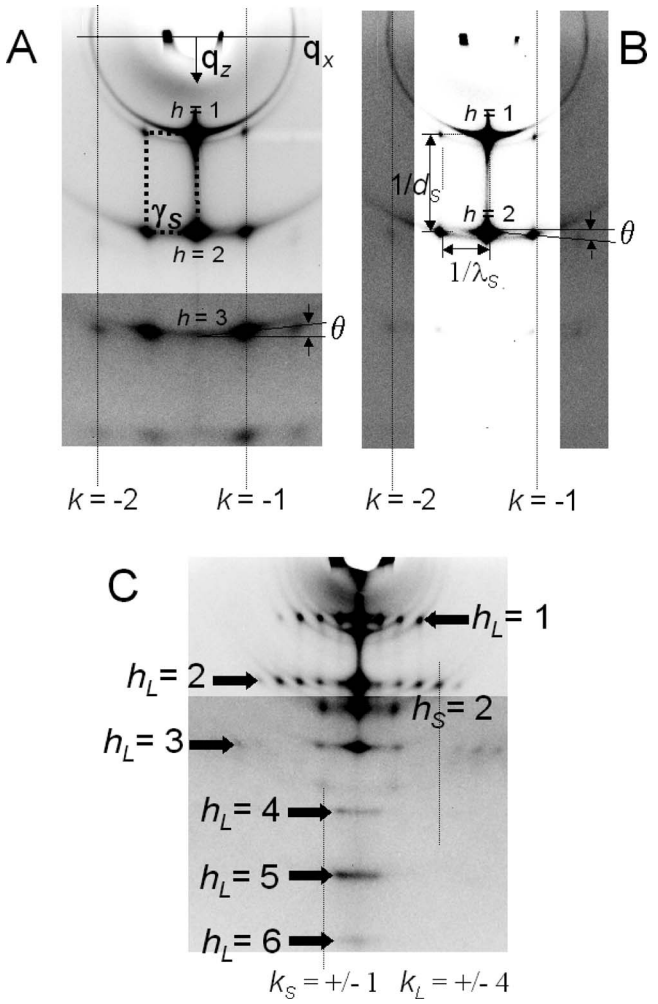

FIG. 2. Two-dimensional x-ray diffraction patterns of oriented (a) *l*-DPPC and (b) *dl*-DPPC rippled bilayes formed on heating from the $L_{\beta'}$ phase. (c) Two-dimensional x-ray diffraction pattern of $P_{\beta'}$ *dl*-DPPC rippled bilayers formed on cooling from the disordered L_α phase. The coexistence of two rippled bilayer populations is depicted by the indices for the short ripple phase (h_S,k_S) and the indices for the long ripple phase (h_L,k_L). The bold arrows identify the family of peaks due to the long ripple. From the diffraction patterns the various lattice parameters were directly obtainable. Cooling *l*-DPPC L_α bilayers also results in two rippled bilayer populations. Figure adapted from Ref. 9.

talline bilayers increases. However, only half of this increase was attributed to a change in bilayer thickness. Pabst *et al.*[54] studied the temperature dependence of the bilayer's elastic properties by analyzing the diffuse scattering from DMPC multilamellar vesicles (MLVs) in order to determine the bilayer's bending modulus (K_c)—which governs bilayer fluctuations—from the measured Caillé fluctuation parameter.[55] However, since the Caillé parameter is the product of the interbilayer compression (B) and in-plane bending moduli, additional osmotic pressure experiments were needed in order to estimate the individual K_c and B contributions.

Unlike powder samples, where only the Caillé parameter $\eta(\pi k_B T/[2d^2(K_cB)^{0.5}])$ is directly obtainable, and which contains the product of the material moduli K_c and B, aligned samples offer the possibility of individually determining K_c and B. This was recognized by Lyatskaya *et al.*[11] (Fig. 3) who determined the material properties of fully hydrated palmitoyl oleoyl PC (16:0–18:1 PC, POPC) bilayers, realizing the potential of aligned samples in individually determining K_c and B. Subsequent analysis of the diffuse scattering from such samples revealed that the anomalous swelling taking place in a certain class of lipid bilayers was the result of bilayer "softening."[56] Although in recent years there have been a number of studies that have analyzed the anisotropic diffuse scattering from aligned samples,[57–60] it should be pointed out that Pabst *et al.*,[61] using a modified Caillé structure factor in combination with a Gaussian representation of the one dimensional (1D) electron density profile, were the first to obtain mesoscopic bilayer information from POPC and dipalmitoyl phosphatidylethanolamine MLV bilayers.

An interesting system in which a membrane's mechanical properties can be determined, was developed by Fragneto *et al.*[62] and consists of two bilayers, one which floats a few angstroms above another that is adsorbed to a solid substrate. This system may provide a direct observation of the balance between energy minimization and entropic repulsion, enabling the determination of the bending modulus.

IV. LOCATION OF CHOLESTEROL IN POLYUNSATURATED FATTY ACID BILAYERS

Neutron and x-ray scattering techniques have over the years been used to determine the location and orientation of molecules of interest within lipid bilayers. Some of these molecules have included fatty acids,[63] antioxidants,[64] cholesterol,[65,66] and a variety of peptides.[67–76] Importantly,

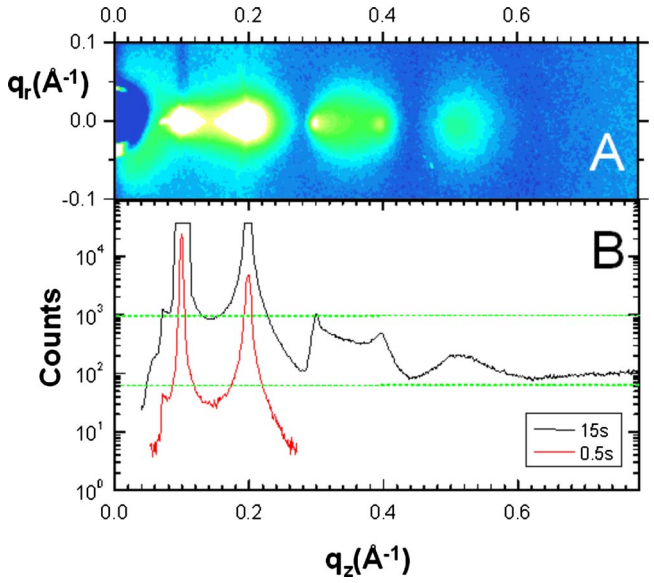

FIG. 3. (a) Intensity as a function of scattering vector from fully hydrated L_α DOPC bilayers oriented on a cylindrical substrate (see Fig. 1), where q_z and q_r represent out-of-plane and in-plane scatterings, respectively. (b) The scattered intensity along q_z for a strip centered at q_r=0. The black and red curves correspond to collection times of 15 and 0.5 s, respectively. Figure adapted from Ref. 11.

some of these studies have yielded intriguing results challenging our preconceived notions of how molecules self-assemble in membranes. An example of this is the location of cholesterol in bilayers made up of lipids containing polyunsaturated fatty acids (PUFAs).[66]

Cholesterol is an essential component of mammalian cells. It can either be obtained from foods of animal origin (e.g., milk, cheese, meat, eggs, etc.), or synthesized in the endoplasmic reticulum.[77] It is required to build and maintain cell membranes, regulates their fluidity and may act as an antioxidant.[78] Recently, cholesterol has also been implicated in cell signaling processes, where it has been suggested that it forms lipid rafts in the plasma membrane,[79,80] and has also been found to reduce the permeability of the plasma membrane to sodium and hydrogen ions.[81]

Aside from cholesterol's many physiological roles, what is also becoming clear is its poor affinity for lipids containing unsaturated fatty acid chains, as opposed to saturated lipids that can more readily form domains of higher conformational order.[82–86] This unequal affinity for cholesterol has also been implicated in sorting different lipid species into membrane domains.[87] In particular, lipid rafts that can serve as platforms for signaling proteins in the plasma membrane is an area of research that over the past decade has received the most attention.[88–90] Liquid ordered (lo) regions enriched in cholesterol and sphingolipids possess predominantly saturated fatty acid chains for which the sterol has high affinity. On the other hand, liquid disordered (ld) domains are enriched in unsaturated phospholipids from which cholesterol is excluded by its aversion for PUFAs, and represent the opposite extreme that is much less well understood.[91]

Over the past couple of years, Harroun *et al.*[66,92] have studied the location of cholesterol in lipid bilayers with varying degree of fatty acid unsaturation [i.e., 16:0–18:1 PC (POPC), di-18:1 PC (DOPC), 18:0–20:4 PC (SAPC), and di-20:4 PC (DAPC)]. Through the use of headgroup and tail labeled cholesterol, aligned multibilayers and neutron diffraction, they have been able to determine the location of the molecule in various bilayers (Fig. 4). With the exception of DAPC bilayers, cholesterol was found to reside in its accepted "upright" orientation as previously determined.[65,93,94] However, in DAPC bilayers cholesterol was found to sequester at the bilayer center. The authors hypothesized that the aversion of cholesterol for PUFA promotes the formation of domains rich in polyunsaturated phospholipids from which the sterol is depleted, resulting in the segregation of the sterol into raftlike domains enriched in saturated sphingolipids[91,95,96]

The recent neutron diffraction results suggest that poor affinity for PUFA may affect the transmembrane as well as the lateral distribution of cholesterol. A tendency to sit at the center of PUFA-containing membranes, tipped over from the usual orientation where the hydroxyl group of the steroid moiety is anchored at the aqueous interface, would facilitate the flip-flop of the sterol from one side of the membrane to the other. Indeed, enhanced rates of cholesterol flip-flop have recently been published,[97] whereby coarse grained molecular dynamics (MD) simulations identified residency times for the sterol in various DAPC bilayer locations. The propensity of cholesterol to sit at the bilayer center was recently assigned to the higher disorder and permeability of these bilayers,[98] while a combined x-ray scattering and molecular dynamics simulations study has suggested that a dynamic network of hydrogen bonds between cholesterol, lipids, and water molecules enables cholesterol to exist in unusual orientations (i.e., bilayer center) when in disordered bilayers.[98]

The importance of these results may be rationalized with what we presently know of biological systems. For example, in plasma membranes sphingolipids are primarily located in the outer monolayer,[89] whereas unsaturated phoshpolipids are more abundant in the inner leaflet.[99] Thus, the presence of PUFA in the inner leaflet may enhance the transfer of cholesterol to the outer layer, potentially modifying raft composition and function. Consistent with this scenario is that the accumulation of PUFA into plasma membranes was seen to result in a substantial redistribution of cholesterol to the outer leaflet.[100]

V. BACTERIAL MEMBRANE

The pathogenic capability of Gram-negative bacteria is usually associated with their unique double walled membrane. Lipopoplysaccharides (LPSs) [Fig. 5(a)] are the major component of the outermost leaflet of these membranes, which while toxic to the host cells endows the bacteria with their resistance. It has thus proven especially difficult to successfully treat these bacteria (e.g., *Pseudomonas aeruginosa*, *Salmonella minnesota*) through conventional antibiotic therapies.

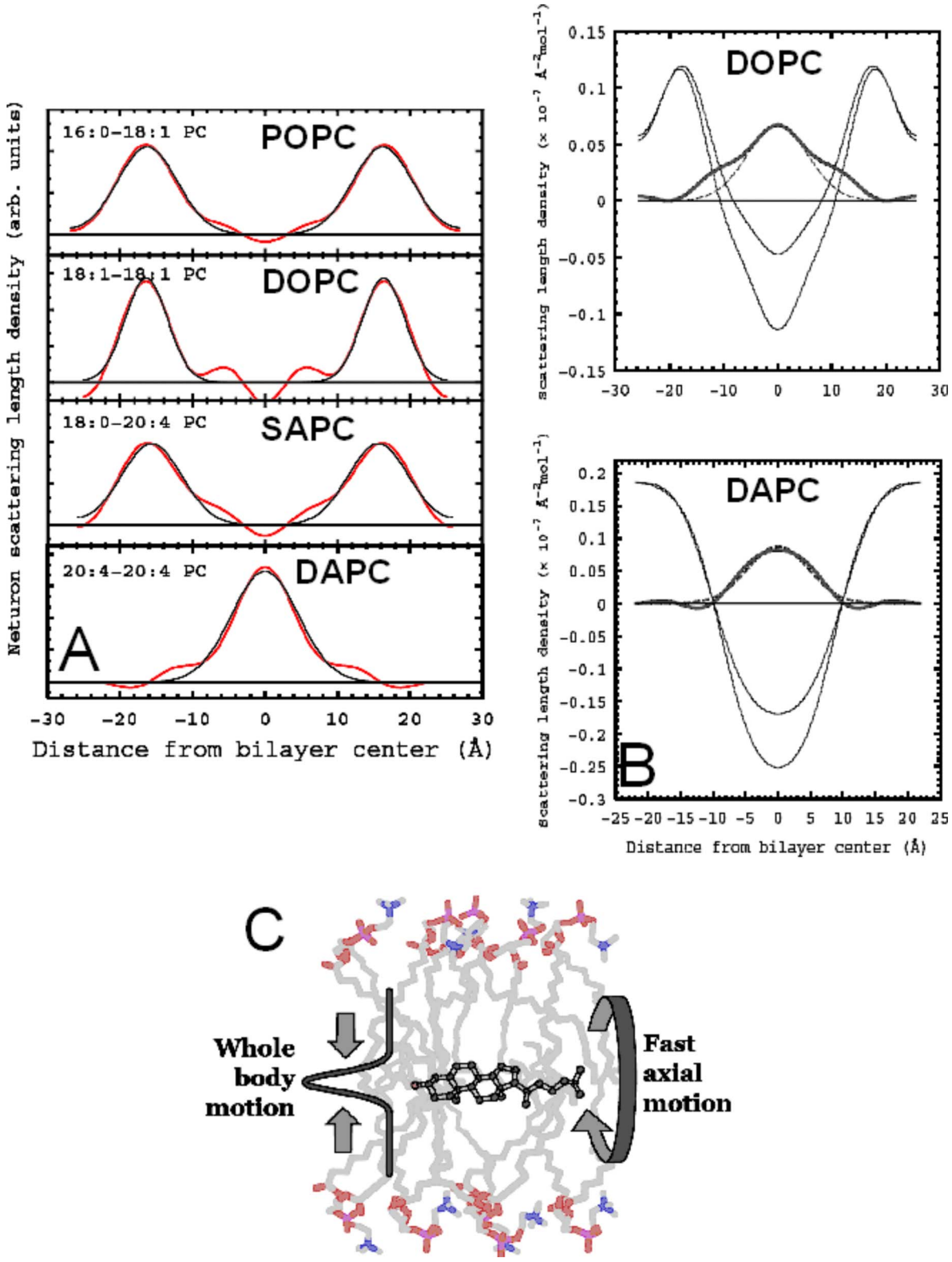

FIG. 4. (a) Difference scattering length density (SLD) profiles of headgroup labeled [2,2,3,4,4,6-^{2}H6] and unlabeled cholesterol in POPC, DOPC, SAPC, and DAPC bilayers. The red lines are the measured data and the black lines are single Gaussian fits to the data. (b) SLD (thick gray lines) and difference SLD (thin black lines) profiles of tail labeled [25,26,26,26,27,27-^{2}H7] cholesterol in DOPC and DAPC bilayers. The dashed lines are fits to the data using a single Gaussian. (c) Schematic depiction of the location of cholesterol in DAPC bilayers. As determined by ^{2}H NMR, the molecule is found to reside at the center of the bilayer and is motionally constrained, while undergoing fast axial rotation. Figure adapted from Refs. 66 and 92.

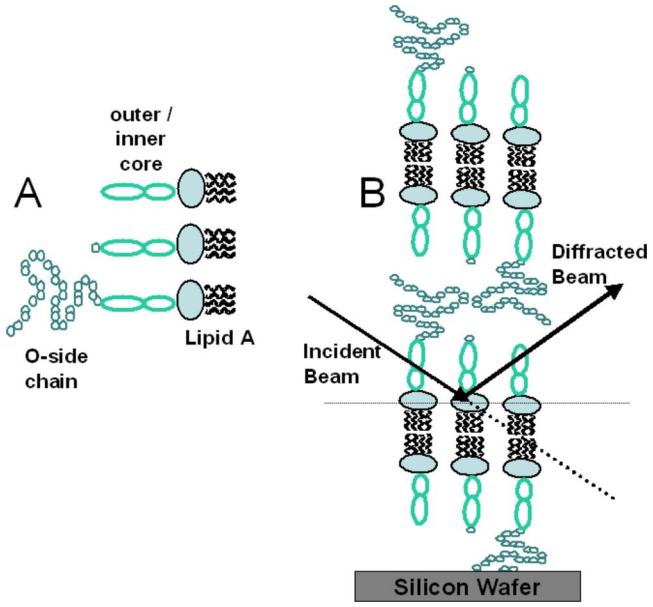

FIG. 5. (a) LPS schematic showing the hydrophobic region formed by lipid A, and an extensive hydrophilic region made up of the inner and outer cores, with additional O-side specific chains. They are generally described as: "rough" (no O-side chains), "semirough" (one repeat unit), and "smooth" (O-side chain made up of up to 50 trisaccharide repeat units) LPS. (b) Schematic of the oriented LPS diffraction geometry. Figure adapted from Ref. 103.

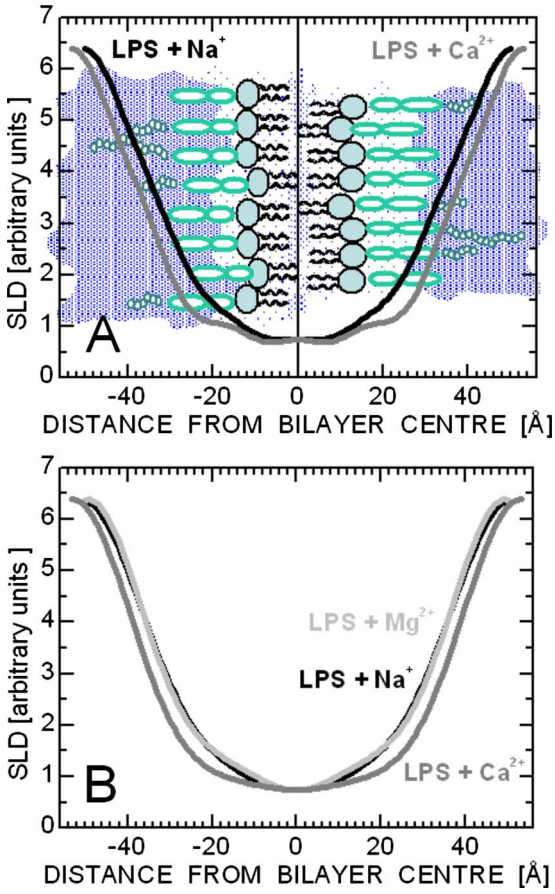

FIG. 6. (a) SLD profiles of LPS bilayers hydrated in 100% D_2O. The solid black line corresponds to Na^+-LPS bilayers, whereas the solid gray line corresponds to Ca^{2+}-LPS bilayers. (b) Lower resolution SLD profiles of Na^+-, Mg^{2+}-, and Ca^{2+}-LPS bilayers hydrated with 100% D_2O. All profiles were reconstructed using three Bragg reflections, instead of the six for the SLD profiles in (a). Figure adapted from Ref. 103.

Although there have been many studies in determining the biological importance of LPS, few have shed information regarding their structural properties. For example, Snyder *et al.*[101] used x-ray diffraction to study osmotically stressed LPS multilayers, and calculated 1D electron density profiles which were used to characterize the in-plane bilayer packing properties. Comparison with typical membrane phospholipids revealed a more compact packing of LPS, offering an explanation to the relatively low permeability of the Gram-negative bacterial membrane to hydrophobic molecules.

Neutron diffraction experiments [Fig. 5(b)] have recently reported[102] that the quasi-Bragg peaks associated with LPS' lamellar structure disappeared when liquid crystalline bilayers were subjected to high levels of hydration and temperature. This suggests either the complete dissolution of the lamellar morphology or the loss of long-range order, implicating hydration an important physical parameter. More important was the fact that the 1D LPS scattering density profiles indicated that water penetrates deep into the bilayer, including the bilayer center (Fig. 6). This observation was made possible due to the differential sensitivity of neutrons to hydrogen (H) and deuterium (D) atoms. While the chemical and structural properties of the biologically relevant systems are, for the most part, not affected by H–D exchange, the sensitivity of neutron scattering experiments is considerably increased by such contrast variation. The distribution of water molecules across the bilayer can then be easily determined with the remarkable resolution of a few angstroms. Subsequently, Kučerka *et al.*[103] studied the effect of cations on LPS bilayers and concluded that, compared to Na^+ and Mg^{2+} counterions, Ca^{2+} resulted in more compact, less hydrated LPS bilayers (Fig. 6), a structural change associated with decreased biological activity.

VI. MEMBRANE DYNAMICS

Although there are myriads of studies published every year with regards to the structure of biomimetic membranes, scattering studies dealing with their dynamical properties have been few and far between. For example, it is only in the past few years that the collective motions of lipid acyl chains using inelastic x-ray[104] and neutron[105] scatterings have been reported. Presently, the factors limiting the study of dynamical processes are intensity, especially at neutron sources, and sample preparation and environments, which when overcome will allow for the study of collective excitations (e.g., phonons) in biomimetic membranes.[106]

Fluctuations in biological membranes cover time and length scales from nanoseconds, involving several hundred lipids, to picoseconds, involving neighboring lipids. In the case of neutron scattering, the dynamics of individual lipid molecules, such as vibrations, full and hindered rotations, and diffusion have been investigated by incoherent quasi-

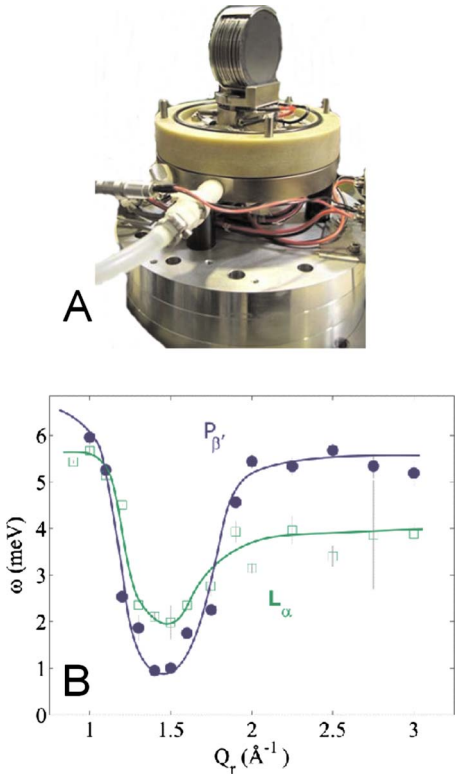

FIG. 7. (a) Temperature and humidity controlled multiwafer sample oven suitable for inelastic neutron scattering studies (photograph courtesy of Rheinstädter). (b) Dispersion curves of gel and fluid phase of the DMPC bilayers. Figure adapted from Ref. 109.

elastic neutron scattering,[107] while spin echo techniques have been applied to the study of collective undulation modes.[108] Precisely, incoherent inelastic neutron scattering techniques capable of determining single particle dynamics (i.e., autocorrelation function) benefit from the use of protonated samples, while coherent inelastic techniques utilize deuterated samples to determine collective dynamics. Of note, is that the first inelastic neutron scattering study to look at the short wavelength dynamics (i.e., nearest neighbor distances) in a phospholipid bilayer was only published in 2004.[105]

As mentioned, factors limiting the study of dynamical processes are small scattering volumes and weak inelastically scattered signals. To address these issues, highly oriented membrane stacks consisting of thousands of lipid bilayers are used [Fig. 7(a)]. Employing this setup, dispersion curves for $P_{\beta'}$ and L_α DMPC bilayers were obtained.[105,109] From the curves it is evident that the lipid chains show a similar behavior to that of a 2D liquid [Fig. 7(b)]. However, unlike liquids the lipid chains are bound to the headgroups and each other, leading to a slower diffusional motion, also explaining why dispersion curves can be measured over such an extended range in reciprocal space (i.e., Q-range).

Although the energy transfers in the high Q-range of $P_{\beta'}$ DMPC bilayers exceeded those of L_α bilayers (due to stiffer lipid chain coupling), they were also lower at lower Q's, something analogous to a soft mode in crystalline systems. In addition, measurements in the gel-liquid coexistence region were made, in which domains were determined to be smaller than ~100 Å, the coherence length of the specific energy neutrons used.[109] Only in this way can the continuous change in parameters in the elastic measurements together with the coexistence of gel and fluid excitation in the spectra be explained. These experimental results were, in part, found to be consistent with MD simulations.[110]

Most recently, elastic x-ray and inelastic neutron scattering techniques, in combination with MD simulations examined the static and dynamic structure factors of DMPC bilayers.[111] From the simultaneous refinement of MD simulations and scattering data, the area per lipid and hydrocarbon chain ordering were described. It was determined that the interchain correlation length decreased linearly with the area per lipid, allowing for the structure factor to be quantitatively related to lipid area. Importantly, the short wavelength dynamics obtained from inelastic neutron scattering and MD simulations were compared, whereby the authors reached the conclusion that the collective dynamics of the lipid hydrocarbon chains cannot be fully described by theories developed for simple liquids.

VII. CONCLUDING REMARKS

In this review some of the state-of-the-art usage of solid supported lipid bilayers was presented. In all cases, the experiments provided unequivocal structural information, while other studies have yielded results challenging our preconceived notions (e.g., cholesterol in PUFA membranes) of membranes. In recent years, the use of newly solid supported membranes[112,113] has expanded into various fields, including biosensors,[114] cell-cell surface interactions,[115] peptide lipid interactions,[116] etc. Solid supported membranes have already provided us with insights into biological phenomena and will, over the next decade, make substantial contributions to biology and biotechnology.

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