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REVIEW

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Floating lipid bilayers: models for physics and biology

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Abstract Progress in the determination of structure and fluctuation spectrum of a floating bilayer system, as well as potential applications for biological studies, is reviewed. The system described here was first introduced by Charitat et al. (Eur Phys J B 8:583–593, 1999) and consists of a planar bilayer floating at 2–3 nm away from an adsorbed one on a solid surface in contact with bulk water. This model has been widely used for surface scattering studies using both neutrons and synchrotron radiation and its use in studies of relevance for physics and biology research areas will be described, together with the progress towards the production of complex biomimetic samples for use with scattering techniques.

Keywords Supported lipid bilayers · Model membranes · Specular reflectometry · Off-specular reflectometry · Neutrons · Synchrotron radiation

Special Issue: Scattering techniques in biology—Marking the contributions to the field from Peter Laggner on the occasion of his 68th birthday.

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Introduction

The cell is the structural and functional unit of most living organisms with about 10^{14} cells present in a human being. Cell sizes vary but they are mostly microscopic (average diameter is of the order of 10 μm). Each cell consists of a mass of protein material (the protoplasm) bound by a cell membrane, which in the cells of plants, fungi, algae and bacteria is surrounded by a cell wall. The cell membrane, or plasma membrane, is partially permeable and consists mostly of proteins and lipids. It regulates the flow of material in and out of the cell.

Lipids are amphiphilic molecules possessing a hydrophobic head and hydrophilic chains. They form a continuous bilayer, which acts as a barrier to water soluble molecules and provides the framework for the incorporation of membrane proteins. In plasma membranes, lipids represent 40–50 % in weight of the membrane composition. The structure and organization of the lipid bilayer component of membranes hold the key to understanding the functioning of membranes.

Because of the complexity of cell membranes, their study as a whole is challenging and a common approach has been to refer to systems mimicking their composition. The most common biomimetic models make use of lipid bilayers (Mouritsen 2005). A considerable effort has led to numerous successes in preparing new types of samples and corresponding structural measurement techniques. These can resolve sub-nanometric details, leading to investigations of lipid–lipid, lipid–peptide or lipid–protein interaction mechanisms. Current efforts are devoted to the exploration of increasingly complex systems like bilayers containing various degrees of insaturation, sterols, gangliosides, as well as natural lipids extracted from cells.

Studies on lipid bilayers are important both from the fundamental point of view and because of the wide variety of possible applications in nano-bio-technology. First and most importantly, the importance of membranes lies in their widespread presence in living matter. For example, a rough estimation indicates that the total surface of membranes covers an area of about 0.03 km^2 in our body that is roughly the area of five football fields. The function of many membrane proteins depends on membrane composition, lipid–protein interaction, and lipid mediated protein–protein interaction. Lipids have a pharmacological interest since drug transport through membranes is dependent on physico-chemical membrane properties. Membranes may play a direct role in signal transduction; they certainly play a direct role in cell adhesion. Finally, many common diseases, as for example, diabetes, schizophrenia, Tay-Sachs syndrome, Alzheimer and Parkinson diseases, are associated with changes in lipid composition.

Nano-bio-technology applications include the development of catalytic biosensors (for example glucose biosensors) and affinity ones (antibodies, DNA, peptides and lectins). Current problems in the development of these include non-specific binding and reproducibility. Biofunctional coatings of artificial organs and implanted medical devices are further areas of interest and application of membrane studies.

The history of membrane models dates back to almost a century ago when Gorter and Grendel (1925) in a simple and elegant experiment on the area of the monolayer formed by spreading lipid extracts of a red blood cell on the surface of water, first suggested that membranes were only two molecules thick. Ten years later Danielli and Davson (1935) suggested the association of proteins with membranes. Finally Singer and Nicolson (1972) introduced the widely used "Fluid Mosaic Model" whereby integral and peripheral proteins "float in a fluid sea". Israelachvili (1978) described thickness variations and pore formation concepts and Sackmann (1995) included cytoskeleton and glycocalix.

The need for a new membrane model has become necessary (Mouritsen 2005). The fluid mosaics model implied a randomness that is now recognized to not exist. The notion of membrane fluidity is important but it is impossible to neglect that liquids or fluids may be structured on length scales in the nanometre range, which are difficult to access experimentally. The structuring in time, i.e. correlated dynamical phenomena, is recently recognized as important features of membrane systems.

Producing a good, biologically relevant model membrane is a challenge (Mouritsen et al. 1998). Issues to be addressed include, for example, the compatibility between the opposing requirements of membrane stability (the membrane must be a permeability barrier) and the bilayer need to adapt to membrane protein conformational changes, the presence of lipid protrusions, instabilities towards non-lamellar symmetries, phase transitions, and the fact that membrane function may be stirred by perturbations by both physical (temperature, pressure) and chemical (drugs) factors.

Moreover, phospholipids like surfactants and other amphiphilic molecules, present a variety of different phases depending on their volume/surface area ratio or temperature (Lipowsky and Sackmann 1995). Going from high to low temperature, lipids may overcome a fluid to gel phase transition at a temperature commonly known as T_m (*melting temperature*). While in the fluid phase, Lα, lipid chains are in a liquid-like conformation and mobile in the lateral direction, in the gel phase, Lβ, chains are stiffer. For some lipids, just below T_m, there is the so-called ripple phase, Pβ', where the lamellae are deformed by a periodic modulation. In nature bilayers are generally fluid.

A variety of sample types have been tried for use in structural studies. They differ by: (1) geometry, e.g. spherical or flat; (2) number of bilayers: one, a few, or hundreds; (3) order: controlled or disordered position and orientation of molecules; (4) phase of the bilayer: gel (e.g. at low temperatures) or fluid; (5) difficulty of the preparation method. The bilayer molecular composition is the first issue. Although bilayers in membranes contain variable amounts of cholesterol, the simplest models make use only of phospholipids. Various lipid chains or heads can be chosen. Di-palmitoyl (16 carbons) and di-stearoyl (18 carbons) chains are often used, since they are dominant in nature (mostly with one to several insaturations in the chains); sample preparations or experimental conditions might sometimes be easier if di-myristoyl (14 carbons) chains are used; 15, 17, 19 or 20 carbon chains are used to investigate the effect of chain length or parity. Molecules with smaller chain lengths are usually soluble in water, so that the bilayer is no more stable. Bilayers with longer chain lengths are always in the gel phase, and too stiff for free out-of-plane fluctuations. Saturated chains, and the use of a single type of molecules, help preparing ordered samples; on the opposite, unsaturated chains, or mixtures of different molecules, favour bilayer fluidity.

As for lipid heads, phosphatidyl-cholines (or PC, or phosphocholines) are widely used; other neutral heads (e.g. phosphatidyl-ethanolamine, PE) are used to investigate the effect of head type or size. Charged heads (e.g. phosphatidyl-serine, PS), often mixed with variable ratios of neutral heads, help controlling the bilayer charge.

Historically, multilamellar systems, including liposomes and stacked bilayers, have been used first. Development in scattering instrumentation has led to a growing interest in the use of lipid monolayers or single bilayers either at solid liquid interfaces or as black lipid membranes (Beerlink et al. 2008). A further approach, used in nanotechnology

for the electrophysiological study of membrane proteins is the reconstitution of planar bilayers on both sides of micron sized holes on silicon substrates (Suzuki et al. 2004).

In this review we will concentrate solely on a model introduced by Charitat et al. (1999) involving a planar bilayer *floating* at 2–3 nm away from an adsorbed on a solid surface in contact with bulk water. This model has been used for surface scattering studies of a highly hydrated, accessible and fluctuating bilayer, where the composition of each leaflet can be chosen separately. Since the second bilayer is only weakly bound, the preparation needs particular care and is not accessible to all lipid compositions. We will point out the interest of this model both for physics and biology studies and report the progress in the last decade in determining its structure and fluctuation spectrum and as well as in the production of complex biomimetic samples for use with scattering techniques.

For *biology* studies, *floating* bilayers are interesting because they are stable in bulk water and in fluid state; they are potentially useful for studies of transport across the membrane and for studying membrane proteins behavior.

For *physics* studies, *floating* bilayers are interesting because reflectivity techniques allow the determination of the structure of a unique bilayer with a fraction of nanometer resolution; direct comparison to theoretical calculations is possible; the fluctuation spectrum can be directly determined by x-ray scattering. In the micrometer range (more easily accessible by x-rays than by optical methods) thermal fluctuations are limited by the bilayer bending modulus (κ) the surface tension (γ) and the attraction potential (U) from the nearby wall. They cross over from a κ-dominated regime to a U-dominated regime according to the in-plane length scale.

Various strategies have been developed to create *floating* bilayers by using different spacer molecules, including grafted layers (Hughes et al. 2002), polymer (El-khouri et al. 2011) and polyelectrolyte cushions (Chen et al. 2009), tethered bilayers (McGillivray et al. 2009). Because of the potential to support transmembrane proteins, all these systems promise to be a better biomimetic model membrane than supported single bilayers.

The wavelengths of neutron and x-ray beams are on the order of a tenth of a nanometre and make them ideal tools for the structural characterisation of lipid bilayers. Biological membrane components, like most soft materials, are rich in hydrogen and neutrons have the unique capability of being scattered differently by hydrogen and deuterium. It is thus possible to choose the H_2O/D_2O water composition so that the water matrix has the same cross section as parts of the sample with the effect that there will be no signal from those regions. This technique is known as *contrast variation*. It is also possible to accentuate or annihilate the scattering from individual parts of a complex system, so, for example, by specific deuterium labeling, it is possible to measure bilayer conformational changes and organization both in the perpendicular and lateral directions.

Neutron and x-ray scattering techniques have been much used as they allow for in situ measurements not only of interfaces, but also of buried (bulk) materials, thanks to the high penetration power of neutrons and the high energies reached at synchrotron facilities for x-rays. They determine structures at different scales simultaneously, thus probing individual molecules as well as collective effects. They provide sub-nanometric details thanks to the small wavelengths of the beam; they are sensitive to density, as well as chemical composition. They thus constitute useful complements to more common laboratory techniques.

Although small angle scattering studies have provided values for the thickness of lipid lamellae as well as their composition and the thickness of the water layer between lamellae, these values are averaged in all directions. Surface sensitive scattering techniques, like reflectivity (Daillant et al. 1999) or grazing incidence diffraction (Solomonov et al. 2009; Watkins et al. 2011), avoid the ambiguities associated with powder averaging. In these cases, information is obtained on single bilayers, either along the chain direction or in the plane of the membrane.

While x-ray and neutron diffraction from stacked bilayers have historically been the source of high resolution structural data of multilamellar model biological membranes, neutron and x-ray reflectometry have provided unique data of single lipid bilayers in contact with bulk water.

For a recent review on supported bilayers for use with neutron reflectometry techniques, see (Wacklin 2010).

Techniques

Sample preparation

Bilayers can be deposited on solid substrates by using modified Langmuir troughs and the so-called *Langmuir–Blodgett* or *Langmuir-Schaefer* techniques (Tamm et al. 1985). After spreading a lipid monolayer on the surface of water and allowing for the evaporation of the solvent, the monolayer is compressed to a chosen surface pressure, usually high enough that lipids are in a liquid crystalline phase and deposition is more efficient. A highly hydrophilic solid substrate, with size of the order of a few centimetres, is immersed in the subphase and by slowly withdrawing it from water a monolayer of lipids is deposited on its surface. Re-immersion will produce a bilayer, and so forth. In the Langmuir-Schaefer modification the solid substrate is rotated by 90° and slowly immersed in water through the monolayer with the large face parallel to the water surface (see Fig. 1).

Peptides, proteins, or other amphiphilic molecules, can be co-deposited or inserted in the water subphase and transferred with the lipids. As shown in Fig. 1, a *floating* bilayer can be produced with these techniques by three vertical Langmuir–Blodgett depositions, followed by a horizontal Langmuir-Schaefer one (Charitat et al. 1999). This method is efficient when lipids are in the gel phase although by raising the temperature the *floating* bilayer overcomes a phase transition becoming fluid and in most cases still stable. The Langmuir-Schaefer step is necessary to obtain a high coverage layer. Successive Langmuir–Blodgett depositions lead to partially covered bilayers in water as demonstrated recently by (DeCaro et al. 2011).

A major advantage of this model compared to others used for structural studies, is the fact that the bilayer is immersed in aqueous solution and that information on the single bilayer can be obtained. Moreover, the composition of each leaflet of the bilayer can be chosen separately. While in adsorbed bilayers the proximity of the substrate limits the position fluctuation, which is a disadvantage when studying the interaction with transmembrane proteins, here the bilayer floats at 2–3 nm away from the surface, so that the interaction with the substrate is reduced. Finally, the *floating* bilayer is accessible to bulk molecules like peptides, proteins, polymers, etc.

For the work reported below, samples were prepared with the following phospholipid species: 1,2-distearoyl-phosphatidyl-choline (DSPC); fully deuterated DSPC (d83-DSPC); 1,2-dipalmitoyl-phosphatidyl-choline (DPPC); 1,2-dipalmitoyl-phospho-ethanolamine (DPPE); 1,2-dipalmitoyl-phospho-serine (DPPS).

Specular reflectometry

Neutron reflectivity is an ideal tool for getting information at the Å level on this buried system, and in the last 15 years the technique has been widely used for the study of single bilayers at solid/aqueous solution interfaces. The use of x-rays was less common until recently since x-rays are adsorbed both by the solid substrate and by the aqueous solution so that studies at the solid/liquid interface are best performed at synchrotron sources.

In a neutron reflectivity measurement the ratio, R, between the intensities of the reflected and incoming beams, is collected, as a function of q_z, the momentum transfer perpendicular to the interface (cf. Fig. 2).

Reflectivity is related to the scattering length density across the interface by the approximate relation: $R(q_z) \approx \frac{16\pi^2}{q_z^2}|\rho(q_z)|^2$, which is the reflectivity in the Born approximation (Crowley et al. 1991). $\rho(q_z)$ is the Fourier transform of the scattering length profile $\rho(z)$ along the normal to the interface, giving information about the composition of each layer and about its local structure. The scattering length density is given by: $\rho(z) = \sum_j b_j n_j = \sum_j b_j n_j$, where n_j is the number of nuclei per unit volume and b_j is the scattering length of nucleus j.

The method of analysis often used for specular reflection data involves the construction of a model of the interface that may be represented by a series of parallel layers of homogeneous material. Each layer is characterised by a scattering length density and a thickness, which are used to calculate a model reflectivity profile by means of the optical matrix method (Born et al. 1989). The interfacial roughness between any two consecutive layers may also be included in the model by the Abeles method (Heavens 1955). The calculated profile is compared to the measured profile and using χ^2 in the least-squares method assesses the quality of the fit.

The spatial resolution of the technique is limited by the high background signal and poor signal/noise ratio. Still, if the technique is appropriately used, can reveal structural details down to the fraction of nanometer scale. The loss of phase information typical of all scattering techniques prevents a direct inversion of the data to determine the scattering length density profile. Although more than one model can be found for a given experimental curve, the number of possible models is greatly reduced by a prior knowledge of the system, which allows us to define upper and lower limits of the parameters used, by the elimination of the physically meaningless parameters, and most importantly by the use of different isotopic contrasts. In fact, the use of a combination of hydrogenated and deuterated material can substantially change the reflectivity curve of a system while maintaining almost the same chemical structure. The same model must describe all the reflectivity curves measured at different isotopic compositions of the same physical system. Experience on this kind of system and on similar ones suggests that the

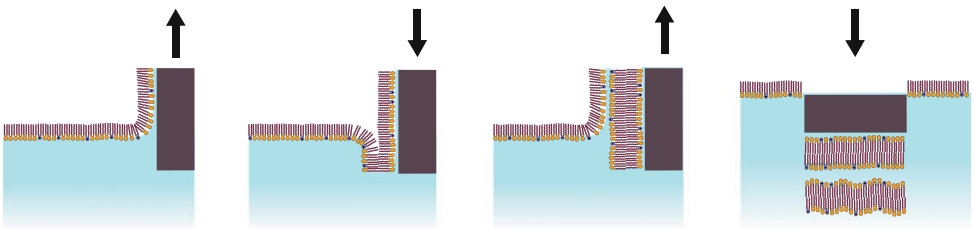

Fig. 1 Schematic cartoon of the technique of preparation of floating bilayers. Adapted with permission from (Charitat et al. 1999)

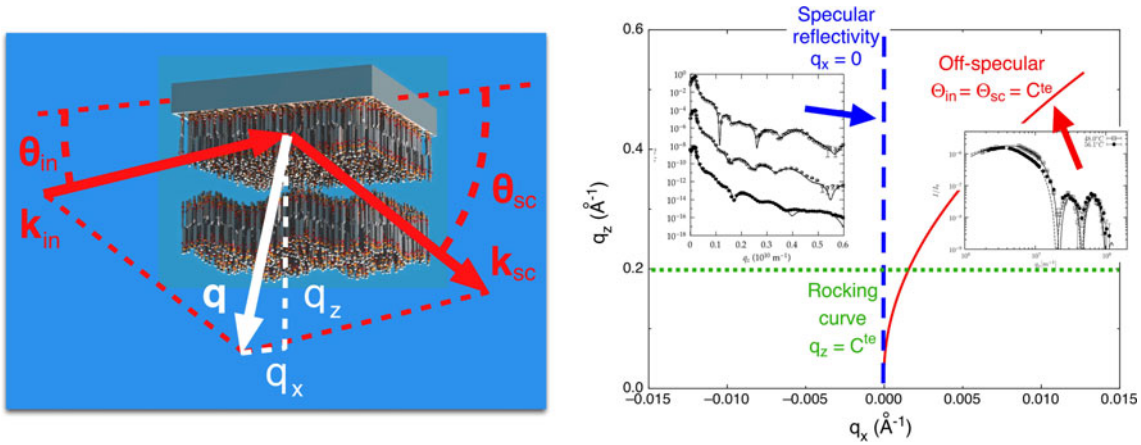

Fig. 2 Simple schematic of the geometry of specular and off-specular x-ray reflectivity from surfaces. Specular reflection occurs for $\theta_{in} = \theta_{sc}$ ($|q| = q_z$). For neutron reflectivity studies the geometry is inversed as the neutron beam strikes the interface from inside the solid substrate

measurement of reflectivity curves from three or more contrasts, combined with standard physical hypotheses, are necessary and sufficient for extracting a unique model of the interface.

X-ray reflectivity follows the same formalism of neutron reflectivity with the scattering length density replaced by the electron density of the material at the interface. Absorption cannot be neglected here as it is often the case for neutrons. The higher flux achievable, especially with synchrotron radiation, allows for a much higher spatial resolution leading to the determination of finer structural details. Figure 3 shows a comparison of specular reflectivity data from the same double bilayer sample obtained with both neutrons and synchrotron radiation (27 keV energy). The possibilities offered by deuterium labeling in neutron reflectometry (see for example the scattering length profiles in the inset of Fig. 3), allow a better determination of the distribution of hydrogenated parts of the system so that the two probes are perfectly complementary.

Off-specular synchrotron radiation reflectometry

Off-specular reflectivity measurements allow the determination of lateral features of thin layers. For layers in the nanometer range, the intensity of off-specular signal is usually much lower than that of the specular one so that the use of synchrotron radiation is more efficient compared to neutron beams. Measurements from *floating* bilayers at the silicon/water interface were performed at the European synchrotron radiation facility (ESRF) in Grenoble at a constant grazing angle of incidence below the critical angle of total external reflection at the Si–water interface (0.83 mrad). The beam traverses 50 mm of water in a Teflon reservoir equipped with micron sized Teflon windows. The sample was translated every 30 min during measurements as no radiation damage was observed below 1 h exposure. Background subtraction as well as the model for calculating the scattering intensity are described in detail in (Daillant et al. 2005). Since the first measurements (an example is shown in Fig. 4), optimization of slit widths allowed us to extend the in-plane wave-vector transfer range $q_{||}$ by one order of magnitude from 2×10^6 m^{-1} to 2×10^5 m^{-1}, allowing a precise determination of interaction potentials (Malaquin et al. 2010). The probed in-plane length scales are in the range of a few tens of microns to a few tens of nanometers while specular reflectivity probes thicknesses in the direction normal to the surface in the range of tenths up to a few hundreds of nanometers.

Floating bilayer structure and fluctuations

Floating bilayers have been studied with both neutron and synchrotron radiation reflectivity techniques. Neutron specular reflectivity studies (Fragneto et al. 2001) enabled the determination of structural changes occurring at the gel to fluid phase transition and in particular the values of the swelling of the water layer between the two bilayers was used to calculate theoretically the bending modulus of the bilayers in both phases (Mecke et al. 2003). Synchrotron radiation data were the first collected on supported single bilayers in water and showed details that cannot be seen with neutrons as for example the position of the CH_3 groups in the bilayers (Daillant et al. 2005). They allowed the determination of the bending modulus and surface tension of adsorbed and *floating* layers as well as the interaction potential between supported *floating* bilayers (Daillant et al. 2005; Malaquin et al. 2010) using the following fluctuation spectrum related to the scattering intensity:

$$\left\langle u_{q_{||}} u_{-q_{||}} \right\rangle = \frac{k_B T}{U'' + \gamma q_{||}^2 + \kappa q_{||}^4}$$

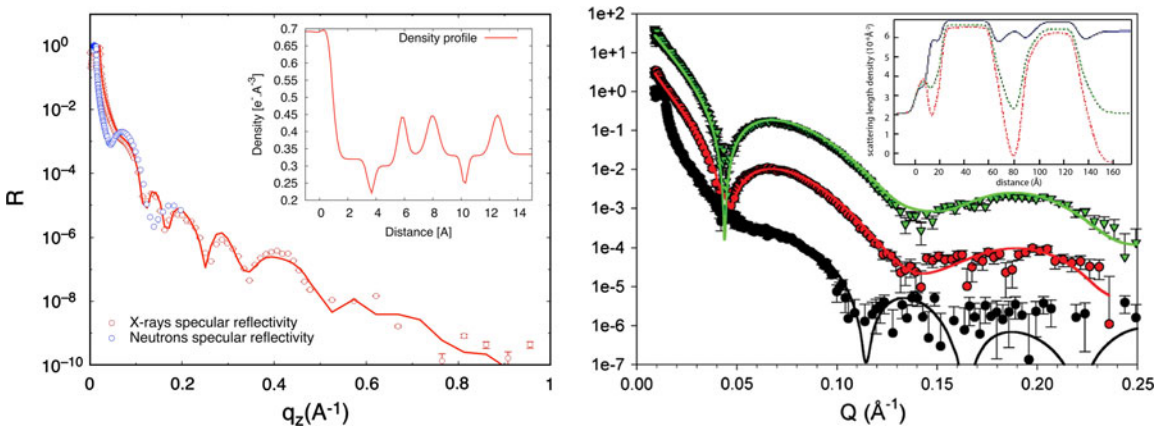

Fig. 3 On the *left* is a comparison of specular x-ray (*red*) and neutron (*blue*) reflectivity from DSPC double bilayers, in the *inset* is the electron density profile describing the curve; on the *right* neutron reflectivity profiles and line fits from d83-DSPC double bilayers in D_2O (*black*), H_2O (*red*) and contrast match silicon (*green*), in the inset the corresponding scattering length density profiles. The neutron reflectivity profiles on the *right* are offset for clarity. *Data* are plotted as a function of the projection in the z direction of the wave vector transfer q in units of $Å^{-1}$

where, $q_{\|} = \sqrt{q_x^2+q_y^2}$,$U''$ is the second derivative of the effective microscopic potential, γ the surface tension and κ the bending modulus.

Figure 4 shows on the left an example of the off-specular data from a DSPC sample in the gel and fluid condition and on the right plots of γ and κ for the *floating* bilayer as a function of temperature for phosphocholine lipids with both 17 and 18 carbons in the saturated chains. While the positive tension is nearly constant in the investigated range, the bending modulus values in the gel and fluid phases of the lipids vary in agreement with literature data. The novelty here is that the values are determined with the same technique and on the same sample.

Effect of chain length

Phospholipid chain length affects the functioning of transmembrane enzymes; notable examples are the membrane bound enzymes Ca^{2+}-ATPase, important for muscle cell action, and Na^{+}-K^{+}-ATPase, responsible for using about a third of all the energy our bodies turn over. The activity of both ion pumps is maximal for a certain lipid type and for a specific membrane thickness (Lee et al. 1998). Geometrical considerations are useful to understand this behavior (the so called hydrophobic matching). Insight on the structure and dynamics dependent phase behavior of lipids as a function of chain length could provide further elements for the understanding of membrane protein functioning.

The behaviour around the phase transition of double bilayers with different chain length was investigated (Fragneto et al. 2003). Lipids were saturated diacyl phosphocholines, with the number of carbon atoms per chain, n, varying from 16 to 20. The average and root-mean-square positions of the floating bilayer were determined by means

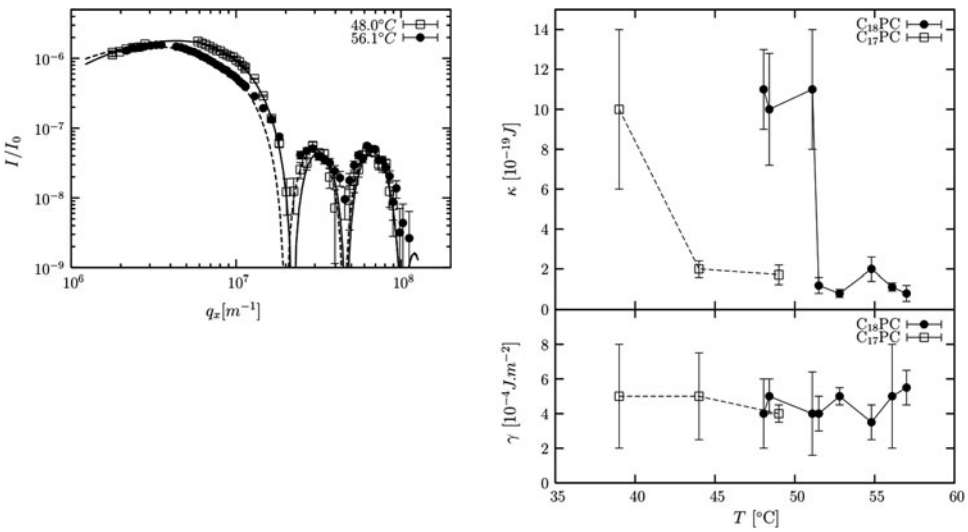

Fig. 4 *Left*: Synchrotron radiation off-specular data collected at the BM32 beamline at the ESRF (Grenoble) from a DSPC floating bilayer in the gel (*squares*) and fluid (*circles*) phases. *Right*: Bending modulus (*top*) and surface tension (*bottom*) for floating bilayers from phosphocholines with 17 and 18 (DSPC) carbons in the saturated chains. The Gel-Fluid phase transition is clearly observable on the bending modulus. Reproduced with permission from (Daillant et al. 2005)

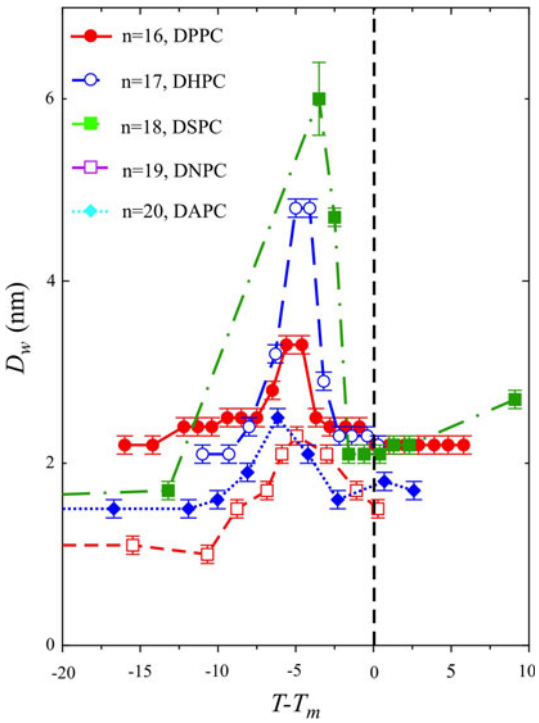

Fig. 5 Distance, Dw, between the two bilayers from measurements from hydrogenated lipids versus the deviation of temperature from the melting temperature, T–T_m. Phosphocholine samples with di-chain lengths, n, from 16 to 20 were used: (*filled circle*) $n = 16$; (*open circle*) $= 17$; (*filled square*) $n = 18$; (*open square*) $n = 19$; (*filled diamond*) $n = 20$

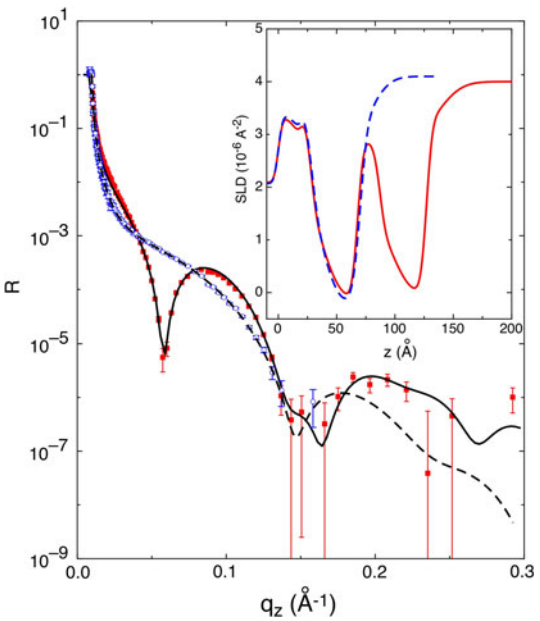

Fig. 6 Specular neutron reflectivity data and line fits from a DPPS single (*blue empty circles*) and double (*red filled circles*) bilayer. In the *inset* are the corresponding neutron scattering length profiles

of neutron specular reflectivity. Samples were prepared at room temperature (i.e., with the lipids in the gel phase), and measurements were performed at various temperatures so that the whole region of transition from gel to fluid phase was explored. In Fig. 5 results are reported from neutron reflectivity measurements showing the existence of a very large swelling of the water layer between the two bilayers for lipids with 17 and 18 carbon atoms in the hydrophobic chain. Results are useful for the physics of fluctuations while biological interest is speculative.

Effect of head-groups

The structure and phase-behavior of single and double bilayers of DPPE were investigated by specular neutron reflectivity for their viability as biomembrane mimics (Stidder et al. 2007). Whilst single bilayer samples were found to exhibit stable gel and fluid structures, double bilayers were found to be intrinsically unstable in the fluid phase as a planar structure. A Bragg peak was observed in the reflectivity data at just above the gel-to-fluid transition temperature, indicating the partial rearrangement of the upper bilayer into a repeat stacked structure. The lower bilayer was structurally stable. The structure and phase-behavior of a double bilayer containing a ratio of 9:1 DPPE/cholesterol was also investigated to assess the stabilizing effect of cholesterol on the upper bilayer. The presence of cholesterol completely destabilized the upper bilayer, causing it to detach 7 °C below the gel-to-fluid transition temperature of DPPE. It is possible that the cholesterol increases the overall conical shape of DPPE molecule by residing in the chain region of the lipid.

Effect of charges

Specular neutron reflectivity (data shown in Fig. 6) and off-specular synchrotron radiation measurements were performed on fully negatively charged double bilayers from DPPS (Malaquin PhD thesis; manuscript in preparation). It was found that bilayers were slightly thinner than DPPC bilayers and less rough in agreement with literature (Petrache et al. 2004). Off-specular scattering analysis suggests that membranes are strongly correlated and a small water layer (<1 nm) is present between the two bilayers: electrostatic interactions are screened over a very short distance.

Effect of alternative current

The study of the influence of electric fields on lipid bilayers has a fundamental interest and is useful for understanding practical mechanisms like vesicle electro-formation. Since they are almost free of defects, *floating* bilayers are good candidates for this investigation. Lecuyer et al. (2006) have reported a pioneering study of the destabilization by an electric field of the *floating* bilayer of a double bilayer

system. The main result of this work was the observation of the complete unbinding of the *floating* bilayer, by application of an electric field on a double DSPC bilayer in a $CaCl_2$ solution. The effect occurs at low frequency (10 Hz) and for voltage amplitude higher than 5 V. After the unbinding, the presence of the first bilayer was still observed, without any major change in structure, but slightly shifted away from the substrate. After removing the bilayer by cleaning the substrate, the silicon oxide layer remained unmodified and, therefore, it was possible to conclude that there was no significant formation of porous silicon oxide. This complete unbinding of the *floating* bilayer was very sudden. However, in a lower-conductivity electrolyte, previous steps of the destabilization were observed. The irreversible destabilization of the *floating* bilayer seems to be preceded by a reversible increase in its roughness. It is possible to describe this roughness in terms of thermal fluctuations as reviewed in (Charitat et al. 2008). No modifications in the bilayer were observed above 100 Hz, whereas the effect became noticeable below 10 Hz, in good agreement with previous observations. Off-specular synchrotron radiation measurements allowed to determine the fluctuation spectrum as a function of applied field (*data not published*).

Floating bilayers as biomimetic systems

Floating bilayers are potentially useful for biology studies already when composition is kept simple and the bilayer is formed by lipids only, but it has been possible to prepare also complex bio-mimetic systems including cholesterol or gangliosides in the lipid scaffold.

An example of application in health areas is the interaction of cationic lipoplexes with bilayers of interest in the field of gene delivery. While many early studies investigated the use of adenoviral and retroviral vectors to effect the required gene delivery, much recent work has been focused on the potential of complexes prepared by mixing cationic lipids, generally in the form of vesicles with plasmid DNA or, more recently, siRNA. The resulting complexes are known as lipoplexes. The interest in lipoplexes as gene delivery vehicles stems from the fact that the use of such complexes to mediate gene transfer provides several important advantages over viral vectors, including: the relatively low toxicity of cationic lipids, the fact that there is no limitation to the size of the gene transported, and the possibility of decorating the surface of the lipoplexes with cell-targeting ligands to allow for site-specific targeting.

The interaction of lipoplexes prepared from vesicles of the dichain cationic lipid, dioctadecyldimethylammonium bromide (DODAB) with DNA, in the presence and absence of a 1:1 molar ratio of a neutral helper lipid (either dioleylphosphatidylethanolamine, DOPE, or cholesterol), with floating supported model membranes composed of DPPC with 10 mol% DPPS has been determined. Regardless of the composition of the lipoplexes, their interactions with the model biomembrane were restricted to the upper *floating* bilayer, and were typified by an accumulation of a layer of lipid from the lipoplex on the upper surface of the *floating* bilayer and, in the case of lipoplexes containing a helper lipid, the addition of some of the lipid from the lipoplex into the *floating* bilayer. Differences were seen in the interaction of the various lipoplexes with the upper bilayer in terms of the rate of the interaction, the amount of material bound to the upper bilayer surface, whether the lipoplexes resulted in any permanent change in the composition of the *floating* bilayer, and the reversibility of the interaction. Lipids from lipoplexes fabricated from DODAB rapidly deposited on the surface of the upper bilayer, covering $\sim$60 % of the surface but were easily removed upon washing the model biomembrane with water. In contrast, lipoplexes prepared from DODAB and cholesterol, although also rapidly depositing on the upper surface of the *floating* bilayer, covered only $\sim$15 % of the surface and also inserted lipid into the *floating* bilayer. In this case the interactions were not reversible (Talbot et al. 2009).

The structural complexity of biomembranes, based on their heterogeneity in composition, involves also the asymmetric disposition of different components, from lipids to proteins, in the transverse and the longitudinal directions. Inhomogeneities in the two leaflets of a membrane can couple, constituting the basis for the structural stabilization and modulation of functional domains involved in transmembrane signaling. Suitable mimics of complex biomembranes, involve the use of asymmetric model systems applicable for structural investigation. The experimental study of asymmetric model membranes is not common, due to the difficulty of preparing artificial membranes with wanted and defined heterogeneous composition. However, some attempts have been made, for example by preparing phospholipid unilamellar vesicles (LUVs) containing small amounts of glycosphingolipids only in their outer layer. The mechanical properties of the membrane were found to be strongly affected by the doping glycolipids, producing a softening that turns to hardening in the case of symmetric redistribution of molecules (Brocca et al. 2004). Fine assessment of structural inhomogeneities requires experimental macroscopic monodispersity.

Initial work by Stidder et al. (2005a) showed the feasibility to insert cholesterol into double bilayers and how this influenced the phase behavior of the bilayer. The effect of the addition of 1, 2, 4, and 6 mol% cholesterol to *floating* bilayers was investigated by neutron reflectivity. All samples exhibited fully stable and reversible gel and fluid

phases. Around the main lipid phase transition temperature, DPPC double bilayers exhibit large increases in the water layer separating the bilayers and the upper bilayer roughness. The inclusion of low amounts of cholesterol reduced the swelling of the water layer between the bilayers (see Fig. 7) and the upper bilayer roughness and progressively widened the temperature range over which swelling occurs. The thickness of the water layer separating the bilayers in the fluid phase goes through a minimum at the value of 2 mol%. The effect of adding cholesterol can be interpreted as an enhancement of the thermal fluctuations in the model membrane system. If cholesterol at these low concentrations makes the membrane more fluid like, the critical behavior at the transition may be somewhat diminished, the membrane fluctuates less, and the swelling is smaller with respect to the pure phospholipid system.

The addition of 10 mol% cholesterol to DPPC *floating* bilayers induced not only structural modifications of the bilayer but also differences in the phase behavior (Stidder et al. 2005b). In particular, the large swelling of the water layer between the two bilayers at the transition resulted practically removed. This confirmed that cholesterol induces a modification in the forces holding the upper bilayer to the lower. The result is in agreement with literature data suggesting that while very low amounts of cholesterol (<4 %) soften the bilayer, higher amounts rigidify it. The structure of the bilayer revealed that in the gel phase cholesterol induces the presence of a high rms roughness that disappears in the other phases.

Grazing incidence diffraction measurement on adsorbed bilayers (Solomonov et al. 2009) showed that in the presence of a very large amount of cholesterol this forms domains within the bilayer two molecules thick. In this work the

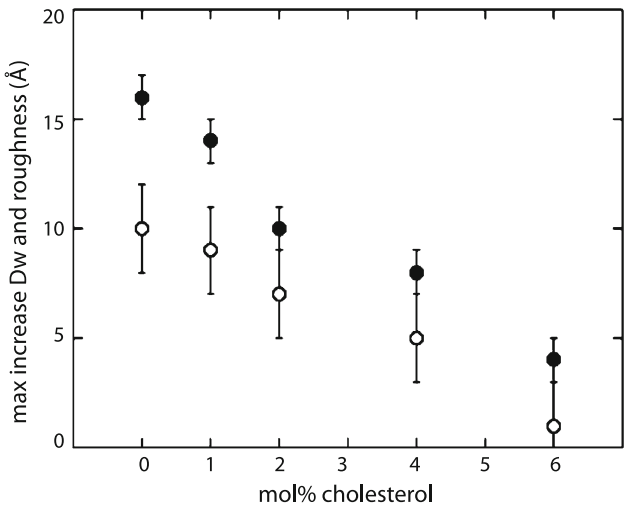

Fig. 7 Maximum increase in the water layer thickness, Dw, between the bilayers (*filled circles*) and upper bilayer roughness, u-Rou (*empty circles*) in the transition region, as a function of cholesterol content

off-specular X-ray reflectivity measurements made on a 1.75:1 mixture of cholesterol and bovine cardiac phosphatidylcholine (Ch:BCPC) deposited as a bilayer on a Si wafer, was interpreted with the presence of a bilayer mixture consistent with possible formation of a crystalline cholesterol bilayer within the hydrated mixed bilayer, which would naturally involve a phase separation of cholesterol and BCPC, as has also been claimed by Tulenko et al. (1998). A driving force for the existence of a crystalline cholesterol bilayer in the 1.75:1 CH:BCPC mixture would be the energetic advantage thereby afforded; the exocyclic moieties of cholesterol, being branched, flexible and with a cross-sectional area less than that of the sterol group (37.5 $Å^2$), would make favorable interlayer contacts that could also lead to partial interleaving between the exocyclic groups.

In small amounts cholesterol presents a transverse distribution that is not symmetric in biomembranes, and may assume specific location in functional asymmetric domains. Recently, Rondelli et al. (2012) succeeded in forcing asymmetry in an "adhering + *floating*" bilayer system composed of phospholipids and cholesterol, in bio-similar mole ratios (11:2.5 mol:mol). They investigated the structure of the bilayers by neutron reflectivity, and presented results on the feasibility of preparation of asymmetric *floating* membranes of desired composition and including cholesterol, and on the effects of common experimental protocols on the overall stability of the bilayer and on the lipid redistribution between different leaflets.

Cholesterol transport is essential to healthy cellular activity and inappropriate transport mechanisms can lead to the appearance of fatal diseases. A complete understanding of cholesterol homeostasis in the cell is still lacking and this is also due to the wide variability in reported values for intra- and intermembrane cholesterol transport rates. In fact, reported half-lives for inter-membrane exchange range from many hours down to tens of minutes, while for trans-membrane cholesterol flipping, the reports vary from several hours to a few seconds down to even a few milliseconds. A recent piece of work (Garg et al. 2011) describes time-resolved small-angle neutron scattering in situ measurements of the cholesterol intermembrane exchange and intramembrane flipping rates. The authors found significantly slower transport kinetics than reported by previous studies, particularly for intramembrane flipping where the measured rates are several orders of magnitude slower. The work described by Rondelli et al. (2012), suggests on the other hand that in the presence of two membranes and in the time-scale of minutes in the fluid phase, while lipids flip from one to the other (see for example Fig. 8), cholesterol flips rapidly only inside a single bilayer. The effects of the presence of a surface are not negligible in these transport phenomena and is currently the subject of further investigation.

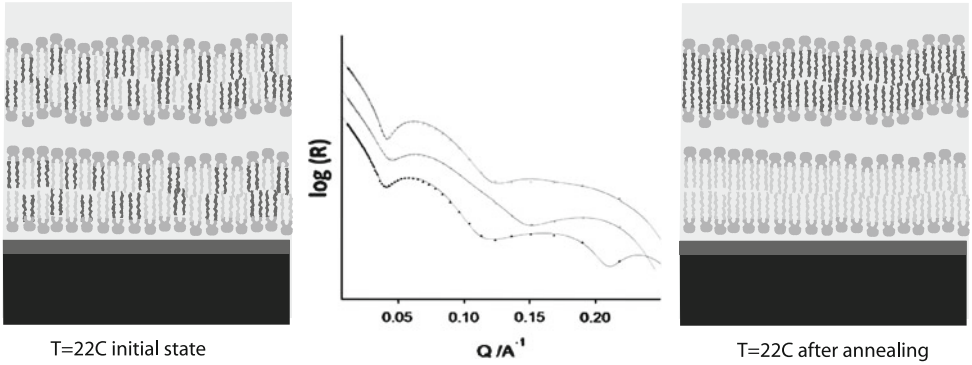

Fig. 8 Neutron reflectivity data from a double bilayer system initially composed of a fully hydrogenated lipid bilayer adsorbed on a silicon substrate and a floating deuterated layer (*top curve* collected from sample as prepared at 22 °C) and two mixed adsorbed and floating layers obtained after annealing (middle curve collected from sample at 54 °C and lower curve at 22 °C after annealing) in contact with bulk H_2O (Rondelli et al. 2012). Data offset for clarity

A very recent piece of work from the Cantù group in Milan aimed at the study of the structural effects brought by the presence of the monosialo GM1 ganglioside to a cholesterol-containing lipid membrane, and the eventual cholesterol-GM1 coupling. In fact, it is often claimed that GM1 and cholesterol constitute a pair affecting the structural properties of their environment in membrane microdomains. Different supported + *floating* bilayers made of phospholipids, cholesterol and GM1 in bio-similar mole ratios were prepared by the Langmuir–Blodgett Langmuir-Schaefer techniques and investigated by neutron reflectivity. It was the first time that such a complex model membrane preparation was attempted. Data analysis suggests that the presence of GM1forces asymmetry in cholesterol distribution, opposite to what happens for a GM1-free membrane, submitted to a similar procedure, resulting in a full symmetrization of cholesterol distribution. It appears also that *preferential* asymmetric distribution of GM1 and cholesterol is attained in a model membrane with biomimetic composition, revealing that a true *coupling* between the two molecules occurs (*data not published*). Off-specular synchrotron radiation data from this complex system are being analyzed.

Conclusions and perspectives

Quantitative measurements of lipid–lipid, lipid–peptide, and lipid–protein interactions can benefit from artificial biomimetic models and lipid bilayers are the most natural substrate on which to test interactions of lipids with other biomolecules. Floating bilayers have become very popular as bio-mimetic systems and have allowed a better insight in the determination of membrane structure and fluctuations. There is ample space for improvement to allow for the preparation of layers in the biologically relevant fluid state and of increasing compositional complexity. A promising area for future development is the in situ measurement of membrane protein conformation in a native like aqueous membrane environment. The determination of the lateral structure of domains, so-called rafts, which are believed to strongly impact membrane properties and functions, is the current focus of much effort in the area. Next obvious step is the use of lipids extracted from natural membranes before moving to the natural membranes themselves.

The planar, substrate-bound topology of supported and floating bilayers provides an advantage for characterizing the structural properties of lipid membranes and membrane proteins. There are also technological advantages of being able to place a biomimetic membrane on semiconductor surfaces, which could facilitate applications in biosensors and biocompatible coatings. The properties of supported bilayers can differ depending on the preparation method and structural studies become of paramount importance.

Scattering techniques have been widely used in the last decades for the in situ characterisation of biological and biomimetic thin films. They simultaneously probe the molecular level, with fractions of nanometre resolution in the determination of composition profiles, and the collective level, with measurements up to the micrometer range. As the deuteration of proteins is becoming an active field of research, the use of fully deuterated or partially deuterated proteins has opened up new possibilities in the study of lipid protein interactions or protein structures at lipid surfaces (Chenal et al. 2009).

Real space techniques including atomic force microscopy, near-field scanning optical microscopy, surface force apparatus, second-harmonic generation, micromanipulation, interference microscopy have largely progressed in the last decades and present many complementary aspects to x-rays or neutrons experiments (*Fourier space*). New promising techniques are emerging like, for example, lensless nanoscale imaging using x-ray waveguides as ultra-small sources for quasi-point-like illumination (Giewekemeyer et al. 2010).

Authors compare one-step holographic and iterative ptychographic reconstruction, both for simulated and experimental data collected on samples illuminated by waveguided beams. They demonstrate that scanning the sample with partial overlap can substantially improve reconstruction quality in holographic imaging, and that divergent beams make efficient use of the limited dynamic range of current detectors, regardless of the reconstruction scheme.

Finally, computer simulations of various lipid membrane systems allow us to elucidate at the molecular level the detailed relations between the chemical structure and physical properties of various lipid molecules and membrane inclusions, to explain individual peculiarities of natural objects, to predict their behavior, etc. An understanding of the molecular basis of various physical properties of lipids and other membrane constituents allows one to narrow down the list of hypotheses under consideration about the possible functions of various components (such as acyl chains) in lipid membranes, e.g., the maintenance of proper bilayer fluidity and permeability, of the activity of membrane-bound enzymes. Together with the continued improvements of force fields and significant development of simulation approaches (methodologies, algorithms and mutually complementary methods), rapid advances in computing power, the long-term prospects of computer simulations in membrane studies is highly promising (Lyubartsev and Rabinovich 2011).

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