Eur. Phys. J. Special Topics **213**, 327–342 (2012)

DOI: 10.1140/epjst/e2012-01680-5

THE EUROPEAN PHYSICAL JOURNAL SPECIAL TOPICS

Review

Neutrons and model membranes

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Received 20 July 2012 / Received in final form 27 September 2012
Published online 3 December 2012

Abstract. Current research in membrane protein biophysics highlights the emerging role of lipids in shaping membrane protein function. Cells and organisms have developed sophisticated mechanisms for controlling the lipid composition and many diseases are related to the failure of these mechanisms. One of the recent advances in the field is the discovery of the existence of coexisting micro-domains within a single membrane, important for regulating some signaling pathways. Many important properties of these domains remain poorly characterized. The characterization and analysis of bio-interfaces represent a challenge. Performing measurements on these few nanometer thick, soft, visco-elastic and dynamic systems is close to the limits of the available tools and methods.

Neutron scattering techniques including small angle scattering, diffraction, reflectometry as well as inelastic methods are rapidly developing for these studies and are attracting an increasing number of biologists and biophysicists at large facilities.

This manuscript will review some recent progress in the field and provide perspectives for future developments. It aims at highlighting neutron reflectometry as a versatile method to tackle questions dealing with the understanding and function of biomembranes and their components. The other important scattering methods are only briefly introduced.

1 Introduction

The cell is the structural and functional unit of most living organisms. Each cell consists of a mass of protein material (the protoplasm) that is differentiated in a substance known as cytoplasm and a nucleus, which contains DNA. The protoplasm is bound by a cell membrane, which 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. The percentage by weight of lipids in membranes varies from 79% in the myelin membrane (because of the high phospholipid content, myelin can electrically insulate the nerve cell from its environment) to 24% in the mitochondrial inner

membrane. In plasma membranes, they represent 40–50% in weight of the membrane composition.

Because of the complexity of cell membranes, their duplication in the laboratory, and the investigation of their interaction with other molecules, are not straightforward. An increasing need for biomimetic models has caused in the last 15 to 20 years the revival of a classical approach: the use of lipid bilayers [1]. 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 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 $0.03\,\mathrm{km}^2$ in our body, that is the area of five football fields [1]. The function of many membrane proteins depends on membrane composition, lipid-protein interaction, 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 modern diseases are associated with changes in lipid composition (as for example diabetes, schizophrenia, Tay-Sachs syndrome, Alzheimer and Parkinson diseases) [1].

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 wavelength of neutron beams is of the order of the tenth of nanometer, therefore they are 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*. Since neutrons interact weakly with atomic nuclei, they are highly penetrating so that samples in complex sample environments can be probed and *in-situ* measurements from buried interfaces are possible. Finally, neutron energies range from the meV to the eV and therefore they are comparable to the energies of atomic and electronic processes. This allows for the study of dynamic properties like translations, vibrations, rotations, lattice modes, exhibited by molecules.

It is also possible to accentuate or annihilate the scattering from individual parts of a macromolecular complex so, for example, by specific deuterium labeling it is possible to measure bilayer conformational changes and organization both in the perpendicular and lateral directions.

In all cases experimental set-ups consist of: a radiation source, a wavelength selector or choppers, a system of collimation, the sample and a system of detection. A collimated incoming beam of wavelength λ interacts with a sample with an angle of incidence. The outgoing beam at an angle θ from the sample is measured by a detector (punctual, mono or bi-dimensional). The angle between the incoming and outgoing beam is defined as the scattering angle, 2θ. The intensity of the

outgoing over that of the incoming beams is recorded vs. the wave vector transfer, $q = 4\pi/\lambda \sin\theta$. This is the modulus of the resultant between the incident and scattered wavevectors.

Different types of experiments can be done: i) in reflection or in transmission; ii) with angular range going from a few mdeg to 100 degrees (q value from 10^{-3} Å^{-1} to 1 Å^{-1}); iii) with a beam of one defined wavelength, by scanning the angle of incidence and the angle of emergence (so called *monochromatic mode*); iv) at a fixed angle of incidence, using a polychromatic incident beam (so called *time-of-flight mode*). Note that only the amplitudes of the scattered waves can be measured while the phase shifts cannot be determined directly. This is the well-known "phase problem" which usually inhibits a direct transformation back into real space.

The techniques, which have been mostly employed in the past for membrane studies, are listed below [2]:

- *Diffraction*, used mainly to determine the structure of stacked lipid lamellar systems: the covered range in q is 0.02–2.5 Å^{-1} and typical time-scales are of the order of the minutes;
- *Small angle scattering (SAS)*, used to gain information on shape, size and interactions of lipid lamellar systems and vesicles: it gives radial averaged information in all directions of space: the covered range in q is 10^{-4}–1 Å^{-1} and typical time-scales are of the order of the minutes but it is possible to go down to seconds;
- *Specular reflectivity*, used to study the structure of bilayers in the direction perpendicular to the plane of the layers in a planar configuration: the covered range in q is 10^{-3}–0.4 Å^{-1} and typical time-scales are of the order of the minutes but it is possible to go down to seconds;
- *Off-specular reflectivity and GISANS*, probe lateral structures from the *nm* to the μm scale. The limited neutron flux is such that a large number of ordered lateral features (domains, holes, ...) have to be present to ensure enough signal and the possibility to do the analysis.
- *Inelastic, quasi-elastic and spin-echo*, used to study the structure and dynamics on length scales ranging from the nearest-neighbor distances of lipid molecules to length scales of more than 100 nm covering time scales from about 0.1 ps to almost 1 μs.

Neutron scattering techniques have been much used as they allow for non-destructive in-situ measurements not only of interfaces, but also of buried (bulk) materials. 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.

The present review presents a few examples of nanometer scale studies of lipid bilayers using neutrons, with emphasis to new possibilities currently emerging. In Sect. 2, some of the model lipid bilayers suitable for studies with scattering techniques are reviewed. Single bilayers, multilamellar stacks, vesicles, are discussed together with the criteria commonly followed for choosing a model adapted to the measurement techniques available. In Sect. 3 the conclusions and perspectives, with special emphasis to current and potential studies on the elucidation of the structure and dynamics of lipid domains.

2 Lipid bilayers as membrane models and neutron reflectivity

The history of membrane models dates back to 1925 when Gorter and Grendel [3] 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 Dawson suggested the association of proteins with membranes [4]. Finally in 1972 Singer and Nicolson [5] introduced the widely used "Fluid Mosaic Model" whereby integral and peripheral proteins "float in a fluid sea". In 1978 Israelachvili [6] described thickness variations and pore formation concepts and in 1995 Sackmann [7] included cytoskeleton and glycocalix. Furthermore, it has recently been determined that to coordinate cell functions, the membrane is able to laterally segregate its constituents forming so-called lipid rafts. This capability is based on dynamic liquid-liquid immiscibility. Lipid rafts are fluctuating nanoscale assemblies of sphingolipid, cholesterol, and proteins that can be stabilized to coalesce and provide platforms for membrane signaling and trafficking [8]. Little is known on the structure of these domains [9].

The need for a new membrane model has become necessary [1]. 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 nanometer range, which are difficult to access experimentally. The structuring in time, i.e. correlated dynamical phenomena, are recently recognized as important features of membrane systems.

Issues to be addressed are 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; protrusions; instabilities towards non-lamellar symmetries; phase transitions: membrane function may be stirred by perturbations by both physical (temperature, pressure) and chemical (drugs) factors.

Producing a good, biologically relevant model membrane is a challenge [10]. Ideally, it should obey different requirements: (i) Its composition should match the content of (at least the lipid part of) the real cell membrane under investigation; (ii) It should be hydrated, i.e. stable in aqueous environment; (iii) It should be able to interact with other biological entities (i.e. peptides, proteins, DNA); (iv) It should be free enough to allow for movements, both out of plane (the bilayer position fluctuates) and in plane (the molecules freely diffuse within the bilayer: fluid phase, as opposed to gel phase, see below).

In practice, compromises are usually made to accommodate for experimental difficulties and scientific choices. For instance, in scattering studies the size of the sample, hence the amount of material (the "scattering volume") should be large enough to obtain a measurable signal. If the technique requires that the bilayer position and orientation is controlled, e.g. for reflectivity, the sample should be ordered enough, which might turn incompatible with free position fluctuations.

A variety of sample types has been tried. They differ by: (i) geometry, e.g. spherical or flat; (ii) number of bilayers: one, a few, or hundreds; (iii) order: controlled or disordered position and orientation of molecules; (iv) phase of the bilayer: gel (e.g. at low temperatures) or fluid; (iv) difficulty of the preparation method (cf. Fig. 1).

Moreover, phospholipids like surfactants and other amphiphilic molecules, present a variety of different phases depending on their volume/surface area ratio or as temperature is changed [11]. Going from high to low temperature, lipids overcome the 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.

The bilayer molecular composition is the first issue. Although bilayers in membranes contain variable amounts of cholesterol, the simplest models make use only

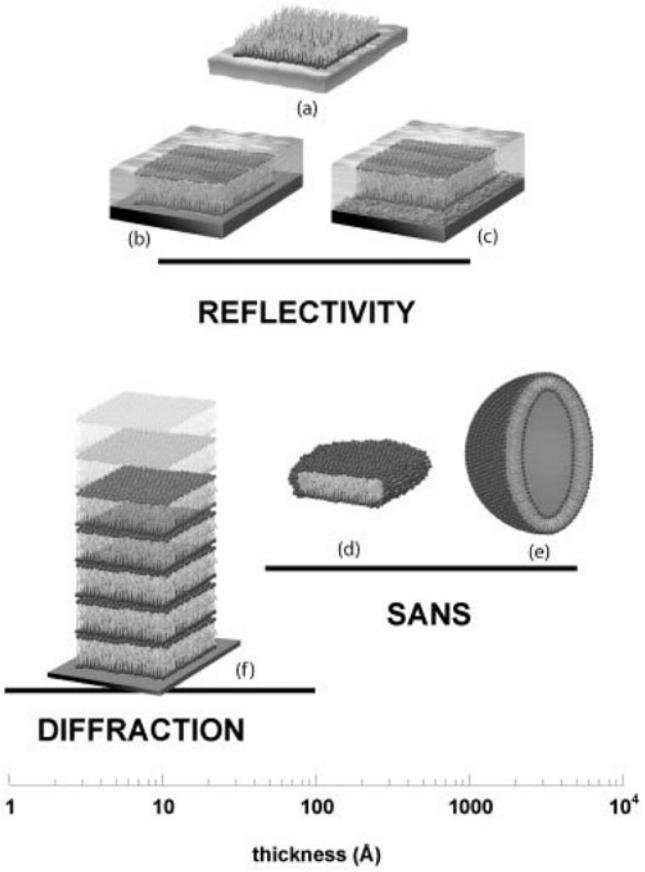

Fig. 1. Examples of common model membrane systems adapted to different neutron scattering techniques and, on the x-axis, size range probed by those techniques. (a) Monolayers; (b) adsorbed bilayers; (c) floating bilayers; (d) bicelles; (e) vesicles; (f) stacked bilayers. Adapted, with permission, from [2].

of phospholipids. Various lipid chains or heads can be chosen. Di-palmitoyl (16 carbons) and di-stearyl (18 carbons) chains are often used, since they are dominant in nature (although often with insaturations in the chains); sample preparations or experimental conditions might sometimes be easier if di-myristoyl (14 carbon) 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 thus usually 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.

In the following paragraphs, the most widely employed bilayer models for scattering studies are introduced. Historically, multilamellar systems, including liposomes and stacked bilayers, have been used first. Developments in scattering instrumentation

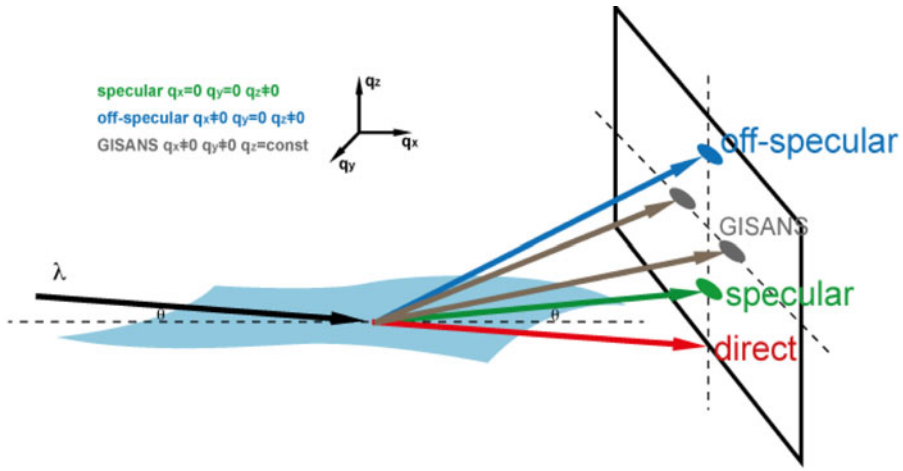

Fig. 2. Simple schematic of the geometry of scattering techniques from surfaces. λ is the wavelength of the incoming beam and θ the angle.

and in the neutron reflectivity technique have led to a growing interest in the use of lipid monolayers or single bilayers.

Neutron reflectivity is indeed an ideal tool for getting information at the Å level and in the last fifteen years the technique has been widely used for the study of single bilayers at solid/aqueous solution interfaces. X-rays have been less used so far since they 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. The advantage with respect to SAS or diffraction studies on multilamellar systems is that the information is not averaged from a large number of bilayers but it can be determined for a bilayer alone.

In a neutron reflectivity measurement R, the ratio 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 [12]. $\rho(q_z)$ 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 (SLD) and a thickness, which are used to calculate a model reflectivity profile by means of the optical matrix method [12]. The interfacial roughness between any two consecutive layers may also be included in the model by the Abeles method. The calculated profile is compared to the measured profile and the quality of the fit is assessed by using χ^2 in the least-squares method.

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 Å. 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 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 materials can substantially change the reflectivity curve of a system while maintaining the same chemical structure. All the reflectivity curves measured at different isotopic compositions of the same physical system must be described by the same model. Experience on this kind of systems and on similar ones suggests that the 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.

2.1 Multilamellar systems

The large body of experimental studies of membrane structure have been conducted on liposomes. Multilamellar vesicles are more suitable than unilamellar vesicles for neutron studies due to stronger scattering. As already stated, since the wavelength of X-ray or neutron beams used for structural determination is of the order of the fraction of nanometer, information on that length scale is obtained. Consequently, 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. All these values are averaged in all directions. Surface sensitive scattering techniques, like reflectivity or grazing incidence diffraction, 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 has historically been the source of high resolution structural data of multilamellar model biological membranes, neutron reflectometry has provided unique data of single lipid bilayers in contact with bulk water.

Vesicles, or liposomes, are bilayers closed in roughly spherical shapes, which enclose a fixed volume of water or solution. They are easy to prepare (e.g. by sonication of lipids in water) in large quantities. It is possible to control not only the composition of the lipid bilayers, but also the outer and inner media. The main advantage of self-assembled vesicles is that the environment can be tailored (pH, ionic strength, etc.) to match that found under physiological conditions. It is e.g. possible to prepare the vesicles in a solution with high osmotic pressure, and rinse them in a solution with a slightly smaller pressure, so that the water flows inwards: the vesicles then swell and become under tension. This dramatically reduces the position fluctuations of the membrane, which amplitude is thus under the experimentalist control.

These vesicles are usually small, typically tens or hundreds of nanometers, and are multilamellar. Due to the absence of any privileged direction, they are suitable for scattering experiments. Forcing the vesicles to flow through a filter with a controlled pore size (an “extruder”) results in a population of small (mostly) unilamellar vesicles, or SUV (Fig. 1(e)). Then all lipid bilayers are accessible to the outer medium, i.e. to injected molecules such as peptides or proteins. Progress in the study of lipid domains by small angle scattering is reported in [12–15].

Multilamellar stacks of bilayers have been prepared and used for a long time (Fig. 1(f)) [16]. They are suitable for diffraction, due to the large amount of material involved. The most common way of aligning lipid multibilayers is to deposit them from a concentrated lipid/solvent solution onto the solid support (typically glass or silicon) and allow the solvent to slowly evaporate in order to get highly aligned samples. Then, they are hydrated in water saturated atmosphere. The current efforts have been particularly successful in two directions. First, by increasing the hydration,

going up to full hydration (100% of the vapor pressure) [17]. Second, in improving the control of both the number of stacked bilayers (from a few units to a few hundreds) and their order (parallelism of bilayers of order of a fraction of degree), making possible to perform simultaneously diffraction and reflectivity on the same sample [18]. Diffraction methods on lipid bilayers have been successfully employed by Huang and co-workers in the last couple of decades [19].

Recently, Del Favero et al. [20] investigated multilamellar-oriented depositions of phospholipids containing minority amounts of ganglioside or sphingomyelin under a low-hydration condition. The minority components are known to form domains within the phospholipid bilayer matrix. The low water content inhibits the lipid exchange among nearby lamellae and strengthens lamella-lamella interactions, allowing for a straightforward comparison with a model presented by the same authors to investigate the effect of lipid clustering on the local interlayer distance in a cluster of interacting lamellae. The model, based on non-equilibrium thermodynamics and linear stability theories, explores the early stages of the lamella-lamella phase separation process where the lateral diffusion is much faster than the inter-lamellar lipid exchange. Small-angle and wide-angle neutron diffraction experiments were performed in order to detect interlayer distances and local chain order, respectively. Lamellar stacking splitting was observed for the ganglioside-containing lamellae, induced by in-phase lipid clustering. In excess water and after long equilibration times, these local structures showed further evolution, leading to coexisting lamellar phases with different lipid compositions and interlayer distances.

The main disadvantage of multilamellar systems is that the information obtained from structural studies is an average of the behaviour of several bilayers. A further disadvantage of stacked bilayers is that it is difficult to keep them in aqueous environment.

2.2 Monolayers on water

Phospholipid monolayers, although less relevant from a biological point of view than bilayers, provide interesting model systems for the study of biological membranes since they enable the investigation of interactions between lipids in well defined arrangements, while allowing a large degree of freedom in parameter variation. Lipid monolayers at the air-water interface [21] (Fig. 1(a)) are commonly referred to as *Langmuir monolayers* and they are very robust and easy to prepare, by spreading a solution of lipids dissolved in a volatile solvent on the surface of water in a Langmuir trough. A pressure captor can monitor the surface pressure and the lipid density can be controlled by a compression barrier. With a slightly more sophisticated apparatus, monolayers can be prepared also at the oil-water interface.

An interesting feature of spread monolayers on water is their versatility as lipid composition may be tailored at will, provided that insoluble molecules are used. The direct access through air, and the good planeity of the layer, facilitates many studies such as X-ray and neutron reflectivity, and other surface techniques such as surface-sensitive infrared spectroscopy. Moreover, many physiologically important interactions take place at the interface between the membrane, for example at the lipid headgroups, and the aqueous compartment.

Amphiphilic cyclodextrins (CDs) are good candidates to functionalize natural membranes, as well as synthetic vesicles. In a recent paper paper, Bauer et al. [22] fully describe and compare the insertion properties of the permethylated mono-cholesteryl α-CD (TASC) and its mono- and di-cholesteryl β-CD analogues (TBSC and TBdSC) in dipalmitoyl-L-α phosphatidylcholine (DPPC) mono- and bi-layers as membrane models from the macroscopic to the molecular scale. By calculating the inverse compressibility moduli and free excess Gibbs energies from the Langmuir isotherms, the

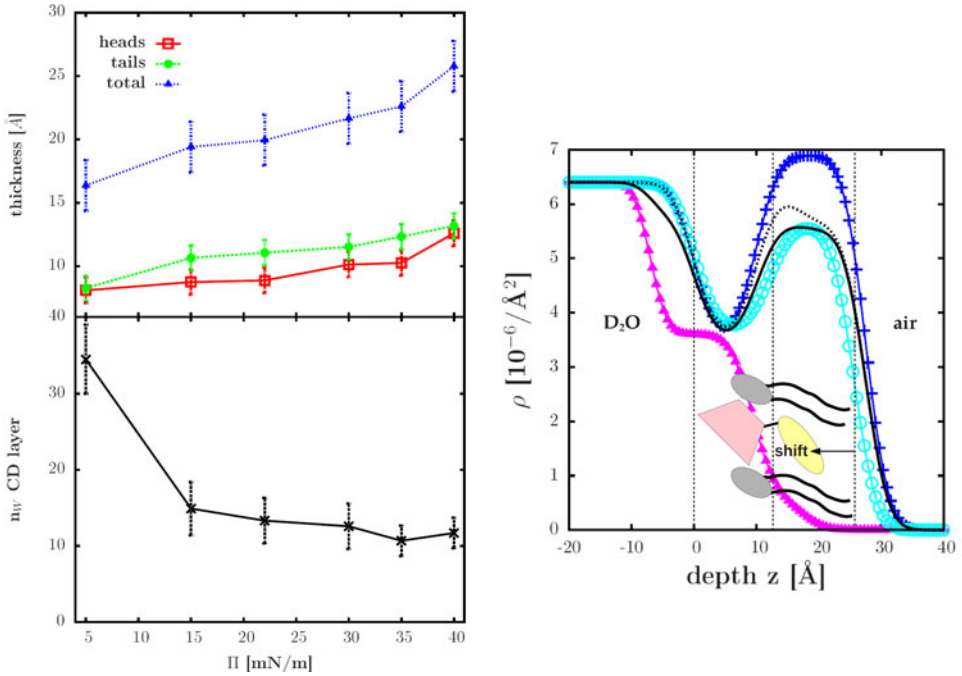

Fig. 3. Thickness of TASC/DPPC monolayer(top) and number of water molecules associated to the headgroups (bottom) as a function of surface pressure, as determined by neutron reflectivity experiments. On the right is the scattering length density profile at 40 mN/m for (+) pure DPPC, (○) 20 mol% TASC and (▲) pure TASC. Adapted with permission from [15].

influence of the CD type, CD ratio and number of cholesteryl anchors on the membrane properties were established. TBdSC, with its two cholesteryl residues, seems to be anchored best to the membrane compared to CD derivatives with only one anchor. Furthermore, TASC appears to be more firmly inserted into the membrane than TBSC. Depending on the compression, full miscibility of the cholesteryl CDs and the phospholipids was observed at low surface pressures and a clear demixing tendency is apparent during compression. The structure of the mixed TASC/DPPC mono- and bi-layers perpendicular to the surface was investigated with Ångstrom resolution by neutron reflectivity. In this way a molecular model of the insertion was established (see Fig. 3), which suggests that the CD cavities partly protrude into the subphase, which should leave them accessible for complex formation.

2.3 Single adsorbed and floating bilayers

Bilayers can be deposited on solid substrates by using modified Langmuir troughs and the so called *Langmuir-Blodgett* or *Langmuir-Schaefer* techniques [23]. 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 block is immersed in the subphase and by slowly withdrawing it from water a monolayer of lipid is deposited on its surface. Reimmersion will produce a bilayer, and so forth. In the Langmuir-Schaefer modification, after depositing the monolayer the solid substrate is rotated by 90° and slowly reimmersed with the large face parallel to the water surface for the formation of the bilayer (see Fig. 1(b)). Pep-

tides or proteins can be co-deposited or inserted in the water subphase and transferred with the lipids on the water surface.

The solid substrates can have sizes up to tens of centimetres, thus being perfect candidates for reflectivity. Supported membranes on solid substrates are interesting as they allow bio-functionalisation of inorganic solids (semiconductors, gold covered surfaces, opto-electronic devices) and polymeric materials; as well as imaging by atomic force microscopy.

A major advantage of this model is the fact that the bilayer is immersed in aqueous solution and that information on the single bilayer is obtained. Moreover, these are well-defined structures, where the composition of each leaflet of the bilayer can be chosen separately. The proximity of the substrate limits the position fluctuation, which is a disadvantage when studying the interaction with transmembrane proteins.

Other approaches for the formation of planar single lipid bilayers less interacting with the solid substrate are being tried. One is vesicle spreading on polymer layers, where the polymer provides a soft cushion screening the influence of the substrate [24].

Charitat et al. [25] have succeeded in the preparation of stable and reproducible double bilayers, in which the second bilayer floats at 20–30 Å on top of the first. This model makes it possible to do reflectivity studies on a highly hydrated, accessible and fluctuating bilayer, where again the composition of each leaflet can be chosen separately. Since the second bilayer is only weakly bound, the preparation is delicate. The current process relies on three vertical Langmuir-Blodgett depositions, followed by a horizontal Langmuir-Schaefer one; this method is efficient when lipids are in the gel phase although by raising the temperature the floating bilayer overcome a phase transition becoming fluid and still stable. This system has been very useful to probe bilayer-bilayer interactions, effect of charges, effect of an electric field, etc. A number of other systems have also been tried to create floating bilayers and these include: grafted [26]; polymer [27] and polyelectrolyte cushions [24]; tethered bilayers [28]. Because of the potential to support transmembrane proteins, all these systems promise to be a better biomimetic model membrane than supported single bilayers.

For a recent review on supported bilayers and scattering techniques, see [29].

Single adsorbed bilayers. The first experiments on supported single lipid bilayers in aqueous solution are described in [30]. DMPC bilayers were adsorbed on quartz from vesicles. The use of chain deuterated and chain protonated bilayers mixed so that the scattering length density of the acyl chains matched that of the quartz, allowed for an independent determination of the headgroup structural parameters. After these early experiments, efforts were made to improve the resolution of the neutron reflectivity technique and also to improve the biological relevance of the models. Krueger et al. [31] were able to obtain reflectivities down to 10^{-8} and Q out to $0.7\,\text{Å}^{-1}$ by decreasing the thickness of the water in contact with a hybrid bilayer (formed by an alkanethiol chemisorbed to a gold surface and a phospholipid layer) down to $15\,\mu$m. In those conditions the resolution of the neutron reflectivity experiments are limited only by the roughness of the supporting substrate.

Progress in protein deuteriation techniques is proving valuable for the determination of lipid-protein interactions from single bilayers. For example, Chenal et al. [32] have investigated the diphtheria toxin T domain membrane insertion upon gradual acidification of the membrane environment to model the uptake of this toxin by cells in endosomes. By using a combination of selective membrane deuteration of one leaflet at a time (achieved by Langmuir-Blodgett Langmuir-Schaefer deposition) and site-

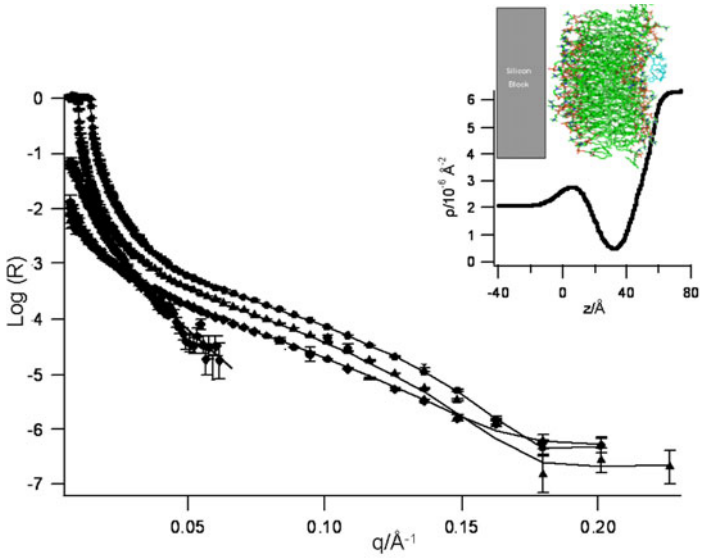

Fig. 4. Neutron reflectivity profiles (points) and best fits (continuous lines) corresponding to POPC bilayer with deuterated C8 peptide in four water contrasts. The inset shows the scattering length density profile for the bilayer in D2O after peptide addition and a system snapshot obtained by MD simulations. Reproduced, with permission, from [33].

specific deuteration of a cluster of 12/13 lysine residues in the N-terminal part of the T domain, they were able to determine the progress of the peptide penetration as a function of membrane acidification as a model of the changing pH environment as the protein is taken up into endosomes, as well as deducing that the N-terminus most likely remained in solution.

The complexity of membrane systems and the limited resolution of the technique is such that often information from complementary measurements as well as molecular dynamics simulations are necessary. A recent example of how the use of different tools, coupled to partial deuteriation of a peptide, has proved efficient is from Merlino et al. [33].

Viral fusion glycoproteins present a membrane-proximal external region (MPER), which is usually rich in aromatic residues and exhibits a marked tendency to stably reside at the membrane interfaces, leading, through unknown mechanisms, to a destabilization of the bilayer structure. This step has been proposed to be fundamental for the fusion process between target membrane and viral envelope. Authors investigated the interaction between an octapeptide (C8) deriving from the MPER domain of gp36 of feline immunodeficiency virus and POPC bilayers by combining experimental results obtained by neutron reflectivity (see Fig. 4), electron spin resonance, circular dichroism, and fluorescence spectroscopy with molecular dynamics simulations. Data from all these complementary methods indicate that C8 binds to the lipid bilayer adsorbing onto the membrane surface without deep penetration. As a consequence of this interaction, the bilayer thickness decreases. The association of the peptide with the lipid membrane is driven by hydrogen bonds as well as hydrophobic interactions that the Trp side chains form with the lipid headgroups. Upon peptide-bilayer interaction, C8 forms transient secondary structures ranging from 310 helices to turn conformations, while acyl chains of the peptide exposed POPC molecules assume a more ordered packing. At the same time, lipid headgroups' hydration increases. The

asymmetric lipid bilayer perturbation is proposed to play a fundamental role in favoring the membrane fusion [33]. In the presence of 10% and 20% of cholesterol, C8 interacts with membrane surface, while peptide-membrane interaction is inhibited with a cholesterol concentration of 30%.

The presence of SM causes variation in the lipid bilayer structure, which favors the interaction with C8 peptide [in preparation].

Floating bilayers. Double bilayers have been studied with both neutron and synchrotron radiation reflectivity techniques. Neutron specular reflectivity studies [34] 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. 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 [35]. Measurements on floating bilayers have allowed determining the bending modulus an surface tension of adsorbed and floating layers [35] as well as the interaction potential between supported floating bilayers [submitted].

The structural complexity of biomembranes, based on their heterogeneity in composition, dramatically involves the asymmetric disposition of different components, from lipids to proteins, in the transverse or the longitudinal directions. Furthermore, 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. In order to build suitable mimics of complex biomembranes, it is then necessary to realize asymmetric model systems applicable for structural investigation. The experimental study of asymmetric model membranes is rare, due to the difficulty of realizing 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 [36]. Fine assessment of structural inhomogeneities requires experimental macroscopic monodispersity. By preparing asymmetric double bilayer samples, it has been possible to test whether a simple imposed asymmetry was kept in time and whether it could stand some standard experimental protocols commonly employed when dealing with model membranes (i.e. annealing and solvent exchange). Rondelli et al. [37] focused on cholesterol, a basic component with a transverse distribution that is not symmetric in biomembranes, and may assume specific location in functional asymmetric domains. The authors forced asymmetry in an "adhering + floating" bilayer system composed of phospholipids and cholesterol in much higher amounts, 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 the desired asymmetric free floating membranes, 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.

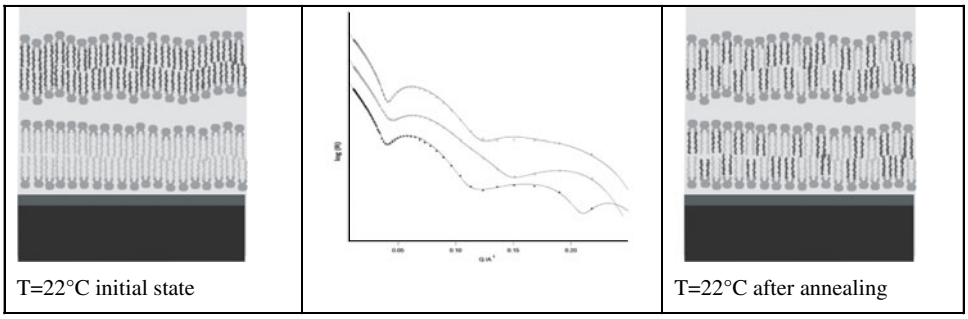

Fig. 5. Neutron reflectivity data from a double bilayer system initially composed of a fully hydrogenated lipid bilayer adsorbed on a silicon substrate and a floating deuterated layer (top curve collected from sample as prepared at 22°C) and two mixed adsorbed and floating layers obtained after annealing (middle curve collected from sample at 54°C and lower curve at 22°C after annealing) in contact with bulk H_2O [37].

A recent piece of work [38] 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. [37], 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. 5), cholesterol flips rapidly only inside a single bilayer. The effects of the presence of a surface are not negligible in these transport phenomena and are currently object of further investigation.

Finally, up to now the experimental observation of lateral nanostructures in membranes has proven difficult, as they are dynamic structures that most likely fluctuate on nano- to microsecond time scales. Using neutron diffraction, Armstrong et al. [40] presented the first direct experimental evidence for the co-existence of gel and fluid domains in a single-component phospholipid membrane as it undergoes its main phase transition. The coherence length of the neutron beam sets a lower limit for the size of structures that can be observed. Neutron coherence lengths between 30 and 242 Å were used in this study, and were obtained by varying the incident neutron energy and the energy resolution of the neutron spectrometer. Authors observed Bragg peaks corresponding to coexisting nanometer sized structures, both in out-of-plane and in-plane scans by tuning the neutron coherence length. During the main phase transition, instead of a continuous transition that shows a pseudo-critical behavior, they observed the co-existence of gel and fluid domains.

3 Conclusions and perspectives

Interactions between proteins and lipids take part to basicly all membrane processes, but on a molecular level they are still poorly understood. Simple model systems comprising designed transmembrane peptides in synthetic lipid bilayers are increasingly being recognized as powerful tools to uncover basic principles of protein–lipid interactions. By means of these model systems it is possible to determine the properties of lipids influence the structure and dynamics of transmembrane helices, how these helices are anchored at the lipid–water interface, and how the length and composition of transmembrane segments influence the organization and dynamics of membrane lipids [41].

Neutron scattering techniques are extremely useful tools for the non-destructive in-situ characterisation of biological and biomimetic thin films. They simultaneously probe the molecular level, with 0.1 nm resolution in the determination of composition profiles, and the collective level, with measurements up to the micrometer range. While some types of experiments deserve their reputation of high sophistication level, it is possible to enter this field without any prior knowledge and quickly acquire basic data. 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 [32].

Near-field microscopies have enjoyed a few decades of successful developments. However, they have not replaced x-rays or neutrons experiments (the so-called "Fourier space" techniques). Direct measurements ("real space" techniques) include atomic force microscopy, near-field scanning optical microscopy, surface force apparatus, second-harmonic generation, micro-manipulation, interference microscopy. The past couple of decades proved how useful are the complementary information obtained in real space and Fourier space: more and more publications actually include simultaneously one technique of each type.

The extensive studies of pure lipid bilayers have provided a firm basis for investigations of lipid bilayers-peptide/protein/DNA interactions, presently the focus of a considerable effort. The mechanism by which some biomolecules insert in membranes or disrupt them is currently addressed using a structural approach.

Complex multi-component membrane structure and composition can be obtained thanks to the developments made in data analysis and measurement scattering techniques, with resolution not currently achievable by other methods. The increased use of complementary characterization techniques has greatly contributed to the improvements in the quality of experiments as well as the interpretation of the results. 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, when the existence of rafts and their role in cell function need still to be elucidated. Cell biologists are aware of the importance to resolve the lipid raft debate because the plasma membrane controls what enters and exits cells and how they send and receive signals. Although several alternatives have been proposed for how the plasma membrane organizes itself, none of them has proved to be the definitive one [9].

Next obvious step in lipid structural studies is the use of lipids extracted from natural membranes before moving to the natural membranes themselves.

Giovanna Fragneto is a Senior Fellow at the ILL where she has worked since 1997. She obtained her PhD from the University of Oxford where she received training on studies at interfaces with the technique of neutron reflectometry. After a post-doc in Paris she arrived at the ILL and in collaboration with a team from the university of Grenoble, Strasbourg and CEA in Paris, she spent the last fifteen years developing models for biological membranes and characterizing their structure and fluctuation spectrum. These models have been applied in a few cases to biological systems (see examples above).

The nature of her past and present position at the ILL has given her the privilege to collaborate with many outstanding scientists from all around the world and she is greatly indebted to the many collaborators whose names can be found in a number of the references cited, including Thierry Charitat, Jean Daillant, Edith Bellet-Amalric, François Graner, Alan Braslau, Tim Salditt, Jayne Lawrence, Maikel Rheinstädter, Steve Roser, Laura Cantú, Tommy Nylander, Hanna Wacklin and many others.

Final thanks to the ILL for awarding beam-time and providing support for all these research activities.

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